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Profil Elemen Jejak dari Unta Bunting (*Camilus dromedaries*), Janin, dan Cairan Ketuban saat Lahir dan Hubungannya dengan Berat Lahir Sapi

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ABSTRAK

Cairan ketuban adalah campuran kompleks dinamis yang membawa komponen yang berkontrinduksi pada pengaturan perkembangan janin. Penelitian ini bertujuan untuk mengukur kadar elemen jejak, seperti Fe, Zn, Cu, Mg, Se, dan Mn dalam cairan ketuban, serum induk, dan serum tali pusat vena saat persalinan. Penelitian lebih lanjut menyelidiki hubungan antara tingkat elemen dalam cairan ketuban (AF), serum induk (MS), dan serum tali pusat vena (VUCS) untuk menilai kemungkinan pemantauan pertumbuhan janin yang abnormal. Sampel darah diambil dari 30 unta betina hamil pada saat persalinan dan VUCS dan AF yang sesuai diperiksa. Fe, Zn, Cu, Mg, Se, dan Mn dianalisis menggunakan spektrofotometer serapan atom. Konsentrasi elemen jejak, Fe, Zn, Cu, Mg, Se, dan Mn dalam VUCS secara signifikan lebih tinggi, dibandingkan dengan MS atau AF. Unsur jejak, Fe, Zn, Cu, Mg, Se, dan Mn hadir dalam konsentrasi AF yang jauh lebih rendah daripada di MS atau VUCS. Berat lahir neonatal tidak berkorelasi dengan tingkat MS dari elemen jejak. Namun, berat badan neonatal berkorelasi positif dengan kadar Fe, Se, dan Zn serum tali vena. Terdapat kelangkaan korelasi antara elemen jejak maternal dan janin pada unta bunting. Kesimpulannya, AF bahkan bisa jadi hasil filtrasi sederhana darah induk. Evaluasi level elemen jejak yang dipilih pada MS tampaknya tidak berguna dalam penilaian pertumbuhan janin. Temuan penelitian ini menunjukkan adanya transpor aktif Fe, Zn, Cu, Mg, Se, dan Mn antara unta bunting dan janin. Berat lahir neonatal tidak berkorelasi dengan tingkat MS dari elemen jejak. Namun, berat badan neonatal berkorelasi positif dengan kadar Fe, Se, dan Zn serum tali vena. Terdapat kelangkaan korelasi antara elemen jejak maternal dan janin pada unta bunting. Kesimpulannya, AF bahkan bisa jadi hasil filtrasi sederhana darah induk. Evaluasi level elemen jejak yang dipilih pada MS tampaknya tidak berguna dalam penilaian pertumbuhan janin. Temuan penelitian ini menunjukkan adanya transpor aktif Fe, Zn, Cu, Mg, Se, dan Mn antara unta bunting dan janin. Berat lahir neonatal tidak berkorelasi dengan tingkat MS dari elemen jejak. Namun, berat badan neonatal berkorelasi positif dengan kadar Fe, Se, dan Zn serum tali vena. Terdapat kelangkaan korelasi antara elemen jejak maternal dan janin pada unta bunting. Kesimpulannya, AF bahkan bisa jadi hasil filtrasi sederhana darah induk. Evaluasi level elemen jejak yang dipilih pada MS tampaknya tidak berguna dalam penilaian pertumbuhan janin. Temuan penelitian ini menunjukkan adanya transport aktif Fe, Zn, Cu, Mg, Se, dan Mn antara unta bunting dan janin. Kesimpulannya, AF bahkan bisa jadi hasil filtrasi sederhana darah induk. Evaluasi level elemen jejak yang dipilih pada MS tampaknya tidak berguna dalam penilaian

Kata kunci: Bobot lahir pedet, Unta Dromedaris, Penghalang plasenta, Kehamilan, Elemen jejak

PENGANTAR

Anak unta dromedaris yang baru lahir menunjukkan kelemahan dan berat badan lahir rendah dalam proporsi yang tinggi sehingga mengakibatkan kerugian ekonomi. Bobot lahir pedet sangat bervariasi, tergantung jenis kelamin janin, ras, paritas (Freetly et al., 2000; Nagy dan Juhász, 2019) dan status gizi induk (Zachara dkk., 1986), terutama pada masa akhir kehamilan dimana kebutuhan janin akan energi, protein dan mineral meningkat. Perkembangan janin yang biasa bergantung pada penyimpanan elemen jejak yang sesuai, termasuk besi (Fe), seng (Zn), dan tembaga (Cu), magnesium (Mg), dan selenium (Se) dan mangan (Nandakumaran dkk., 2016). Peran trace mineral dalam perkembangan janin dan pertumbuhannya baru-baru ini disarankan serta hubungannya dengan berat lahir pedet (Graham et al., 1994). Kekurangan trace mineral seperti Fe dan Zn dapat menghambat pertumbuhan janin (Mitchell

dkk., 1998). Demikian juga, Se (Black, 2001; Mitchell et al., 1998) dan defisiensi Cu (Mills dan Davies, 1979) diimplikasikan sebagai kemungkinan faktor yang dapat mengganggu perkembangan janin. Kadar yang lebih rendah dari trace mineral seperti Cu, Fe, dan Zn dinyatakan pada janin yang diaborsi, menunjukkan kemungkinan peran mineral ini dalam pertumbuhan dan perkembangan janin (Graham et al., 1994). Kehamilan memberi beban yang cukup besar pada homeostasis elemen jejak pada mamalia (Hitam, 2001), di mana fisiologi hewan bunting, dan persyaratan janin yang sedang tumbuh berubah (Faye dan Bengoumi, 1994). Ada bukti bahwa unta rentan terhadap gangguan elemen jejak serupa dengan yang dimiliki hewan ruminansia lain (Faye dkk., 1992). Ada beberapa laporan cacat mineral klinis pada unta (Faye dkk., 1992; Faye dan Bengoumi, 1994; Zong-Ping dkk., 1994; Chuka Ozegbe, 2005), dan prevalensi serta kepentingannya kemungkinan besar salah menilai karena tanda-tanda defisiensi subklinis mungkin tidak terdeteksi dalam jangka waktu yang lama.

Pada semua spesies mamalia, cairan ketuban (AF) menumpuk lebih awal, dan kemudian berkurang seiring dengan pertumbuhan embrio (Sulindukrska dkk., 2016). Peran elemen jejak yang terdapat dalam AF tidak jelas. Disarankan bahwa cairan ini dapat menjadi sumber nutrisi janin yang penting (Abdelrahman dan Kincaid, 1993). Setiap janin bergantung sepenuhnya pada induknya melalui plasenta untuk suplai elemen jejak (Perveen et al., 2002). Itu Kepekaan janin terhadap penyerapan elemen-elemen kecil tidak hanya sangat dipengaruhi oleh penyerapan oleh induk, tahap kehamilan dan pengangkutan plasenta, tetapi juga oleh kemampuan yang melekat pada organ-organ janin untuk mengakumulasi cadangan (Hitam, 2001). Pengangkutan elemen jejak dari hewan bunting ke janin bervariasi selama kehamilan, dan secara bertahap meningkat selama periode waktu ini (Tibary dan Anouassi, 1997). Beberapa elemen jejak diangkut melintasi plasenta dalam proses aktif, sementara yang lain tampak diangkut secara pasif (Romeu et al., 1986). Unta memiliki jenis plasenta epitheliochorial dimana selaput janin tidak menginvasi lapisan endometrium uterus (Tibary dan Anouassi, 1997). Mekanisme yang terlibat dalam pengangkutan transplasental elemen jejak dari unta bunting ke janin masih belum diketahui. Untuk lebih memahami transfer elemen jejak dari bendungan ke janin; konsentrasi elemen jejak dalam serum induk, tali pusat vena dan cairan ketuban diselidiki. Meskipun beberapa data tersedia untuk sejumlah spesies, termasuk kelinci (Kriesten Schmidtmann et al., 1986), tikus (Romeu Alemany dkk., 1986), tikus (McArdle dan Erlich, 1991), domba (Langlands Bowles dkk., 1982), ternak (Van Wouwe dkk., 1991) dan manusia (Hurley, 1976; Zhou dkk., 2019), sedikit referensi untuk unta. Tujuan dari penelitian ini adalah untuk mengetahui konsentrasi unsur Fe, Zn, Cu, Mg, Se dan Mn pada induk, darah tali pusat dan cairan ketuban saat lahir. Selain itu, korelasi antara elemen serum tali pusat, cairan ketuban, dan darah induk juga ditentukan. Penelitian ini bertujuan untuk memberikan kontrinduksi untuk pemahaman yang lebih baik tentang mekanisme transportasi elemen jejak dari bendungan hamil ke janin, dan pengaruh elemen tertentu terhadap berat lahir.

BAHAN DAN METODE

Persetujuan etis

Semua pedoman kelembagaan dan nasional untuk perawatan dan penggunaan hewan diikuti sesuai dengan pedoman yang disetujui.Semua prosedur yang melibatkan perawatan dan penggunaan hewan telah disetujui oleh komite etik dari fakultas kedokteran hewan, komite perawatan dan penggunaan hewan kelembagaan, Universitas Aswan, Mesir.

Hewan dan area belajar

Penelitian ini dilakukan terhadap 30 ekor unta bunting (unta dromedari) selama September 2016 hingga Februari 2018 di sebuah peternakan swasta di desa Daraw, provinsi Aswan, Mesir. Unta bebas dari penyakit brucellosis dan tuberculosis (rata-rata umur: 7 sampai 10 tahun; berat: 450 sampai 550 kg), yang divaksinasi untuk penyakit Camel pox, Brucella dan Rift Valley Fever. Semua hewan dibesarkan di bawah sistem semi-intensif di mana mereka diberi makan dengan sedikit dan jerami alfalfa dengan penggembalaan yang sangat terbatas, dan dengan suplemen garam biasa, dan memiliki akses gratis ke air minum. Unta-unta itu ditempatkan di halaman terbuka. Unta yang bunting dipilih setelah berkonsultasi dengan unta yang mencatat riwayat kawinnya, dan kehamilannya dikonfirmasi dengan palpasi rektal.

Contoh

Sepuluh mililiter darah diambil dari setiap unta yang bunting segera setelah melahirkan melalui vena jugularis dan dari vena tali pusat bayi baru lahir saat plasenta masih belum terlepas. Vena tali pusat mengandung serum yang diperkaya dengan semua nutrisi yang diperlukan dari darah induk di dalam plasenta untuk memasok janin. Sampel darah dibiarkan menggumpal, dan disentrifugasi pada putaran 3000 per menit (rpm) selama 15 menit; serum dipisahkan dan disimpan pada -20 ° C untuk analisis lebih lanjut. Selama tahap pertama persalinan, kantung amino-korionik (transparan, amnion vaskular) muncul di dalam vulva dengan bagian janin terlihat di dalam kantung air, jarum suntik sepuluh mililiter yang dilengkapi dengan jarum ukuran dua puluh tidak biasa dikumpulkan. sampel cairan ketuban yang keruh, kekuningan dan berair dengan cara menembus amnion, berhati-hati untuk menghindari kontaminasi dari darah atau cairan allantois. Setelah pecahnya amnion, sampel darah tali pusat diambil dari vena tali pusat. Bobot lahir pedet diukur sebelum kolostrum diambil. Semua serum darah dan sampel cairan ketuban dianalisis

untuk Fe, Zn, Cu, Mg, Se dan Mn dengan menggunakan spektrofotometer serapan atom (Shimadzu, Model AA-6601, Jepang).

Analisis statistik

Semua data yang diperoleh dianalisis menggunakan Paket Statistik untuk Ilmu Sosial (SPSS) versi 25 (Armonk, NY: IBM Corp). Perbedaan unsur kandungan dalam serum induk, tali pusat vena dan cairan ketuban dianalisis secara statistik dengan Analysis of variance (ANOVA). Korelasi Pearson digunakan untuk menguji hubungan antara masing-masing elemen jejak dan berat lahir pedet. Demikian juga, korelasi tingkat elemen jejak antara induk, serum tali pusat vena dan cairan ketuban dianalisis. Nilai rata-rata pada baris yang sama dengan huruf yang berbeda signifikan secara statistik, dan nilai tertinggi ditunjukkan dengan huruf (a). Signifikansi statistik dinyatakan pada tingkat $p \leq 0,05$ dan data disajikan sebagai mean ± Standard Error (SE).

HASIL

Jejak konten elemen dalam serum induk, tali pusat vena dan cairan ketuban

Besi

Seperti yang ditunjukkan pada tabel 1, rata-rata kadar zat besi adalah 0,66 ± 0,12 bagian per juta (ppm) dalam serum induk, 0,37 ± 0,09 ppm dalam cairan ketuban dan 2,98 ± 0,66 ppm dalam serum tali pusat vena. Kadar besi serum tali pusat vena secara signifikan lebih tinggi dibandingkan serum induk (P <0,05) dan cairan ketuban; dan, kadar besi serum induk juga lebih tinggi secara signifikan dibandingkan dengan cairan ketuban (P <0,05).

Seng

Kadar seng dalam serum induk, tali pusat vena, dan cairan ketuban adalah 0,78 ± 0,10 ppm, 1,08 ± 0,29 ppm dan, 0,53 ± 0,09 ppm. Tali pusat vena memiliki konsentrasi seng yang lebih tinggi secara signifikan dibandingkan serum induk dan cairan ketuban (P <0,05). Selain itu, kadar seng juga lebih tinggi secara signifikan pada serum induk dibandingkan pada cairan ketuban (P <0,05).

Tembaga

Kadar tembaga rata-rata dalam serum induk dan tali pusat serta cairan ketuban adalah 0,75 ± 0,08 ppm, 0,91 ± 0,14 ppm dan 0,61 ± 0,09 ppm; masing-masing. Ada perbedaan yang signifikan antara kadar tembaga pada induk, tali pusat dan cairan ketuban (P <0,05).

Magnesium

Rata-rata kadar magnesium dalam serum tali pusat induk dan vena dan cairan ketuban adalah 7,21 ± 0,75 ppm, 8,42 ± 0,46 ppm dan 6,78 ± 0,44 ppm. Kadar magnesium rata-rata pada serum tali pusat induk dan vena secara signifikan lebih tinggi dibandingkan dengan cairan ketuban (P <0,05).

Selenium

Seperti yang ditunjukkan pada tabel 1, rata-rata kadar selenium adalah 1,06 ± 0,24 ppm, 0,79 ± 0,15ppm dan 0,66 ± 0,06 ppm dalam serum tali pusat vena, serum induk, dan cairan ketuban. Kandungan selenium dalam cairan ketuban secara signifikan lebih rendah dibandingkan dengan serum maternal dan vena (P <0,05); dan kadar selenium serum induk juga secara signifikan lebih rendah dibandingkan serum tali pusat vena (P <0,05).

Mangan

Rata-rata kadar mangan dalam serum induk, cairan ketuban dan serum tali pusat vena adalah 0,27 ± 0.15ppm, 0.18 ± 0.04 ppm dan 0.52 ± 0.13ppm (Tabel 1). Rata-rata kadar mangan dalam serum induk secara signifikan lebih rendah dibandingkan dengan serum tali pusat vena (P <0,05). Selain itu, kadar magnesium juga secara signifikan lebih tinggi pada serum induk dibandingkan pada cairan ketuban (P<0,05).

Korelasi antara serum induk dan kandungan elemen jejak cairan ketuban

Beberapa elemen di AF secara efektif dipengaruhi oleh serum induk (Tabel 2). Konsentrasi Fe, Cu, Mg, Se dan Mn di AF berkorelasi positif dengan serum induk (r: 0,09, 0,08, 0,01, 0,41 dan 0,12). Sebaliknya, terdapat korelasi negatif kandungan Zn antara AF dan serum induk (MS) dengan r: - 0,19.

Korelasi antara konten elemen jejak serum tali pusat induk dan vena

Konsentrasi serum induk dari Fe, Cu dan se berkorelasi negatif dengan serum tali pusat vena (VUCS) (r: - 0.04, - 0.11 dan - 0.07), tetapi terdapat korelasi positif antara kandungan Zn, Mg dan Mn antara MS dan VUCS dengan r: 0,01, 0,07 dan 0,08 masing-masing (Tabel 3).

Hubungan Kadar Jejak Serum Induk, Serum Tali Pusar Venus dan Cairan Ketuban dengan Berat Badan Lahir Pedet

Berat lahir rata-rata unta adalah 25,5 ± 0,08 kg. Korelasi Pearson menunjukkan korelasi positif yang signifikan (P <0,05) antara kadar Fe, Zn dan Se VUCS dan berat lahir pedet (Tabel 4). Kadar Fe di AF menunjukkan korelasi negatif

yang signifikan (tabel 5) dengan bobot lahir pedet (P <0,05). Tidak ada korelasi yang signifikan (P > 0,05) dapat ditunjukkan antara bobot lahir pedet dan tingkat elemen jejak dalam sampel se rum induk (Tabel).

Tabel 1. Kadar unsur dalam serum induk, serum tali pusat vena, dan cairan ketuban unta saat nifas

Parameter (ppm)	Serum induk (n = 30)	Cairan ketuban (n = 30)	Serum tali pusat (n = 30)
Fe	0,66 ± 0,12 b	0,37 ± 0,09 c	2.98 ± 0.66 a
Zn Cu Mg	0,78 ± 0,10 b	0,53 ± 0,09 c	1.08 ± 0.29 a
Se	0,75 ± 0,08 b	0,61 ± 0,09 c	0,91 ± 0,14 a
	7,21 ± 0,75 b	6.78 ± 0.44 c	8,42 ± 0,46 a
	0,79 ± 0,15 b	0,66 ± 0,06 c	1.06 ± 0.24 a
M N	0,27 ± 0,15 b	0,18 ± 0,04 c	0,52 ± 0,13 a

Data dinyatakan sebagai nilai rata-rata ± Standard error (SE); jumlah sampel yang dipelajari di setiap fluida ditampilkan dalam tanda kurung. a, b, c Mean ± SE pada baris yang sama dengan superskrip yang berbeda secara signifikan (P <0,05) berbeda. n: jumlah sampel yang diteliti.

Tabel 2. Korelasi antara konsentrasi elemen jejak dalam serum induk versus cairan ketuban unta saat nifas

Parameter (ppm)	Koefisien korelasi Pearson (r)	Korelasi signifikan pada p = 0,05
Fe	0,09	0.65
Zn	- 0.19	0.31
Cu	0,08	0.69
Mg	0,01	0.97
Se	0.41	0,03
M N	0.12	0,53

Tabel 3. Korelasi antara konsentrasi elemen jejak dalam serum induk versus serum tali pusat vena unta saat nifas

Parameter (ppm)	Koefisien korelasi Pearson (r)	Korelasi signifikan pada p = 0,05
Fe	- 0,04	0.84
Zn	0,01	0.94
Cu	- 0.11	0,55
Mg	0,07	0.70
Se	- 0,07	0.71
M N	0,08	0.60

Tabel 4. Hubungan antara konsentrasi trace elemen dalam serum induk dan berat lahir pedet

Elemen (ppm)	Koefisien korelasi Pearson (r)	Korelasi signifikan pada p = 0,05
Fe	- 0.23	0.23
Zn	0.11	0,57
Cu	- 0,09	0.76
Mg	- 0.15	0.43
Se	0.11	0,56
M N	- 0,02	0.92

Tabel 5. Hubungan konsentrasi trace element dalam cairan ketuban dengan berat lahir pedet

Parameter (ppm)	Koefisien korelasi Pearson (r)	Korelasi signifikan pada p = 0,05
Fe	- 0.43	0,02
Zn	0,07	0.70
Cu	0.12	0,58
Mg	0.19	0.32
Se	- 0,35	0.87
M N	0.15	0.44

Tabel 6. Hubungan konsentrasi trace elemen pada serum tali pusat vena dan berat lahir pedet

Parameter (ppm)	Koefisien korelasi Pearson (r)	Korelasi signifikan pada p = 0,05
Fe	0.37	0,04
Zn	0.37	0,04
Cu	- 0.24	0.21
Mg	- 0.11	0,56
Se	0.48	0,01
M N	0.24	0.20

DISKUSI

Untuk pengetahuan terbaik, studi saat ini adalah studi pertama yang diterapkan. Ini sering menjadi studi utama untuk mengkarakterisasi profil elemen jejak di MS, VUCS dan AF unta dromedaris hamil, dan untuk menyelidiki korelasi antara konsentrasi tingkat elemen di MS , VUCS dan AF, dan bobot lahir pedet. apakah korelasi antara level elemen di MS, VUCS dan AF ada atau tidak. Ada dua alasan utama untuk mempelajari hubungan fisiologis antara profil elemen jejak darah di bendungan dan berat badan bayi baru lahir. Pertama, bendungan yang melahirkan anak sapi yang berat memiliki risiko distosia yang lebih besar. Kedua, anak sapi yang terlalu lemah saat melahirkan mungkin memiliki lebih banyak masalah vitalitas. Untuk perkembangan intrauterin yang baik, Rossipal dkk., 2000). Dalam penelitian ini, fungsi plasenta sebagai penghalang janin-induk dalam transfer beberapa mineral dari bendungan ke janin selama masa gestasi telah diselidiki dengan baik. Dalam studi ini signifikansi terbukti konsentrasi Zn dan Mn yang lebih tinggi di VUCS dibandingkan di MS. Penelitian saat ini sesuai dengan penelitian sebelumnya yang membandingkan kadar unsur logam dalam darah induk dan tali pusat (Baig et al., 2003; Nandakumaran, 2016; Zhou et al., 201 9) pada manusia. Diduga bahwa Zn dan Mn dapat melintasi plasenta melalui transpor aktif, dan janin memberikan permintaan khusus untuk Zn dan Mn. Namun, klarifikasi lain untuk konsentrasi Mn yang lebih tinggi dalam darah tali pusat juga disarankan, seperti pembuangan Mn yang lebih rendah atau terbatas oleh janin atau ketidakmampuan janin untuk menggunakan Mn (Widdowson et al., 1974).

Permintaan elemen jejak meningkat dengan cepat selama kehamilan, dan ini dapat menyebabkan penurunan simpanan induk dan / atau janin (Al-Saleh dkk., 2004). Dalam penelitian ini, konsentrasi Fe, Cu, Se dan Mg serum tali pusat vena secara signifikan lebih tinggi dibandingkan dengan serum dam yang sesuai.

Zat besi adalah elemen terpenting dalam darah yang berkontrinduksi pada komposisi hemoglobin, dan juga penting untuk keberhasilan perkembangan janin. Dalam penelitian ini, kadar Fe di VUCS lebih tinggi dibandingkan di MS. Temuan ini sesuai dengan yang dilaporkan sebelumnya pada manusia (Rallis dan Papasteriadis, 1987) dan dalam ovine (Gooneratne dan Christensen, 1989), dan menunjukkan pengangkutan aktif elemen ini melintasi plasenta unta. Tren perubahan selama gestasi pada unta tampak terlalu sesuai dengan pengamatan pada sapi bunting, di mana penurunan transfer plasenta Fe tampak terjadi pada sepertiga terakhir kehamilan (Richards, 1999).

Ada peningkatan konstan dalam deposisi Cu sepanjang periode janin dan, oleh karena itu, peningkatan permintaan Cu oleh janin (Eltohamy dkk.,1986). Pada penelitian ini, kadar Cu pada VUCS lebih tinggi dibandingkan pada MS, sedangkan pada penelitian lain menunjukkan hasil yang berlawanan (Zhou dkk., 2019 Interaksi ini menyiratkan bahwa janin memiliki kapasitas untuk menyerap Cu induk, bahkan ketika bendungannya kekurangan Cu (Graham et al., 1994). Berdasarkan Seboussi dkk. (2010) dan Eltohamy dkk. (1986), Konsentrasi Cu dalam serum menurun pada akhir kehamilan karena transfer aktif dari penyimpanan hepar bendungan ke janinnya. Korelasi yang signifikan antara Se dan Cu diamati pada unta yang menerima suplementasi selenium (Koller et al., 1984).

Dalam penelitian ini, tingkat Se di VUCS lebih tinggi daripada di MS. Ini mungkin karena tingkat selenium yang dapat dengan mudah melintasi plasenta unta seperti yang diamati pada sapi (Wooten dkk., 1996) dan manusia (Baig et al., 2003). Seperti yang ditunjukkan dalam penelitian ini, kadar Mg dalam VUCS secara signifikan lebih tinggi daripada di MS, yang sesuai dengan penelitian terbaru pada manusia (Nandakumaran dkk., 2016). Hasil penelitian menunjukkan bahwa konsentrasi elemen jejak di AF ternyata lebih rendah daripada MS. Data ini menunjukkan bahwa elemen jejak dalam AF mungkin berasal dari darah. Peran elemen jejak yang terkandung dalam AF tidak jelas. Karena AF ditelan oleh janin, disarankan bahwa cairan ini mungkin menjadi sumber penting elemen jejak tertentu untuk nutrisi janin (Wooten dkk., 1996).

Dalam penelitian ini, kami tidak mengamati korelasi antara konsentrasi berbagai elemen yang diteliti dalam serum induk dan berat lahir anak sapi. Kadar serum induk dari berbagai elemen yang diteliti tidak berkorelasi positif atau negatif dengan berat lahir pedet. Bobot lahir pedet merupakan salah satu masalah dasar untuk menilai pengelolaan unta yang bunting, dan untuk mengharapkan kemungkinan ketebalan atau kematian baru. Menurut berbagai penelitian sebelumnya (Barhat dkk., 1979; Al Mutairi, 2000; Bissa, 2002; Nagy dan Juhász, 2019), berat lahir anak unta bervariasi antara 19 sampai 52 kg. Bobot lahir pedet dalam penelitian ini berkisar antara 18 dan 45 kg, dan tidak adanya korelasi level elemen jejak dengan bobot pedet menyebabkan asumsi bahwa level elemen ini dalam MS bukanlah nilai yang berguna untuk penilaian berat janin. Namun, kadar Fe dalam AF ditemukan berkorelasi negatif dengan berat lahir pedet, sedangkan kadar unsur lain tidak menunjukkan korelasi yang signifikan. Menariknya, kadar Fe, Zn dan Se di VUCS ditemukan berkorelasi positif dengan berat lahir pedet. Di antara faktor variasi, breed, pa rity dan bobot induk bunting, induk, serta tahun dan bulan lahir dilaporkan berpengaruh nyata terhadap bobot anak pedet (Barhat dkk., 1979; Al Mutairi, 2000; Bissa, 2002; Nagy dan Juhász, 2019). Untuk pengetahuan terbaik, tidak ada penelitian yang menunjukkan hubungan antara konsentrasi elemen jejak dan berat lahir anak unta. Pada sapi, Graham dkk. (1994) melaporkan bahwa ukuran janin meningkat seiring dengan peningkatan Cu janin, dan kurang dari atau sama dengan Cu induk. Gooneratne dan Christensen (1989) menunjukkan bahwa baik Mn induk maupun janin tidak berkorelasi dengan ukuran janin. Jelas, penelitian lebih lanjut diperlukan untuk memeriksa efek elemen jejak ini pada berat lahir pedet.

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Beberapa elemen di AF dan VUCS secara efektif dipengaruhi oleh elemen di MS (Tabel 2 dan 3). Untuk Hasil VUCS menunjukkan korelasi positif antara konsentrasi Zn, Mg dan Mn di VUCS dan di MS. Sebaliknya, terdapat korelasi negatif kandungan Fe, Cu dan Se antara VUCS dan MS. Di sisi lain, konsentrasi Fe, Cu, Mg, Se dan Mn di AF berkorelasi positif dengan MS, sedangkan, korelasi negatif kandungan Zn antara AF dan MS dilaporkan. Tidak ada korelasi yang signifikan antara semua mineral di MS dan VUCS atau AF. Sayangnya, tidak banyak penelitian sebelumnya yang melaporkan tentang aspek ini pada unta. Mitchell dkk. (1998) melaporkan korelasi positif antara induk dan janin Cu, induk dan janin Mn, dan induk dan janin Zn. Sebaliknya, ada kurangnya korelasi antara dua belas induk dan Fe janin pada sapi. Korelasi positif antara elemen jejak induk dan janin menunjukkan ketergantungan janin pada bendungan untuk suplai nutrisinya, termasuk elemen jejak. Kurangnya korelasi menunjukkan kemandirian janin. Mekanisme yang mengatur interaksi antar nutrien masih kurang dijelaskan Mekanisme yang mengatur retensi, ekskresi atau interaksi antara nutrien di lokasi pengangkutan sel atau penyimpanan protein memerlukan penyelidikan lebih lanjut, tetapi penelitian seperti yang disajikan di sini dapat mengarahkan penelitian di masa mendatang terhadap deskripsi biokimia dari interaksi nutrien.

KESIMPULAN

Hasil saat ini menunjukkan pengangkutan plasenta yang hidup dari Fe, Zn, Mg, Mn, Cu dan Se tampaknya dipertukarkan secara aktif antara bendungan dan janin. Fe, Zn, Cu, Mg, Se dan Mn dipertukarkan secara pasif antara bendungan dan cairan ketuban. Evaluasi Fe, Zn, Cu, Mg, Se dan Mn dalam serum induk tampaknya tidak berguna dalam penilaian pertumbuhan janin.

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REFERENSI

Abdelrahman M and Kincaid R (1993). Deposition of copper, manganese, zinc, and selenium in bovine fetal tissue at different stages of gestation. Journal of dairy science. 76(11): 3588-3593. DOI: https://doi.org/10.3168/jds.S00220302(93)77698-5.

Al-Saleh E, Nandakumaran M, Al-Shammari M, Al-Falah F and Al-Harouny A (2004). Assessment of maternal-fetal status of some essential trace elements in pregnant women in late gestation: relationship with birth weight and placental weight. Journal of Maternal-Fetal and Neonatal Medicine, 16(1): 9-14. DOI: https://doi.org/10.1080/14767050412331283157.

Al Mutairi SE (2000). Evaluation of Saudi camel calves' performance under an improved management system. Review of breeding and veterinary medicine in tropical countries (Revue d'élevage et de médecine vétérinaire des pays tropicaux), 53(2): 219-222. DOI:http://dx.doi.org/10.19182/remvt.9757.

Baig S, Hasnain N and Ud-Din Q (2003). Studies on Zn, Cu, Mg, Ca and phosphorus in maternal and cord blood. Journal of Pakistan Medical Association, 53(9): Sep 2003. Available at: https://www.jpma.org.pk/article-details/263.

Barhat N, Chowdhary M and Gupta A (1979). Note on relationship among gestation length, birth weight, placental weight and intrauterine development index in Bikaneri camel. Indian journal of animal research, 13(2):115-117. Available at: https://arccjournals.com.

Bissa UK (2002). Selectivity, Longevity and Productivity in Indian Camels (Camelus dromedarius), Rajasthan University of Veterinary and Animal Sciences, Bikaner, Available at: http://krishikosh.egranth.ac.in/handle/1/5810136975.

Black RE (2001). Micronutrients in pregnancy. British Journal of Nutrition, 85(2):193-197. DOI: https://doi.org/10.1079/bjn2000314.

Chuka Ozegbe P (2005). Comparative biochemical assessment of the amniotic fluid and maternal plasma of pregnant rabbits, Veterinary Archive, 75(5): 431-437. Available at: https://hrcak.srce.hr/31916.

Eltohamy M, Salama A and Yousef A (1986). Blood constituents in relation to the reproductive state in she-camel (Camelus dromedarius). Contributions to Tropical Agriculture and Veterinary Medicine, 24(4): 425-430. Available at: https://europepmc.org/article/med/3566702.

Faye B and Bengoumi M (1994) Trace-elements status in camels. Biological trace element research, 41(1-2): 1-11. DOI:https://doi.org/ 10.1007/bf02917213.

Faye B, Saint-Martin G, Cherrier R and Ruffa A (1992). The influence of high dietary protein, energy and mineral intake on deficient young camel (*Camelus dromedarius*)—II. Changes in mineral status. Comparative Biochemistry and Physiology Part A: Physiology, 102(2): 417-424. DOI: https://doi.org/10.1016/0300-9629(92)90155-j.

Freetly H, Ferrell C and Jenkins T (2000). Timing of realimentation of mature cows that were feed-restricted during pregnancy influences calf birth weights and growth rates. Journal of Animal Science, 78(11): 2790-2796. DOI: https://doi.org/10.2527/2000.78112790x

Gooneratne S and Christensen D (1989). A survey of maternal and fetal tissue zinc, iron, manganese and selenium concentrations in bovine. Canadian Journal of Animal Science, 69(1): 151-159. DOI: https://doi.org/10.4141/cjas89-018.

Graham TW, Thurmond MC, Mohr FC, Holmberg CA, Anderson ML and Keen CL (1994). Relationships between maternal and fetal liver copper, iron, manganese, and zinc concentrations and fetal development in California Holstein dairy cows. Journal of Veterinary Diagnostic Investigation, 6(1): 77-87. DOI: https://doi.org/10.1177/104063879400600114.

Hurley LS (1976). Perinatal effects of trace element deficiencies. Trace elements in human health and disease, 2: 301-314. Available at: https://www.elsevier.com/en-xm.

Kriesten K, Schmidtmann Wand Murawski U (1986). Iron and copper concentrations in the maternal and fetal serum, placenta and amniotic fluid during the reproductive stadium as well as in the milk of rabbits. Comparative biochemistry and physiology. Comparative physiology, 83(2): 291-296. DOI: https://doi.org/ 10.1016/0300-9629(86)90576-1.

Langlands J, Bowles J, Donald D, Smith AJ, Paull DR and Davies HI (1982). Deposition of copper, manganese, selenium and zinc in the ovine fetus and associated tissues. Australian journal of agricultural research, 33(3): 591-605. DOI: https://doi.org/10.1071/AR9820591.

McArdle H and Erlich R (1991). Copper uptake and transfer to the mouse fetus during pregnancy. The Journal of nutrition, 121(2):208-214. DOI: https://doi.org/10.1093/jn/121.2.208.

Mills C and Davies N (1979). perinatal changes in the absorption of trace elements. Development of mammalian absorptive processes. Ciba Foundafion Series, 70: 247-266. DOI: https://doi.org/10.1002/9780470720530.ch14.

Mitchell J, Nicol F and Beckett G (1998). Selenoprotein expression and brain development in preweanling selenium-and iodine-deficient rats. Journal of Molecular Endocrinology, 20(2): 203-210. DOI: https://doi.org/10.1677/jme.0.0200203.

Nagy P and Juhász J (2019). Pregnancy and parturition in dromedary camels I. Factors affecting gestation length, calf birth weight and timing of delivery. Theriogenology, 134: 24-33. DOI: https://doi.org/10.1016/j.theriogenology.2019.05.017.

Nandakumaran M, Al-Sannan B, Al-Sarraf H and Al-Shammari M (2016). Maternal–fetal transport kinetics of manganese in perfused human placental lobule in vitro. The Journal of Maternal-Fetal and Neonatal Medicine, 29(2): 274-278. Available at: https://www.tandfonline.com/doi/full/10.3109/14767058.2014.998193.

Perveen S, Altaf W, Vohra N, Bautista ML, Harper RG and Wapnir RA (2002). Effect of gestational age on cord blood plasma copper, zinc, magnesium and albumin. Early human development, 69(1-2): 15-23. DO: https://doi.org/10.1016/S0378-3782(02)00024-5

Rallis T and Papasteriadis A (1987). A study on iron and copper concentrations of foetal liver in sheep. Journal of Veterinary Medicine Series A, 34(1-10): 582-584. DOI: https://doi.org/10.1111/j.1439-0442.1987.tb00318.x.

Richards MP (1999). Zinc, copper, and iron metabolism during porcine fetal development. Biological trace element research. 69(1):27-44. DOI. https://doi.org/10.1007/BF02783913.

Romeu A, Alemany M and Arola L (1986). Net transfer of essential metals from mother to fetus in the second half of pregnancy in the rat. Neonatology, 49(4): 204-210. DOI. https://doi.org/10.1159/000242532.

Rossipal E, Krachler M, Li F and Micetic-Turk D (2000) Investigation of the transport of trace elements across barriers in humans:studies of placental and mammary transfer. Acta Paediatrica, 89(10): 1190-1195. DOI: https://doi.org/10.1080/080352500750027556.

Seboussi R, Faye B, Alhadrami G, Askar M, Ibrahim W, Mahjoub B, Hassan K, Moustafa T and Elkhouly A (2010). Selenium distribution in camel blood and organs after different level of dietary selenium supplementation. Biological trace element research, 133(1): 34-50. DOI. https://doi.org/10.1007/s12011-009-8410-1.

Suliburska J, Kocyłowski R, Komorowicz I, Grzesiak M, Bogdański P and Barałkiewicz D (2016). Concentrations of mineral in amniotic fluid and their relations to selected maternal and fetal parameters. Biological trace element research, 172(1): 37-45. DOI: https://doi.org/10.1007/s12011-015-0557-3.

Tibary A and Anouassi A (1997) Theriogenology in Camelidae: Anatomy, Physiology, Pathology and Artificial Breeding. Available at: https://openlibrary.org/books/OL17569021M.

Van Wouwe JP, Hoogenkamp S and Van den Hamer CJ (1991). Zinc and copper of fetal organs during the second trimester of pregnancy. Biological trace element research, 29(3): 175. DOI: https://doi.org/10.1007/BF03032675.

Wapnir R, Gyasi I, Harper R, Moyse J and Teichberg S (1996). Placental copper transport in the rat. II: Effect of maternal copper deficiency. Placenta, 17(7): 479-486. DOI: https://doi.org/10.1016/S0143-4004(96)90030-2.

Widdowson EM, Dauncey J and Shaw J (1974). Trace elements in fetal and early postnatal development. Proceedings of the Nutrition Society, 33(3): 275-284. DOI: https://doi.org/10.1079/PNS19740050.

Wooten L, Shulze RA, Lancey RW, Lietzow M and Linder MC (1996). Ceruloplasmin is found in milk and amniotic fluid and may have a nutritional role. The Journal of Nutritional Biochemistry, 7(11): 632-639. DOI: https://doi.org/10.1016/S0955-2863(96)00127-1.

Zachara BA, Wąsowicz W, Gromadzińska J, Skłodowska M and Krasomski G (1986). Glutathione peroxidase activity, selenium, and lipid peroxide concentrations in blood from a healthy polish population. Biological trace element research, 10(3): 175-187. DOI:https://doi.org/10.1007/BF02795616.

Zhou C, Zhang R, Cai X, Xiao R and Yu H (2019). Trace elements profiles of maternal blood, umbilical cord blood, and placenta in Beijing, China. The Journal of Maternal-Fetal and Neonatal Medicine, 32(11): 1755-1761. Available at: https://www.tandfonline.com/doi/abs/10.1080/14767058.2017.1416602.

Zong-Ping L, Zhuo M and You-Jia Z (1994). Studies on the relationship between sway disease of Bactrian camels and copper status in Gansu province. Veterinary research communications, 18(4): 251-260. DOI:https://doi.org/10.1007/BF01839191.

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Pengaruh Suplementasi Kurkumin terhadap Viabilitas dan Kapasitas Antioksidan Sel Granulosa Kerbau di bawah Kondisi Kultur *In Vitro*

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ABSTRAK

Penelitian saat ini dilakukan untuk menyelidiki kemungkinan efek perlindungan dari suplementasi kurkumin pada sel granulosa kerbau (GCs) di bawah in vitro kondisi kultur. Ovarium kerbau dikumpulkan dari rumah potong hewan lokal dalam larutan garam fisiologis dan diangkut langsung ke laboratorium. Cairan folikel yang mengandung GC dan kompleks kumulusoosit disedot dari folikel antral dengan diameter 2-8 mm. GC yang terkumpul diunggulkan (Sekitar 375.000 sel yang layak) di piring kultur 8-sumur yang berisi media kultur jaringan- 199 (TCM-199) dan disimpan pada 37 ° C dalam suasana lembab dengan CO 5% 2. Kurkumin ditambahkan ke media TCM pada level 1, 2.5, 5 dan 10 µM selama 24 dan 48 jam. Pada 37 ° C atau disimpan tanpa perlakuan sebagai kelompok kontrol. Kelangsungan hidup sel itu ditentukan menggunakan tes biru tripan. Tingkat spesies oksigen reaktif intraseluler (ROS) dinilai dengan mengukur intensitas fluoresen 6-carboxy-2 ′, 7′-dichlorodihydro fluorescein diacetate (H 2 DCFDA). Selain itu, aktivitas mitokondria GC ditentukan. Hasil penelitian ini menunjukkan bahwa GCs dapat bertahan dalam kondisi kultur menurun secara signifikan pada kelompok yang diobati dengan kurkumin 1, 2.5, 5 dan 10 µM (86.0%, 86,26%, 83,0% dan 74,0%, masing-masing) dibandingkan dengan kelompok kontrol (93,60%). Dua kelompok sel granulosa yang dibiakkan dengan kurkumin 2,5 dan 5 µM mencatat tingkat aktivitas mitokondria yang lebih tinggi daripada kelompok yang dikultur dengan 1 µM dan 10 µM kurkumin. Apalagi ada a penting peningkatan kadar ROS pada kelompok yang dibudidayakan dengan 10 µM kurkumin, dibandingkan dengan kelompok kontrol dan kelompok eksperimen lainnya. Aktivitas enzim katalase (CAT), superoksida dismutase (SOD), glutathione (GSH) dan 1, 1-difenil-2-pikrilhidrazil (DPPH) meningkat setelah perawatan. in vitro sel granulosa berkultur dengan 5 µM kurkumin. Namun, aktivitas enzimatik CAT, SOD, GSH dan DPPH menurun secara signifikan 48 jam setelah pengobatan kurkumin. Kesimpulannya, suplementasi kurkumin pada konsentrasi rendah (2,5 µM) selama 24 jam sampai in vitro GC yang dibudidayakan meningkatkan aktivitas metabolik intraseluler dan sistem perlindungan antioksidan, padahal tidak bisa pertahankan tindakan ini selama 48 jam. Selain itu, suplementasi kurkumin pada konsentrasi tinggi dan untuk durasi yang lama dapat berdampak negatif pada viabilitas GC di bawah in vitro kondisi kultur melalui induksi stres oksidatif.

Kata kunci: Antioksidan, Kerbau, sel Granulosa, Stres oksidatif, Viabilitas.

PENGANTAR

Stres oksidatif, dimediasi oleh radikal bebas yang diturunkan dari oksigen (juga dikenal sebagai spesies oksigen reaktif, ROS) adalah keadaan yang sering mempengaruhi hampir semua organisme hidup karena kondisi lingkungan yang kurang optimal. Dalam situasi homeostatis, terdapat stabilitas antara produksi ROS dan daya pemulungan sel melalui sistem antioksidan seluler (Panieri dkk., 2016). Namun demikian, ketika produksi ROS mengatasi kemampuan antioksidan seluler, hal itu mungkin berkontribusi pada masalah yang disebut stres oksidatif (Agarwal et al., 2005). Tingkat ROS dapat meningkat secara endogen selama banyak prosedur fisiologis dan reproduksi termasuk ovulasi (Agarwal dkk., 2005; Gupta dkk., 2010). Selain itu, penggunaan oksigen sebagai substrat pernapasan dilaporkan menghasilkan stres oksidatif selama proses metabolisme aerobik dan produksi energi (Frisard dan Ravussin, 2006). Selain itu, sumber endogen lainnya (mitokondria, aktivasi sel inflamasi, membran plasma nicotinamide adenine dinucleotide phosphate, oksidase, lisosom, dan peroksisom) dapat mempengaruhi produksi ROS dalam sel mamalia (Klaunig dkk., 2009). Insiden stres oksidatif yang dimediasi oleh ROS ternyata memiliki efek negatif pada sistem reproduksi wanita dan akhirnya menyebabkan infertilitas (Agarwal et al., 2012). Sel granulosa ovarium (GC), penyusun seluler utama dalam folikel, memiliki dua fungsi vital dalam reproduksi wanita: produksi steroid dan

mempertahankan oosit selama ovulasi (Yada et al., 1999; Sohel dkk., 2013; Cinar dan Sohel, 2015). Pada akhir pertumbuhan folikel, GC di folikel dominan dibedakan menjadi sel luteal oleh lonjakan hormon luteinizing ovulatori (LH) (Duffy dan Stouffer, 2003). Cara ini sangat penting untuk keberhasilan ovulasi dan pembentukan korpus luteum untuk menjaga kehamilan. Di sisi lain, selama ovulasi setelah peningkatan pra-ovulasi LH, sel-sel inflamasi terutama neutrofil dan makrofag banyak digunakan untuk memproduksi ROS untuk memfasilitasi ruptur folikel dan pelepasan oosit (Shkolnik dkk., 2011), menunjukkan paparan GC terhadap semacam stres oksidatif selama ovulasi. Selain sumber endogen, sumber lingkungan dari ROS dapat membuat situasi menjadi lebih kompleks.

Salah satu tanaman yang efektif digunakan dalam pengobatan tradisional adalah temulawak longa Linn (Hatcher et al., 2008). Dalam ramuan ini, komponen kurkumin memiliki proporsi tertinggi (Aggarwal et al., 2007). Kurkumin adalah senyawa polifenol kuning yang ditemukan dalam kunyit (Esatbeyoglu dkk., 2012), dan struktur kimianya adalah 1, 7-bis (4-hidroksi-3- metoksifenil) -1, 6eptadiena-3, 5-dione (Nadkarni, 2007; Kádasi dkk., 2012). Ini telah terbukti menjadi anti-karsinogenik, antivirus, antioksidan yang sangat efektif (Steward dkk., 2008; Correa dkk., 2013; Tapia dkk., 2013), dan zat anti-inflamasi pada model manusia dan hewan (Epstein dkk., 2010; Sung dkk., 2012).

Kurkumin bertindak sebagai antioksidan karena mengais oksigen reaktif dan spesies nitrogen (Barzegar dan MoosaviMovahedi, 2011; Trujillo dkk., 2013; Mohebbati dkk. 201 7) dan menginduksi enzim sitoprotektif seperti glutathioneStransferase (GST), •- glutamyl cysteine ligase (•- GCL) dan heme oxygenase-1 (HO-1) (Dinkova - Kostova dkk., 2008; Reyes-Fermín dkk., 2012). Ia mampu mengais hidrogen peroksida, radikal peroksil, anion superoksida, radikal hidroksil, oksigen singlet, oksida nitrat, dan anion peroksinitrit (Trujillo dkk., 2013). Telah terungkap bahwa kurkumin menyebabkan sistem pertahanan antioksidan endogen dengan memodulasi faktor transkripsi seperti faktor nuklir (erythroidderived 2) -like 2 (Nrf2) (Tapia dkk., 2012; Liu et al. 2016; Zhang dkk., 2019a; Zhang dkk., 2019b; Zhu dkk., 2020), aktivator protein-1 (AP-1), dan faktor inti kappa B (NF • B) (Pinkus et al., 1996). Oleh karena itu, tujuan dari penelitian ini adalah untuk mengetahui efek suplementasi kurkumin terhadap in vitro media kultur GCs kerbau tentang viabilitas dan sistem pertahanan enzimatisnya.

BAHAN DAN METODE

Kelompok eksperimental

Kultur utama GC digunakan sebagai teknik dasar untuk mempelajari efek suplementasi kurkumin pada kultur GCs kerbau. in vitro dalam media TCM-199. Kultur utama GC ditanam dalam enam kelompok. Kelompok dibagi sebagai berikut: kelompok 1: tidak diobati (kontrol), kelompok 2: kontrol tanpa perlakuan dan menambahkan dimetil sulfoksida (DMSO) (itu adalah larutan pelarut kurkumin), kelompok 3: hanya dengan 1 µM kurkumin, kelompok 4 : hanya diobati dengan kurkumin 2,5 µM, kelompok 5: hanya diobati dengan kurkumin 5 µM dan kelompok 6: hanya diobati dengan kurkumin 10 µM. Sel-sel yang dipulihkan ditumbuhkan sampai mencapai 40-50% pertemuan sebelum dialokasikan ke dalam kelompok perlakuan yang berbeda. Minimal 10 ovarium digunakan dalam setiap replikasi biologis. Tiga ulangan biologis GC digunakan untuk setiap uji eksperimental yang dilakukan dalam penelitian ini.

Kumpulan ovarium dan sel granulosa

Sel granulosa dikumpulkan dan dikultur sesuai dengan prosedur yang dijelaskan oleh Sohel dkk. (2017). Sebanyak 120 ovarium kerbau diperoleh dari rumah pemotongan hewan lokal, dan diangkut dalam larutan garam 0,9% pada suhu 37 ° C dalam waktu 2 jam setelah pengambilan. Minimal 10 ovarium digunakan di setiap ulangan untuk mendapatkan jumlah GC yang cukup untuk pengujian yang berbeda. Ovari dicuci dua kali dengan larutan garam 0,9% dan kemudian dicuci sekali dengan etanol 70%. Isi folikel (cairan folikel yang mengandung GC dan kompleks kumulus-oosit) disedot dari folikel antral berdiameter 2-8 mm dengan jarum ukuran 18 yang dipasang pada semprit 5 mL dan ditempatkan dalam tabung elang steril 50 mL yang berisi 10-mL TCM-199 medium (Sigma-Aldrich, M5017, Steinheim, Jerman). Setelah pengumpulan, tabung dibiarkan selama 15 menit pada suhu 37 ° C untuk memungkinkan kompleks oosit-kumulus dan puing-puing seluler mengendap di bagian bawah tabung. Cairan bagian atas yang mengandung GC kemudian dikumpulkan dalam tabung elang 15-mL, dan disentrifugasi pada 1800 rpm selama 5 menit untuk mendapatkan GC. GC yang terkumpul dicuci dengan 5 ml larutan garam dapar fosfat (PBS) yang bebas dari kalsium magnesium dengan pipet berulang diikuti dengan sentrifugasi pada 1500 rpm selama 10 menit. Akhirnya, 3 ml tripsin ditambahkan dan tabung diinkubasi pada suhu 37 ° C selama 3 menit, kemudian 5 ml TCM-199 ditambahkan untuk menonaktifkan tripsin dengan pipet berulang GC diikuti dengan sentrifugasi pada 1500 rpm selama 10 menit.

In vitro kultur dan pengobatan sel granulosa

Sekitar 375.000 sel yang layak disemai di piring kultur 8-sumur (Corning Incorporated, Kennebunk, ME, USA), in vitro biakan dalam medium yang mengandung medium TCM-199 (Sigma-Aldrich, D6046, Steinheim, Germany) dilengkapi dengan 10% serum janin sapi (vol / vol), penisilin (100 U / mL) dan streptomisin (100 µg / mL) (Sigma

Aldrich, P4333, Steinheim, Jerman) dan disimpan pada suhu 37 ° C dalam atmosfer yang dilembabkan dengan CO 5% 2 hingga mencapai pertemuan 40–50%. Kurkumin ditambahkan ke media TCM-199 pada level berikut (Kontrol, DMSO, 1 µM, 2.5 µM, 5 µM dan 10 µM) selama 48 jam dengan mengganti media satu kali setelah 24 jam.

Morfologi dan viabilitas sel

Setelah pengobatan, GC dari kelompok perlakuan yang berbeda diamati menggunakan mikroskop terbalik untuk mengetahui pertemuan dan perubahan morfologi. Viabilitas sel ditentukan dengan menggunakan uji eksklusi tripan biru seperti yang dijelaskan oleh
Strober (2015) dengan beberapa modifikasi. Secara singkat, setelah perawatan, baik sel yang melekat maupun yang mengambang dari masing-masing kelompok perlakuan dikumpulkan dan disuspensi kembali dalam satu mL in vitro media kultur. Setelah itu, 100 µL suspensi sel dan 100 µL dari 0,4% trypan blue dicampur ke dalam tabung sentrifus mikro dan diinkubasi selama 1–2 menit pada suhu kamar. Sepuluh mikroliter campuran sel / tripan biru diaplikasikan ke hemositometer dan ditempatkan di bawah mikroskop (Mikroskop Terbalik, Leica DMI 3000B, Wentzler, Jerman) pada perbesaran 20X untuk menghitung sel hidup dan mati. Viabilitas GC dihitung sebagai persentase sel yang layak dari jumlah sel total.

Uji sitotoksisitas

Dalam penelitian ini, uji serapan merah netral memberikan perkiraan kuantitatif tentang berapa banyak sel yang layak dalam kultur. Ini adalah salah satu tes sitotoksisitas yang paling banyak diterapkan dengan banyak aplikasi biomedis dan lingkungan. Ini didasarkan pada kemampuan sel yang layak untuk menggabungkan dan mengikat pewarna merah netral supravital di lisosom. Sel-sel granulosa diunggulkan di 96-piring kultur jaringan dan dirawat selama periode yang sesuai. Plat diinkubasi selama 2 jam dengan media yang mengandung warna merah netral. Sel selanjutnya dibilas dengan media, pewarna dihilangkan dengan baik dan dibaca absorbansi menggunakan spektrofotometer. Setelah sel dirawat, pengujian dapat diselesaikan dalam <3 jam (Repetto dkk., 2008).

Intraseluler spesies oksigen reaktif deteksi

Akumulasi ROS intraseluler dalam perlakuan yang berbeda dan kelompok kontrol dinilai dengan 6-carboxy-2 ′, 7′- dichlorodihydro fluorescein diacetate (H. 2 DCFDA, Sigma-Aldrich, USA) menurut protokol yang dijelaskan oleh Sohel et Al. (2017). GC dari masing-masing kelompok diinkubasi dengan 400 µL 15 µMH 2 DCFDA selama 20 menit dalam gelap pada 37 ° C. Sel kemudian dicuci dua kali dengan PBS dan gambar segera ditangkap dengan mikroskop Nikon Eclipse Ti-S (Nikon Instruments Inc., Tokyo, Jepang) menggunakan filter fluoresensi hijau pada eksitasi / emisi: ~ 492–495 / 517–527 nm dan gambar diperoleh dengan perangkat lunak NIS Elements. Untuk analisis kuantitatif, intensitas fluoresensi rata-rata dari lima bidang yang tidak tumpang tindih di setiap sumur diukur menggunakan perangkat lunak Image J (Rueden dkk., 2017). Data disajikan sebagai mean ± standar deviasi.

Aktivitas mitokondria

Aktivitas mitokondria GC kerbau ditentukan menggunakan MitoTracker Red CMXRos (M7512, Invitrogen, Karlsruhe, Jerman) sesuai dengan protokol yang diterbitkan sebelumnya (Prastowo et al., 2017) dengan sedikit modifikasi. GC dari masing-masing kelompok diinkubasi dengan 15 µL pewarna merah MitoTracker 200 nM selama 45 menit, dilanjutkan dengan dua kali pencucian dengan PBS dan kemudian difiksasi semalaman pada suhu 4 ° C dengan 4% formaldehida. Aktivitas mitokondria GC sampel divisualisasikan di bawah mikroskop confocal pemindaian laser (LSM 710; Carl Zeiss, Jerman) menggunakan laser eksitasi spesifik pada 579-599 nm. Tingkat penguatan laser yang konstan (keuntungan master = 700), lubang jarum (1 µm) dan ukuran piksel (1024 × 1024) diterapkan selama tujuan akuisisi gambar untuk memungkinkan perbandingan sinyal fluoresensi gambar. Gambar yang dihasilkan kemudian diolah menggunakan software ZEN 2011 (Carl Zeiss, Jerman). Untuk analisis kuantitatif, intensitas fluoresensi rata-rata dari lima bidang yang tidak tumpang tindih di setiap sumur diukur menggunakan perangkat lunak Image J. Data disajikan sebagai mean ± SD.

Aktivitas enzim

1, 1-Difenil-2-pikrilhidrazil (DPPH •) uji pemulungan radikal

Kemampuan ekstrak yang berbeda untuk bertindak sebagai donor hidrogen diukur dengan aktivitas pemulung radikal DPPH. Pengujian dilakukan sesuai dengan metode Blois (1958). DPPH, radikal bebas yang stabil, mengandung elektron ganjil, yang bertanggung jawab atas absorbansi pada 515-517 nm dan menghasilkan warna ungu tua yang terlihat. Ketika DPPH menerima elektron dari senyawa antioksidan, ia direduksi menjadi 1,1-difenil-2-pikrilhidrazin (dekolorisasi non-radikal, DPPH2).

Penentuan aktivitas superoksida dismutase (SOD)

Aktivitas SOD diuji di jaringan hati dengan metode Marklund dan Marklund, (1974) pada 420 nm selama 1 menit pada spektrofotometer Shimadzu UV-2450 (Shimadzu, Kyoto, Jepang). Aktivitas dinyatakan sebagai jumlah enzim yang menghambat autoksidasi pyrogallol sebesar 50%, yang setara dengan 1 U / mg protein.

Penentuan aktivitas katalase (CAT)

Aktivitas CAT diukur dengan metode spektrofotometri berdasarkan dekomposisi H. 2 HAI 2 seperti yang dijelaskan oleh Aebi (1984).

Penentuan profil GSSG dan GSH oleh HPLC

Senyawa tiol dari glutathione teroksidasi dan tereduksi dideteksi dengan HPLC menggunakan metode Jayatilleke dan Shaw (1993). Standar referensi glutathione (teroksidasi dan tereduksi) yang dibeli dari Sigma-Aldrich Chemical Co (G4376, USA). Bubuk glutathione dilarutkan dalam 75% metanol dalam stok 1 mg / ml dan diencerkan sebelum diaplikasikan ke HPLC. Sistem HPLC Agilent (Santa Clara, USA) terdiri dari pompa kuartener, oven kolom, Injektor reodine dan loop 20µl, detektor panjang gelombang variabel UV. Laporan dan kromatogram diambil dari program Chemstation yang dibeli dari Agilent. Synerji RP Max kolom 3.9 pada panjang gelombang 210 nm dengan debit aliran 2ml / menit digunakan. Pot. Buffer fosfat - asetonitril pada pH 2,7 digunakan sebagai fase gerak isokratik.

Penentuan kandungan adenosin tri-fosfat dalam media dengan HPLC

Deteksi adenosin tri-fosfat (ATP) dengan HPLC dilakukan sesuai dengan metode Teerlink et al. (1993)

Analisis statistik

Minimal tiga ulangan biologis digunakan dalam setiap percobaan per setiap esai (jumlah setiap ulangan = 3n dan jumlah total ulangan = 72n). Perbedaan statistik rata-rata dibandingkan antara kelompok eksperimen yang berbeda dan dianalisis dengan menerapkan ANOVA satu arah, diikuti dengan uji jarak berganda Duncan yang digunakan untuk mendeteksi perbedaan antar rata-rata. Perbedaan nilai mean dianggap signifikan pada $P \leq 0,05$. Prosedur General Linear Model (GLM) pada SAS Software (SAS, 2004) digunakan untuk analisis statistik. Data seharusnya terdistribusi normal dan dinyatakan sebagai mean ± SD dari tiga ulangan biologis.

Parameter dianalisis menurut model berikut:

Y ijk = µ + A i + e aku j.

Y ijk = sifat yang diukur. µ = Rata-rata keseluruhan.

SEBUAH i = Pengaruh tingkat kurkumin yang berbeda. e ij = Kesalahan eksperimental.

HASIL

Kelangsungan hidup

Kelangsungan hidup in vitro kultur sel granulosa (gambar 3) menurun secara signifikan ($p \leq 0,05$) pada kelompok yang diobati dengan DMSO (88,0 ± 1,6%), 1 µM kurkumin (86,0 ± 1,6%) , 2.5 µM kurkumin (86,26 ± 1,6%) , 5 µM kurkumin (83,0 ± 1,6%) dan 10 µM kurkumin (74,0 ± 1,6%) dibandingkan dengan kelompok control (93,60 ± 1,6%).

Aktivitas mitokondria

Tidak ada perbedaan yang signifikan dalam aktivitas mitokondria sel granulosa yang dikultur dengan DMSO, 2.5 µM kurkumin dan kelompok kontrol (Gambar 2 dan 4). Selain itu, tidak terdapat perbedaan yang signifikan pada aktivitas mitokondria antara kelompok yang dibudidayakan dengan kurkumin 2.5 µM dan 5 µM. µM kurkumin. Apalagi kedua kelompok itu berkultur dengan 1 µM kurkumin dan 10 µM kurkumin tidak menunjukkan perbedaan aktivitas mitokondria. Namun kedua kelompok sel granulosa dikultur dengan 2,5 µM dan 5 µM kurkumin mencatat tingkat aktivitas mitokondria yang lebih tinggi daripada kelompok yang dibudidayakan dengan 1 orang µM dan 10 µM kurkumin.

Tingkat spesies oksigen reaktif (ROS)

Tidak ada perbedaan yang signifikan pada kadar ROS sel granulosa yang dikultur dengan kelompok kurkumin DMSO, 1 µM dan 2,5µM (Gambar 1 dan 5). Namun, ada yang signifikan ($p \leq 0,05$) peningkatan kadar ROS pada kelompok kultur dengan 10µM kurkumin dibandingkan dengan kontrol dan perlakuan lainnya. Selain itu, kelompok sel granulosa yang dibiakkan dengan kurkumin 5µM mencatat tingkat ROS yang lebih tinggi daripada kelompok yang dikultur dengan kurkumin 1µM, 2.5µM dan 10µM.

Aktivitas 1, 1-difenil-2-pikrilhidrazil (DPPH) dalam sel granulosa yang dikultur secara in vitro

Tidak ada perbedaan yang signifikan aktivitas pemulung radikal DPPH dari sel granulosa yang dikultur dengan DMSO. 2.5µM setelah 48 jam, 5µM setelah 48 jam, 10µM setelah 48 tangan 1µM setelah 24 jam kultur kurkumin (Gambar 6). Selain itu, tidak ada perbedaan yang signifikan antara sel granulosa yang dikultur dengan 1µM setelah 48 jam, 10 µM setelah 24 jam dan 2,5 µM setelah 24 jam kultur kurkumin. Selain itu, terdapat perbedaan yang signifikan antara sel granulosa yang dikultur sebagai kelompok kontrol dan kelompok kurkumin 5µM selama 24 jam. Namun, dua kelompok sel granulosa yang dikultur dengan 5µM kurkumin selama 24 jam dan 1 µM kurkumin selama 48 jam mencatat aktivitas pemulung radikal DPPH yang tinggi dibandingkan dengan kelompok lainnya.

Aktivitas superoksida dismutase (SOD) dalam kultur granulosa in vitro

Ada perbedaan yang signifikan pada aktivitas SOD antara sel granulosa yang dikultur pada kelompok kontrol dan kelompok eksperimen yang diberi kurkumin (Gambar 7). Aktivitas maksimum SOD tercatat pada sel granulosa yang dikultur dengan kurkumin 5 µM selama 24 jam dibandingkan dengan kelompok kontrol yang memiliki aktivitas enzim terendah. Selain itu, tidak terdapat perbedaan yang signifikan pada aktivitas SOD sel granulosa yang dikultur dengan

DMSO, kurkumin 1µM selama 24 jam, kurkumin 2,5 µM, 5 µM, dan 10 µM selama 48 jam. Selain itu, terlalu tidak ada perbedaan yang signifikan dari sel granulosa yang dikultur dengan kurkumin 2,5 µM selama 24 jam, kurkumin 5 µM selama 24 jam dan kurkumin 1 µM selama 48 jam, Sebagaimana dicatat kelompok sel granulosa yang dikultur dengan kurkumin 5 µM selama 24 jam mencatat tingkat yang lebih tinggi aktivitas SOD dibandingkan kelompok eksperimen lainnya.

Aktivitas katalase dalam sel granulosa berkultur in vitro

Ada perbedaan yang signifikan pada aktivitas CAT antara sel granulosa berkultur sebagai kelompok kontrol, DMSO dan 5 µM kelompok kurkumin selama 24 jam (Gambar 8). Selain itu, tidak terdapat perbedaan yang signifikan pada aktivitas CAT sel granulosa yang dikultur DMSO, 1 µM kurkumin selama 24 jam, kurkumin 2.5 µM selama 24 jam, kurkumin 10 µM selama 24 jam, 1 µM, 2.5 µM, 5 µM, dan 10 µM kurkumin untuk 48 jam Sebagaimana dicatat, kelompok sel granulosa yang dikultur dengan 5 µM kurkumin selama 24 jam mencatat tingkat aktivitas SOD yang lebih tinggi dibandingkan kelompok lainnya.

Aktivitas glutathione dalam sel granulosa berkultur in vitro

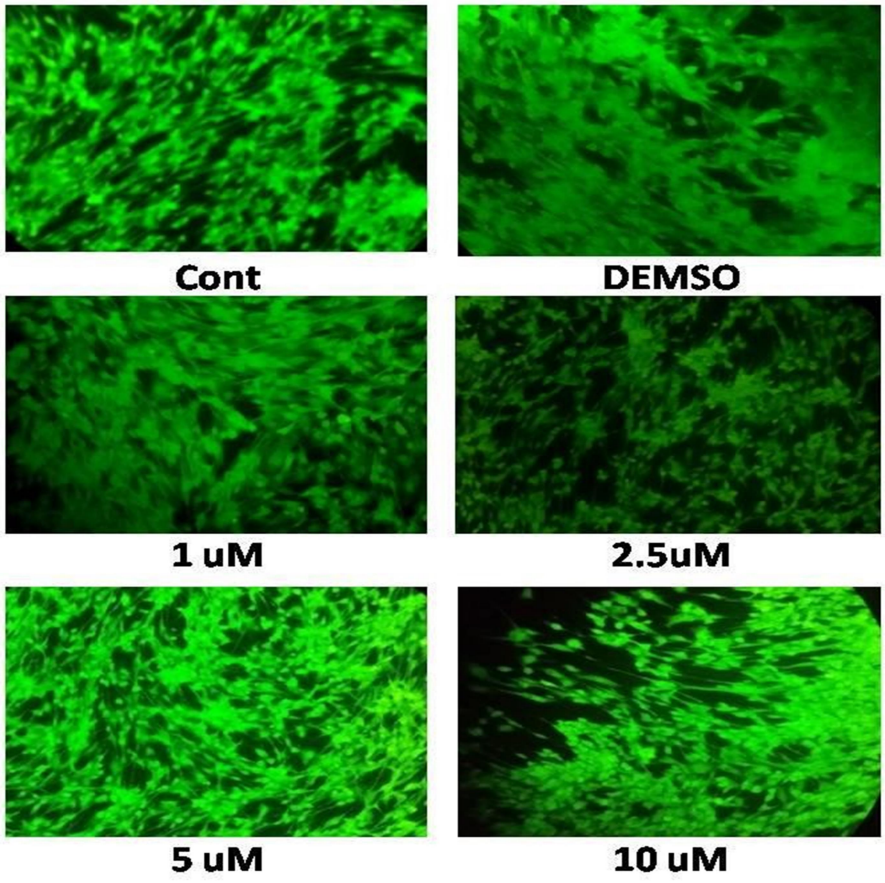
Ada perbedaan yang signifikan (P <0,05) pada aktivitas GSH antara kelompok kontrol berkultur sel granulosa, DMSO dan 5 µM kurkumin selama 24 jam (Gambar 9). Sebagaimana dicatat, kelompok sel granulosa yang dikultur dengan 5 µM kurkumin selama 24 jam mencatat tingkat aktivitas SOD yang lebih tinggi dibandingkan kelompok lainnya.

Aktivitas glutathione teroksidasi dalam sel granulosa berkultur in vitro

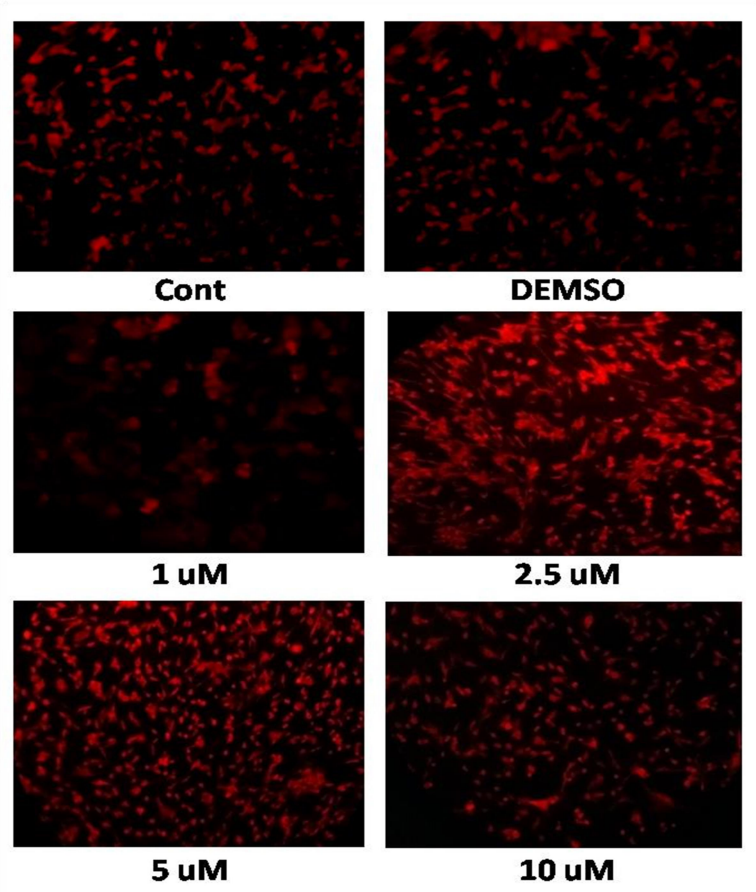
Terdapat penurunan yang signifikan (P <0,05) pada kadar GSSG pada kelompok yang diberi kurkumin pada konsentrasi 5 µM dan 10 µM setelah 24 jam kultur selain itu tren yang sama terlihat pada sel granulosa yang diberi kurkumin pada konsentrasi 1 µM setelah kultur. 48 jam dibandingkan dengan semua kelompok eksperimen (Gambar 10). Namun, level tertinggi dari enzim ini tercatat pada kelompok kontrol.

Kandungan adenosin trifosfat intraseluler dalam sel granulosa berkultur in vitro

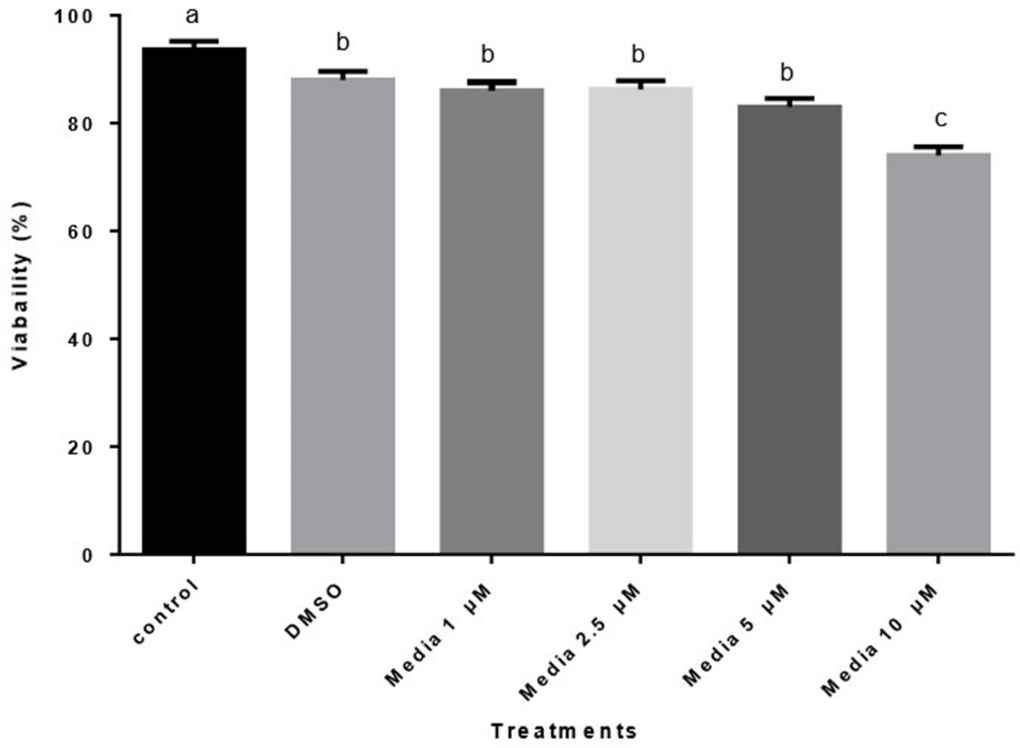
Ada perbedaan yang signifikan (P <0,05) pada aktivitas CAT antara sel granulosa yang dikultur dalam kelompok kontrol, DMSO dan 5 µM kurkumin selama 24 jam (Gambar 11). Kandungan ATP meningkat secara bertahap dan signifikan (P <0,05) dalam pola menaik di GCs dan mencapai profil maksimum setelah 24 H pada kelompok yang dikultur dengan 5 µM. Setelah itu, profil ATP diturunkan (P <0,05) pada GC yang dibudidayakan dengan kurkumin pada konsentrasi 5 dan 10 µM selama 48 h. Profil terendah ATP tercatat pada kelompok kontrol.



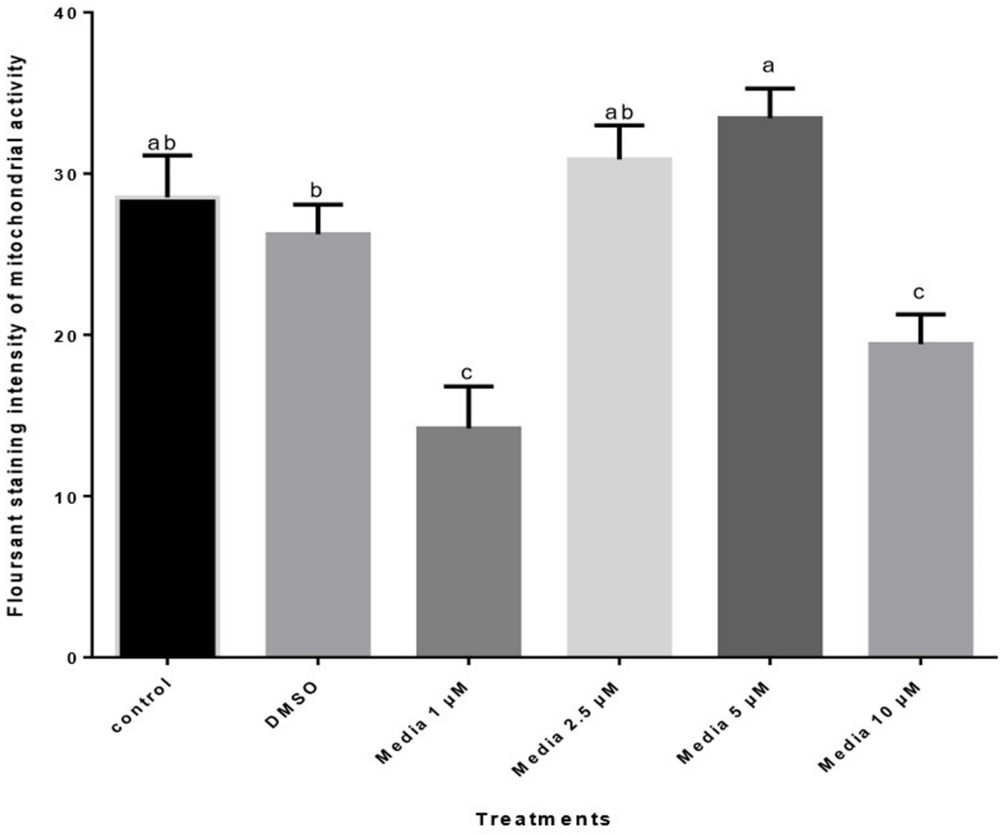
Gambar 1. Gambar dari in vitro sel granulosa yang dikultur diwarnai dengan H. 2 DCFDA mengukur tingkat spesies oksigen reaktif (ROS) setelah suplementasi dengan konsentrasi kurkumin yang berbeda (1, 2.5, 5 dan 10 µM) setelah 24 jam. Itu Gambar diambil dengan mikroskop terbalik (Leica DMI 3000B, Wentzler, Jerman) pada perbesaran 20X.



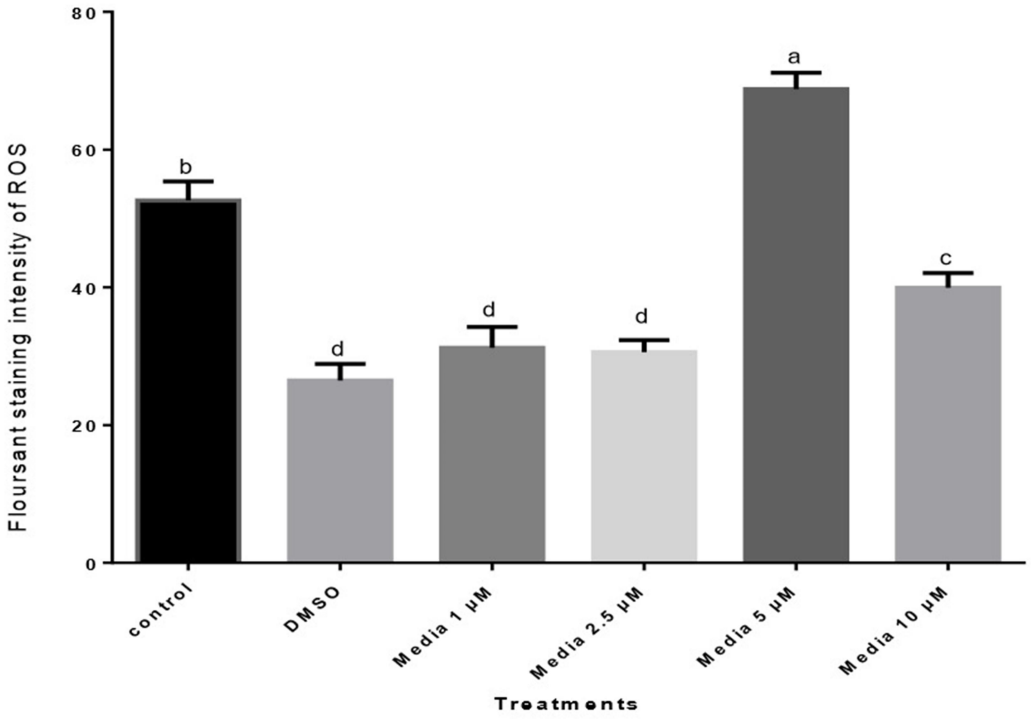
Gambar 2. Gambar dari in vitro sel granulosa yang dikultur diwarnai dengan mitotraker merah mengukur aktivitas mitokondria setelah suplementasi dengan konsentrasi kurkumin yang berbeda (1, 2.5, 5 dan 10 µM) setelah 24 jam. Gambar diambil dengan mikroskop terbalik (Leica DMI 3000B, Wentzler, Jerman) perbesaran 20X.



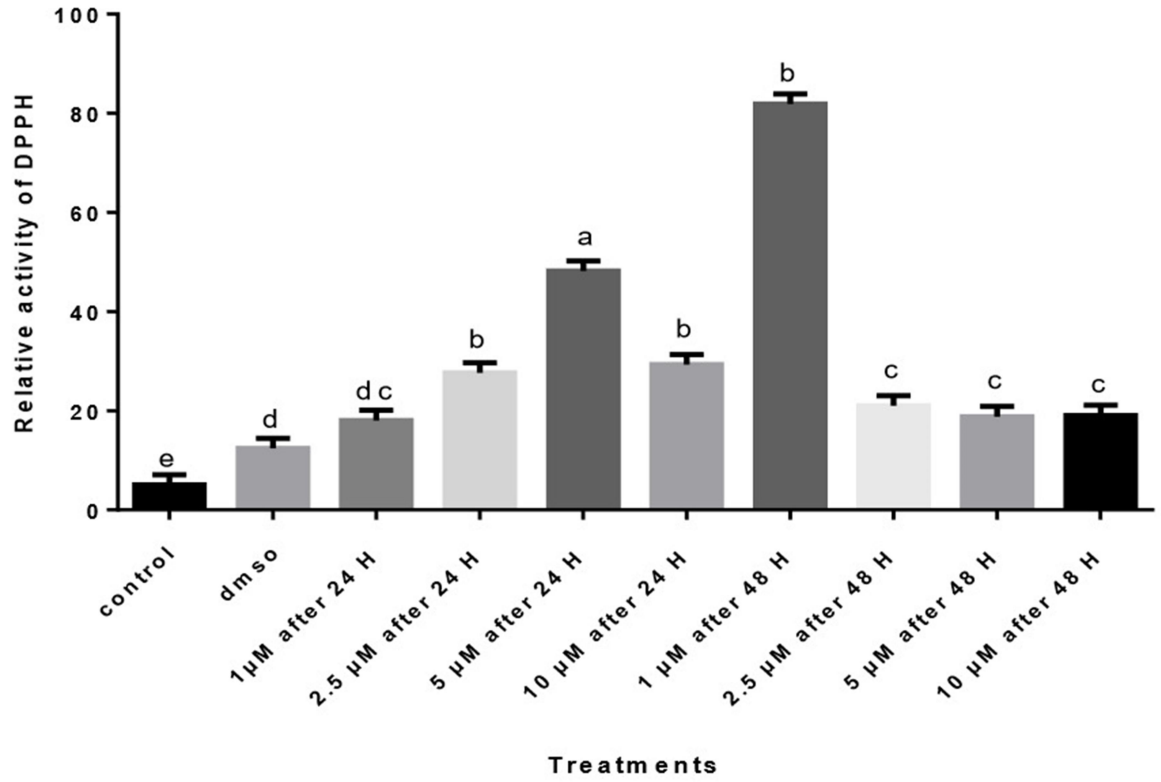
Gambar 3. Kelayakan in vitro sel-sel granulosa yang dikultur diperlakukan dengan konsentrasi kurkumin yang berbeda (1, 2.5, 5 dan 10 µM) setelah 24 jam.



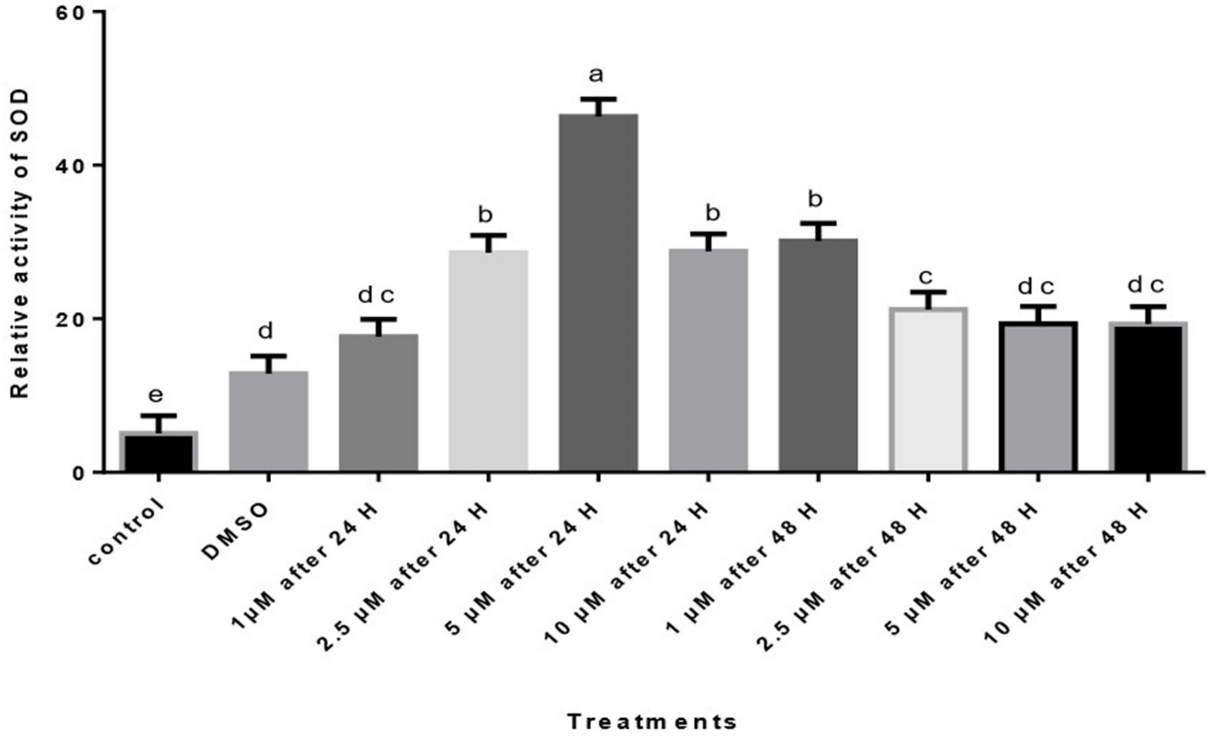
Gambar 4. Aktivitas mitokondria i n vitro sel granulosa berkultur diperlakukan dengan konsentrasi kurkumin yang berbeda (1, 2.5, 5 dan 10 µM) setelah 24 jam.



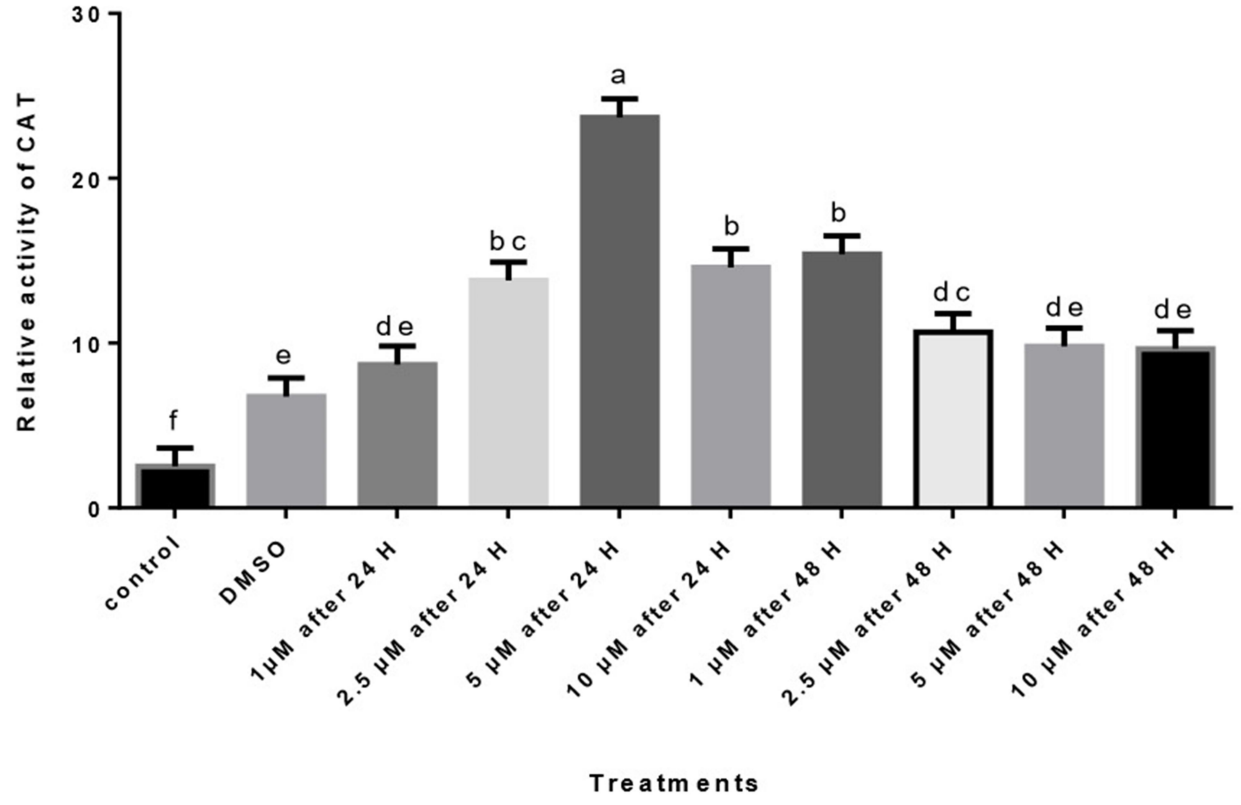
Gambar 5. Tingkat spesies oksigen reaktif intraselual in vitro sel-sel granulosa yang dikultur diperlakukan dengan konsentrasi kurkumin yang berbeda (1, 2.5, 5 dan 10 µM) setelah 24 jam.



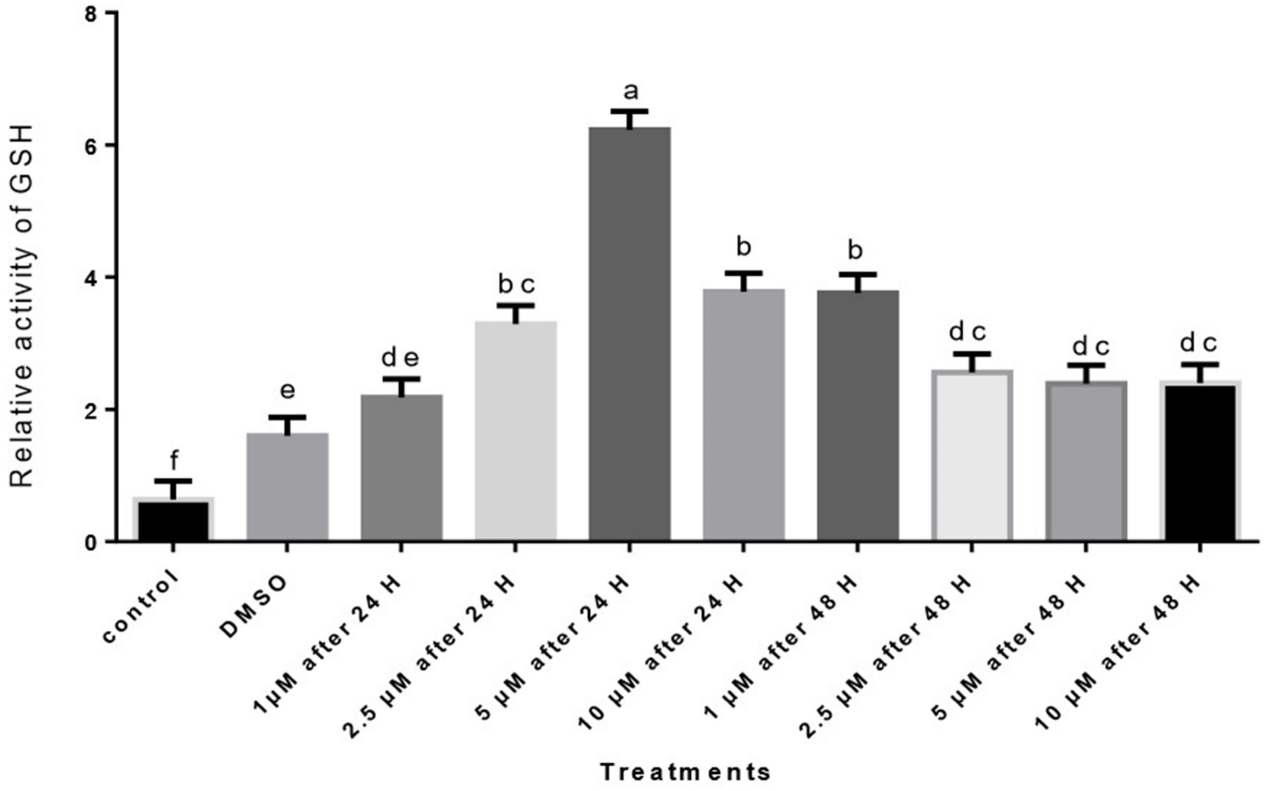
Gambar 6. Aktivitas enzimatis 1,1-difenil-2-pikrilhidrazil (DPPH) di in vitro sel granulosa yang dikultur diperlakukan dengan konsentrasi kurkumin yang berbeda (1, 2.5, 5 dan 10 µM) setelah 24 jam dan 48 jam.



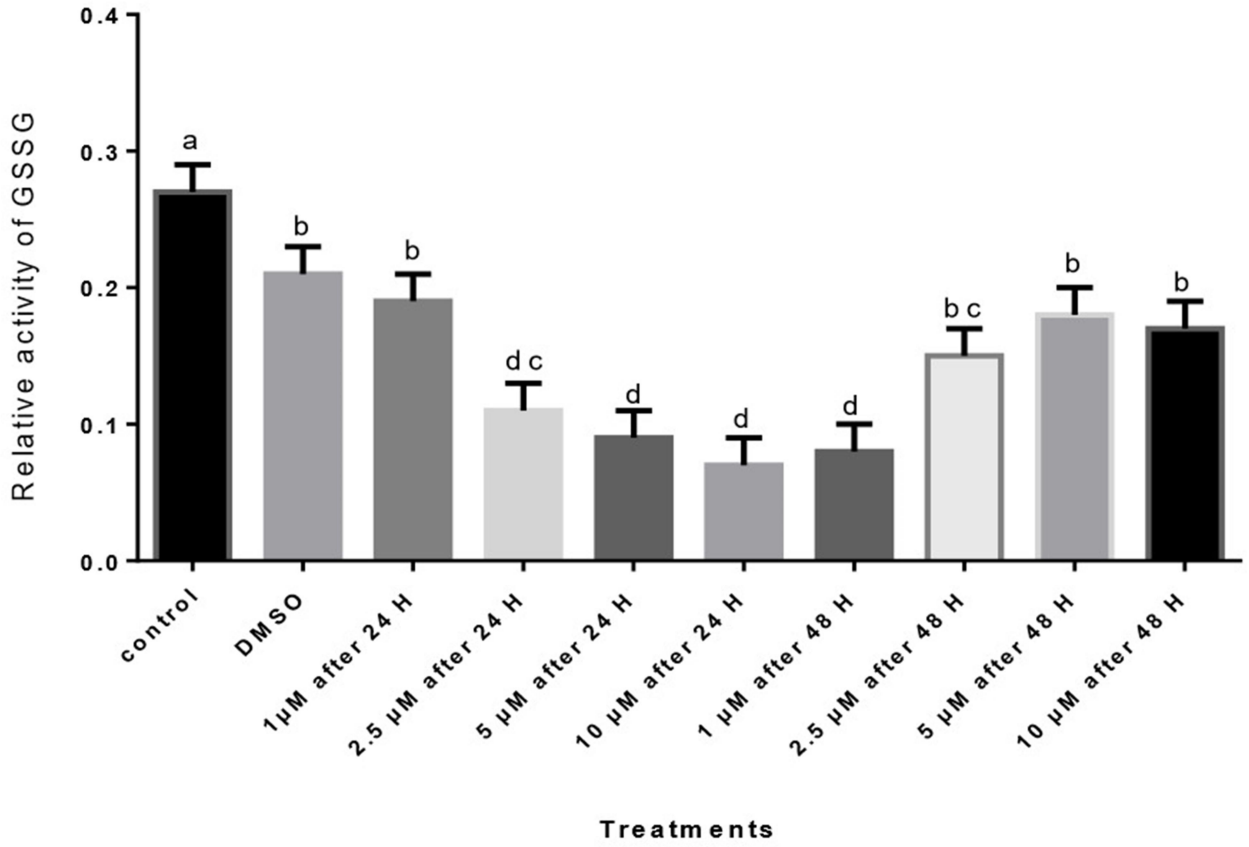
Gambar 7. Aktivitas enzimatis superoksida dismutase (SOD) di in vitro sel granulosa yang dikultur diperlakukan dengan konsentrasi kurkumin yang berbeda (1, 2.5, 5 dan 10 µM) setelah 24 jam dan 48 jam.



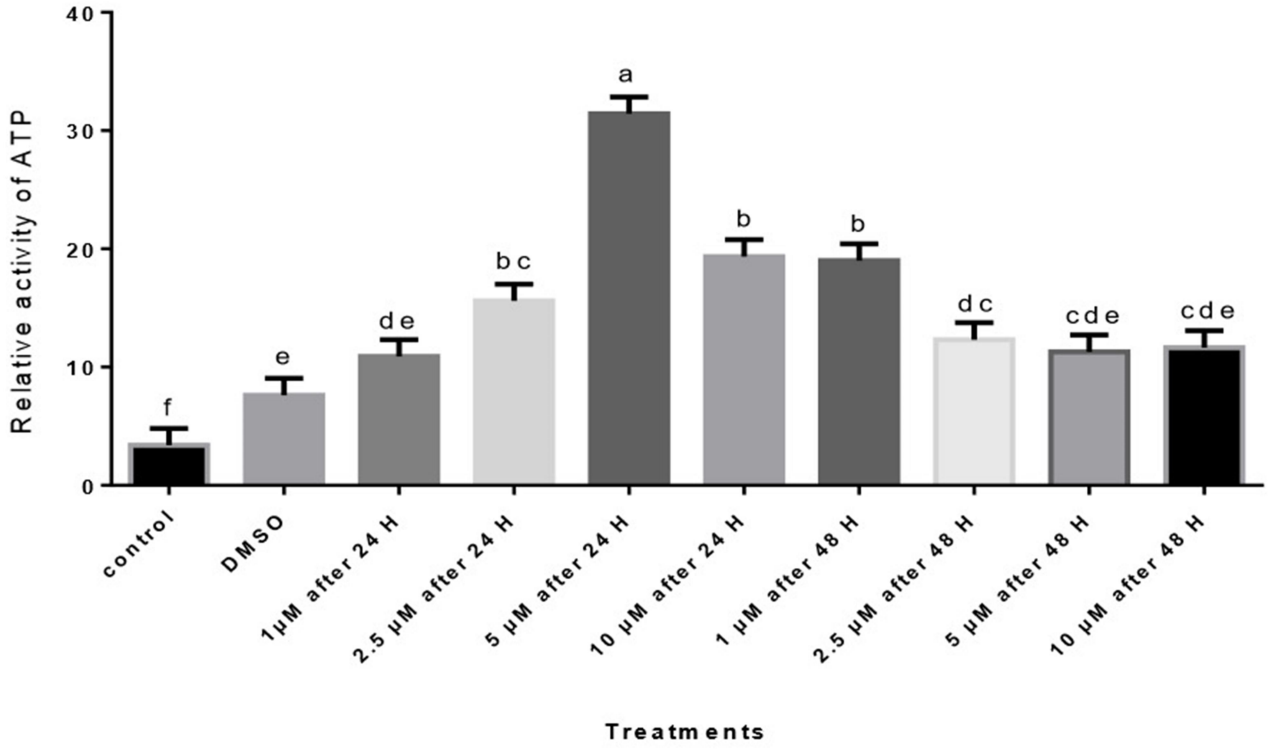
Gambar 8. Aktivitas enzimatik katalase (CAT) di in vitro sel granulosa yang dikultur diperlakukan dengan konsentrasi kurkumin yang berbeda (1, 2.5, 5 dan 10 µM) setelah 24 jam dan 48 jam.



Gambar 9. Aktivitas enzimatis glutatione (GSH) di in vitro sel granulosa yang dikultur diperlakukan dengan konsentrasi kurkumin yang berbeda (1, 2.5, 5 dan 10 µM) setelah 24 jam dan 48 jam.



Gambar 10. Aktivitas enzimatis glutatione teroksidasi (GSSG) di in vitro sel granulosa yang dikultur diperlakukan dengan konsentrasi kurkumin yang berbeda (1, 2.5, 5 dan 10 µM) setelah 24 jam dan 48 jam.



Gambar 11. Konten adenosin trifosfat (ATP) intraseluler di in vitro sel granulosa yang dikultur diperlakukan dengan konsentrasi kurkumin yang berbeda (1, 2.5, 5 dan 10 µM) selama 24 jam dan 48 jam.

DISKUSI

Sel granulosa merupakan sel somatik ovarium yang bersentuhan langsung dengan oosit. GC mendukung oosit melalui aktivitas sekretori, efek perlindungan dan nutrisi. Oleh karena itu, GC berperan besar dalam perolehan potensi perkembangan oosit dan proses ovulasi (Buccione et al., 1990; Joyce dkk., 2001; Su et al., 2009). Namun, sel-sel ini juga dipengaruhi oleh stres oksidatif yang dapat diinduksi oleh ROS yang dihasilkan oleh aktivitas metabolisme normal atau sebagai akibat dari in vitro kondisi kultur (Aggarwal et al., 2005). Induksi stres oksidatif selanjutnya dapat menyebabkan apoptosis sel-sel hidup (Al Dhaheri dkk., 2014).

Dalam penelitian saat ini, media kultur dilengkapi dengan konsentrasi kurkumin yang berbeda untuk mengurangi akumulasi ROS yang berlebihan dalam GC di bawah in vitro kondisi kultur. Hasil penelitian ini menunjukkan penurunan viabilitas sel granulosa yang dikultur pada kelompok yang diobati dengan DMSO (88,0%), 1 µM kurkumin (86,0%) , 2.5 µM kurkumin (86,26%) , 5 µM kurkumin (83,0%) dan 10 µM kurkumin (74,0%) dibandingkan dengan kelompok kontrol (93,60%). Sesuai dengan hasil kami, Kádasi dkk. (2012 dan 2017) melaporkan penurunan pertumbuhan in vitro kultur sel granulosa babi setelah suplementasi kurkumin dengan 10 dan 100 µg.mL- 1 dibandingkan dengan kontrol dan 1 µg.mL- 1. Selain itu, dilaporkan bahwa kurkumin menurunkan proliferasi sel kanker usus besar (Hanif et al., 1997). Efek negatif kurkumin dalam sel kultur ini diberikan melalui induksi apoptosis (Bhaumik dkk., 1999; Liduan et al., 2004; Voznesens 'ka dkk., 2010). Sebaliknya, Aktas dkk. (2012) telah menunjukkan efek proliferatif positif kurkumin pada sel folikel ovarium tikus dengan mencegah apoptosis. Memang, variasi efek kurkumin yang diamati dalam penelitian kami dan penyelidikan lainnya dapat dijelaskan oleh jenis sel yang sedang diteliti, kondisi kultur, dosis dan durasi pengobatan (Kádasi et al., 2012 dan 2017).

Penurunan viabilitas sel granulosa ditambah dengan peningkatan kadar ROS pada kelompok yang diberi 5 µM kurkumin dibandingkan dengan kelompok eksperimen lain dalam penelitian ini. Meskipun, kurkumin adalah antioksidan terkenal (Mantzorou dkk., 2018) yang digunakan untuk mengurangi timbulnya stres oksidatif (Santos-Parker dkk., 2017). Namun, konsentrasi kurkumin yang tinggi dapat menyebabkan kematian sel (Raza dkk., 2008). Namun demikian, ketika produksi ROS mengatasi kemampuan antioksidan seluler, ini dapat menyebabkan masalah yang disebut stress oksidatif (Agarwal et al., 2005). Tingkat ROS dapat meningkat secara endogen selama banyak prosedur fisiologis termasuk ovulasi (Agarwal et al., 2005). Namun, selama in vitro kultur sel, ROS dapat ditingkatkan ke tingkat yang menyebabkan stres oksidatif (Rizzo dkk., 2012; Castro dkk., 2014; Hatami dkk., 2014). Menariknya, hasil kami menunjukkan bahwa tingkat ROS dan aktivitas mitokondria meningkat pada sel granulosa yang dibiakkan dengan 5 µM kurkumin yang dikaitkan dengan penurunan viabilitas kelompok ini, menegaskan efek samping berbahaya dari peningkatan kadar senyawa ini selama kultur sel. Stres oksidatif yang terjadi akibat suplementasi oksidan eksogen telah menyebabkan apoptosis pada berbagai jenis sel mamalia, termasuk hepatosit (Haidara dkk., 2002), sel epitel (Jungas et al., 2002), dan fibroblas (Ran et al., 2004). Di sisi lain, kurkumin telah mempertahankan fungsi pernapasan mitokondria serta status redoks garis sel PC12 tanpa mempengaruhi ROS dan viabilitas sel (Raza dkk., 2008). Ini sesuai dengan hasil kami yang menunjukkan peningkatan aktivitas mitokondria dan konten ATP di GC yang dilengkapi dengan kurkumin selama 24 jam selama in vitro kultur bagaimanapun, itu ditambah dengan penurunan viabilitas seluler.

Beberapa enzim intraseluler terdiri dari sistem pertahanan sel mamalia. Misalnya, SOD, GPX1 dan CAT, GSSG dan DPPH berkontribusi pada kapasitas pembersihan sel untuk mengurangi efek berbahaya dari stres oksidatif yang disebabkan oleh ROS (Qin dkk., 2015). Dalam penelitian ini, aktivitas enzim CAT, SOD, GSH dan DPPH meningkat setelah merawat sel-sel granulosa yang dibiakkan dengan 5 µM kurkumin, namun semua enzim ini menurun secara signifikan setelah 48 jam. Sebuah studi terbaru yang dilakukan oleh Qin et al., (2015) mendemonstrasikan efek perlindungan kurkumin dalam mengurangi stres oksidatif sel granulosa babi dengan menyelamatkan aktivitas enzim antioksidan. Namun, penelitian ini menunjukkan bahwa meskipun kurkumin meningkatkan tingkat enzim antioksidan yang berbeda setelah 24 jam in vitro kultur sel tetapi tidak dapat mempertahankan tindakan biologis ini setelah 48 jam dan viabilitas sel berkurang karena peningkatan level ROS.

KESIMPULAN

Penemuan ini menunjukkan efek negatif dari in vitro kultur pada viabilitas sel granulosa dan status redoks. Senyawa antioksidan yaitu kurkumin meningkatkan efek negatif in vitro kultur bila ditambahkan pada konsentrasi yang lebih tinggi (10 µM). Namun, kurkumin dengan konsentrasi rendah (2,5 µM) dapat mempertahankan aktivitas metabolisme serta sistem pertahanan dengan meningkatkan regulasi enzim antioksidan untuk waktu yang singkat.

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REFERENSI

Aebi H (1984). Catalase in vitro, In: methods in enzymology. Elsevier, pp. 121-126.

Agarwal A, Aponte-Mellado A, Premkumar BJ, Shaman A and Gupta S (2012). The effects of oxidative stress on female reproduction: a review. Reproductive Biology and Endocrinology, 10 (1): 49. DOI: http://dx.doi.org/10.1186/1477-7827-10-49.

Agarwal A, Gupta S and Sharma RK (2005). Role of oxidative stress in female reproduction. Reproductive Biology and Endocrinology, 3 (1): 28. DOI: http://dx.doi.org/10.1186/1477-7827-3-28.

Aggarwal BB, Kumar A, Aggarwal MS and Shishodia S (2005). Curcumin derived from turmeric (Curcuma longa): a spice for all seasons. Phytopharmaceuticals in Cancer Chemoprevention, 23: 351-387. Available at: http://www.sciepub.com/reference/38129

Aggarwal BB ,Sundaram C, Malani N and Ichikawa H (2007). Curcumin: the Indian solid gold, In: The molecular targets and therapeutic uses of curcumin in health and disease. Springer, pp. 1-75. DOI: http://dx.doi.org/10.1007/978-0-387-46401-5_1

Aktas C, Kanter M and Kocak Z (2012). Antiapoptotic and proliferative activity of curcumin on ovarian follicles in mice exposed to whole body ionizing radiation. Toxicology and Industrial Health, 28 (9): 852-863. DOI: http://dx.doi.org/10.1177/0748233711425080

Al Dhaheri Y, Attoub S, Ramadan G, Arafat K, Bajbouj K, Karuvantevida N , AbuQamar S, Eid A and Iratni R (2014). Carnosol induces ROS-mediated beclin1-independent autophagy and apoptosis in triple negative breast cancer. PLoS One, 9 (10): e109630. DOI: http://dx.doi.org/10.1371/journal.pone.0109630.

Barzegar A and Moosavi-Movahedi AA (2011). Intracellular ROS protection efficiency and free radical-scavenging activity of curcumin, PLoS One, 6 (10): e26012. DOI: http://dx.doi.org/10.1371/journal.pone.0026012.

Bhaumik S, Anjum R, Rangaraj N, Pardhasaradhi BV and Khar A (1999). Curcumin mediated apoptosis in AK-5 tumor cells involves the production of reactive oxygen intermediates. Federation of European Biochemical Societies, 456 (2): 311-314. DOI: http://dx.doi.org/10.1016/s0014-5793(99)00969-2.

Blois MS (1958). Antioxidant determinations by the use of a stable free radical. Nature, 181 (4617): 1199-1200. DOI: http://dx.doi.org/10.1371/journal.pone.0026012

Buccione R, Vanderhyden B, Caron PJ and Eppig JJ (1990). FSH-induced expansion of the mouse cumulus oophorus in vitro is dependent upon a specific factor(s) secreted by the oocyte. Developmental Biology, 138 (1): 16-25. DOI: http://dx.doi.org/10.1016/0012-1606(90)90172-f.

Castro SV, Carvalho AA, Silva CM, Santos FW, Campello CC, de Figueiredo JR and Rodrigues AP (2014). Frozen and fresh ovarian tissue require different culture media to promote in vitro development of bovine preantral follicles. Biopreservation and Biobanking, 12 (5): 317-324. DOI: http://dx.doi.org/10.1089/bio.2014.0020.

Cinar MU and Sohel MMH (2015). Advancement in Molecular Genetics to understand the molecular reproduction of Livestock – follicular development. Research in Agriculture Livestock and Fisheries, 1 (1): 47-60. DOI: http://dx.doi.org/10.3329/ralf.v1i1.22355.

Correa F, Buelna-Chontal M, Hernandez-Resendiz S, Garcia-Nino WR, Roldan FJ ,Soto V, Silva-Palacios A, Amador A, Pedraza-Chaverri J, Tapia E and Zazueta C (2013). Curcumin maintains cardiac and mitochondrial function in chronic kidney disease. Free Radical Biology and Medicine, 61: 119-129. DOI: http://dx.doi.org/10.1016/j.freeradbiomed.2013.03.017.

Dinkova-Kostova AT and Talalay P (2008). Direct and indirect antioxidant properties of inducers of cytoprotective proteins. Molecular Nutrition Food Research, 52(S1): S128-S138. DOI: https://doi.org/10.1002/mnfr.200700195.

Duffy DM and Stouffer RL (2003). Luteinizing hormone acts directly at granulosa cells to stimulate periovulatory processes: modulation of luteinizing hormone effects by prostaglandins. Endocrine, 22 (3): 249-256. DOI: http://dx.doi.org/10.1385/endo:22:3:249.

Epstein J ,Sanderson IR and Macdonald TT (2010). Curcumin as a therapeutic agent: the evidence from in vitro, animal and human studies. British Journal of Nutrition, 103 (11): 1545-1557. DOI: http://dx.doi.org/10.1017/S0007114509993667.

Esatbeyoglu T, Huebbe P, Ernst IM, Chin D, Wagner AE and Rimbach G (2012). Curcumin--from molecule to biological function. Angewandte Chemmie International Edition, 51 (22): 5308-5332. DOI: http://dx.doi.org/10.1002/anie.201107724.

Frisard M and Ravussin E (2006). Energy metabolism and oxidative stress: impact on the metabolic syndrome and the aging process. Endocrine, 29 (1): 27-32. DOI: http://dx.doi.org/10.1385/ENDO:29:1:27.

Haidara K, Morel I, Abalea V, Gascon Barre M and Denizeau F (2002). Mechanism of tert-butylhydroperoxide induced apoptosis in rat hepatocytes: involvement of mitochondria and endoplasmic reticulum. Biochimica et Biophysica Acta (BBA) . Molecular Cell Research, 1542(1-3): 173-185. DOI: http://dx.doi.org/10.1016/s0167-4889(01)00178-1.

Hanif R, Qiao L, Shiff SJ and Rigas B (1997). Curcumin, a natural plant phenolic food additive, inhibits cell proliferation and induces cell c ycle changes in colon adenocarcinoma cell lines by a prostaglandin-independent pathway. Journal of Laboratory and Clinical Medicine, 130 (6): 576-584. DOI: http://dx.doi.org/10.1016/s0022-2143(97)90107-4.

Hatami S, Zavareh S, Salehnia M, Lashkarbolouki T and Karimi I (2014). Comparison of oxidative status of mouse pre-antral follicles derived from vitrified whole ovarian tissue and vitrified pre-antral follicles in the presence of alpha lipoic acid. Journal of Obstetrics and Gynaecology Research, 40 (6): 1680-1688. DOI: http://dx.doi.org/10.1111/jog.12394.

Hatcher H, Planalp R, Cho J, Torti FM, and Torti SV (2008). Curcumin: from ancient medicine to current clinical trials. Cellular and Molecular Life Sciences, 65(11): 1631–1652. https://doi.org/10.1007/s00018-008-7452-4.

Jayatilleke E and Shaw S (1993). A high-performance liquid chromatographic assay for reduced and oxidized glutathione in biological samples. Analytical Biochemistry, 214 (2): 452-457. DOI: http://dx.doi.org/10.1006/abio.1993.1522.

Joyce IM, Pendola FL, O'Brien M and Eppig JJ (2001). Regulation of prostaglandin-endoperoxide synthase 2 messenger ribonucleic acid expression in mouse granulosa cells during ovulation. Endocrinology, 142 (7): 3187-3197. DOI: http://dx.doi.org/10.1210/endo.142.7.8268.

Jungas T, Motta I, Duffieux F, Fanen P, Stoven V and Ojcius DM (2002). Glutathione levels and BAX activation during apoptosis due to oxidative stress in cells expressing wild-type and mutant cystic fibrosis transmembrane conductance regulator. The Journal of Biological Chemistry, 277 (31): 27912-27918. DOI: http://dx.doi.org/10.1074/jbc.M110288200.

Kádasi A, Maruniakova N, Stochmalova A, Bauer M, Grossmann R, Harrath AH, Kolesarova A and Sirotkin AV (2017). Direct effect of curcumin on porcine ovarian cell functions. Animal Reproduction Science, 182: 77-83. DOI: http://dx.doi.org/10.1016/j.anireprosci.2017.05.001.

Kádasi A, Sirotkin AV, Maruniaková N, Kolesárová A, Bulla J and Grossmann R (2012). The effect of curcumin on secretory activity, proliferation and apoptosis of the porcine ovarian granulosa cells. The Journal of Microbiology, Biotechnology and Food Sciences, 2(1): 349. DOI: http://dx.doi.org/10.1016/j.anireprosci.2017.05.001.

Klaunig JE, Kamendulis LM and Hocevar BA (2010). Oxidative stress and oxidative damage in carcinogenesis. Toxicologic Pathology, 38 (1): 96-109. DOI: http://dx.doi.org/10.1177/0192623309356453.

Liu G, Xiang T, Wu QF and Wang WX (2016). Curcumin suppresses the proliferation of gastric cancer cells by downregulating H19. Oncology Letters, 12(6): 5156-5162. DOI: http://dx.doi.org/10.3892/ol.2016.5354.

Mantzorou M, Pavlidou E, Vasios G,Tsagalioti E and Giaginis C (2018). Effects of curcumin consumption on human chronic diseases: A narrative review of the most recent clinical data. Phytotherapy Research, 32 (6): 957-975. DOI: http://dx.doi.org/10.1002/ptr.6037.

Marklund S and Marklund G (1974). Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. European Journal of Biochemistry, 47 (3): 469-474. DOI: http://dx.doi.org/10.1111/j.1432-1033.1974.tb03714.x.

Mohebbati R, Anaeigoudari A, Khazdair MR (2017). The effects of Curcuma longa and curcumin on reproductive systems. Endocrine Regulations, 51(4): 220-228. DOI: http://dx.doi.org/10.1515/enr-2017-0024

Nadkarni K (2007). Indian Materia Medica, Ed. Bombay Popular Mumbai, 1: 691-694. Available at: https://trove.nla.gov.au/work/18643838?q&versionId=21887031.

Panieri E and Santoro M (2016). ROS homeostasis and metabolism: a dangerous liason in cancer cells. Cell Death & Disease 7, e2253. DOI: https://doi.org/10.1038/cddis.2016.105

Pinkus R ,Weiner LM and Daniel V (1996). Role of oxidants and antioxidants in the induction of AP-1, NF-kappaB, and glutathione S-transferase gene expression. The Journal of Biological Chemistry, 271 (23): 13422-13429. DOI: http://dx.doi.org/10.1074/jbc.271.23.13422.

Prastowo S, Amin A, Rings F, Held E, Wondim DS, Gad A, Neuhoff C, Tholen E, Looft C, Schellander K and Tesfaye D (2016). Fateful triad of reactive oxygen species, mitochondrial dysfunction and lipid accumulation is associated with expression outline of the AMP-activated protein kinase pathway in bovine blastocysts. Reproduction, Fertility and Development, 29 (5): 890-905. DOI: http://dx.doi.org/10.1071/RD15319.

Qin X, Cao M, Lai F, Yang F, Ge W, Zhang X, Cheng S, Sun X, Qin G, Shen W and Li L (2015). Oxidative stress induced by zearalenone in porcine granulosa cells and its rescue by curcumin in vitro. PLoS One, 10 (6): e0127551. DOI: http://dx.doi.org/10.1371/journal.pone.0127551.

Ran Q, Liang H, Gu M, Qi W, Walter CA, Roberts LJ, Herman B, Richardson A and Van Remmen H (2004). Transgenic mice overexpressing glutathione peroxidase 4 are protected against oxidative stress-induced apoptosis. The journal of Biological Chemistry, 279 (53): 55137-55146. DOI: http://dx.doi.org/10.1074/jbc.M410387200.

Raza H, John A, Brown EM, Benedict S and Kambal A (2008). Alterations in mitochondrial respiratory functions, redox metabolism and apoptosis by oxidant 4-hydroxynonenal and antioxidants curcumin and melatonin in PC12 cells. Toxicology and Applied Pharmacology, 226 (2): 161-168. DOI: http://dx.doi.org/10.1016/j.taap.2007.09.002.

Repetto G, Del Peso A and Zurita JL (2008). Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nature Protocols, 3 (7): 1125-1131. DOI: http://dx.doi.org/10.1038/nprot.2008.75.

Reyes-Fermin LM, Gonzalez-Reyes S, Tarco-Alvarez NG, Hernandez-Nava M, Orozco-Ibarra M and Pedraza-Chaverri J (2012). Neuroprotective effect of alpha-mangostin and curcumin against iodoacetate-induced cell death. Nutritional Neuroscience, 15 (5): 34-41. DOI: http://dx.doi.org/10.1179/1476830512Y.0000000011.

Rueden CT, Schindelin J, Hiner MC, DeZonia BE, Walter AE, Arena ET and Eliceiri KW (2017). ImageJ2: ImageJ for the next generation of scientific image data. BMC Bioinformatics, 18(1): 529. doi:10.1186/s12859-017-1934-z

Santos-Parker JR, Strahler TR, Bassett CJ, Bispham NZ, Chonchol MB and Seals DR (2017). Curcumin supplementation improves vascular endothelial function in healthy middle-aged and older adults by increasing nitric oxide bioavailability and reducing oxidative stress. Aging (Albany NY), 9 (1): 187-208. DOI: http://dx.doi.org/10.18632/aging.101149.

Shkolnik K, Tadmor A, Ben-Dor S, Nevo N, Galiani D and Dekel N (2011). Reactive oxygen species are indispensable in ovulation. Proceedings of the National Academy of Sciences, 108 (4): 1462-1467. DOI: http://dx.doi.org/10.1073/pnas.1017213108.

Sohel MM, Hoelker M, Noferesti SS, Salilew-Wondim D, Tholen E, Looft C , Rings F, Uddin MJ, Spencer TE, Schellander K and Tesfaye D (2013). Exosomal and Non-Exosomal Transport of Extra-Cellular microRNAs in Follicular Fluid: Implications for Bovine Oocyte Developmental Competence. PLoS One, 8 (11): e78505. DOI: http://dx.doi.org/10.1371/journal.pone.0078505.

Sohel MMH, Konca Y, Akyuz B, Arslan K, Sariozkan S and Cinar MU (2017). Concentration dependent antioxidative and apoptotic effects of sulforaphane on bovine granulosa cells in vitro. Theriogenology, 97:17-26. DOI: 10.1016/j.theriogenology.2017.04.015.

Steward WP and Gescher AJ (2008). Curcumin in cancer management: recent results of analogue design and clinical studies and desirable future research. Molecular Nutrition & Food research, 52 (9): 1005-1009. DOI: http://dx.doi.org/10.1002/mnfr.200700148.

Strober W (2015). Trypan blue exclusion test of cell viability. Current protocols in immunology, 111:A3.B.1-A3.B.3. DOI: http://dx.doi.org/10.1002/0471142735.ima03bs111.

Su YQ, Sugiura K and Eppig JJ (2009). Mouse oocyte control of granulosa cell development and function: paracrine regulation of cumulus cell metabolism, In: Seminars in Reproductive Medicine, 27 (1): 32-42. DOI: https://doi.org/10.1055/s-0028-1108008

Sung B, Prasad S, Yadav VR and Aggarwal BB (2012). Cancer cell signaling pathways targeted by spice-derived nutraceuticals. Nutrition and cancer, 64 (2): 173-197. DOI: http://dx.doi.org/10.1080/01635581.2012.630551.

Tapia E, Soto V, Ortiz-Vega K M, Zarco-Marquez G, Molina-Jijon E, Cristobal-Garcia M, Santamaria J, Garcia-Nino WR, Correa F, Zazueta C and Pedraza-Chaverri J (2012). Curcumin induces Nrf2 nuclear translocation and prevents glomerular hypertension, hyperfiltration, oxidant stress, and the decrease in antioxidant enzymes in 5/6 nephrectomized rats. Oxidative medicine and cellular longevity, 2012: 269039. DOI: http://dx.doi.org/10.1155/2012/269039.

Tapia E, Zatarain-Barron ZL, Hernandez-Pando R, Zarco-Marquez G, Molina-Jijon E, Cristobal-Garcia M, Santamaria J and Pedraza-Chaverri J (2013). Curcumin reverses glomerular hemodynamic alterations and oxidant stress in 5/6 nephrectomized rats. Phytomedicine, 20 (3-4): 359-366. DOI: http://dx.doi.org/10.1016/j.phymed.2012.11.014.

Teerlink T, Hennekes M, Bussemaker J and Groeneveld J (1993). Simultaneous determination of creatine compounds and adenine nucleotides in myocardial tissue by high-performance liquid chromatography. Analytical Biochemistry, 214 (1): 278-283. DOI: http://dx.doi.org/10.1006/abio.1993.1488.

Trujillo J, Chirino YI, Molina-Jijon E, Anderica-Romero AC, Tapia E and Pedraza-Chaverri J (2013). Renoprotective effect of the antioxidant curcumin: Recent findings. Redox Biology, 1 (1): 448-456. DOI: http://dx.doi.org/10.1016/j.redox.2013.09.003.

Voznesens'ka T, Bryzhina TM, Sukhina VS, Makohon NV and Aleksieieva IM (2010). Effect of NF-kappaB activation inhibitor curcumin on the oogenesis and follicular cell death in immune ovarian failure in mice. Fiziolohichnyi Zhurnal, 56 (4): 96-101. DOI: http://dx.doi.org/10.15407/fz56.04.096.

Yada H, Hosokawa K, Tajima K, Hasegawa Y and Kotsuji F (1999). Role of ovarian theca and granulosa cell interaction in hormone productionand cell growth during the bovine follicular maturation process. Biology of Reproduction, 61 (6): 1480-1486. DOI: http://dx.doi.org/10.1095/biolreprod61.6.1480.

Zheng L, Tong Q and Wu C (2004). Growth-inhibitory effects of curcumin on ovary cancer cells and its mechanisms. Journal of Huazhong University of Science and Technology, 24 (1): 55-58. DOI: http://dx.doi.org/10.1007/bf02830706.

Zhang Y, Huang Y, Deng H and Huang X (2019a). Curcumin attenuates oxidative stress in RAW264.7 cells by increasing the activity of antioxidant enzymes and activating the Nrf2-Keap1 pathway. PLoS One, 14(5): e0216711. DOI: http://dx.doi.org/10.1371/journal.pone.0216711.

Zhang Y, Lin XY, Zhang JH, Xie ZL, Deng H, Huang YF and Huang XH (2019b). Apoptosis of mouse myeloma cells induced by curcumin via the Notch3-p53 signaling axis. Oncology Letters, 17(1):127-134. DOI:http://dx.doi.org/ 10.3892/ol.2018.9591.

Zhu G, Shen Q, Jiang H, Ji O, Zhu L and Zhang L (2020). Curcumin inhibited the growth and invasion of human monocytic leukaemia SHI-1 cells in vivo by altering MAPK and MMP signalling. Pharmacy Biology, 58(1)

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Investigasi In Vitro tentang Efek Antibakteri dari Nanopartikel Perak pada *E. coli* dan *Klebsiella* spp penghasil ESBL. Terisolasi dari Hewan Peliharaan

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ABSTRAK

Meskipun sudah ada obat antibakteri modern, infeksi bakteri masih menjadi masalah utama yang mengancam karena peningkatan yang sangat besar pada bakteri yang resistan terhadap beberapa obat. Nanopartikel telah banyak digunakan sebagai alternatif yang dapat diterapkan dan aman untuk antibiotik. Penelitian ini bertujuan untuk mengeksplorasi efek penghambatan nanopartikel perak pada Extended Spectrum Beta lactamase (ESBL) yang memproduksi *E. coli* dan *Klebsiella* spp. in vitro serta pengaruhnya terhadap ekspresi gen resistensi antibiotik. Sampel yang berbeda (mis., Usap luka, usap tinja, dan sampel urin) dikumpulkan dari anjing dan kucing. Identifikasi fenotipe dan molekuler, uji kepekaan antibiotik, dan uji sinergi cakram ganda dilakukan untuk mengidentifikasi *E. coli* penghasil ESBL dan *Klebsiella* spp. Nanopartikel perak diuji untuk potensi antibakteri in vitro dan terdapat laporan konsentrasi hambat minimum dan konsentrasi bakterisidal minimum. Selain itu, efek nanopartikel perak pada ekspresi gen resistensi antibiotik (yaitu, blaTEM, blaSHV, dan blaCTX) dinilai serta pengaruhnya terhadap integritas struktural sel bakteri menggunakan Scanning Electron Microscope (SEM). Hasil penelitian menunjukkan bahwa 23 isolat (19,16%) (*E. coli* = 17, *Klebsiella* spp. = 6) dikonfirmasi sebagai penghasil ESBL. Nanopartikel perak menunjukkan efek antibakteri yang menjanjikan dimana konsentrasi hambat minimum AgNP untuk *E. coli* penghasil ESBL diukur sebagai 0,31 mg / ml, dan 0,62 mg / ml untuk *Klebsiella* spp penghasil ESBL, sedangkan konsentrasi bakterisidal minimum penghasil ESBL. *E. coli* dan *Klebsiella* spp. dilaporkan masing-masing sebagai 0,15 mg / ml dan 0,3 mg / ml. Akibatnya, ekspresi gen resistensi antibiotik diturunkan regulasi pada kedua spesies bakteri dan ada efek toksik yang nyata dari AgNPs pada *E. coli* dan *Klebsiella* spp. sel yang diselidiki menggunakan SEM. Dapat disimpulkan bahwa nanopartikel perak memiliki aktivitas antibakteri yang menjanjikan dan dapat dianggap sebagai alternatif yang dapat diterapkan untuk pengendalian bakteri penghasil ESBL.

Kata kunci: *E. coli*, ESBLs, *Klebsiella* spp., Hewan peliharaan, nanopartikel perak

PENGANTAR

Dalam dekade saat ini, pencarian alternatif antibiotik telah menjadi salah satu masalah paling klasik karena meningkatnya resistensi antibiotik. Penggunaan nanopartikel perak telah dikenal luas karena tindakan bakterisidal serta bakteriostatiknya yang diketahui terhadap berbagai jenis patogen bakteri dan jamur.(Saeb et al., 2014). Nanopartikel perak (AgNPs) memiliki efek antimikroba spektrum luas karena luas permukaannya yang besar menyediakan kesempatan untuk kontak yang lebih baik dengan mikroba (Li et al., 2010). Selain itu, nanopartikel perak memiliki kecenderungan lebih rendah untuk menginduksi resistensi mikroba dibandingkan agen antimikroba lainnya.(Ansari et al., 2014). Perlu juga disebutkan bahwa nanopartikel ini memiliki efek tidak beracun pada manusia pada konsentrasi rendah(Bindu et al., 2015). Mereka dicirikan oleh efek antioksidan dan antibakteri yang kuat karena molekul bioaktif pada permukaan luar nanopartikel perak(Keshari, 2020).

Bakteri Penghasil Extended Spectrum β-Lactamase (ESBL) adalah jenis bakteri yang menunjukkan resistensi terhadap beberapa jenis antibiotik melalui hidrolisis cincin b-laktam antibiotik (Kizilca et al., 2012) dan mereka dapat mentransfer resistensi terhadap penisilin, sefalosporin generasi ketiga, dan monobaktam (Ejaz et al., 2011). Selain itu, sebagian besar tidak dihambat oleh antibiotik non-b-laktam lainnya karena gen pengkode resistensi dari kelas antibiotik lain juga dapat dibawa oleh plasmid yang mengandung gen pengkode ESBL.(Alyamani et al., 2017; Fan et al., 2014). Escherichia coli dan *Klebsiella* spp. tetap menjadi mikroorganisme penghasil ESBL utama yang diisolasi di seluruh dunia. Mereka dianggap sebagai Extended-spectrum β-lactamase (ESBL) yang paling muncul dengan efek yang serius pada komunitas(Devrim et al., 2011). Enzim tipe CTX-M adalah kelompok ESBL terbesar yang menyebar secara global, diikuti oleh kelompok TEM dan SHV(Sukmawinata dkk, 2020). Peneliti dari berbagai negara telah berhasil mengisolasi dan mengidentifikasi bakteri penghasil ESBL dari berbagai jenis sampel, termasuk isolat dari manusia di Bahrain.(Shahid et al., 2014), sampel yang berbeda (mis., usapan tinja, pakan ternak, air, dan kotoran) dari hewan yang berbeda (mis., anjing, kucing, domba,

kambing, ayam, kalkun, bebek, dan manusia, (Okapara dkk., 2018), mengisolasi sampel urin dari anjing dan kucing di AS dan Swiss (Thungrat et al., 2015; Zogg et al., 2018), juga mengisolasi usapan feses dari anjing dan kucing di Newzeland (Karkaba dkk., 2019). Nanopartikel dikenal terutama oleh aksi langsungnya pada dinding sel bakteri yang berbeda dari mekanisme resistensi antibiotik lain dan tidak memerlukan penetrasi sel bakteri (Wang et al., 2019). Selanjutnya, mereka kurang cenderung mengembangkan resistansi, dibandingkan dengan antibiotik. AgNps telah membuktikan aktivitas antibakteri dan antioksidan yang baik terhadap isolat *E. coli* dan *Klebsiella* pneumoniae.(Khan et al., 2020). Selain itu, telah dibuktikan bahwa AgNPs memiliki aksi toksik yang kuat pada gen *Klebsiella* Pneumoniae yang resisten terhadap ampisilin dan protein bakteri serta kerusakan membran bakteri dan stres oksidatif.(Hamida dkk., 2020). Oleh karena itu, penelitian ini bertujuan untuk mengetahui efek penghambatan nanopartikel perak terhadap *E. coli* dan *Klebsiella* spp penghasil ESBL. in vitro serta pengaruhnya terhadap ekspresi gen resistensi. Identifikasi fenotipik dan molekuler ESBL menghasilkan *E. coli* dan *Klebsiella* spp. dilakukan pada sampel berbeda yang dikumpulkan dari anjing dan kucing yang tinggal di berbagai tempat di Mesir. Aktivitas bakterisidal dari nanopartikel Ag dinilai melalui teknik mikrobiologi dan molekuler yang berbeda.

BAHAN DAN METODE

Persetujuan etis

Penelitian dilakukan sesuai dengan pedoman etika yang disetujui oleh Fakultas Kedokteran Hewan, Universitas Kairo. Tidak ada eksperimen yang diterapkan pada peserta manusia.

Pengumpulan dan persiapan sampel

Sebanyak 120 sampel dikumpulkan dari anjing yang sakit (n = 55) dan kucing (n = 65). Semua sampel dikumpulkan sesuai dengan pedoman dari Komite Perawatan dan Penggunaan Hewan Institusional di Universitas Kairo dan disetujui oleh Vet-CU-IACUC (Vet CU 16072020198), Kairo, Mesir. Persetujuan tertulis diperoleh dari pemilik hewan setelah mereka diberitahu tentang penggunaan sampel hewan mereka dalam penelitian. Sampel termasuk usap feses (n = 61), usap luka (n = 17), dan sampel urin (n = 42). Pengambilan sampel dilakukan di Rumah Sakit Hewan Al-Shaab, Departemen Bedah dan Kedokteran di Fakultas Kedokteran Hewan Universitas Kairo serta laboratorium hewan di Kairo dan Giza dari bulan Maret hingga Desember 2019. Pengambilan sampel dari hewan yang menderita gangguan saluran cerna, dan akut. atau sistitis kronis. Tambahan,(Huber et al., 2013). Semua sampel yang diselidiki dikumpulkan dalam kondisi aseptik dan tindakan pencegahan keamanan. Sampel langsung diinokulasi ke dalam 9 ml larutan garam fisiologis steril(Okapara dkk., 2018)**.** Sampel urin dikumpulkan dari setiap kasus melalui kateter dan urin dikumpulkan dari distal bagian dalam kondisi aseptik dengan pengumpulan sampel urin aliran tengah (Cystocentesis) seperti yang dilaporkan oleh Huber dkk. (2013). Sampel diberi label yang tepat dan diangkut tanpa penundaan ke laboratorium dan segera diproses.

Karakterisasi Fenotipik dan Pengujian Antibiogram untuk *E. coli* dan *Klebsiella* spp. mengisolasi

Usap luka dan feses diinokulasi ke MacConkey agar (Oxoid) ditambah dengan ampisilin (100 mg / L; Mac-AMP100, Oxoid) sesuai dengan Okapara dkk. (2018). Sampel urin disentrifugasi, dan sedimen diinokulasi langsung pada agar MacConkey (Oxoid). Semua pelat yang diinokulasi diinkubasi pada suhu 37˚c selama 18-24 jam dan diperiksa untuk pertumbuhan bakteri. Koloni fermentor laktosa dan koloni fermentasi laktosa akhir dipilih untuk pemeriksaan lebih lanjut. Isolat yang telah dimurnikan akhirnya dikonfirmasi secara biokimia dengan uji sitrat, oksidase, indol, katalase, Voges Proskauer, metil merah, urease, dan triple sugar iron (TSI) menurutCruickshank dkk. (1975).Semua isolat diuji kerentanannya terhadap obat antimikroba dan antibiotik yang berbeda (Tabel 1). Uji kepekaan antimikroba dilakukan dengan metode difusi cakram (metode Kibry-Bauer) pada pelat agar Muller-Hinton (Oksoid) dan interpretasi dilakukan berdasarkanCLSI (2018).

Tes sinergi cakram ganda

Produksi ESBL diidentifikasi menggunakan Double Disk Synergy Test (DDST) menurut Iqbal dkk. (2017). Tiga antibiotik digunakan untuk DDST ceftriaxone (30µg), Amoxicillin-clavulanic acid (20 / 10µg), dan ceftazidime (30µg, Oxoid). Disk ditempatkan pada jarak 1,5 cm. Organisme ESBL positif menunjukkan perkembangan zona hambat menuju cakram klavulanat pada suhu 37 ° C setelah inkubasi 24 jam.

Karakterisasi molekuler dari gen ESBL pada *E. coli* dan *Klebsiella* spp. mengisolasi

Ekstraksi DNA dilakukan dengan menggunakan instruksi QIAmp DNA Mini Kit (QIAGEN, Jerman). Primer khusus digunakan untuk amplifikasi blaTEM, blaSHV dan gen blaCTX (Tabel 2). Penyusunan PCR Master Mix dilakukan sesuai dengan Emerald Amp GT PCR Master Mix (Takara). Campuran reaksi terdiri dari 12,5µl Emerald Amp GT PCR Master Mix (2x premix), 4,5 µl air kadar PCR, 1µl masing-masing primer dalam konsentrasi dari (20 pmol), 6µl DNA Template, dan mengarah ke total 25µl. Kondisi bersepeda seperti dulu(Hasman et al., 2005). Tangga dicampur secara perlahan dengan memipet ke atas dan ke bawah, dan 6 µl tangga yang dibutuhkan adalah langsung dimuat. Produk PCR diselesaikan dengan elektroforesis pada gel agarosa sesuai denganSambrook dkk. (1989) dengan beberapa modifikasi.

Tabel 1. Antibiotik digunakan dalam uji kepekaan antimikroba dan pola resistensinya terhadap *E. coli* dan *Klebsiella* spp. mengisolasi

Antibiotika	Cakram kandungan	Perlawanan pola *E. coli*			Pola resistensi *Klebsiella* spp.		
		Peka (%)	Menengah (%)	Tahan (%)	Peka (%)	Menengah (%)	Tahan (%)
Aminoglikosida							
Amikacin	30 µg	89	5	6	86	6	8
Gentamycin	10 µg	53	18	29	72	0	28
Streptomisin	10 µg	47	0	53	28	20	52
Kanamycin	10 µg	31	13	56	60	12	28
Sefalosporin (1st generasi)							
Cephalexin	30 µg	8	0	92	18	6	76
Sefalosporin (3rd generasi)							
Cefotaxime	30 µg	9	4	87	52	0	48
Ceftazidime	30 µg	19	0	81	36	4	60
Ceftriaxone	30 µg	23	6	71	38	22	40
Antibiotik lain-lain							
Kloramfenikol	10 µg	49	27	34	45	31	24
Nitrofurinasi	300 µg	69	13	18	58	24	28
B laktam lainnya							
Aztreonam	30 µg	41	6	53	66	10	24
Penisilin							
Ampisilin	10 µg	0	0	100	0	0	100
Amoksisilin Asam klavulanat	(20/10) µg	37	0	63	32	8	60
Kuinolon							
Ciprofloxacin	5 µg	71	11	18	88	0	12
Asam nalidixic	30 µg	68	3	29	92	0	8
Tetrasiklin							
Tetrasiklin	30 µg	34	6	60	42	22	36

Tabel 2. Primer dan probe oligonukleotida yang digunakan dalam PCR dan SYBR Green real-time PCR

Gen	Urutan primer (5'-3 ')	Amplifikasi ukuran	Referensi
***bla*$_{TEM}$**	ATCAGCAATAAACCAGC	516 bp	Colom dkk. (2003)
	CCCCGAAGAACGTTTTC		
***bla*$_{SHV}$**	AGGATTGACTGCCTTTTTG	392 bp	
	ATTTGCTGATTTCGCTCG		
blaCTX	ATG TGC AGY ACC AGT AAR GTK ATG GC	593 bp	Archambault et Al. (2006)
	TGG GTR AAR TAR GTS ACC AGA AYC AGC GG		
***gyrA (Klebsiella* spp.)**	CGC GTA CTA TAC GCC ATG AAC GTA	-	Brisse dan Verhoef (2001)
	ACC GTT GAT CAC TTC GGT CAG G		
16S rRNA (E. coli)	GCTGACGAGTGGCGGACGGG	-	Tivendale dkk. (2004)
	TAGGAGTCTGGACCGTGTCT		

Penilaian in vitro dari efek antibakteri suspensi Ag NPs

Bubuk nanopartikel perak dibeli dari National Research Center, Mesir. Proses dispersi dilakukan dengan menggunakan prosesor ultrasonik (instrumen Cole-Parmer, Illinois USA). Untuk preparasi nanofluida, 10 mg Ag NP dengan ukuran rata-rata 58 nm disebarkan dalam 1ml kaldu Muller Hinton steril dan disonikasi selama 5 menit pada frekuensi 20000 HZ dari 3 hingga 5 kali untuk menghindari agregasi(Tayel et al., 2010). Koloni murni dari *E. coli* dan *Klebsiella* spp. isolat diambil dan disuspensikan dalam kaldu Muller Hinton. Suspensi disesuaikan agar sesuai dengan standar McFarland 0,5 ($1{,}5\times10^{8}$CFU / ml) agar siap untuk pengujian antimikroba. Tes disesuaikan dengan CLSI (2018).Konsentrasi hambat minimum (MIC) dari AgNP ditentukan dalam pelat mikrotiter 96-sumur steril. 100 µL kaldu muller steril Hinton dipipet ke dalam sumur No. 1 sampai kolom No.12. Pada langkah selanjutnya ditambahkan 100 µL suspensi Ag NPs (10mg / ml) ke dalam sumur No. 1 kolom (A), hingga mencapai volume total 200 µL dengan

konsentrasi (5 mg / ml). Pengenceran serial dua kali lipat diaplikasikan dimulai dengan konsentrasi yang disebutkan sebelumnya. Kemudian, 100 µL suspensi *E. coli* (McFarland 0.5) ditambahkan ke dalam kolom sumur, kemudian konsentrasi awal diubah menjadi 2.5 mg / ml. Sumur No. 11 berfungsi sebagai pertumbuhan negatif (kontrol sterilitas) yang mengandung suspensi Ag NP ditambah kaldu steril saja sedangkan sumur No. 12 berfungsi sebagai kontrol bakteri positif yang mengandung kaldu ditambah inokulum bakteri saja. Langkah-langkah ini diulangi dengan *Klebsiella* spp. di piring mikrotiter lain. Plat mikrotiter diinkubasi pada suhu 37 ° C selama 24 jam dalam inkubator shaker. Konsentrasi Ag NPs terendah dalam seri penghambat pertumbuhan bakteri in vitro diambil sebagai MIC. Untuk penentuan MBC, 50 µL dari masing-masing sumur disebarkan pada piring agar MacConkey dan diinkubasi pada suhu 37 ° C selama 48 jam. Pelat bebas pertumbuhan memvalidasi bahwa konsentrasi yang digunakan menghambat pertumbuhan bakteri. Tes ini dilakukan dalam rangkap tiga. 50 µL dari masing-masing sumur disebarkan pada piring agar MacConkey dan diinkubasi pada suhu 37 ° C selama 48 jam. Pelat bebas pertumbuhan memvalidasi bahwa konsentrasi yang digunakan menghambat pertumbuhan bakteri. Tes ini dilakukan dalam rangkap tiga. 50 µL dari masing-masing sumur disebarkan pada piring agar MacConkey dan diinkubasi pada suhu 37 ° C selama 48 jam. Pelat bebas pertumbuhan memvalidasi bahwa konsentrasi yang digunakan menghambat pertumbuhan bakteri. Tes ini dilakukan dalam rangkap tiga.

Penilaian efek penghambatan AgNPs pada ekspresi gen resistensi di *E. coli* dan *Klebsiella* spp. menggunakan SYBR Green RT- PCR

Pengaruh dosis sub-MIC (1/2) dari AgNPs pada ekspresi gen blaTEM, blaSHV, dan blaCTX dipelajari dengan adanya 16s rRNA untuk *E. coli* dan gyrA untuk *Klebsiella* spp. sebagai gen rumah tangga(Brisse dan Verhoef, 2001; Tivendal et al., 2004).Prosedur RT-PCR dilakukan di Laboratorium Referensi Kualitas Veteriner Pengendalian Produksi Unggas, Institut Penelitian Kesehatan Hewan, Mesir. Ekstraksi RNA dilakukan sesuai dengan instruksi RNeasy Mini Kit (QIAGEN, Jerman, GmbH). Primer dan probe oligonukleotida yang digunakan dalam PCR real-time SYBR Green ditunjukkan pada Tabel 2. PCR Master Mix QuantiTect SYBR Green PCR Kit digunakan. Campuran reaksi terdiri dari 12.5µl 2x QuantiTect SYBR Green PCR Master Mix, 0.25µl Revert Aid Reverse Transcriptase (ThermoFisher, 200 U / µL), 0.5µl setiap primer (20 pmol), 8.25µl RNase Free Water, 3µl Template RNA, mengarah ke total 25µl. Kondisi bersepeda dilakukan menurut penelitian sebelumnya(Brisse dan Verhoef, 2001; Colom dkk., 2003; Tivendale et al., 2004; Archambault et al., 2006)**.** Kurva amplifikasi dan nilai CT ditentukan dengan software strata gene MX3005P. Untuk memperkirakan variasi ekspresi gen dari sampel yang berbeda, CT dari setiap sampel dibandingkan dengan kelompok kontrol menurut "CT metode ΔΔCt" yang dinyatakan olehYuan dkk. (2006)dan sampel diuji dalam rangkap tiga. Kurva disosiasi dari sampel yang berbeda dibandingkan untuk mengecualikan hasil positif palsu.

Evaluasi perubahan morfologi pada *E. coli* dan *Klebsiella* spp. atas interaksi mereka dengan nanopartikel perak

Sampel termasuk sampel yang tidak diobati (kontrol) dan AgNPs yang diobati *E. coli* dan *Klebsiella* spp. koloni. Sampel difiksasi dengan glutaraldehyde 2,5% dan didehidrasi dengan pengenceran etanol serial dengan agitasi menggunakan prosesor jaringan otomatis (Leica EM TP, Leica Microsystems: Austria). Langkah selanjutnya dikeringkan menggunakan CO_2titik kritis pengering (Model: Audosamdri-815, Tousimis; Rockville, Maryland, USA). Sampel dilapisi dengan sputter coater emas (SPI-Module, USA). Mereka diperiksa dengan Scanning electron microscopy (Model: JSM-5500 LV; JEOL Ltd -Japan) menggunakan mode vakum tinggi di Pusat Regional Mikologi dan Bioteknologi, Kairo, Mesir.

Analisis statistik

Analisis statistik dilakukan dengan menggunakan R-program. ANOVA satu arah dijalankan untuk mengevaluasi signifikansi statistik antara sampel kontrol dan sampel yang dirawat. Nilai P kurang dari 0,05 dianggap signifikan secara statistik.

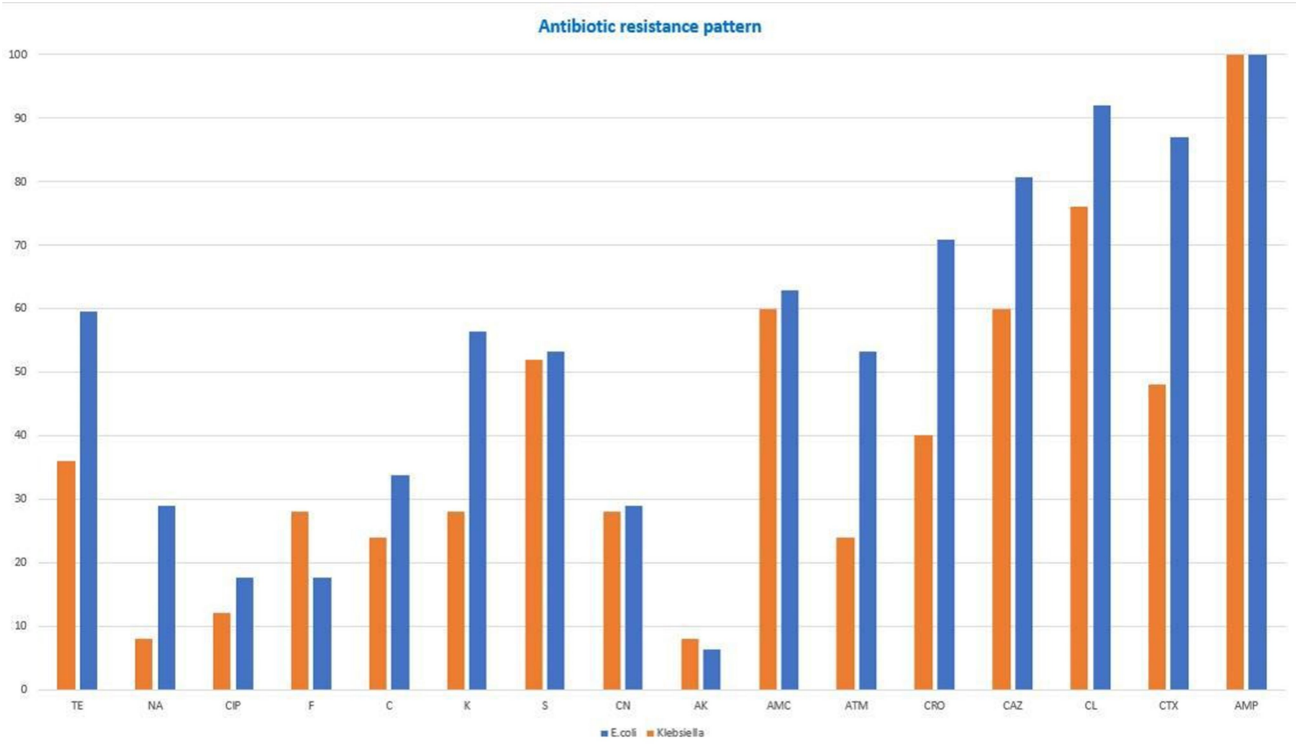
HASIL

Identifikasi fenotipik *E. coli* dan *Klebsiella* spp. mengisolasi

Dari 120 sampel, *E. coli* dan *Klebsiella* spp. terdeteksi di 62 (51,6%) dan 25 (20,8%) kasus, masing-masing. Pada agar MacConkey, *E. coli* muncul sebagai koloni berukuran sedang, halus, bulat, berfermentasi laktosa, merah jambu hingga merah dengan endapan garam empedu yang mengelilingi koloni. Di sisi lain, *Klebsiella* spp. diamati sebagai koloni berukuran sedang, merah muda, berfermentasi laktosa, bulat, berkilau, dan berlendir. Pemeriksaan mikroskopis koloni murni *E. coli* dan *Klebsiella* spp. isolat adalah bakteri berbentuk batang gram negatif. Isolat dikonfirmasi secara biokimia. *E. coli* negatif dalam urease, oksidase, Voges Proskauer (vp), uji sitrat sedangkan positif untuk katalase, metil merah (MR), uji Indole dan A / A dengan produksi gas dan H negatif$_2$Produksi S untuk uji TSI. *Klebsiella* spp. positif pada urease, oksidase, Voges Proskauer, uji katalase dan sitrat dan A / A dengan produksi gas dan indol negatif, Metil merah, dan H_2Produksi S untuk uji TSI.

Pengujian kerentanan antimikroba

Menggunakan kelompok antibiotik yang berbeda (Tabel 1), antibiogram dinilai untuk *E. coli* dan *Klebsiella* spp. mengisolasi. Ampisilin menunjukkan persentase resistensi tertinggi pada *E. coli* dan *Klebsiella* spp. seperti yang ditunjukkan pada pola resistensi (Gambar 1). Kelompok pola resistensi yang paling menonjol adalah Cephalosporin generasi pertama dan ketiga diwakili oleh Cephalexin (92%, 76%), Cefotaxime (87%, 48%), Ceftazidime (81%, 60%), Ceftriaxone (71%, 40%) untuk *E. coli* dan *Klebsiella* spp., masing-masing. Selain itu, asam klavulanat Amoksisilin menunjukkan resistensi 63% terhadap isolat *E. coli* dan 60% untuk *Klebsiella* spp. Hasil keseluruhan dari antibiogram menunjukkan bahwa sekitar 29 isolat (*E. coli* = 20, *Klebsiella* spp. = 9) diduga sebagai isolat penghasil ESBL.



Gambar 1. Pola resistensi antibiotik penggunaan antibiotik terhadap *E. coli* dan *Klebsiella* spp. mengisolasi

Metode uji sinergi cakram ganda

Deteksi isolat yang dicurigai dengan DDST menunjukkan bahwa 23 isolat (*E. coli* = 17, *Klebsiella* = 6) merupakan isolat penghasil ESBL. Banyaknya isolat penghasil ESBL *E. coli* dan *Klebsiella* spp. untuk setiap jenis sampel ditunjukkan pada Tabel 3.

Deteksi molekuler gen penyandi ESBL

Skrining PCR dari gen yang mengkode ESBL mengungkapkan adanya gen blaCTX, blaSHV, dan blaTEM di semua isolat yang diuji kecuali satu *Klebsiella* spp. isolat yang tidak mengandung gen blaCTX (Gambar 2).

Evaluasi in vitro dari efek antibakteri dari Ag NPs

Scanning Electron Microscopy dari AgNPs (Gambar 3) mengungkapkan bahwa Ag NPs berbentuk bola dengan ukuran rata-rata 58 nm. MIC AgNPs yang diuji untuk *E. coli* penghasil ESBL adalah 0,31 mg / ml, dan 0,62 mg / ml untuk *Klebsiella* spp penghasil ESBL. Konsentrasi bakterisidal minimum (MBC) dari *E. coli* penghasil ESBL dan *Klebsiella* spp. adalah 0,15 mg / ml dan 0,3 mg / ml.

Pengaruh konsentrasi sub MIC AgNPs pada ekspresi gen resistensi.

Ekspresi gen blaTEM, blaSHV, dan blaCTX diturunkan regulasi dengan dosis sub-MIC AgNPs (150 µg / ml untuk *E. coli* dan 310 µg / ml untuk *Klebsiella* spp.), Dibandingkan dengan sampel yang tidak diberi perlakuan seperti yang ditunjukkan pada (Gambar 4).

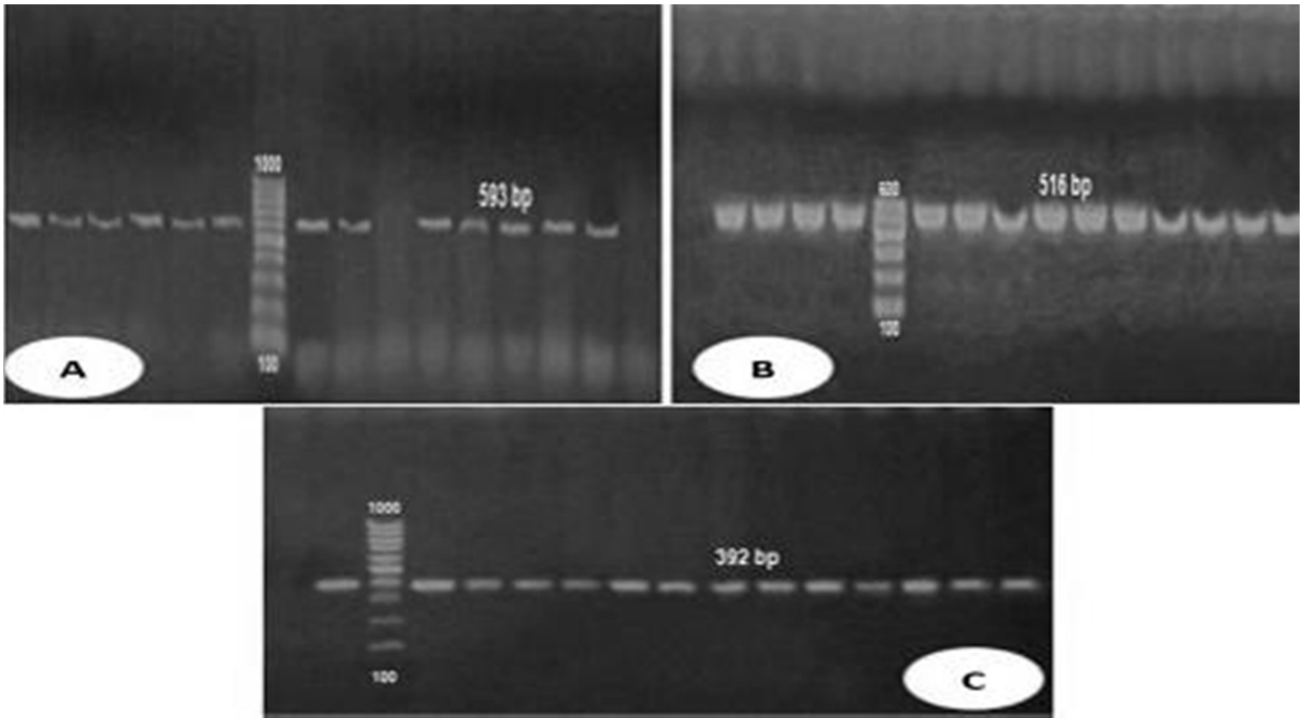
Pengaruh nanopartikel perak pada integritas sel

Citra SEM sel yang tidak diberi perlakuan (kontrol) menunjukkan sel sehat dengan membran sel bening yang belum tertembus sedangkan sel yang diberi perlakuan menunjukkan kerusakan membran sel akibat menempelnya AgNPs dengan membran sel bakteri dan penetrasi ke dalam sel sehingga menyebabkan kematian sel (Gambar 5).

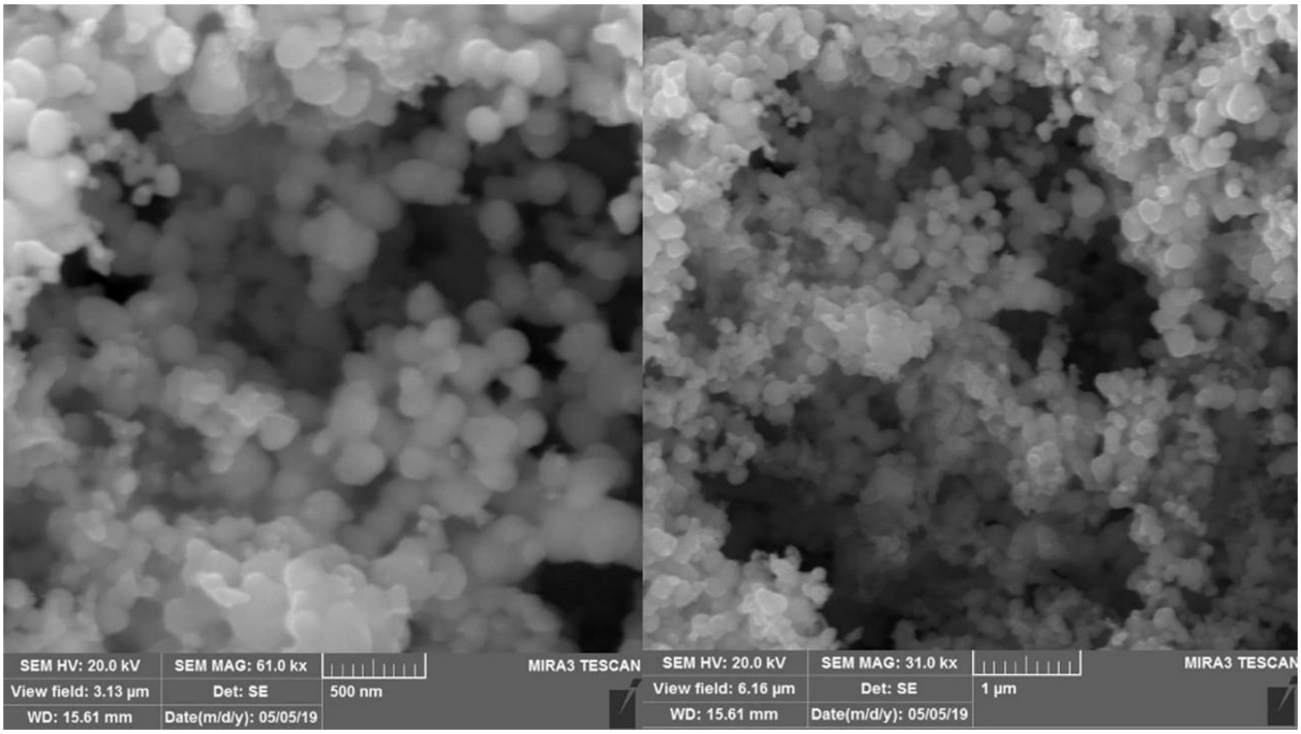
Tabel 3. Prevalensi produsen ESBL dari anjing dan kucing di Mesir

Asal	Jumlah sampel dengan produsen ESBL (%)		
	EC	KS	Total
Usap luka (n = 17)	-	2 (11,7)	2 (11,7%)
Usap tinja (n = 61)	10 (16,4%)	1 (1,6%)	11 (18%)
Sampel urin (n = 42)	7(16,7%)	3 (7,1%)	10 (23,8%)
Jumlah (n = 120)	17(14,16%)	6 (5%)	23 (19,6%)

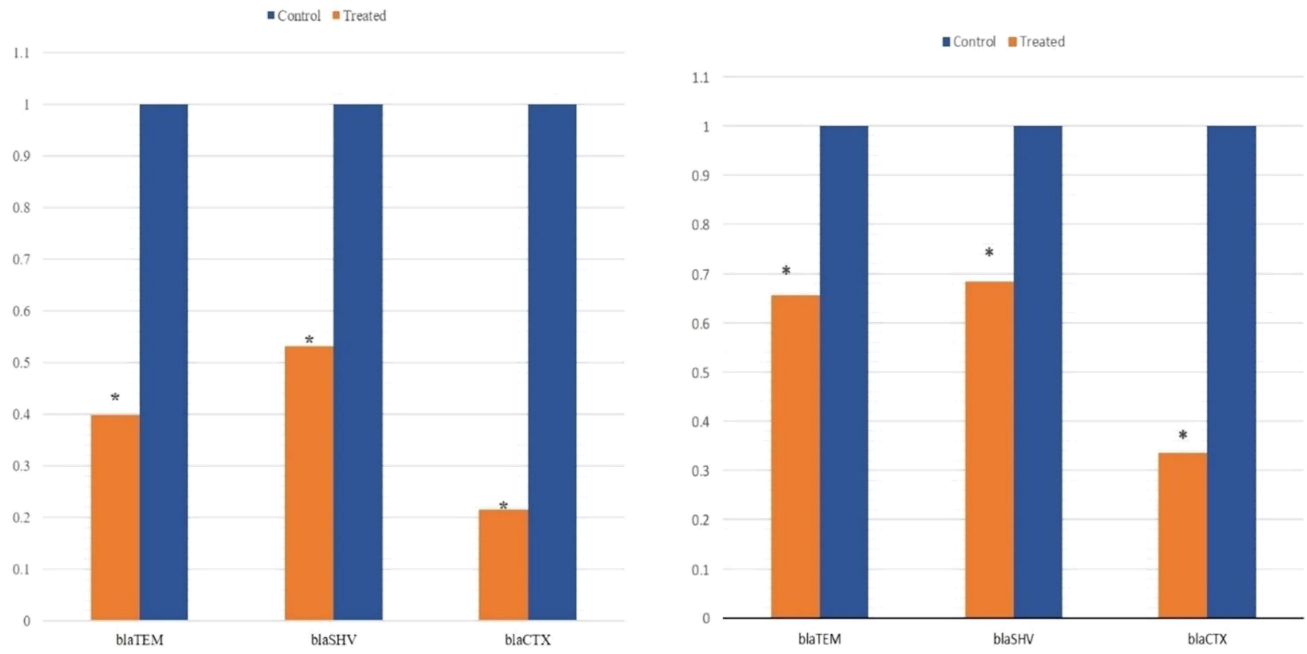
EC: Escherichia coli; KS: *Klebsiella* spp.



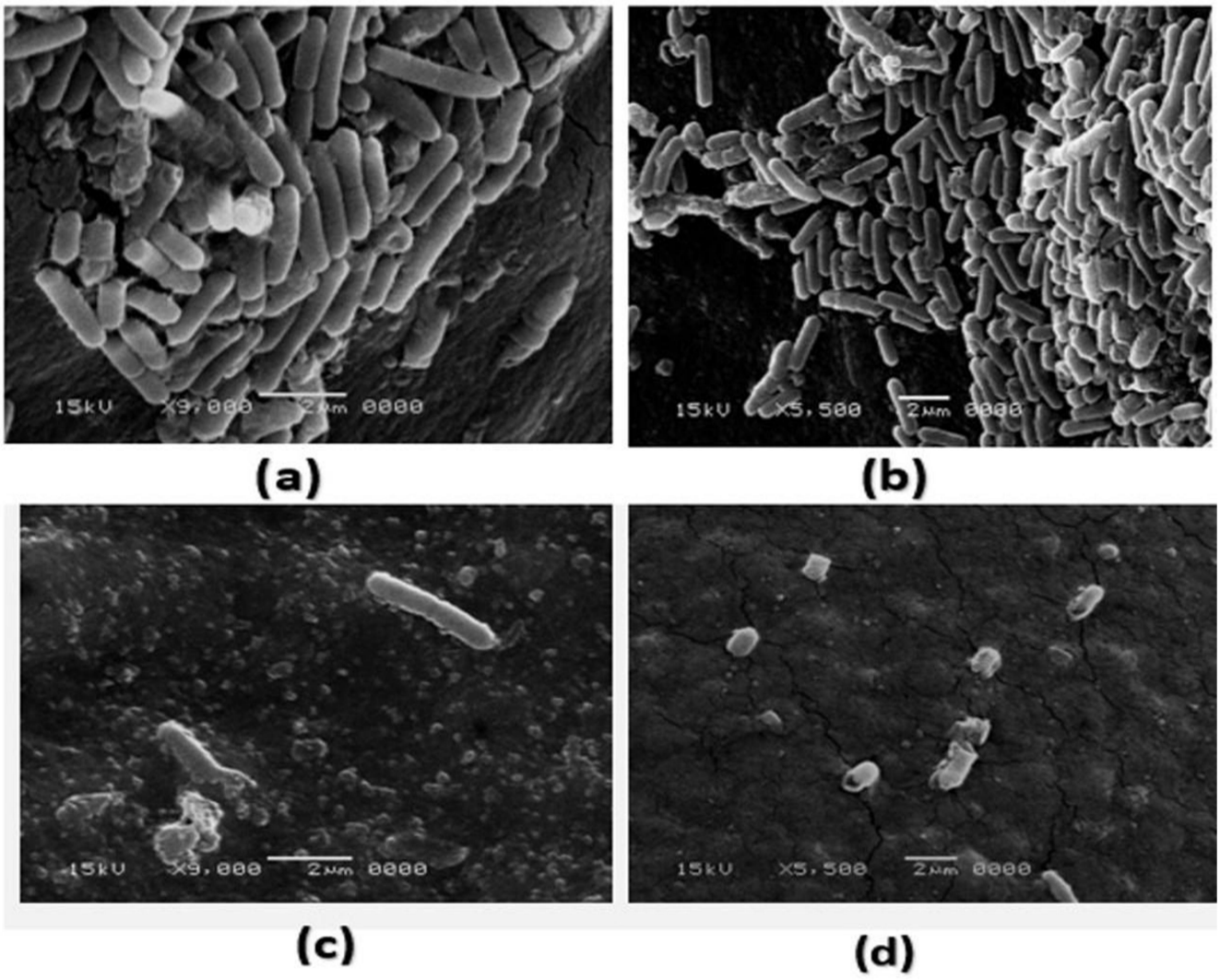
Gambar 2. Elektroforesis gel agarose dari gen A- blaCTX (ukuran Amplicon593 bp), gen B- blaTEM (ukuran Amplicon 516 bp), gen C-blaSHV (ukuran Amplicon 392 bp) Tangga [Gelpilot100 bp plus tangga (Qiagen, 100-1500 bp)]



Gambar 3. SEM dari AgNPs menunjukkan bahwa Ag NP berbentuk bulat dan rata-rata berukuran 58 nm.



Gambar 4. Pengaruh nanopartikel perak terhadap ekspresi gen resistensi antibiotik pada *E. coli* Kiri, *Klebsiella* spp Kanan. Bintang menunjukkan perbedaan yang signifikan antara sampel kontrol dan sampel yang dirawat untuk setiap gen.



Gambar 5. Pemindaian gambar mikroskopis dari AgNPs yang dirawat dan yang tidak dirawat *E. coli* dan *Klebsiella* spp. Sel kontrol menunjukkan membran sel normal, bening dan tidak tertembus (a, b). Sel yang diobati AgNps menunjukkan penetrasi membran sel dan perforasi sel yang menyebabkan ekskresi metabolit sel dan kematian sel (c, d)

DISKUSI

Meskipun terdapat berbagai agen antimikroba modern dan obat antibakteri, infeksi bakteri masih menjadi masalah utama yang mengancam karena peningkatan yang sangat besar pada bakteri yang resistan terhadap beberapa obat. Elemen genetik seluler, seperti plasmid, mampu mentransfer elemen determinan resistensi antimikroba di antara populasi bakteri yang berbeda dan memainkan peran penting dalam epidemiologi resistensi antimikroba(Carattoli, 2013). Penyalahgunaan antibiotik yang ekstensif telah menjadi penyebab utama munculnya beberapa bahaya bagi kesehatan masyarakat, seperti bakteri super, yang melawan semua obat yang ada saat ini.(Khameneh et al., 2016). Efektivitas klinis beta-laktam telah berkurang karena peningkatan besar-besaran pada bakteri resisten dan pemulihan pasien yang berkepanjangan.(Denisuik et al., 2013; Mathers et al., 2015). Beta-laktamase spektrum luas (ESBL) pada hewan peliharaan merupakan masalah yang mengancam yang telah muncul di seluruh dunia. Sejak kemunculannya, ESBL paling sering ditemukan pada Escherichia coli dan *Klebsiella* pneumonia(Bonnet 2004; Livermore dkk. 2006; Mathers dkk. 2015).Dalam penelitian ini, prevalensi produksi ESBL *Escherichia coli* dan *Klebsiella* spp. diselidiki dalam sampel berbeda yang diperoleh dari hewan peliharaan. Agar MacConkey (Oksoid) ditambah dengan Ampisilin digunakan untuk isolasi isolat penghasil ESBL yang memfasilitasi skrining isolat daripada Macconkey yang dilengkapi dengan Cephalosporine seperti yang dilaporkan olehOkapara dkk. (2018). *E. coli* dan *Klebsiella* spp penghasil ESBL. terdeteksi di 23 dari 120 sampel penelitian saat ini yang mewakili 19,6% dari total sampel. Dalam penelitian sebelumnya, K.pneumoniae penghasil ESBL tercatat 41%.(Okapara dkk., 2018) dan 7,5% (Liu et al., 2017). Penghasil ESBL terisolasi *E. coli* terdeteksi pada 17 (14,16%) sampel, 7 dari urin, dan 10 lainnya dari feses sampel. Hasil yang diperoleh dari penelitian ini menunjukkan persentase yang lebih tinggi, dibandingkan dengan *E. coli* yang diisolasi ESBL yang diperoleh dari anjing dan kucing di Swiss 8%.(Huber et al., 2013) dan Selandia Baru 6,4%, (Karkaba et al., 2019) dan lebih rendah dari yang diperoleh dari hewan peliharaan di Swiss 54,7%, (Zogg dkk., 2018) dan dari produk susu pertanian di Jerman 75,6%, (Odenthal dkk., 2016)**.** Tiga gen (yaitu TEM, SHV, dan CTX-M) adalah yang paling dominan dalam bakteri penghasil ESBL (Paterson dan Bonomo, 2005). BlaSHV dan blaTEM terdeteksi pada semua isolat sedangkan blaCTX-M ditemukan pada 92,3%.Huber dkk. (2013)menyelidiki gen ESBL dari isolat *E. coli* penghasil ESBL dimana blaCTX-M ditemukan pada 100% isolat dan blaTEM pada 87,5%. Mencari alternatif bakterisida baru yang efektif telah menjadi masalah mendesak untuk memerangi resistensi obat. Nanopartikel perak telah ditetapkan sebagai pendekatan yang menjanjikan sebagai alternatif untuk agen antimikroba dalam pengobatan beberapa masalah pengobatan.(Beyth dkk., 2015; Hassanen dan Ragab, 2020). Mereka telah menarik perhatian bersama yang besar dan telah digunakan secara luas dalam berbagai macam aplikasi sebagai agen antibakteri / antijamur dalam beragam produk, termasuk semprotan pembersih udara, bantal, respirator, tisu basah, deterjen, sabun, sampo, pasta gigi, filter udara, pelapis lemari es, penyedot debu, mesin cuci, wadah penyimpanan makanan, telepon seluler (Sun et al., 2001). Selain itu, mereka tidak menyebabkan toksisitas tingkat tinggi pada manusia serta memiliki aksi antibakteri spektrum luas(Chandran et al., 2006).

Dalam penelitian ini, AgNPs diuji terhadap *E. coli* penghasil ESBL dan *Klebsiella* spp. mengisolasi secara in vitro dengan mendeteksi MIC dan MBC.Manikprabhu dan Lingappa (2014)menentukan efek antibakteri AgNPs dengan ukuran (28-50nm) terhadap *E. coli* penghasil ESBL di mana MIC dan MBC ditemukan (dalam kisaran 0,11 dan 0,22 mg / ml). Selain itu, nanosilver biogenik (20-70nm) digunakan melawan k penghasil ESBL. pneumoniae dan *E. coli*, dimana MIC dan MBC yang tercatat masing-masing adalah 1,4µg dan 2µg(Subashini et al., 2014). Hal ini menunjukkan bahwa AgNPS mempunyai efek bakteriostatik yang baik menurut MIC dan efek bakterisidal yang baik menurut MBC terhadap *E. coli* dan *Klebsiella* spp penghasil ESBL. Seperti yang dilaporkan, ukuran nanopartikel yang kecil mempotensiasi efek antibakteri pada mikroorganisme(Smekalova dkk., 2016). Juga, AgNPs memiliki efek yang sama terhadap bakteri penghasil ESBL dan non ESBL(Ansari et al., 2014).

Perlu dicatat bahwa tidak mudah untuk membandingkan hasil yang diperoleh dari efek antibakteri AgNPs dalam penelitian saat ini dengan penelitian sebelumnya karena peneliti yang berbeda menggunakan metode yang berbeda untuk mempelajari efek antibakteri AgNP terhadap berbagai jenis bakteri. Selain itu, pengaruh AgNPs terhadap mikroorganisme dipengaruhi oleh ukuran, bentuk, stabilitas, dan konsentrasi AgNPs.(Bandyopadhyay et al., 2018). Kami menemukan bahwa konsentrasi AgNP yang digunakan untuk menghambat atau membunuh mikroorganisme berbeda satu sama lain karena nilai MIC dan MBC dari AgNPs terhadap *E. coli* lebih rendah daripada *Klebsiella* spp.

Dalam penelitian ini, SYBR Green RT-PCR digunakan untuk menyelidiki pengaruh nanopartikel perak terhadap *E. coli* dan *Klebsiella* spp penghasil ESBL. gen resistensi (blaCTX-M, blaTEM, dan blaSHV). Dosis sub-MIC (150µg) nanopartikel perak dengan ukuran rata-rata 58 nm diuji terhadap *E. coli* dan menunjukkan regulasi turun dari gen blaCTX-M, blaTEM, dan blaSHV dengan perubahan lipat masing-masing sekitar 0,21, 0,39, 0,53, untuk E . coli. Selanjutnya, dosis sub-MIC (310µg) nanopartikel perak diuji terhadap *Klebsiella* spp. dan menunjukkan penurunan regulasi ekspresi gen dengan 0,33, 0,65, 0,68 untuk gen resistensi blaCTX-M, blaTEM, dan blaSHV. Hasil ini

menunjukkan bahwa AgNPs lebih efektif mempengaruhi ekspresi gen *E. coli* (blaCTX-M, blaTEM, dan blaSHV) dibandingkan dengan *Klebsiella* spp.

Dalam penelitian ini, perubahan morfologi pada *E. coli* dan *Klebsiella* spp. sel dievaluasi sebelum dan sesudah perlakuan dengan nanopartikel perak menggunakan SEM. Pengamatan SEM dalam sel yang dirawat mengkonfirmasi membran sel

kerusakan akibat menempelnya AgNPs dengan membran sel bakteri dan penetrasi ke dalam sel menyebabkan kematian sel. Di sisi lain, sel yang tidak dirawat menunjukkan sel sehat dengan membran sel jelas yang tidak tertembus. Efek bakterisidal dari nanopartikel perak masih belum diketahui mekanismenya. Banyak penelitian menunjukkan bahwa pengikatannya pada membran sel bakteri dapat mengganggu permeabilitas sel(Kvítek dkk., 2008) sementara penelitian lain mengusulkan bahwa efek bakterisidal tidak hanya disebabkan oleh kontak dengan membran sel tetapi juga karena penetrasi ke dalam sel bakteri yang menyebabkan inaktivasi replikasi DNA dan menyebabkan kematian sel. (Morones et al., 2005).

KESIMPULAN

Dalam penelitian ini, efek antibakteri dari nanopartikel perak diselidiki secara in vitro terhadap Extended Spectrum Beta lactamase yang memproduksi *E. coli* dan *Klebsiella* spp. Temuan menunjukkan bahwa penggunaan nanopartikel perak sebagai alternatif agen antimikroba memiliki efek yang jelas pada konsentrasi hambat minimum, konsentrasi bakterisidal minimum, integritas dinding sel bakteri serta secara genetik pada ekspresi gen resistensi antibiotik. Percobaan ini sangat menggembirakan untuk pengendalian bakteri yang kebal antibiotik.

DEKLARASI

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REFERENSI

Alyamani EJ, Khiyami AM, Booq RY, Majrashi MA, Bahwerth FS, and Rechkina E (2017). The occurrence of ESBL-producing *Escherichia coli* carrying aminoglycoside resistance genes in urinary tract infections in Saudi Arabia. Annals of Clinical Microbiology and Antimicrobials, 16(1):DOI: https://www.doi.org/10.1186/s12941-016-0177-6

Ansari MA, Khan HM, Khan AA, Cameotra SS, Saquib Q, and Musarrat J (2014). Interaction of A l2 O 3 nanoparticles with E scherichia coli and their cell envelope biomolecules. Journal of applied microbiology, 116(4): 772-783. DOI: https://www.doi.org/10.1111/jam.12423

Archambault M, Petrov P, Hendriksen RS, Asseva G, Bangtrakulnonth A, Hasman H, and Aarestrup FM (2006). Molecular characterization and occurrence of extended-spectrum beta-lactamase resistance genes among Salmonella enterica serovar Corvallis Thailand, Bulgaria, and Denmark. Microbial Drug Resistance, 12(3): 192-198. DOI: https://www.doi.org/10.1089/mdr.2006.12.192

Bandyopadhyay S, Banerjee J, Bhattacharyya D, Samanta I, Mahanti A, Dutta TK, Ghosh S, Nanda PK, Dandapat P and Bandyopadhyay S (2018). Genomic identity of fluoroquinolone-resistant bla CTX-M-15-Type ESBL and pMAmpC β-lactamase producing *Klebsiella pneumoniae* from buffalo milk, India. Microbial Drug Resistance, 1: 24(9): 1345-1353. DOI: https://doi.org/10.1089/mdr.2017.0368

Beyth N, Houri-Haddad Y, Domb A, Khan W, and Hazan R (2015). Alternative antimicrobial approach: nano-antimicrobial materials. *Evidence-Based Complementary* and *Alternative Medicine*, 2015: 246012. DOI: http://dx.doi.org/10.1155/2015/246012

Bindu D, Mythili S, Inthira KS, palanisamy R, Lingesh KT, and Thandapani S (2015). New Generation of Silver Nanoparticles against Extended Spectrum Beta Lactamase Producing Organisms. International Journal of Drug Development & Research, 7(3): 001-003. Available at: https://www.ijddr.in/drug-development/new-generation-of-silver-nanoparticles-against-extended-spectrum-betalactamase-producing-organisms.pdf

Brisse S, and Verhoef J (2001). Phylogenetic diversity of *Klebsiella pneumonia* and *Klebsiella oxytoca* clinical isolates revealed by randomly amplified polymorphic DNA, gyrA and parC gene sequencing and automated ribotyping. *International Journal* of Systematic and Evolutionary *Microbiology*, 51: 915-924.DOI:: https://www.doi.org/10.1099/00207713-51-3-915

Carattoli A. Plasmids and the spread of resistance(2013) . International Journal of Medical Microbiology 1;303(6-7):298-304. DOI :https://doi.org/10.1016/j.ijmm.2013.02.001

Chandran SP, Chaudhary M, Pasricha R, Ahmad A, and Sastry M (2006). Synthesis of gold nanotriangles and silver nanoparticles using Aloe vera plant extract. Biotechnology Progress, 22: 577-583. DOI: https://www.doi.org/10.1021/bp0501423

Clinical and Laboratory Standards Institute (CLSI) (2018). Performance standards for antimicrobial susceptibility testing. 27th Informational Supplement Document M100- S27, CLSI, Wayne, 37(1). Available at :https://clsi.org/media/1469/m100s27_sample.pdf

Colom K, PèrezJ, Alonso R, Fernández-Aranguiz A, Lariño E, and Cisterna R (2003). Simple and reliable multiplex PCR assay for detection of blaTEM,blaSHV and blaOXA-1 genes in Enterobacteriaceae. FEMS Microbiology Letters, 223: 147-151. DOI:https://www.doi.org/10.1016/S0378-1097(03)00306-9

Cruickshank R, Duguid RP, Marmion BP, and Swain RH (1975). Medical Microbiology.New York: Churchill Livingstone, Available at: https://agris.fao.org/agris-search/search.do?recordID=XF2015010062

Denisuik AJ, Lagacé-Wiens PR, Pitout JD, Mulvey MR, Simner PJ, Tailor F, Karlowsky JA, Hoban DJ, Adam HJ, and Zhanel GG (2013), Canadian Antimicrobial Resistance Alliance (CARA). Molecular epidemiology of extended-spectrum β-lactamase-, AmpC β-lactamase-and carbapenemase-producing *Escherichia coli* and *Klebsiella pneumoniae* isolated from Canadian hospitals over a 5 year period: CANWARD 2007–11. Journal of Antimicrobial Chemotherapy, 1: 68(suppl_1): 57-65. DOI: https://doi.org/10.1093/jac/dkt027

Devrim I, Gulfidan G, Gunay I, Agin H, Güven B, Yilmazer MM, and Dizdarer C (2011). Comparison of in vitro activity of ertapenemwith other carbapenems against extended- spectrum beta-lactamase-producing Escherichia coli and Kleibsella species isolated in a tertiary children's hospital. *Expert Opinion* on *Pharmacotherapy*, 12: 845–849. DOI: https://www.doi.org/10.1517/14656566.2011.559460

Ejaz H, Zafa A, Mahmood S, and Javed MM (2011). Urinary tract infections caused by extended spectrum β-lactamase (ESBL) producing *Escherichia coli* and *Klebsiella pneumoniae*. African Journal of Biotechnology, 10(73): 16661-16666. DOI https://www.doi.org/10.5897/AJB11.2449

Fan NC, Chen HH , Chen CL Ou LS, Lin TY, Tsai MH and Chiu CH (2014). Rise of community-onset urinary tract infection caused by extended-spectrum β-lactamase-producing *Escherichia coli* in children. Journal of microbiology,Immunology and Infection 47(5): 399-405. DOI: https://www.doi.org/10.1016/j.jmii.2013.05.006

Hamida RS, Ali MA, Goda DA, Khalil MI, and Redhwan A (2020). Cytotoxic effect of green silver nanoparticles against ampicillin resistant *Klebsiella pneumoniae*. Royal Socitey of Chemistry Advances, 10: 21136-21146. DOI: https://www.doi.org/10.1039/D0RA03580G

Hasman H, Mevius D, Veldman K, Olesen I and Aarestrup FM (2005). β-Lactamases among extended-spectrum β-lactamase (ESBL)-resistant Salmonella from poultry, poultry products and human patients in The Netherlands. Journal of Antimicrobial Chemotherapy. 1;56(1):115-21. Doi: https://doi.org/10.1093/jac/dki190

Hassanen EI, and Ragab E (2020). In Vivo and In Vitro Assessments of the Antibacterial Potential of Chitosan-Silver Nanocomposite Against Methicillin-Resistant Staphylococcus aureus–Induced Infection in Rats. Biological Trace Element Research, Apr 18: 1-4. DOI: https: www.doi.org/10.1007/s12011-020-02143-6

Huber H, Zweifel C, Wittenbrink MM, and Stephan R (2013). ESBL-producing uropathogenic Escherichia coli isolated from dogs and cats in Switzerland.Veterinary Microbiology, 162: 992-996. DOI: https://www.doi.org/10.1016/j.vetmic.2012.10.029.

Iqbal R, Ikram N, Shoaib M, Asad MJ, Tahir R, Mehmood, Niazi A, Asghar A, Bushra Ishfaq and Faiza Naseer (2017). Phenotypic cofirmatory disc diffusion test (PCDDT), double disc synergy test (DDST), E-test OS diagnostic tool for detection of extended spectrum beta lactamase (ESBL)

producing Uropathogens. Journal of Applied Biotechnology and Bioengineering, 3(3): 344–349 DOI: https://www. doi.org/10.15406/jabb.2017.03.00068

Karkaba A, Hillb K, Benschop J, Pleydellc E, and Grinberg A (2019). Carriage and population genetics of extended spectrum β -lactamaseproducing

Escherichia coli in cats and dogs in New Zealand.Veterinary Microbiology, 233: 61-67. DOI:https://www.doi.org/10.1016/j.vetmic.2019.04.015.

Keshari AK, Srivastava R, Singh P, Yadav B, and Nath G (2020). Antioxidant and antibacterial activity of silver nanoparticles synthesized by Cestrum nocturnum. Journal of Ayurveda and Integrative Medicine, 11(1): 37-44. DOI: https://www.doi.org/10.1016/j.jaim.2017.11.003

Khameneh B, Diab R, Ghazvini K, and Fazly Bazzaz BS (2016). Breakthroughs in bacterial resistance mechanisms and the potential ways to combat them. Microbial Pathogenesis, 95: 32–42. DOI: https://www.doi.org/10.1016/j.micpath.2016.02.009

Khan T, Yasmin A, and Townley HE (2020). An evaluation of the activity of biologically synthesized silver nanoparticles against bacteria, fungi and mammalian cell lines. Colloids and surfaces B: Biointerfaces, 194: 111156. DOI: https://www.doi.org/10.1016/j.colsurfb.2020.111156

Kizilca O, Siraneci R, and Yilmaz A (2012). Risk factors for community-acquired urinary tract infection caused by ESBL-producing bacteria in children. Pediatrics *International*, 54(6): 858-862. DOI: https://www.doi.org/10.1111/j.1442-200X.2012.03709.x

Kvítek L, Panáček A, Soukupova J, Kolář M, Večeřová R, Prucek R, Holecova M, and Zbořil R (2008). Effect of surfactants and polymers on stability and antibacterial activity of silver nanoparticles (NPs). The Journal of Physical Chemistry C, 17: 112(15): 5825-5834. DOI: https://www.doi.org/10.1021/jp711616v

Li WR, Xie XB, Shi QS, Zeng HY, OU-Yang YS, and Chen Y (2010). Antibacterial activity and mechanism of silver nanoparticles on Escherichia coli. *Applied Microbiology* and *Biotechnology*, 85: 1115-1122. DOI 10.1007/s00253-009-2159-5

Liu Y, Yang Y, Chen Y, and Xia Z (2017). Antimicrobial resistance profiles and genotypes of extended-spectrum b-lactamase- and AmpC b-lactamase-producing *Klebsiella pneumoniae* isolated from dogs in Beijing, China. Journal of Global Antimicrobial Resistance, 10: 219–222. DOI: https://www.doi.org/10.1016/j.jgar.2017.06.006

Livermore DM, Hope R, Fagan EJ, Warner M, Woodford N, and Potz N (2006). Activity of temocillin against prevalent ESBL-and AmpC-producing Enterobacteriaceae from south-east England. Journal of Antimicrobial Chemotherapy. 1:57(5):1012-4. DOI : https://doi.org/10.1093/jac/dkl043

Manikprabhu D, and Lingappa K (2014). Synthesis of silver nanoparticles using the Streptomycces Coelicolor Klmp33 pigment: An antibacterial agent against extended-spectrum beta-lactamase (ESBL) producing Escherichia Coli. Materials Science and Engineering C, 45: 434-437. DOI: https://www.doi.org/10.1016/j.msec.2014.09.034

Mathers AJ, Peirano G, and Pitout JD (2015). The role of epidemic resistance plasmids and international high-risk clones in the spread of multidrug-resistant Enterobacteriaceae. Clinical microbiology reviews. 28(3):565-591. DOI: https://www.doi.org/10.1128/CMR.00116-14

Morones JR, Elechiguerra JL, Camacho A, Holt K, Kouri JB, Ramírez JT, and Yacaman MJ (2005). The bactericidal effect of silver nanoparticles. Nanotechnology, Aug 26; 16(10): 2346. DOI: https://www.doi.org/10.1088/0957-4484/16/10/059

Odenthal S, Akineden Ö and Usleber E (2016). Extended-spectrum β-lactamase producing Enterobacteriaceae in bulk tank milk from German dairy farms. International Journal of Food Microbiology, 238: 72–78. DOI: https://www.doi.org/10.1016/j.ijfoodmicro.2016.08.036

Okapara EO, Ojo EO, Awoyomi OJ, Dipeolu MA, Oyekunle MA, and Schwarz S (2018). Antimicrobial usage and presence of extended-spectrum- β-lactamase-producing Enterobactereaceae in animal-rearing households of selected rural and peri-urban communities.Veterinary Microbiology,

218: 31-39. DOI: https://www.doi.org/10.1016/j.vetmic.2018.03.013 PatersonDL and Bonomo RA (2005). Extended_spectrumbeta_lactamases: a clinical update. Clinical Microbiology Review,18: 657-686. DOI: https://www.doi.org/10.1128/CMR.18.4.657-686.2005

Saeb ATM, Alshammari AS, Al-Brahim H, and Al-Rubeaan KA (2014). Production of silver nanoparticles with strong and stable antimicrobial activity against highly pathogenic and multidrug resistant bacteria. Scientific World Journal, 1: 704-708. DOI: https://www.doi.org/10.1155/2014/704708

Sambrook J, Fritsch EF, Maniatis T, and Sambrook-Fritsch-Maniatis (1989). Molecular Cloning: A Laboratory Manual ;[prepared for Use in the CSH Courses on the Molecular Cloning of Eukaryotic Genes]. Cold Spring Harbor Laboratory Press, Available at: https://www.cshlpress.com/pdf/sample/2013/MC4/MC4FM.pdf

Shahid M, Al-Mahmeed A, Murtadha MM, Qareeballa A, Eltahir MA, Tabbara KS, Ismaeel AY, Dar FK, Giha HA and Bindayna KM (2014). Characterization of cephalosporin-resistant clinical Enterobacteriaceae for CTX-M ESBLs in Bahrain. Asian Pacific Journal of Tropical Medicine, 7(1): S212-S216. DOI:https://www.doi.org/10.1016/s1995-7645(14)60234-0

Smekalova M, Aragon V, Panacek A, Prucek R, Zboril R and Kvitek L (2016). Enhanced antibacterial effect of antibiotics in combination with silver nanoparticles against animal pathogens. The Veterinary Journal, 209: 174-179. DOI: https://www.doi.org/10.1016/j.tvjl.2015.10.032

Subashini J, Khanna VG, and Kannabiran K (2014). Anti-ESBL activity of silver nanoparticles biosynthesized using soil Streptomyces species *Bioprocess* and *Biosystems Engineering*, 37: 999–1006. DOI: https://www.doi.org/10.1007/s00449-013-1070-8

Sukmawinata E, Uemura R, Sato W, Thu Htun M, and Sueyoshi M (2020) . Multidrug-Resistant ESBL/AmpC-Producing *Klebsiella pneumoniae* Isolated from Healthy Thoroughbred Racehorses in Japan. Animals 10(3):369. DOI: https://doi.org/10.3390/ani10030369

Sun YP, Atorngitjawat P, and Meziani MJ (2001). Preparation of silver nanoparticles via rapid expansion of water in carbon dioxide micro-emulsiuon into reductant solution. Langmuir 17: 5707-5710. DOI: https:// www.doi.org/10.1021/la0103057

Tayel AA, El-Tras WF, Moussa S, El-Baz AF, Mahrous H, Salem MF, and Brimer L (2010). Antibacterial action of zinc oxide nanoparticles against foodborne pathogens. Journal of Food Safety, 31: 211-218. DOI: https://www.doi.org/10.1111/j.1745-4565.2010.00287.x

Thungrat K, price SB, Carpeter DM, and Boothe DM (2015). Antimicrobial susceptibility patterns of clinical Escherichia coli isolates from dogs and cats in the united states:january 2008 through january 2013.Veterinary Microbiology. 179(3-4) 287-295. DOI: https://www.doi.org/10.1016/j.vetmic.2015.06.012.

Tivendale KA, Allen JL,Ginns CA, Crabb BS, and Browning GF (2004). Association of iss and iucA, but not tsh, with plasmid - mediated virulence of avian pathogenic Escherichia coli. Infection and Immunity, 72(11): 6554-6560. DOI: https://www.doi.org/10.1128/IAI.72.11.6554-6560.2004

Wang Y, Hu Y, Cao J, Bi Y, Lv N, Liu F, Liang S, Shi Y,Jiao X, Gao GF, et al. (2019). Antibiotic resistance gene reservoir in live poultry markets. Journal of infection 78(6): 11-53. DOI: https://www.doi.org/10.1016/j.jinf.2019.03.012

YuanJS, Reed A,Chen F, and Stewart CN (2006). Statistical analysis of real-time PCR data. BMC Bioinformatics, 7-85. Available at: https://bmcbioinformatics.biomedcentral.com/articles/10.1186/1471-2105-7-

Zogg AL, Zurfluha K, Schmittb S, Nüesch-Inderbinena M, and Stephana R (2018). Antimicrobial resistance, multilocus sequence types and virulence profiles of ESBL producing and non-ESBL producing uropathogenic Escherichia coli isolated from cats and dogs in Switzerland.Veterinary Microbiology, 216: 79-84. DOI: https://www.doi.org/10.1016/j.vetmic.2018.02.011.

2020, Scienceline Publication
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Studi Mikrobiologi pada Bakteri Secara Alami di Susu Unta dan Kerbau

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ABSTRAK

Tujuan dari penelitian ini adalah untuk mengisolasi dan mengidentifikasi spesies Lactobacillus probiotik alami dalam susu kerbau, susu unta, dan urin unta untuk mengetahui kerentanan mereka terhadap antibiotik dan aktivitas antibakterinya terhadap bakteri patogen. Sebanyak tujuh sampel yang terdiri dari tiga sampel susu dari kerbau, tiga sampel susu dari unta, dan satu sampel urin dari unta dikumpulkan dan digunakan dalam penelitian ini. Sampel dikultur, dan 18 strain yang diisolasi diidentifikasi dengan menggunakan analisis Reaksi Rantai Polimerase multipleks 16S rRNA, yang dilakukan setelah ekstraksi DNA dari bakteri yang diisolasi. Kerbau dan susu unta memiliki kandungan Lactobacilli yang berbeda. Semua strain Lactobacilli yang ditemukan pada susu unta dan urin unta, juga ditemukan pada susu kerbau, Strain lactobacilli dalam susu unta dan urin umumnya lebih resisten terhadap antibiotik. Lactobacilli yang diisolasi dari susu kerbau, susu unta, dan juga urine unta menunjukkan tingkat aktivitas antibakteri yang bervariasi terhadap bakteri patogen. Penelitian lebih lanjut harus dilakukan dengan lebih banyak sampel untuk mendapatkan lebih banyak informasi di bidang aktivitas antibakteri lactobacilli probiotik dan untuk memahami mekanisme aktivitasnya. Mudah-mudahan bisa digunakan sebagai alternatif alami daripada antibiotik sintetis. Penelitian lebih lanjut harus dilakukan dengan lebih banyak sampel untuk mendapatkan lebih banyak informasi di bidang aktivitas antibakteri lactobacilli probiotik dan untuk memahami mekanisme aktivitasnya. Mudah-mudahan bisa digunakan sebagai alternatif alami sebagai pengganti antibiotik sintetis. Penelitian lebih lanjut harus dilakukan dengan lebih banyak sampel untuk mendapatkan lebih banyak informasi di bidang aktivitas antibakteri lactobacilli probiotik dan untuk memahami mekanisme aktivitasnya. Mudah-mudahan bisa digunakan sebagai alternatif alami sebagai pengganti antibiotik sintetis.

Kata kunci: Antibakteri, Antibiotik, Lactobacillus, Probiotik

PENGANTAR

Resistensi antibiotik dianggap sebagai krisis kesehatan global yang mengancam kehidupan manusia dan hewan. Banyak bakteri patogen yang terisolasi secara klinis menjadi semakin resisten terhadap antibiotik dan disinfektan yang membuat infeksi bakteri ini sulit diobati. Selama evolusinya, bakteri telah mengembangkan beberapa mekanisme canggih dari resistensi antibiotik terhadap semua jenis antibiotik tanpa kecuali (Davies dan Davies, 2010). Ancaman yang berkembang dari resistensi antibiotik memerlukan penggunaan pendekatan kreatif menuju penemuan alternatif baru untuk antibiotik. Penggunaan probiotik merupakan salah satu pilihan yang sedang dibicarakan oleh komunitas medis untuk digunakan sebagai alternatif pengganti antibiotik (Brunel dan Guery, 2017).

Probiotik adalah mikroorganisme hidup yang memberikan manfaat kesehatan bagi inang setelah pemberiannya dalam jumlah yang sesuai (FAO / WHO, 2011). Keseimbangan menguntungkan dari mikrobiota usus adalah salah satu sifat peningkat kesehatan yang dapat disajikan oleh mikroorganisme probiotik. Probiotik telah diresepkan untuk pasien dengan penyakit saluran cerna dan keluhan (Williams et al., 2010). Terdapat sekumpulan bukti kumulatif yang mendukung penggunaan probiotik, baik dalam produk makanan maupun suplemen untuk memberikan perlindungan terhadap penyakit infeksi termasuk infeksi saluran pernafasan.(Hao et al., 2011; Ozen et al., 2015). Lactobacilli, Enterococci, dan Bifidobacteria adalah famili dari Lactic Acid Bacteria (BAL) dan merupakan strain probiotik yang paling sering digunakan (Fijan, 2014). BAL merupakan kelompok beragam mikroorganisme yang secara alami ada dalam makanan manusia dan di saluran pencernaan dan urogenital hewan (Ruiz Rodriguez dkk., 2019Tujuan utama dari penelitian ini adalah untuk mengisolasi dan mengidentifikasi Lactobacilli probiotik yang terjadi secara alami dalam susu kerbau serta susu unta dan urin untuk menyelidiki kerentanan mereka terhadap antibiotik serta aktivitas antibakterinya terhadap strain bakteri patogen yang representatif baik Gram-positif maupun Gram-positif. Bakteri gram negatif untuk menilai potensi penggunaannya sebagai alternatif alami untuk antibiotik sintetis.

BAHAN DAN METODE

Persetujuan etis

Komite Etika Hewan Institusional, hukum dan peraturan setempat dipertimbangkan dalam melakukan percobaan kami. Semua prosedur yang melibatkan penggunaan hewan disetujui oleh komite etika Pusat Penelitian Nasional, Mesir.

Pengumpulan sampel

Sebanyak tujuh sampel termasuk tiga sampel susu dari tiga kerbau berbeda, tiga sampel susu dari tiga unta berbeda dan satu sampel urin dari unta terpisah dikumpulkan selama musim panas 2016 dari pribadi, hewan sehat yang dimiliki secara individu di Giza, Mesir. Sampel dikumpulkan dalam kondisi aseptik dalam wadah steril dan disimpan di atas es. Lactobacillus spp. diisolasi dari sampel yang dikumpulkan dengan menggunakan media MRS sebagai media selektif. Sebanyak 1 ml sampel susu serta 1 ml sampel urin dilarutkan dalam 100 ml MRS broth (pH 6,5) dan diinkubasi pada suhu 37 ° C selama 24 jam dalam kondisi aerobik. Kultur awal disubkultur sebanyak tujuh kali pada suhu 37 ° C di bawah pH rendah (pH 4,5) dan kondisi anaerobik dengan adanya 10% CO_2 untuk menghilangkan bakteri yang tidak diinginkan. Koloni tunggal dipilih dan digoreskan pada media agar MRS pada pH 4,8 untuk mendapatkan koloni murni. Akhirnya, koloni murni tunggal Lactobacillus dipilih untuk karakterisasi dan identifikasi lebih lanjut (Shokryazdan dkk., 2014).

Karakterisasi bakteri yang diisolasi

Bakteri yang diisolasi dievaluasi dengan uji biokimia dan molekuler yang berbeda termasuk pewarnaan Gram dan uji Katalase serta morfologi bakteri. Isolat bakteri diidentifikasi sebagai Lactobacilli berdasarkan Gram-positif, Katalase-negatif dan berbentuk batang di bawah mikroskop cahaya. Identifikasi bakteri Lactobacilli yang diisolasi selanjutnya dikonfirmasi dengan menggunakan analisis 16S rRNA multiplex polymerase chain reaction (PCR).

Pewarnaan Gram

Sediaan apusan dari 24 jam bakteri yang dikultur dipanaskan dengan kaca objek. Pewarnaan Gram berdasarkan teknik standar kemudian dilakukan dan kemudian slide diamati di bawah mikroskop cahaya (Bergey et al., 1994).

Tes katalase

Biakan cair segar yang berisi kultur tumbuh semalam dari koloni tunggal terpilih digunakan untuk uji Katalase. Sejumlah larutan hidrogen peroksida 3% diteteskan ke dalam 1 ml kultur. Pembentukan gelembung gas dianggap sebagai uji Katalase positif dan sampel ini diabaikan sedangkan sampel lain dengan uji Katalase negatif dipilih karena Lactobacilli diketahui bersifat Katalase-negatif.

Identifikasi molekuler dari strain probiotik

DNA diekstraksi dari bakteri yang diisolasi dan strain Lactobacillus dikonfirmasi dengan menggunakan analisis PCR multipleks 16S rRNA (Kwon et al., 2004). Campuran reaksi (25 µl) mengandung 12,5 µl Master Mix PCR, 5 µl campuran primer yang terdiri dari masing-masing primer 50 pmol, 4,5 µl air, dan 3 µl cetakan DNA. Amplifikasi PCR dilakukan di Applied Biosystem 2720 thermal cycler, dan fragmen DNA diamplifikasi sebagai berikut. Pemanasan awal pada
94 ° C selama 2 menit, 35 siklus terdiri dari denaturasi pada 94 ° C selama 20 detik, anil pada 51 ° C selama 40 detik, ekstensi pada 68
° C selama 30 detik, dan langkah ekstensi terakhir dalam 7 menit pada 68 ° C. Produk PCR dipisahkan pada gel agarosa 1,5% dengan elektroforesis dan dianalisis dengan RedSafe Nucleic Acid Staining Solution (Intron Biotechnology, Korea).

Kerentanan antibiotik Lactobacilli

Sebuah panel lebar 14 disk antibiotik diuji terhadap 7 kultur campuran Lactobacilli probiotik yang diisolasi dari sampel kerbau dan unta (Gambar 2-8). Uji kepekaan antibiotik dilakukan dengan metode difusi cakram dengan beberapa modifikasi (ISO, 2010). Kultur yang diaktivasi lactobacilli diseka pada pelat agar MRS, bukan pada pelat Agar Muller Hinton. Empat belas disk antibiotik yang berbeda digunakan untuk uji kerentanan termasuk Trimethoprim / sulfamethoxazole (SXT-25), Ofloxacin (OFX5), Cefuroxime (CXM-30), Amoxicillin with clavulanic acid (AmC-30), Cefotaxime (CTX-30), Cefaclor (CEC-30), Rifampisin (RD-5), Eritromisin (E-15), Vankomisin (Va-30), Amikasin (AK-30), Ampisilin dengan sulbaktam (SAM-20), Cefadroxil (CFR-30), Azitromisin (AZM-15), dan Doxycycline (DO-30). Semua piring diinkubasi selama 24 jam pada 37^{Hai}C dan zona hambat diukur.

Aktivitas antibakteri Lactobacilli

Kemampuan tujuh kultur campuran Lactobacilli probiotik yang diisolasi untuk menghambat pertumbuhan bakteri patogen telah diteliti terhadap sembilan strain standar patogen baik dari bakteri Gram-positif dan Gram-negatif (Gambar 9-15). Strain Gram-positif diwakili oleh Staphylococcus aureus (ATCC 26923), Staphylococcus aureus (ATCC 29213), Staphylococcus epidermidis (ATCC 12228), Streptococcus pneumoniae (ATCC 29619), dan Enterococcus faecalis ATCC (29212). Strain gram negatif diwakili oleh Pseudomonas aeruginosa ATCC (27853), Escherichia coli ATCC (25922), Escherichia coli ATCC (10536), dan Klebsiella pneumoniae ATCC (700603). Uji aktivitas antibakteri probiotik Lactobacilli menggunakan metode difusi agar-well dengan beberapa modifikasi (Bauer et al., 1966; Sgouras et al., 2004). Sumur dengan diameter 7 mm dibuat pada pelat agar Muller-Hinton. Setiap pelat diseka dengan patogen uji masing-masing. Dari masing-masing galur probiotik Lactobacillus yang sebelumnya diinkubasi dalam kondisi anaerobik selama 24 jam pada 37^{Hai}C, 70 µl kultur cair MRS ditempatkan di masing-masing sumur. Setelah 24 jam inkubasi pada 37^{Hai}C, zona hambat diukur dan dicatat dalam cm.

Analisis statistik

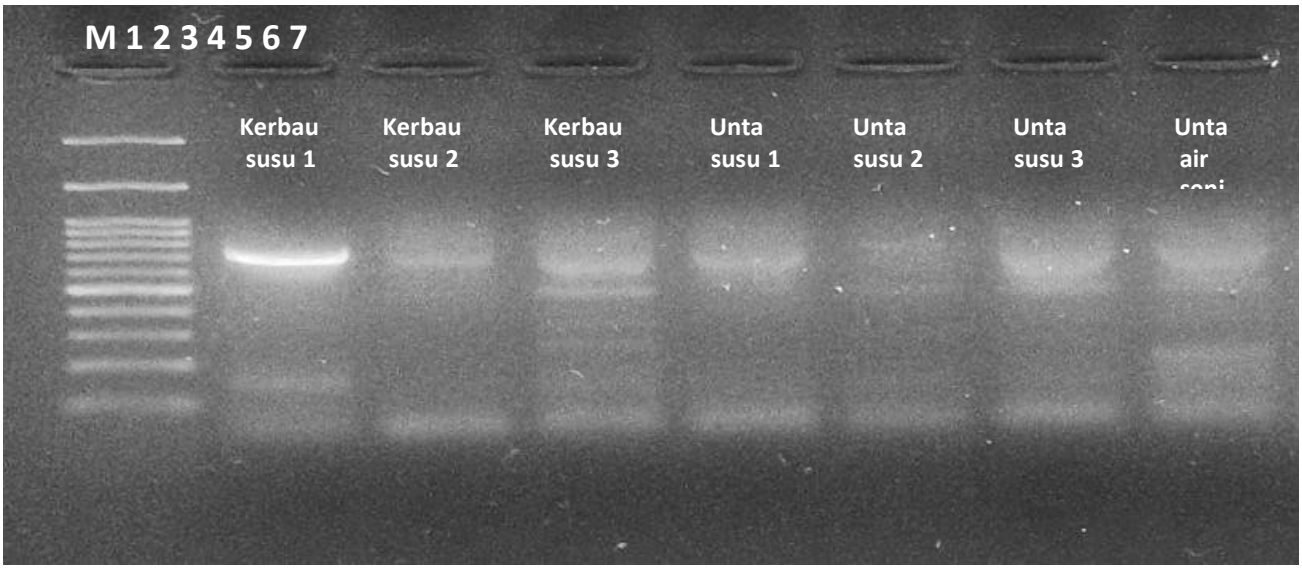
Aktivitas antibakteri in vitro dilakukan dalam rangkap tiga. Semua data kemudian tunduk pada SPSS Versi 21 (IBM, New York, AS). Analisis statistik dilakukan dengan menggunakan ANOVA dua arah dilanjutkan dengan Uji Jarak Berganda Duncan untuk mengetahui perbedaan yang signifikan. Nilai yang diberikan mewakili mean ± Standar Deviasi (SD). Nilai probabilitas P <0,05 dianggap sebagai perbedaan yang signifikan (Steel dan Torrie, 1980).

HASIL DAN DISKUSI

Lactobacilli Hasil isolasi dari susu kerbau, susu unta, dan urine unta dilakukan karakterisasi dan identifikasi menggunakan metode identifikasi biokimia dan molekuler yang berbeda. Total diambil tujuh sampel yang terdiri dari tiga sampel susu dan satu sampel urin dari unta dan tiga sampel susu dari kerbau. Lactobacilli diisolasi dengan cara menumbuhkan kandungan bakteri pada sampel pada media MRS sebagai media selektif. Koloni bakteri awalnya diidentifikasi sebagai Lactobacilli berdasarkan Gram-positif dan Katalase-negatif serta berbentuk batang di bawah mikroskop. Masing-masing sampel dalam medium MRS broth digunakan koloni campuran untuk mengekstraksi DNA untuk identifikasi molekuler menggunakan analisis PCR multipleks 16S rRNA.

Analisis multipleks PCR

Hasil analisis PCR multipleks 16S rRNA telah ditunjukkan pada gambar 1. Sebanyak 18 bakteri yang diisolasi dari susu kerbau, susu unta, dan urin unta diidentifikasi sebagai Lactobacilli. Spesies Lactobacillus diidentifikasi berdasarkan ukuran produk PCR (Kwon et al., 2004). Hasil penelitian menunjukkan bahwa susu kerbau dan unta memiliki kandungan Lactobacilli yang berbeda. Ada juga perbedaan kandungan Lactobacilli pada sampel susu berbeda yang dikumpulkan dari spesies yang sama. Hasil penelitian menunjukkan adanya L. casei, L. acidophilus, L. rhamnosus, L. plantarum, L. gasseri dan L. delbrueckii pada sampel susu kerbau. Sedangkan sampel susu unta dan urine unta mengungkapkan keberadaan L. casei, L. acidophilus dan L. plantarum.

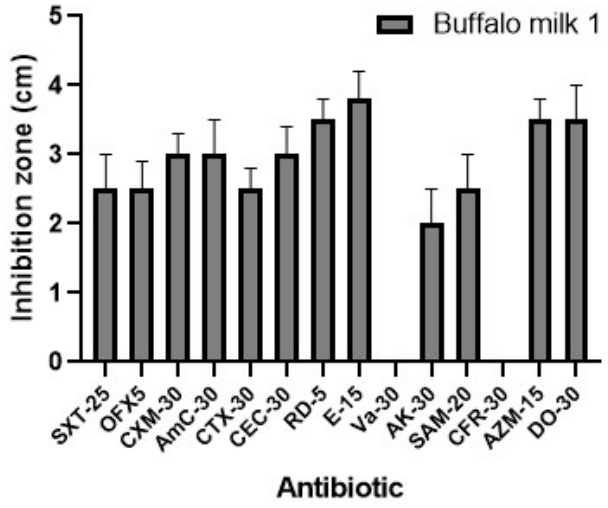


Gambar 1. Elektroforesis gel agarose produk PCR dari uji PCR multipleks.Uji PCR multipleks dilakukan dengan campuran primer spesifik tujuh spesies atau kelompok spesifik untuk L. acidophilus, L. bulgaricus (sama dengan L. delbrueckii subsp. Bulgaricus), L. casei-group L. gasseri, L. plantarum, L. reuteri dan L. rhamnosus dan dua primer yang diawetkan oleh bakteri. Jalur 1–7 menunjukkan produk PCR dari setiap DNA genom yang diekstraksi dari suspensi sel tunggal atau campuran yang diisolasi dari inang perwakilan yang digunakan sebagai templat PCR. Jalur 1: L. casei, L. delbrueckii; jalur 2: L. casei; jalur 3: L. casei, L. acidophilus, L. rhamnosus, L. plantarum dan L. gasseri; jalur 4: L. casei; jalur 5: L. plantarum; Jalur 6: L. plantarum; jalur 7: L. plantarum; jalur M: tangga 100 bp-DNA.

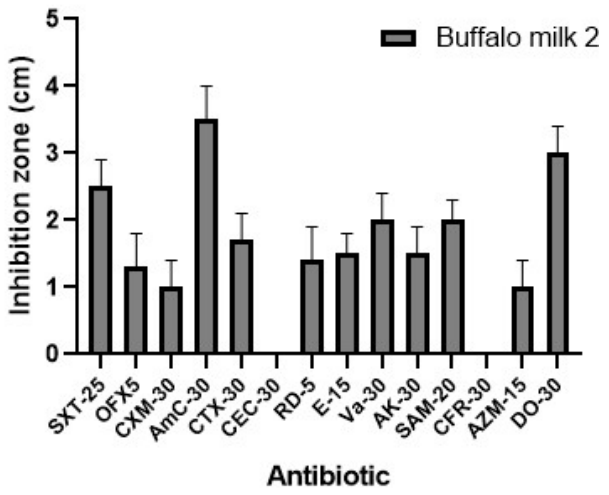
Kerentanan antibiotik Lactobacilli

Kerentanan antibiotik strain Lactobacillus diuji dengan menggunakan panel 14 antibiotik (Gambar 2-8). Jelas bahwa semua sampel memiliki resistensi yang sangat signifikan (p <0,0001) terhadap Cefadroxil (CFR-30) dengan zona hambat 0,0 cm. Cefaclor (CEC-30) secara eksklusif tidak menunjukkan adanya penghambatan terhadap pertumbuhan salah satu sampel susu kerbau (susu kerbau 2) dan semua sampel susu dan urin unta. Sedangkan Vancomycin (Va-30) tidak menyebabkan hambatan hanya pada satu sampel susu kerbau (susu kerbau 1). Antibiotik lainnya secara eksklusif tidak menunjukkan adanya penghambatan pada sampel unta termasuk Cefuroxime (CXM-30), Cefotaxime (CTX-30), Erythromycin (E-15), Ampicillin dengan sulbactam (SAM-20), dan Azitromisin (AZM-15). Selain itu, sampel urin unta adalah satu-satunya yang benar-benar resisten terhadap (AmC-30).

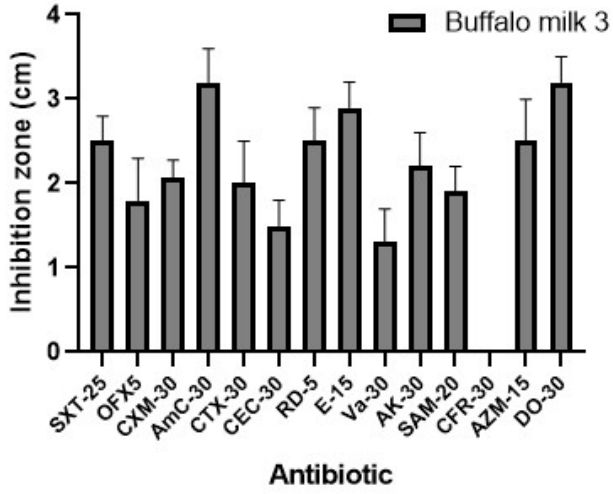
dengan hambatan pertumbuhan 0,0 cm. Di sisi lain, semua sampel sensitif terhadap Trimethoprim / sulfamethoxazole (SXT-25), Ofloxacin (OFX5), Rifampicin (RD-5) dan Doxycycline (DO-30) dengan berbagai tingkat penghambatan.



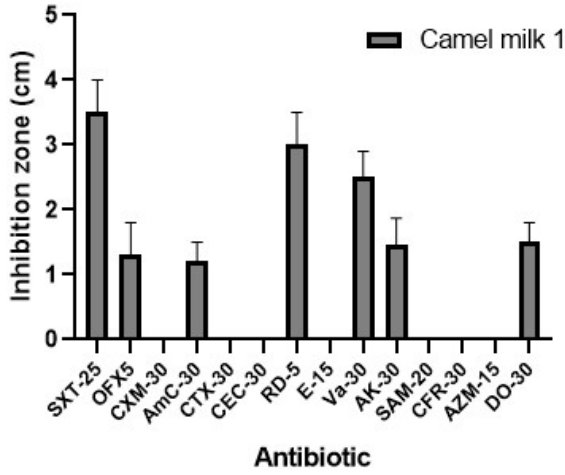
Gambar 2



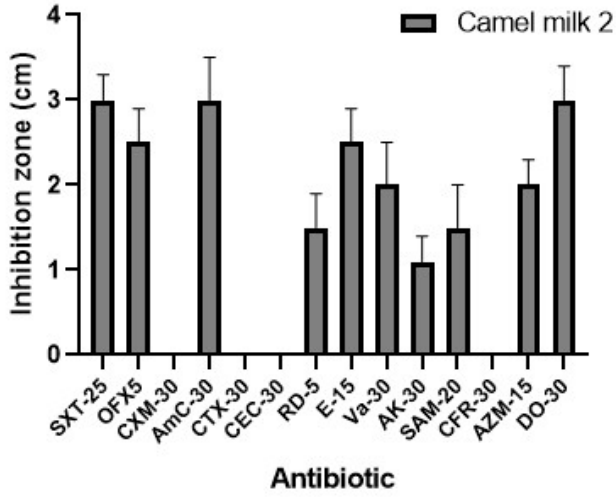
Gambar 3



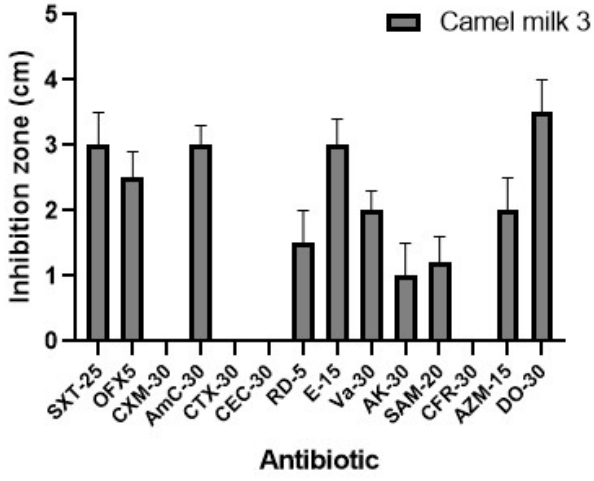
Gambar 4



Gambar 5

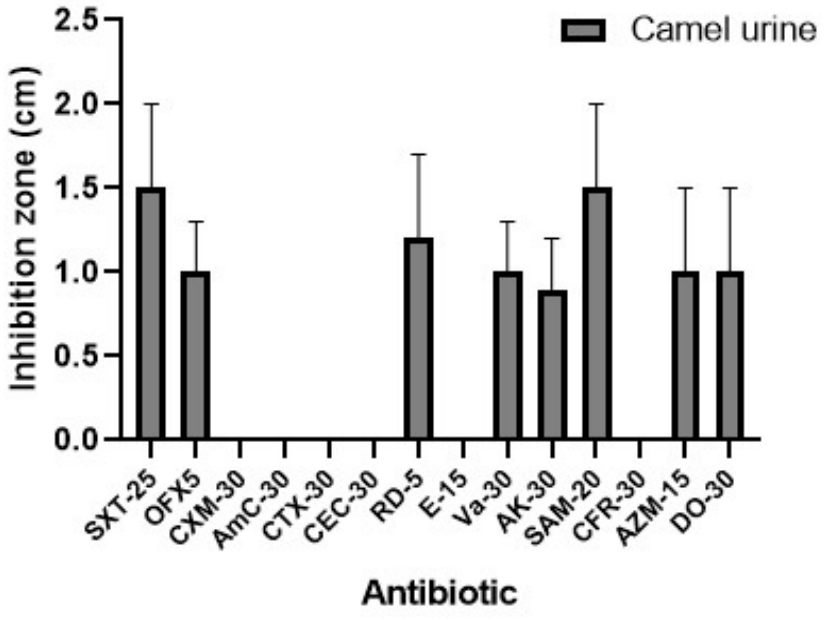


Gambar 6



Gambar 7

Urine unta

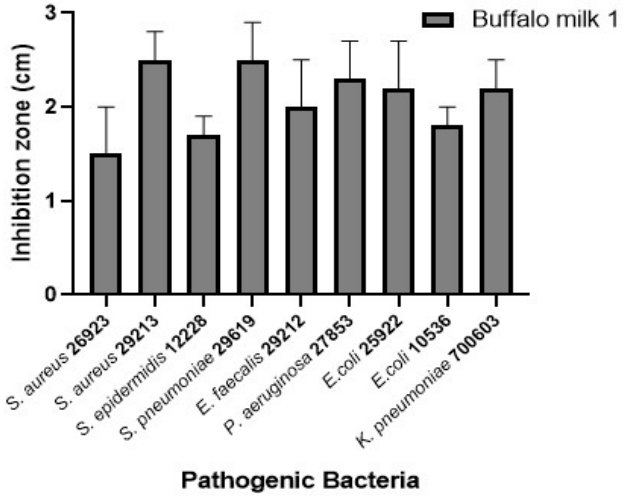


Gambar 8

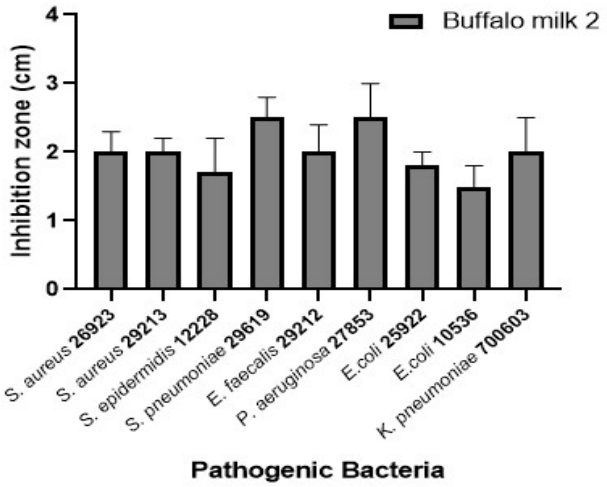
Gambar 2-8. Kerentanan antibiotik dari probiotik Lactobacilli yang diisolasi dari susu kerbau, susu unta atau air seni unta. Empat belas disk antibiotik digunakan untuk uji kepekaan termasuk Trimethoprim / sulfamethoxazole (SXT-25), Ofloxacin (OFX5), Cefuroxime (CXM-30), Amoxicillin with clavulanic acid (AmC-30), Cefotaxime (CTX-30), Cefaclor (CEC-30), Rifampisin (RD-5), Eritromisin (E-15), Vankomisin (Va-30), Amikasin (AK-30), Ampisilin dengan ctam (SAM-20), Cefadroxil (CFR-30), Azitromisin (AZM-15), dan Doxycycline (DO-30).

Aktivitas antibakteri Lactobacilli

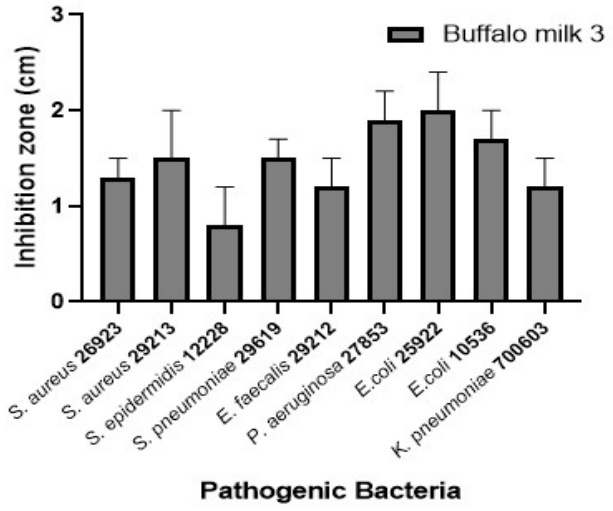
Aktivitas antibakteri dari probiotik Lactobacilli yang diisolasi diselidiki terhadap sembilan strain standar patogen dari bakteri Gram positif dan Gram negatif (Gambar 9-15). Hasil penelitian menunjukkan bahwa aktivitas antibakteri Lactobacilli yang diisolasi dari urin unta secara umum jauh lebih rendah signifikan (p <0,0001) dibandingkan aktivitas antibakteri Lactobacilli dari susu kerbau dan susu unta terhadap semua strain bakteri yang diuji. Efek antibakteri susu kerbau tiga secara signifikan lebih tinggi (P = 0,0045) dibandingkan semua sampel lain terhadap E. coli 25922 sedangkan aktivitas antibakteri susu unta dua secara signifikan lebih tinggi terhadap S. aureus 29213 (P = 0,0014), *S. pneumonia* 29619 (P = 0,0014) dan E. faecalis 29212 (P = 0,0014) jika dibandingkan pengaruhnya terhadap E. coli 10536.



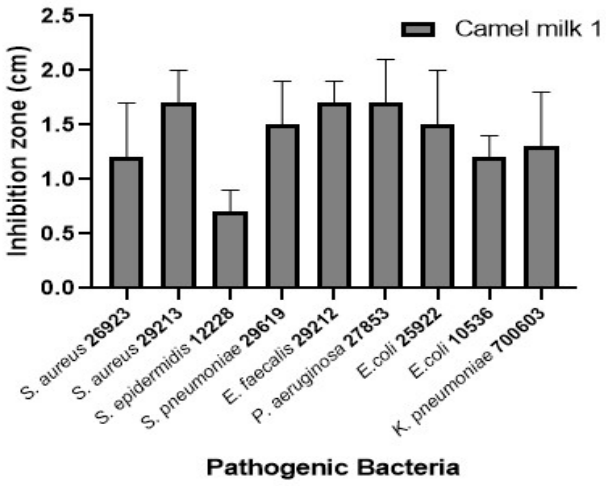
Gambar 9



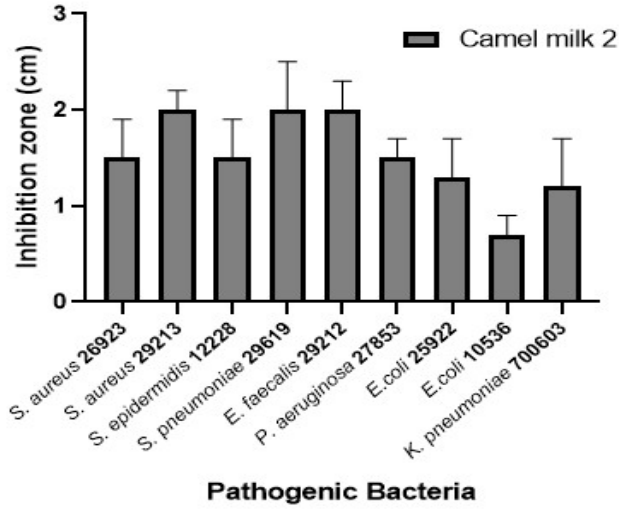
Gambar 10



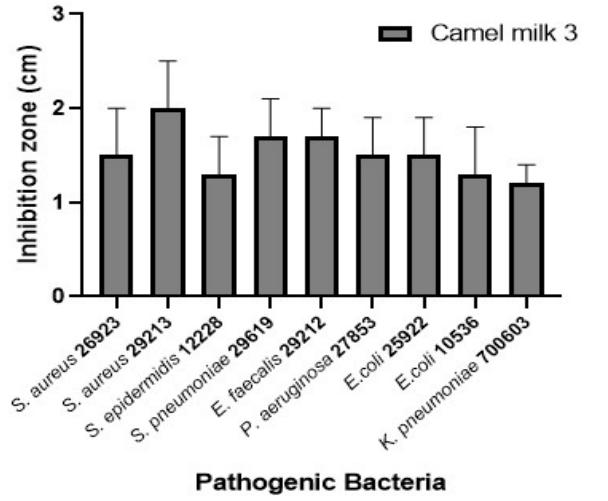
Gambar 11



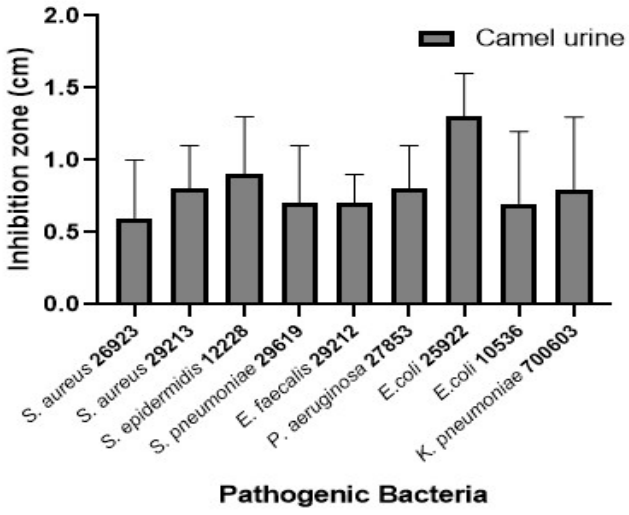
Gambar 12



Gambar 13



Gambar 14



Gambar 15

Gambar 9-15: Aktivitas antibakteri probiotik Lactobacilli yang diisolasi dari susu kerbau, susu unta atau urine unta. Aktivitas antibakteri diteliti terhadap bakteri Gram-positif dan Gram-negatif menggunakan metode difusi agar-well. Strain Gram-positif diwakili oleh S. aureus (ATCC 26923), S. aureus (ATCC 29213), S. epidermidis (ATCC 12228), S. pneumoniae (ATCC 29619), dan E. faecalis ATCC (29212). Strain gram negatif diwakili oleh P. aeruginosa ATCC (27853), E. coli ATCC (25922), E. coli ATCC (10536), dan K. pneumoniae ATCC (700603).

DISKUSI

Bakteri probiotik telah dikenal karena efek kesehatannya yang menguntungkan pada manusia dan hewan. Konsumsi mereka dalam makanan tradisional dikaitkan dengan umur panjang dan perlindungan terhadap penyakit (Kechagia et al., 2013). Mekanisme efek menguntungkannya termasuk perlindungan terhadap penyakit menular baik dengan persaingan langsung dengan mikroorganisme patogen atau dengan modulasi sistem kekebalan dan meningkatkan pencernaan dan pengurangan gangguan metabolisme (Azad et al., 2018; Ghosh et al., 2019 ; Yousefi et al., 2019).

Sumber utama probiotik adalah makanan fermentasi termasuk susu fermentasi, keju, dan produk susu lainnya. Probiotik juga diisolasi dari saluran pencernaan manusia dan hewan. Selanjutnya, strain probiotik telah diisolasi dari substrat fermentasi non-susu termasuk daging dan buah-buahan. Anehnya, strain probiotik juga hadir dalam susu manusia dan hewan yang awalnya diharapkan steril (McGuire dan McGuire, 2015). Temuan ini sesuai dengan temuan bahwa bayi yang diberi ASI lebih sedikit terpengaruh oleh infeksi saluran cerna dan memiliki lebih sedikit alergi dibandingkan bayi yang diberi susu formula (Fontana et al., 2013).

Keragaman probiotik Lactobacilli yang diisolasi dari spesies hewan yang berbeda telah didokumentasikan (Abdou et al., 2018; Abdou et al., 2019). Keragaman ini merupakan hasil dari beberapa faktor antara lain nutrisi, infeksi, antibiotik, stres dan berbagai kondisi penyakit. Variasi strain probiotik menyebabkan berbagai jenis manfaat bagi inang.

Jelas dari temuan ini bahwa strain Lactobacilli yang diisolasi dari susu unta dan urin unta lebih resisten terhadap efek antibiotik daripada Lactobacilli yang diisolasi dari susu kerbau. Ini bisa berguna untuk memulihkan mikrobiota usus setelah perawatan antibiotik (Gueimonde et al., 2013). Meskipun semua strain Lactobacilli yang ditemukan dalam susu unta dan urin unta juga ditemukan dalam susu kerbau, dua yang pertama menunjukkan resistensi yang lebih umum terhadap antibiotik. Hal ini bisa disebabkan karena akuisisi plasmid dari bakteri lain (Gueimonde et al., 2013). Susu unta dan urine telah digunakan dalam pengobatan tradisional selama beberapa tahun untuk mengobati banyak penyakit (Hu et al., 2017). Terlepas dari popularitas kerbau dan susu sapi dan preferensi mereka di kalangan masyarakat umum, susu unta adalah pengganti yang sangat penting di daerah kering dan semi-kering dimana susu kerbau dan sapi sangat kurang. Investigasi susu unta untuk kandungan bakteri ternyata kaya akan BAL (Bin Masalam et al., 2018). Dalam studi saat ini susu kerbau dan unta berbeda dalam kandungan Lactobacilli mereka. Perbedaan ini mungkin disebabkan oleh perbedaan komposisi susu (Yoganandi et al., 2014), yang memungkinkan tumbuhnya strain lactobacillus yang berbeda. Lactobacillus plantarum diisolasi dari susu unta dan merupakan salah satu BAL yang sering diisolasi dari susu unta mentah (Khedid et al., 2009; Edalati et al., 2019). Perbedaan ini mungkin disebabkan oleh perbedaan komposisi susu (Yoganandi et al., 2014), yang memungkinkan tumbuhnya strain lactobacillus yang berbeda. Lactobacillus plantarum diisolasi dari susu unta dan merupakan salah satu BAL yang sering diisolasi dari susu unta mentah (Khedid et al., 2009; Edalati et al., 2019). Perbedaan ini mungkin disebabkan oleh perbedaan komposisi susu (Yoganandi et al., 2014), yang memungkinkan tumbuhnya strain lactobacillus yang berbeda. Lactobacillus plantarum diisolasi dari susu unta dan merupakan salah satu BAL yang sering diisolasi dari susu unta mentah (Khedid et al., 2009; Edalati et al., 2019).

Probiotik Lactobacilli berpotensi untuk digunakan sebagai alternatif alami dari antibiotik sintetis yang saat ini digunakan karena aktivitas antagonisnya terhadap berbagai bakteri patogen (Prabhurajeshwar dan Chandrakanth, 2017). Dalam penelitian ini, telah diindikasikan bahwa Lactobacilli yang diisolasi dari susu kerbau, susu unta serta urin unta menunjukkan aktivitas antibakteri yang bervariasi terhadap bakteri patogen. Meskipun data saat ini menunjukkan bahwa Lactobacilli yang diisolasi dari urin unta memiliki aktivitas antibakteri paling sedikit bila dibandingkan dengan kerbau dan susu unta, aktivitas antibakteri, antijamur dan antivirus dari susu unta dan urin dilaporkan sebelumnya (Al-Bashan, 2011; Hu et al ., 2017). Salah satu alasan aktivitas antibakteri paling sedikit pada urine unta adalah karena hanya menggunakan satu sampel.

KESIMPULAN

Penelitian ini menunjukkan adanya variabilitas kandungan strain lactobacillus yang diisolasi dari susu kerbau, susu unta, dan urine unta. Meskipun beberapa strain serupa di antara sampel-sampel ini, mereka menunjukkan kerentanan yang berbeda terhadap antibiotik dan memiliki aktivitas antibakteri yang berbeda terhadap bakteri patogen. Penelitian lebih lanjut harus dilakukan dengan lebih banyak sampel untuk mendapatkan lebih banyak informasi di bidang aktivitas antibakteri lactobacilli probiotik dan untuk memahami mekanisme aktivitasnya. Mudah-mudahan, mereka akan digunakan sebagai alternatif alami daripada antibiotik sintetis.

DEKLARASI

Kontribusi penulis

Amr M. Abdou berpartisipasi dalam identifikasi molekuler dari strain probiotik, melakukan analisis statistik dan menyusun naskah. Riham H. Hedia berpartisipasi dalam karakterisasi bakteri yang diisolasi, identifikasi molekuler dari strain probiotik dan kerentanan antibiotik Lactobacilli. Shimaa T. Omara berpartisipasi dalam karakterisasi bakteri yang diisolasi, identifikasi molekuler dari strain probiotik, dan aktivitas antibakteri Lactobacilli. Mai M. Kandil

berpartisipasi dalam pengumpulan sampel dan berpartisipasi dalam identifikasi molekuler dari strain probiotik. MA Bakry berpartisipasi dalam pengambilan sampel dan karakterisasi bakteri yang diisolasi. Mohammad M. Effat mengusulkan gagasan studi saat ini, dan berpartisipasi dalam desain dan koordinasi serta membantu menyusun naskah. Semua penulis membaca dan menyetujui naskah akhir.

Minat yang bersaing

Para penulis menyatakan bahwa mereka tidak memiliki kepentingan yang bersaing.

REFERENSI

Abdou AM, Fouad EA, Alam SS, and Hakim AS (2019). Isolation and Identification of Probiotic Lactobacilli from Non-ruminant Animals. International Journal of Veterinary Science, 8(4): 349-354. Available at: http://www.ijvets.com/.../349-354.pdf

Abdou AM, Hedia RH, Omara ST, Mahmoud MAE, Kandil MM, and Bakry MA (2018). Interspecies comparison of probiotics isolated from different animals. Veterinary World, 11: 227-230. DOI: https://doi.org/10.14202/vetworld.2018.227-230

Akgul T, and Karakan T (2018). The role of probiotics in women with recurrent urinary tract infections. Turkish Journal of Urology, 44: 377-383. DOI: https://doi.org/10.5152/tud.2018.48742

Al-Bashan MM (2011). In vitro Assessment of the Antimicrobial Activity and Biochemical Properties of Camel's Urine Against Some Human Pathogenic Microbes. Middle-East Journal of Scientific Research, **7**: 947-958. Available at: https://bengreenfieldfitness.com/wp-content/uploads/2017/02/In-vitro-Assessment-of-the-Antimicrobial-Activity-and-Biochemical-Properties-of-camels-urine.pdf

Azad MAK, Sarker M, and Wan D (2018). Immunomodulatory Effects of Probiotics on Cytokine Profiles. BioMed Research International, 2018: 8063647. DOI: https://doi.org/10.1155/2018/8063647

Bauer AW, Kirby WM, Sherris JC, and Turck M (1966). Antibiotic susceptibility testing by a standardized single disk method. American Journal of Clinical Pathology, 45(4): 493-496. DOI: https://doi.org/10.1093/ajcp/45.4_ts.493

Bergey DHJ, John GH, Noel RK, and Peter HAS (1994). Bergey's Manual of Determinative Bacteriology, 9 th ed. Avaliable at:

https://books.google.com.eg/books?id=jtMLzaa5ONcC&printsec=frontcover&hl=ar&source=gbs_ge_summary_r&cad=0#v=onepage&q&f=false

Bin Masalam MS, Bahieldin A, Alharbi MG, Al-Masaudi S, Al-Jaouni SK, Harakeh SM, and Al-Hindi RR (2018). Isolation, Molecular Characterization and Probiotic Potential of Lactic Acid Bacteria in Saudi Raw and Fermented Milk. Evidence-Based Complementary and Alternative Medicine, 2018, 7970463. DOI: https://doi.org/10.1155/2018/7970463

Brunel AS, and Guery B (2017). Multidrug resistant (or antimicrobial-resistant) pathogens - alternatives to new antibiotics? Swiss Medical Weekly, 147: w14553. DOI: https://doi.org/10.4414/smw.2017.14553

Davies J, and Davies D (2010). Origins and evolution of antibiotic resistance. Microbiology and Molecular Biology Reviews, 74: 417-433. DOI: https://doi.org/10.1128/MMBR.00016-10

Edalati E, Saneei B, Alizadeh M, Hosseini SS, Zahedi Bialvaei A, and Taheri K (2019). Isolation of probiotic bacteria from raw camel's milk and their antagonistic effects on two bacteria causing food poisoning. New Microbes and New Infections, 27: 64-68. DOI: https://doi.org/10.1016/j.nmni.2018.11.008

FAO/WHO (2011). ICMR-DBT guidelines for evaluation of probiotics in food. Indian Journal of Medical Research, 134(1): 22-25. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3171912/

Fijan S (2014). Microorganisms with claimed probiotic properties: an overview of recent literature. Int International Journal of Environmental Research and Public Health, 11(5): 4745-4767. https://doi.org/10.3390/ijerph110504745

Fontana L, Bermudez-Brito M, Plaza-Diaz J, Munoz-Quezada S, and Gil A (2013). Sources, isolation, characterisation and evaluation of probiotics. British Journal of Nutrition, 109 Suppl 2: S35-50. DOI: https://doi.org/10.1017/S0007114512004011

Ghosh T, Beniwal A, Semwal A, and Navani NK (2019). Mechanistic Insights Into Probiotic Properties of Lactic Acid Bacteria Associated With Ethnic Fermented Dairy Products. Frontiers in Microbiology, 10: 502. DOI: https://doi.org/10.3389/fmicb.2019.00502

Gueimonde M, Sánchez B, G de Los Reyes-Gavilán C, and Margolles A (2013). Antibiotic resistance in probiotic bacteria. Frontiers in microbiology, *4*, 202. https://doi.org/10.3389/fmicb.2013.00202

Hao Q, Lu Z, Dong BR, Huang CQ, and Wu T (2011). Probiotics for preventing acute upper respiratory tract infections. Cochrane Database of Systematic Reviews, CD006895. DOI: https://doi.org/10.1002/14651858.CD006895.pub2

Hu Z, Chang X, Pan Q, Gu K, and Okechukwu PN (2017). Gastroprotective and Ulcer Healing Effects of Camel Milk and Urine in HCl/EtOH, Non-steroidal Anti-inflammatory Drugs (Indomethacin), and Water-Restraint Stress-induced Ulcer in Rats. Pharmacognosy Magazine, 13: 559-565. DOI: https://doi.org/10.4103/pm.pm_135_17

International Organization for Standardization (ISO) (2010). Milk and milk products—determination of the minimal inhibitory concentration (MIC) of antibiotics applicable to bifidobacteria and non-enterococcal lactic acid bacteria (LAB). ISO 10932/IDF 233 standard. Available at: https://www.iso.org/standard/46434.html

Kechagia M, Basoulis D, Konstantopoulou S, Dimitriadi D, Gyftopoulou K, Skarmoutsou N, and Fakiri EM (2013). Health benefits of probiotics: a review. ISRN Nutrition, Article ID: 481651. DOI: https://doi.org/10.5402/2013/481651

Khedid K, Faid M, Mokhtari A, Soulaymani A, and Zinedine A (2009). Characterization of lactic acid bacteria isolated from the one humped camel milk produced in Morocco. Microbiological Research, 164: 81-91. DOI: https://doi.org/10.1016/j.micres.2006.10.008

Kwon HS, Yang EH, Yeon SW, Kang BH, and Kim TY (2004). Rapid identification of probiotic Lactobacillus species by multiplex PCR using species-specific primers based on the region extending from 16S rRNA through 23S rRNA. FEMS Microbiology Letters, 239: 267-275. DOI: https://doi.org/10.1016/j.femsle.2004.08.049

McGuire MK, and McGuire MA (2015). Human milk: mother nature's prototypical probiotic food? Adv Nutr. Jan 15; 6(1): 112-123. DOI: https://doi.org/10.3945/an.114.007435.

Ozen M, Kocabas Sandal G, and Dinleyici EC (2015). Probiotics for the prevention of pediatric upper respiratory tract infections: a systematic review. Expert Opinion on Biological Therapy, 15: 9-20. DOI: https://doi.org/10.1517/14712598.2015.980233

Prabhurajeshwar C, and Chandrakanth RK (2017). Probiotic potential of Lactobacilli with antagonistic activity against pathogenic strains: An in vitro validation for the production of inhibitory substances. Biomedical journal, 40(5): 270–283. DOI: https://doi.org/10.1016/j.bj.2017.06.008

Ruiz Rodriguez LG, Mohamed F, Bleckwedel J, Medina R, De Vuyst L, Hebert EM, and Mozzi F (2019). Diversity and Functional Properties of Lactic Acid Bacteria Isolated From Wild Fruits and Flowers Present in Northern Argentina. Frontiers in Microbiology, 10: 1091. DOI: https://doi.org/10.3389/fmicb.2019.01091

Sgouras D, Maragkoudakis P, Petraki K, Martinez-Gonzalez B, Eriotou E, Michopoulos S, Kalantzopoulos G, Tsakalidou E, and Mentis A (2004). In vitro and in vivo inhibition of Helicobacter pylori by Lactobacillus casei strain Shirota. Applied and Environmental Microbiology, 70(1): 518-526. DOI: https://doi.org/10.1128/aem.70.1.518-526.2004

Shokryazdan P, Sieo CC, Kalavathy R, Liang JB, Alitheen NB, Faseleh Jahromi M, and Ho YW (2014). Probiotic potential of Lactobacillus strains with antimicrobial activity against some human pathogenic strains. BioMed Research International, 927268. DOI: https://doi.org/10.1155/2014/927268

Steel RGD, and Torrie JH (1980). Principles and Procedures of Statistics. 2nd ed. McGraw Hill Book Company, New York.

Williams MD, Ha CY, and Ciorba MA (2010). Probiotics as therapy in gastroenterology: a study of physician opinions and recommendations. Journal of Clinical Gastroenterology, 44: 631-636. DOI: https://doi.org/10.1097/MCG.0b013e3181d47f5b

Yoganandi J, Mehta BM, Wadhwani KN, Darji VB, and Aparnathi KD (2014). Evaluation and Comparison of Camel Milk with Cow Milk and Buffalo Milk for Gross Composition. Journal of Camel Practice and Research, 21(2): 259-265. DOI: https://doi.org/10.5958/2277-8934.2014.00046.0

Yousefi B, Eslami M, Ghasemian A, Kokhaei P, Salek Farrokhi A, and Darabi N (2019). Probiotics importance and their immunomodulatory properties. Journal of Cellular Physiology, 234 (6): 8008-8018. DOI: https://doi.org/10.1002/jcp.27559

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Perlindungan Silang Protein Yolk Immunoglobulin Anti-Hemagglutinin Subtipe H5N1 Influenza A Patogen Tinggi yang Diberikan pada Ayam Yang Terinfeksi Avian Influenza A Subtipe H5N1 Patogenik Tinggi

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ABSTRAK

Yolk Immunoglobulin (IgY) melawan Avian Influenza (AI) biasa digunakan sebagai teknik imunoterapi dan imunodiagnostik. Penerapan IgY yang dicampur dalam air minum diketahui efektif menghambat replikasi AI. Uji efektivitas IgY anti-Hemagglutinin Protein (anti-HA) dari High Pathogenic Avian Influenza (HPAI) clade 2.1 (A / Ayam / Blitar / 2003) telah diuji terhadap infeksi High Pathogenic Avian Influenza clade 2.3.2 (A / Duck / Sidoarjo / 2012). Aktivitas penghambatan diamati melalui Imunohistokimia. Enam puluh ayam terinfeksi 10 ekor^{5} EID_{50}/ ml HPAI clade 2.3.2 (A / Bebek / Sidoarjo / 2012). Imunoglobulin kuning telur dengan jumlah yang berbeda (0 µg, 100 µg, 200 µg dan 400 µg) diberikan pada tiga waktu yang berbeda yaitu 24 jam sebelum infeksi, saat infeksi, dan 24 jam setelah infeksi. Pengamatan dilakukan selama 7 hari. Selama pengamatan pasca infeksi, ayam mati dikelola untuk uji imunohistokimia untuk mengamati keberadaan virion dan IgY sialic acid 2,3-alfa galactosa (SA α 2,3 gal) aktivitas pemblokiran di septa alveoli. Pada akhir pengamatan semua ayam di eutanasia untuk uji imunohistokimia. Hasil penelitian menunjukkan bahwa anti-HA IgY yang diperoleh dari HPAI clade 2.1 dapat melindungi infeksi HPAI clade 2.3.2. Menurut uji imunohistokimia, Pemberian IgY dapat menetralkan virus yang menginfeksi yang ditandai dengan banyaknya virion yang diamati pada septa alveoli paru. Berdasarkan uji tersebut, dosis 200 µg dan 400 µg IgY yang diaplikasikan 24 jam sebelum infeksi, dapat menurunkan gejala klinis dan kematian ayam yang terinfeksi (80-100%). Dosis IgY terbaik untuk melindunginya dari infeksi clade 2.3.2 (A / Duck / Sidoarjo / 2012) adalah 400 µg yang diberikan 24 jam sebelum infeksi. Dapat disimpulkan bahwa pemberian IgY anti-Haemaglutinin Protein (anti-HA) dari High Pathogenic Avian Influenza (HPAI) clade 2.1 (A / Ayam / Blitar / 2003) dapat melindungi ayam dari infeksi HPAI clade 2.3.2 (A / Duck / Sidoarjo / 2012), meski memiliki kelas yang berbeda. Tingkat proteksi adalah 80-100%.

Kata kunci: Virus flu burung, IgY anti-HA, Imunoterapi, Produktivitas.

PENGANTAR

Avian Influenza (AI) biasa dikenal dengan fowl plaque yang merupakan penyakit yang disebabkan oleh infeksi virus Influenza A yang tergolong dalam famili Orthomyxoviridae. Penyakit ini rentan terhadap banyak spesies burung (Bouma dkk., 2009; Webby dan Webster, 2003; De Jong et al., 1997). Menurut genotipe, itu diklasifikasikan menjadi 16 Haemagglutinin dan 9 subtipe Neuraminidase (Bergervoet dkk., 2019). Menurut virulensinya, penyakit ini diklasifikasikan menjadi dua kelompok yaitu Low Pathogenic Avian Influenza (LPAI) dan High Pathogenic Avian Influenza (HPAI) (OIE, 2016). Baik LPAI dan HPAI berasal dari subtipe H5 dan H7 (Bouma dkk., 2009; Webby dan Webster, 2003; De Jong dkk., 1997), dan telah menjadi perhatian komunitas perdagangan internasional karena HPAI menyebabkan kerugian besar bagi wabah, dan LPAI menyebabkan masalah tahunan dan berpotensi bermutasi menjadi HPAI (MacLachlan dkk., 2016). Ini telah menjadi endemik di banyak negara seperti Indonesia (Daniel et al., 2012).

Pencegahan telah dilakukan seperti vaksinasi rutin dan pengelolaan biosekuriti tetapi wabah tahunan tetap berlangsung. Perusahaan peternakan unggas di Indonesia telah melakukan vaksinasi lebih dari 400 juta dosis sejak tahun 2004 (Bouma dkk., 2009). AI adalah virus RNA sense negatif beruntai tunggal tersegmentasi yang terbungkus. Di bawah mikroskop elektronik, ia terlihat pada bentuk pleomorfik, bola, atau velamen. Virionnya terdiri dari 10-14,6 kb genom yang terbagi menjadi delapan segmen tersusun secara heliks-simetris. Ini memiliki tujuh protein struktural seperti protein Haemagglutinin (HA), protein Neuraminidase (N), dua protein Matriks (M1 dan M2), dan tiga protein Polimerase (PB1, PB2, dan PA). HA dan N merupakan lapisan protein yang terbungkus pada paku bentuk membran yang memiliki peran penting pada patogenisitas, klasifikasi dan netralisasi virus (MacLachlan dkk., 2016; Knipe dan Howley, 2013). Antibodi spesifik biasanya digunakan sebagai aturan diagnostik atau sebagai pencegahan penyakit tertentu. Antibodi yang diperoleh dari hewan memerlukan prosedur produksi yang baik terkait dengan instruksi kesejahteraan hewan (Hau dan Hendriksen, 2005). Antibodi yang diperoleh dari Yolk Immunoglobulin (IgY) bersifat homolog terhadap Immunoglobulin G (IgG) yang diperoleh dari mamalia. Saat ini aplikasi IgY yang diperoleh dari telur sebagai imunoterapi semakin meningkat karena konsentrasi imunoglobulin lebih tinggi dibandingkan IgG yang diperoleh dari mamalia. Salah satu keistimewaan memperoleh imunoglobulin dari ayam adalah ayam memiliki

sensitivitas yang tinggi terhadap paparan antigen, sehingga respon imun dan produksi IgY tetap ada (Hau dan Hendriksen, 2005).

Virus AI berpindah melalui jalan nafas atau secara oral, kemudian protein Haemagglutinin dari virus terikat pada reseptor protein sialic acid alfa 2 dan 3- galactosa (SA α 2,3 gal). Pengikatan ini memicu fusi virus ke dalam sel (Knipe dan Howley, 2013). Pengikatan ini bisa gagal jika antibodi spesifik melawan protein HA memblokir proses tersebut. Antibodi HA diperoleh dari Yolk Immunoglobulin mungkin memiliki potensi untuk memblokir proses ini untuk mencegah infeksi AI pada ayam. Penelitian ini dilakukan untuk mengetahui efektivitas anti-HA dari HPAI clade 2.1 (A / Ayam / Blitar / 2003) terhadap infeksi HPAI clade 2.3.2 (A / Itik / Sidoarjo / 2012).

BAHAN DAN METODE

Enam puluh ekor ayam (umur 21 hari) digunakan dalam percobaan ini. Mereka dibagi menjadi tiga kelompok secara acak. Setiap kelompok terdiri dari empat sub kelompok perlakuan yang masing-masing terdiri dari lima ekor ayam. IgY diperoleh dengan menginfeksi protein HA HPAI clade 2.1 (A / Ayam / Blitar / 2003) ke dalam ayam petelur Specific Pathogen Free (SPF). IgY diekstraksi dari telur, dan disimpan pada -20 ° C (Narat, 2003). Imunoglobulin kuning telur diberikan kepada setiap kelompok utama pada tiga waktu yang berbeda. IgY diberikan 24 jam sebelum infeksi, 24 jam setelah infeksi, dan pada saat infeksi masing-masing pada Kelompok I, II, dan III. Jumlah IgY yang diberikan pada masing-masing sub kelompok adalah 0 µg, 100 µg, 200 µg, dan 400 µg. Masing-masing dosis diencerkan dengan akuades hingga volume total adalah satu ml. Penelitian dilakukan di Biosecurity Level-2 (BSL-2) Fakultas Kedokteran Hewan Universitas Airlangga Indonesia. Suhu disetel $16^{Hai}C$.

Ayam-ayam itu terinfeksi 10 ekor^{5} EID_{50}/ ml dosis antigen (A / Bebek / Sidoarjo / 2012). Pengamatan dimulai pada saat infeksi berlangsung selama 7 hari. Selama periode ini semua ayam yang mati dicatat, kemudian disiapkan uji imunohistokimia untuk menentukan derajat kerusakan sel dan asam sialat 2,3-alfa galactosa (SA α 2,3 gal) aktivitas penghambatan dari anti-HA pada tropisme sel menurut antigen (deteksi virion) dan deteksi antibodi (anti-anti HA). Pada akhir pengamatan, sisa ayam yang masih hidup dimatikan dengan metode dislokasi serviks. Sampel untuk imunohistokimia dikumpulkan dari paru. Paru-paru dicelupkan ke dalam buffer formalin 10%, kemudian diolah menjadi slide (Damayanti dkk., 2004). Sebelum prosedur imunohistokimia diterapkan pada slide, slide disiapkan untuk deparaffinisasi untuk menghilangkan lilinnya. Setelah slide dibersihkan, ditambahkan 250 µl antibodi primer (anti H5N1 dan anti HA) yang telah diencerkan (1: 1600), kemudian diinkubasi selama 60 menit. Kemudian aktivitas peroksidase diblok dengan menambahkan tiga tetes hidrogen peroksidase (H_2HAI_2), kemudian diinkubasi selama 20 menit. Slide tersebut kemudian dibilas menggunakan PBS sebanyak tiga kali. Selanjutnya ditambahkan konjugat anti kelinci berlabel Biotin-Streptavidin diikuti dengan substrat DAB. Setelah itu slide dibilas, kemudian dicelupkan ke dalam Haematoxilyn selama dua menit. Mereka dipindahkan ke larutan Scott, kemudian diinkubasi selama 2 menit. Mereka dibilas, lalu ditutup dengan kaca penutup. Hasil positif ditandai dengan hadirnya warna coklat pada slide (Damayanti dkk., 2004). Data yang diperoleh dianalisis menggunakan ANOVA (Analysis of Variance) pada program Program Statistik untuk Ilmu Sosial (SPSS). Hasil yang mungkin dianalisis menurut analisis Least Significance Different (LSD) (Kusriningrum, 2012).

Persetujuan etis

Susunan penelitian ini telah mendapat persetujuan dari komisi etik hewan coba Fakultas Kedokteran Hewan Universitas Airlangga Indonesia.

HASIL DAN DISKUSI

Pengamatan sudah dilakukan selama tujuh hari setelah infeksi. Terungkap bahwa setiap perlakuan menunjukkan efek yang berbeda menurut angka kematian; adanya virion yang ditangkap pada septa alveoli, dan adanya IgY pada septa alveoli ayam. Pada kelompok I ayam yang tidak diberi anti HA mati pada hari ke 2 sampai hari ke 3 setelah tertular (A / Itik / Sidoarjo / 2012) (Diagram 1). Sebaliknya, ayam yang diberi anti-HA (dengan jumlah 100µg, 200µg, dan 400µg) menunjukkan kondisi sehat, dan tidak ada tanda klinis. Meskipun tidak ada tanda klinis, salah satu ayam yang diberi 100µg antibodi mati pada hari kedua setelah infeksi sementara yang lain tetap utuh sampai akhir periode pengamatan (Diagram 1).

Kelompok II yang diobati dengan anti-HA pada saat infeksi menunjukkan hasil yang berbeda. Pemberian anti-HA telah melindungi ayam dari kematian hanya pada hari ke-1 setelah terinfeksi. Pada hari kedua infeksi, kematian ayam terlihat pada setiap subkelompok; terutama pada subkelompok yang tidak diobati dengan anti-HA. Subkelompok yang tidak diobati dengan anti-AH (0 µg) telah memulai kematian sebelum subkelompok diobati dengan 100 µg anti-HA. Kematian masih dapat terlihat bahkan pada subkelompok yang diobati dengan 400 µg anti-HA (Diagram 2). Berdasarkan tingkat perlindungan, pemberian anti-HA pada saat yang bersamaan dapat memberikan perlindungan 40-80% (Tabel 1).

Sementara pemberian anti-HA pada Kelompok III telah sepenuhnya melindungi ayam dari kematian hanya pada hari ke-1 setelah infeksi, Kematian terjadi pada semua subkelompok bahkan pada subkelompok yang diberi 400 µg anti-HA. Angka kematian secara signifikan sekitar 60% dari populasi kelompok (Diagram 3). Angka ini adalah yang terbesar di antara kelompok perlakuan lainnya. Pada akhir pengamatan, hanya subkelompok ayam yang diberi perlakuan 200 µg dan 400 µg anti-HA yang bertahan hidup, sedangkan semua ayam di subkelompok lainnya mati. Dapat disimpulkan

bahwa anti-HA yang diberikan 24 jam setelah terinfeksi memberikan perlindungan sebesar 40% (Tabel 1). Mengenai dosis anti-HA, pemberian 200 µg dan 400 µg anti-HA lebih protektif daripada 100 µg anti-HA. Mereka bisa memberikan perlindungan sekitar 40-100%. Pemberian kedua dosis 24 jam sebelum infeksi menunjukkan perlindungan yang bertahan lebih lama dari waktu pemberian lainnya (Diagram 1-3). Semua data dikumpulkan, kemudian diolah menjadi analisis ANOVA. Menurut analisis ANOVA, hasil menunjukkan perbedaan yang signifikan ($p < 0,05$) (Tabel 2). Sehingga diolah menjadi analisis LSD. Hasil analisis menunjukkan bahwa pemberian anti-HA dipengaruhi oleh waktu pemberian dan dosis. Pemberian anti-HA 24 jam sebelum infeksi dapat memberikan perlindungan yang sesuai lebih lama dan lebih lama dari waktu pemberian lainnya. Penemuan ini didukung oleh hasil uji imun-histokimia (IHC). Berdasarkan hasil IHC, tidak ditemukan virus AI pada septa intra-alveola dari ayam yang diberi 400µg anti-HA pada Grup I. Hal ini ditandai dengan tidak adanya warna coklat tua seperti formasi yang terlihat pada slide IHC (Gambar 1). Sebaliknya, keberadaan virus AI diamati pada ayam yang diberi anti-HA dengan 200µg dan 100µg anti-HA pada kelompok I. Keberadaan virus AI juga telah diamati pada ayam yang tidak diberi anti-HA antibodi. (Gambar 1). Pemberian anti-HA pada kelompok II dan kelompok III tampaknya tidak dapat menetralkan virus sebaik kelompok I, sehingga jumlah virion meningkat pada kedua kelompok (Gambar 1). Keberadaan virion di dalam septa-alveoli paru-paru dapat mengganggu pernapasan ayam yang terinfeksi (Keberadaan virus AI juga telah diamati pada ayam yang tidak diberi antibodi anti-HA (Gambar 1). Pemberian anti-HA pada kelompok II dan kelompok III tampaknya tidak dapat menetralkan virus sebaik kelompok I, sehingga jumlah virion meningkat pada kedua kelompok (Gambar 1). Keberadaan virion di dalam septa-alveoli paru-paru dapat mengganggu pernapasan ayam yang terinfeksi (Keberadaan virus AI juga telah diamati pada ayam yang tidak diberi antibodi anti-HA (Gambar 1). Pemberian anti-HA pada kelompok II dan kelompok III tampaknya tidak dapat menetralkan virus sebaik kelompok I, sehingga jumlah virion meningkat pada kedua kelompok (Gambar 1). Keberadaan virion di dalam septa-alveoli paru-paru dapat mengganggu pernapasan ayam yang terinfeksi (OIE, 2016). Mengenai dosis proteksi yang efektif, 400 µg anti-HA memberikan proteksi terbaik diantara lainnya. Pada unggas, virus AI masuk ke tubuh inang melalui sistem pernafasan dan secara oral, kemudian menempel pada protein reseptor sialic acid alfa 2,3-galactosa (SA α 2,3 gal) yang terletak pada epitel mukosa yang terletak pada saluran pernafasan dan saluran pencernaan (Costahurtado et al., 2014; Webby dan Webster, 2003). Penularan biasanya terjadi melalui sumber air yang terkontaminasi, konsumsi pakan yang terkontaminasi dan kotoran hidung (Achenbach dan Bowen, 2011).

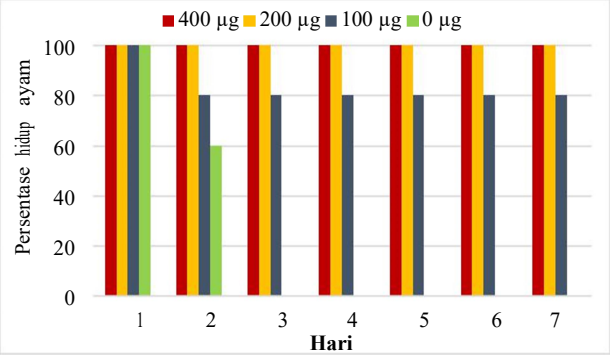


Diagram 1. Persentase ayam hidup setelah pemberian anti HA 24 jam sebelum infeksi

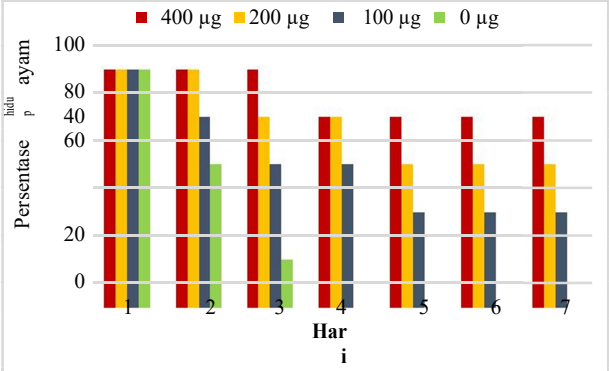


Diagram 2. Persentase ayam hidup setelah pemberian anti-HA pada waktu infeksi

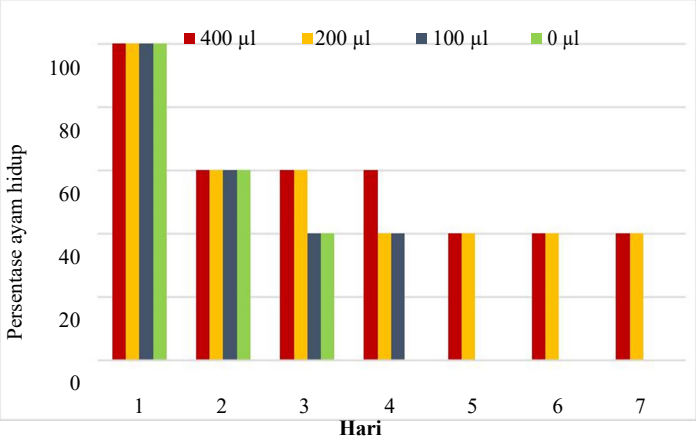


Diagram 3. Persentase ayam hidup setelah pemberian anti-HA 24 jam setelah infeksi

Tabel 1. Laju proteksi antibodi anti HA diperoleh dari kuning telur (IgY).

	Penerapan Antibodi Anti-HA (IgY)		
Dosis	**24 jam sebelum infeksi (%)**	**0 jam sebelum infeksi (%)**	**24 jam setelah infeksi (%)**
0 µg / ekor	0	0	0
100 µg / ekor	80	40	0
200 µg / ekor	100	60	40
400 µg / ekor	100	80	40

%: berarti tingkat proteksi

Tabel 2. Jumlah IgY dan waktu pemberian berpengaruh terhadap protektifitas ayam

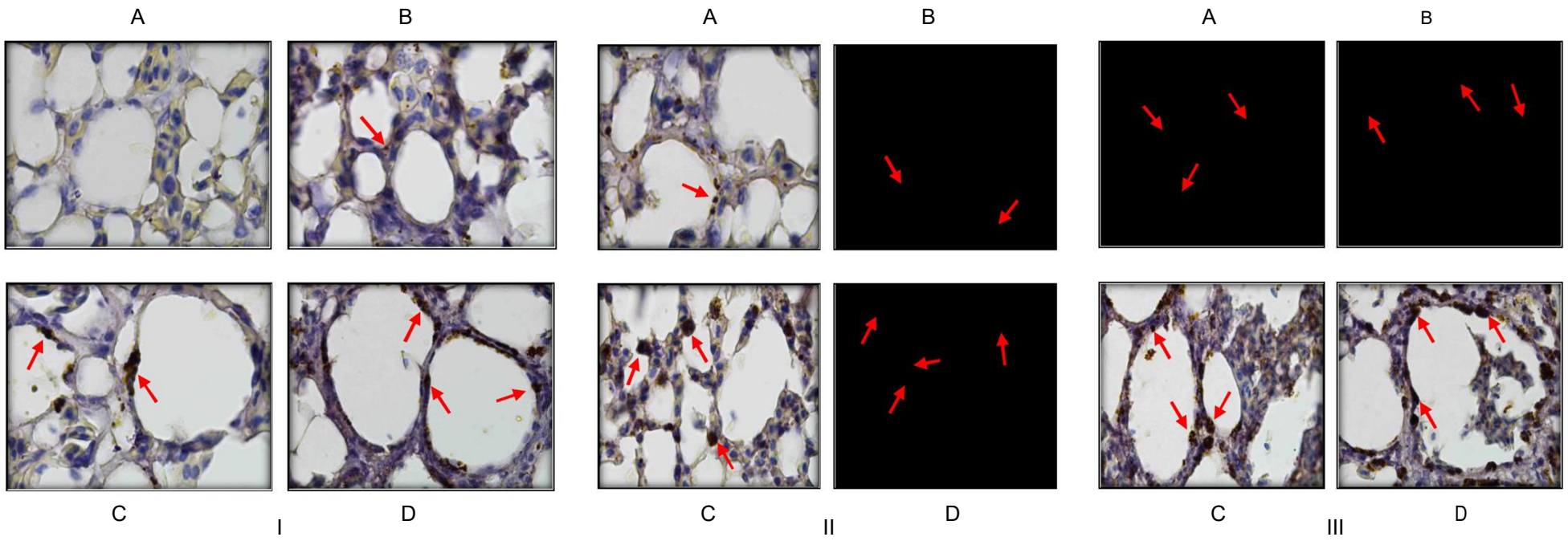
Jumlah	**Waktu penerapan anti-HA**	**(Rata-rata ± SD)**
400	H-1	$4,8^{a} \pm 0,83$
	D-0	$10^{b} \pm 1,41$
	D + 1	$23,2^{c} \pm 1,09$
200	H-1	$12,8^{d} \pm 1,09$
	D-0	$18,8^{e} \pm 1,09$
	D + 1	$35,2^{f} \pm 1,09$
100	H-1	$30,8^{g} \pm 1,09$
	D-0	$35,6^{f} \pm 0,89$
	D + 1	$41,2^{h} \pm 1,09$
0	H-1	$47,6^{i} \pm 0,89$
	D-0	$47,2^{i} \pm 1,09$
	D + 1	$47,6^{i} \pm 0,89$

Superskrip berbeda pada kolom yang sama menunjukkan perbedaan bermakna ($p < 0,05$). H-1: 24 jam sebelum infeksi, H-0: saat infeksi, H + 1: 24 jam setelah infeksi.

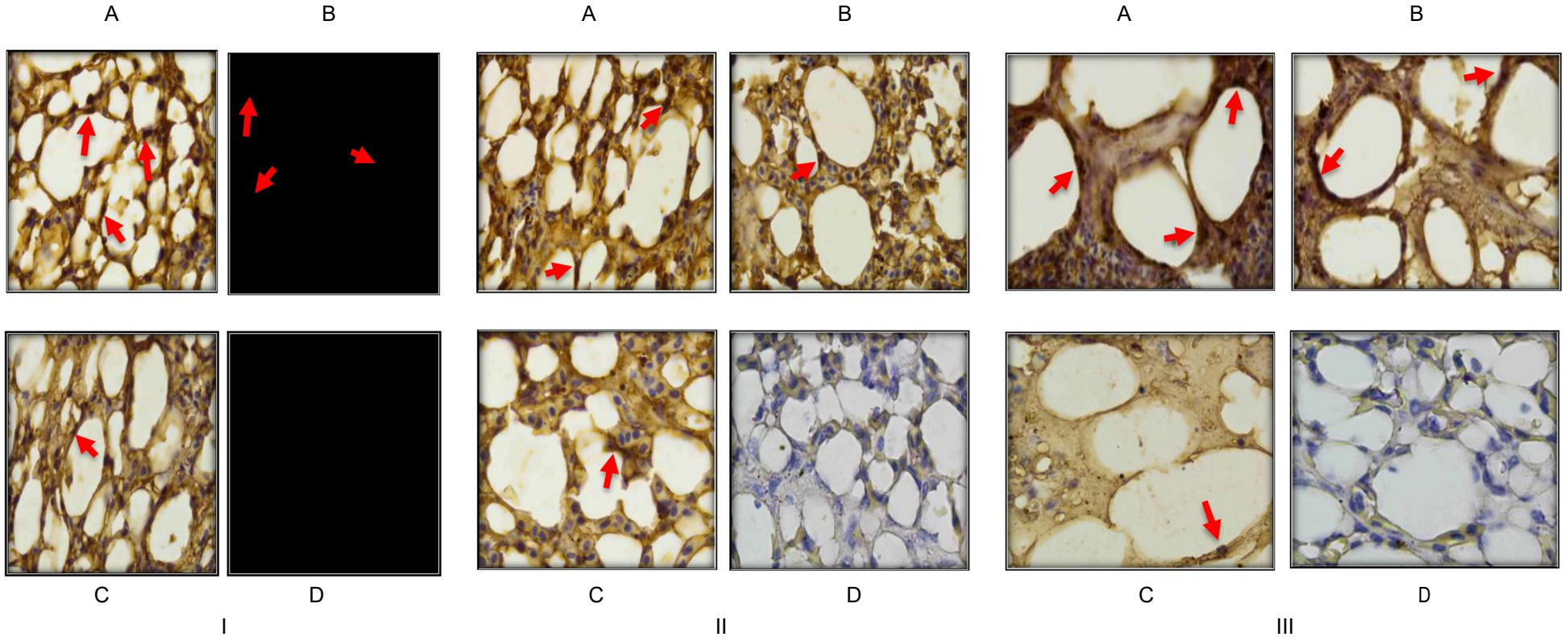
Dalam penelitian ini digunakan anti HA yang diperoleh dari kuning telur yang disebut IgY yang setara dengan Immunoglobulin G (IgG) mamalia, karena dibedakan sebagai nenek moyang. Karena IgY setara dengan IgG mamalia, ia memiliki fungsi yang mirip dengan sistem kekebalan humoral utama untuk membasmi antigen (Agrawal dkk., 2016). IgY sering digunakan sebagai substitusi dari antibodi mamalia karena proses produksinya lebih mengutamakan kesejahteraan hewan. Selain itu, lebih mudah dilakukan dan jumlah imunoglobulin yang diperoleh lebih banyak pada hewan berukuran kecil (Narat, 2003; Ko dan Ahn, 2007; Wen dkk., 2012; Agrawal dkk., 2016). Kemampuannya dalam mengikat dan menargetkan spesifitas yang lebih tinggi dibandingkan dengan Immunoglobulin G (IgG) mamalia sehingga berpotensi sebagai terapi terapeutik untuk infeksi saluran pernafasan (Abbas et.al., 2018). Penerapan IgY diketahui mampu mencegah infeksi bakteri dan virus (Narat, 2003; Ko dan Ahn, 2007; Wen dkk., 2012; Agrawal dkk., 2016). Bisa juga diterapkan pada manusia yang memberi banyak keuntungan (Pereira et.al., 2019; Constantin et. al., 2020). Menurut IHC deteksi anti-HA, anti-HA yang diberikan secara oral dapat dilihat pada septa alveoli paru-paru yang berwarna coklat (Gambar 2). Imunoterapi yang diberikan secara oral mampu memblokir reseptor pada epitel mukosa pada sistem pencernaan. Ini langsung diangkut ke seluruh tubuh diserap oleh usus ke kapiler, kemudian diangkut ke vena portal di hati dan kemudian vena cava di jantung (Rahimi et al., 2007). Pemberian anti-HA 24 jam sebelum terinfeksi memberi kesan bahwa itu dapat bersaing mengikat virus untuk reseptor protein SA α 2,3 gal. Pemberian anti-HA yang didapat dari kuda yang diberikan pada tikus secara intraperitoneal sebelum infeksi dapat memberikan perlindungan 100% (Lu dkk., 2006).

Penelitian ini menggunakan anti-AH yang diperoleh HPAI clade 2.1 (A / Ayam / Blitar / 2003) terhadap infeksi HPAI clade 2.3.2 (A / Itik / Sidoarjo / 2012). Kedua virus termasuk dalam klade yang berbeda, host yang berbeda, dan isolasi waktu yang berbeda. Menurut hasil, bahkan kedua virus itu berbeda; perlindungan silang diamati. Temuan ini memperluas fakta bahwa terjadi reaktivitas silang di antara klade subtipe H5 (Dharmayanti dkk., 2017; Ducatez dkk., 2011). Bukti ini tidak hanya terjadi di antara subtipe H5. Reaktivitas silang juga terjadi pada subtipe H7 dengan subtipe H3 dan H4. Terlihat bahwa reaktivitas silang antara H7 dan H3 lebih kuat dari H7 dan H4 (Guo dkk., 2016). Reaktivitas silang antar subspesies dalam famili yang sama tidak hanya terjadi pada AI. Itu juga terjadi pada Penyakit Newcastle (Aldous dkk., 2016). Hal yang berbeda adalah reaktivitas silang pada Penyakit Newcastle tampak lebih kuat daripada AI. Kedua virus itu tunggal virus RNA sense negatif yang terdampar. Virus RNA mudah bermutasi karena enzim polimerase mereka kekurangan pembacaan bukti. Diantaranya, tingkat mutasi AI lebih tinggi karena genomnya tersusun pada beberapa segmen yang mengarah pada antigenic shift dan antigenic drift (MacLachlan dkk., 2016).

Temuan ini mengungkapkan kemungkinan aplikasi serum hiperimun anti-HA pada pencegahan AI. Vaksinasi rutin sebagai salah satu metode pencegahan utama mungkin dapat dioptimalkan dengan aplikasi serum anti-HA secara oral. Penelitian lebih lanjut perlu dilakukan karena penelitian ini dilakukan pada lingkungan variabel dan variabel.



Gambar 1. Imunohisokimia paru-paru ayam. Tanda panah menunjukkan adanya Virus Avian Influenza pada septa alveoli. SAYA; ayam yang diberikan anti-HA IgY 24 jam sebelum infeksi. II; ayam yang diberikan anti-HA IgY 24 jam setelah infeksi. AKU AKU AKU; ayam yang diberikan anti HA IgY 24 pada saat infeksi. SEBUAH; Jumlah IgY yang diberikan adalah 400 µg. B; Jumlah IgY yang diberikan adalah 200 µg. C; Jumlah IgY yang diberikan adalah 100 µg. D; Jumlah IgY yang diberikan adalah 0µg.



Gambar 2. Imunohisokimia paru-paru ayam. Tanda panah menunjukkan aktivitas pemblokiran IgY anti-HA pada Avian Influenza Virus pada septa alveoli. SAYA; ayam yang diberikan anti-HA IgY 24 jam sebelum infeksi. II; ayam yang diberikan anti-HA IgY 24 jam setelah infeksi. AKU AKU AKU; ayam yang diberikan anti HA IgY 24 pada saat infeksi. SEBUAH; Jumlah IgY yang diberikan adalah 400 µg. B; Jumlah IgY yang diberikan adalah 0µg.

KESIMPULAN

Dapat disimpulkan bahwa aplikasi anti-HA yang diperoleh High Pathogenic Avian Influenza (HPAI) clade 2.1 (A / Ayam / Blitar / 2003) dapat memberikan perlindungan dari infeksi HPAI clade 2.3.2 (A / Duck / Sidoarjo / 2012). , meskipun mereka berasal dari kelas yang berbeda. Tingkat perlindungan 80-100% diterapkan 24 jam sebelum infeksi.

DEKLARASI

Kontribusi penulis

Suwarno berkontribusi dalam analisis data dan penulisan naskah. Saya juga berkontribusi pada produksi IgY dan memformulasi dosis IgY yang diberikan dan memproses sampel pada uji Immunohistochemistry. Rahaju Ernawati dan Nanik Sianita Widjaya berkontribusi pada analisis data dan penulisan naskah serta penghitungan dosis PIB_{50}/ ml dan melakukan uji tantangan. Semua penulis membaca dan menyetujui draf akhir naskah.

Minat yang bersaing

Penulis belum menyatakan konflik kepentingan apa pun.

Persetujuan publikasi

Semua penulis setuju untuk menerbitkan manuskrip ini di World`s Veterinary Journal.

Ucapan Terima Kasih

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REFERENSI

World Organization for Animal Health (OIE) (2016). Avian influenza (infection with avian influenza viruses). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, pp. 1–23. Available at: https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.03.04_AI.pdf

Achenbach JE and Bowen RA (2011).Transmission of avian influenza a viruses among species in an artificial barnyard. PLoS ONE, 6(3):1-7. DOI: https://doi.org/10.1371/journal.pone.0017643

Agrawal R Hirpurkar SD Sannat C and Gupta AK (2016). Comparative study on immunoglobulin Y transfer from breeding hens to egg yolk and progeny chicks in different breeds of poultry. Veterinary World, 9(4): 425–431. DOI: https://dx.doi.org/10.14202%2Fvetworld.2016.425-431

Aldous EW and Alexander DJ (2016). Detection and differentiation of newcastle disease virus (avian paramyxovirus type 1).Avian Pathology,30:117-128/ DOI: https://doi.org/10.1080/03079450120044515

Bergervoet SA, Pritz-Verschuren SBE, Gonzales JL, Bossers A, Poen MJ, Dutta J, Khan Z, Kriti D, Van Bakel H, Bouwstra R et al. (2019).Circulation of low pathogenic avian influenza (LPAI) viruses in wild birds and poultry in the Netherlands 2006–2016. Scientific Reports, 9(1):1–12. DOI: https://doi.org/10.1038/s41598-019-50170-8

Bouma A Claassen I Natih K Klinkenberg D Donnelly CA Koch G and Boven MV (2009). Estimation of transmission parameters of H5N1 avian influenza virus in chickens. PLoS Pathogens, 5(1):1-13. DOI: https://doi.org/10.1371/journal.ppat.1000281

Costa-hurtado M, Afonso CL, Miller PJ, Spackman E, Kapczynski DR, Swayne DE, Shepherd E, Smith D, Zsak A and Pantin-Jackwood M (2014). Virus interference between H7N2 low pathogenic avian influenza virus and lentogenic newcastle disease virus in experimental co-infections in chickens and turkeys. Veterinary Research, 45(1): 1–11. DOI: https://dx.doi.org/10.1186%2F1297-9716-45-1

Wright PF Neumann G and Kawaoka Y (2013). Orthomyxoviruses. In Knipe DM and Howley PM (Editor in Chief), Fields Virology 6th Edition, Philadelphia, PA 19103, pp. 1186-1243.

Guo L, Wang D, Zhou H, Wu C, Gao X, Xiao Y, Ren L, Paranhos-Baccalà G, ShuY, Jin Q and Wang J (2016). Cross-reactivity between avian influenza A (H7N9) virus and divergent H7 subtypic- and heterosubtypic influenza A viruses. Scientific Reports, 6: 1-8. DOI: https://dx.doi.org/10.1038%2Fsrep22045

Hau J and Hendriksen CFM (2005). Refinement of polyclonal antibody production by combining oral immunization of chickens with harvest of antibodies from the egg yolk. ILAR Journal, 46(3): 294–299. DOI: https://dx.doi.org/10.1093/ilar.46.3.294.

Indriani R, Dharmayanti NLPI and Martindah E (2017). Cross - reaction of duck and chicken sera against avian influenza H5N1 virus clades 2.1.3 and 2.3.2 antigens by hemagglutination inhibition test. Jurnal Biologi Indonesia, 13(2): 325–329. DOI: https://dx.doi.org/10.14203/jbi.v13i2.3407

de Jong JC, Claas ECJ, Osterhaus ADME, Webster RG and Lim WL (1997). A pandemic warning?.Nature, 389: 554-555. DOI: https://dx.doi.org/10.1038%2F39218

Ko KY and Ahn DU (2007).Preparation of immunoglobulin Y from egg yolk using ammonium sulfate precipitation and ion exchange chromatography. Poultry Science, 86(2): 400–407. DOI: https://doi.org/10.1093/ps/86.2.400

Lu J, Guo Z, Pan X, Wanf G, Zhang D, Li Y, Tan B, Ouyang L and Yu X (2006). Passive immunotherapy for influenza A H5N1 virus infection with equine hyperimmune globulin F(ab′1)2 in mice. Respiratory Research, 7: 1–7. DOI: https://doi.org/10.1186/1465-9921-7-43

Narat M (2003). Production of Antibodies in Chickens.Food Technology and Biotechnology, 41(3): 259–267.

Rahimi S, Zahra MS, Salehi TZ, Torshizi MAK and Grimes JL (2007). Prevention of Salmonella infection in poultry by specific egg-derived antibody. International Journal of Poultry Science, 6(4): 230–235. DOI: http://dx.doi.org/10.3923/ijps.2007.230.235

Webby RJ and Webster RG (2003). Are we ready for pandemic influenza?. Science, 302(5650): 1519–1522. DOI: https://doi.org/10.1126/science.1090350

Wen J, Zhao S, He D, Yang Y, Li Y and Zhu S (2012). Preparation and characterization of egg yolk immunoglobulin Y specific to influenza B virus. Antiviral Research, 93(1): 154–159. DOI: https://doi.org/10.1016/j.antiviral.2011.11.005

Abbas AT, El-Kafrawy SA, Sohrab SS and Azhar EIA (2019). IgY antibodies for the immunoprophylaxis and therapy of respiratory infections. Human Vaccines and Immunotherapeutics, 15(1): 264-275. DOI: https://doi.org/10.1080/21645515.2018.1514224

Constantin C, Neagu M, Supeanu TD, Chiurciu V and Spandidos DA (2020). IgY-turning the psge toward passive immunization in COVID-19 infection (Review). EXPERIMENTAL AND THERAPEUTIC MEDICINE, 20:151-158. DOI: https://doi.org/10.3892/etm.2020.8704

Pereira EPV, van Tillburg MF, Florean EOPT and Guedes MIF (2019). Egg yolk antibodies (IgY) and their applications in human and veterinary health:review. International Immunopharmacology, 73: 293-303. DOI: https://doi.org/10.1016/j.intimp.2019.05.015

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Profil Distribusi dan Fungsi Residu Karbohidrat pada Testis Landak Sunda Muda dan Dewasa (*Hystrix javanica*)

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ABSTRAK

Populasi landak Sunda (Hystrix javanica) setiap tahun semakin menurun karena jarang ditemukan di alam. Penelitian ini bertujuan untuk memperoleh informasi tentang sebaran residu karbohidrat yang terdapat pada testis landak sunda yang belum matang dan matang serta membahas fungsi-fungsinya yang relevan. Penelitian ini menggunakan enam buah testis yang diperoleh dari empat buah testis yang belum menghasilkan dan dua buah landak Sunda dewasa asal Kabupaten Ngawi, Provinsi Jawa Timur, Indonesia. Jaringan testis diwarnai dengan hematoksilin dan eosin serta lektin histokimia dari Lens culinaris agglutinin (LCA), Phaseolus vulgaris leucoagglutinin (PHA-L), Pisum sativum agglutinin (PSA), Sophora japonica agglutinin (SJA), dan Wheat germ agglutinin (WGA). Data dianalisis dengan metode deskriptif dan semi kuantitatif. Pewarnaan histokimia lektin dengan LCA, PHA-L, SJA, PSA, dan WGA menunjukkan adanya alpha-D-mannose dan alpha-D-glukosa, N-acetylgalactosamine, mannose, dan residu N-acetylglucosamine pada testis yang belum matang dan matang dengan intensitas lemah hingga sangat kuat. Pada testis landak Sunda yang belum matang terdapat reaktivitas positif dengan PHA-L untuk sel Leydig dan Sertoli, N-acetylgalactosamine berperan penting dalam perkembangan dan pematangan sel Leydig dan Sertoli. Testis yang matang menunjukkan reaksi positif yang kuat terhadap residu LCA, SJA, PSA, dan WGA yang menunjukkan peran signifikan alfa-D-manosa dan alfa-D-glukosa, N-asetilgalaktosamin, manosa, dan residu N-asetilglukosamin pada proses pematangan. dari spermatid awal hingga spermatid akhir. Hasil ini dapat dijadikan sebagai data dasar untuk diterapkan dalam upaya pelestarian landak sunda.

Kata Kunci : Residu Karbohidrat, Lektin, Spermatogenesis, Landak Sunda, Testis

PENDAHULUAN

Hystrix javanica merupakan landak endemik indonesia, biasa disebut landak sunda. Taksonomi landak Sunda adalah: kingdom Animalia, filum Chordata, kelas Mamalia, ordo Rodentia, famili Hystricidae, genus Hystrix dan spesies H. javanica. Berdasarkan International Union for Conservation of Nature (IUCN), landak Sunda diklasifikasikankategori perhatian yang artinya masih relatif tersebar, melimpah dan tidak menjadi ancaman besar bagi keberadaan landak sunda. The Convention on International Trade in Endangered Species (CITES) yang mengatur tentang perdagangan satwa dan tumbuhan langka termasuk landak Sunda dalam daftar lampiran III yang artinya belum termasuk kategori terancam punah.(Aplin, 2016).

Testes adalah alat reproduksi yang berjumlah satu pasang. Testis berada di skrotum dan diselimuti oleh tunika albuginea. Tubulus seminiferus terdapat di testis yang dibatasi oleh epitel kompleks sel spermatogenik dan sel Sertoli. Sel spermatogenik menghasilkan spermatozoa(Bacha Jr dan Bacha, 2012). Sel Sertoli terletak di segmen terminal tubulus seminiferus yang berfungsi sebagai transpor cairan dan aktivitas sekretori serta fagositosis dan degradasi spermatozoa intrasitoplasma.(Ahmed, 2005). Sel Sertoli memiliki inti pucat atau segitiga yaitu sel tinggi yang memanjang dari membran basal hingga lumen tubular.(Bacha Jr dan Bacha, 2012). Jaringan interstisial mengisi ruang antara tubulus seminiferus dengan pembuluh darah, pembuluh getah bening, dan saraf parenkim testis(Setchell, 1986). Sel leydig merupakan sumber androgen yang sangat penting. Lebih dari 90% androgen dalam organisme diproduksi di testis(Ahmed, 2005).

Lektin dapat didefinisikan sebagai protein pengikat karbohidrat non-imun yang dapat menggumpalkan dan atau membentuk endapan glikokonjugat. Glikokonjugat berperan dalam diferensiasi sel, pematangan sel, pengenalan sel, adhesi sel, dan interaksi sel. Distribusi glikokonjugat pada jaringan hewan dapat diteliti dengan pewarnaan histokimia lektin(Dias et al., 2015).

InformaPenemuan biologi testis pada landak Sunda belum pernah dilaporkan sebelumnya. Keterbatasan data mengenai biologi reproduksi landak sunda menjadi alasan penting untuk penelitian ini. Informasi ini dapat digunakan untuk mendukung upaya penangkaran landak sunda di Indonesia. Tujuan penelitian ini adalah untuk memberikan informasi sisa karbohidrat testis muda dan dewasa landak sunda untuk mengetahui peran glikokonjugat dalam proses spermatogenesis.

MATERIAL DAN METODE

Penelitian ini menggunakan testis dari landak Sunda jantan (dua dewasa dan empat belum dewasa) yang berasal dari Ngawi, Jawa Timur, Indonesia. Sampel testis dipotong dengan membagi tiga bagian testis sesuai dengan letak epididimis yaitu caput, corpus, dan cauda.

Hematoksipewarnaan lin dan eosin

Konvpewarnaan Hematoxylin dan Eosin (H&E) entional, yang telah digunakan oleh ahli histologi selama lebih dari 100 tahun, adalah standar emas struktur histologi(Li et al., 2018). Tahap pertama pewarnaan H&E adalah deparaffinization. Slide dicelupkan ke dalam larutan Harris Hematoxylin kemudian dicelupkan ke dalam larutan eosin. Langkah selanjutnya adalah dehidrasi. Proses pembersihan dilakukan dengan memasukkan slide ke dalam xylene. Proses pemasangan dilakukan dengan menutup tisu menggunakan glass decks dan entellan sebagai perekat.

Lpewarnaan histokimia ektin

Tahap pertama pewarnaan histokimia lektin adalah deparaffinisasi dengan xylene dan rehidrasi dengan etanol. Slide diinkubasi dalam larutan 3% H2O2 dalam metanol sebagai penghambat peroksida endogen selama 30 menit dan dicuci dengan PBS, kemudian background sniper diaplikasikan untuk memblokir protein non-spesifik selama 30 menit. Lima belas mikroliter larutan Lens culinaris agglutinin (LCA), wheat germ agglutinin (WGA), Sophora japonica agglutinin (SJA), Phaseolus vulgaris leucoagglutinin (PHA-L), Pissum sativum agglutinin (PSA) dan PBS dijatuhkan sebagai kontrol negatif, kemudian diinkubasi semalaman dalam suhu 40 C. Reaksi positif lektin divisualisasikan dengan substrat diaminobenzidine (DAB), diikuti dengan larutan Hematoxylin Harris untuk counterstain. Kemudian slide didehidrasi dengan etanol, dibersihkan dengan xylene dan dipasang.

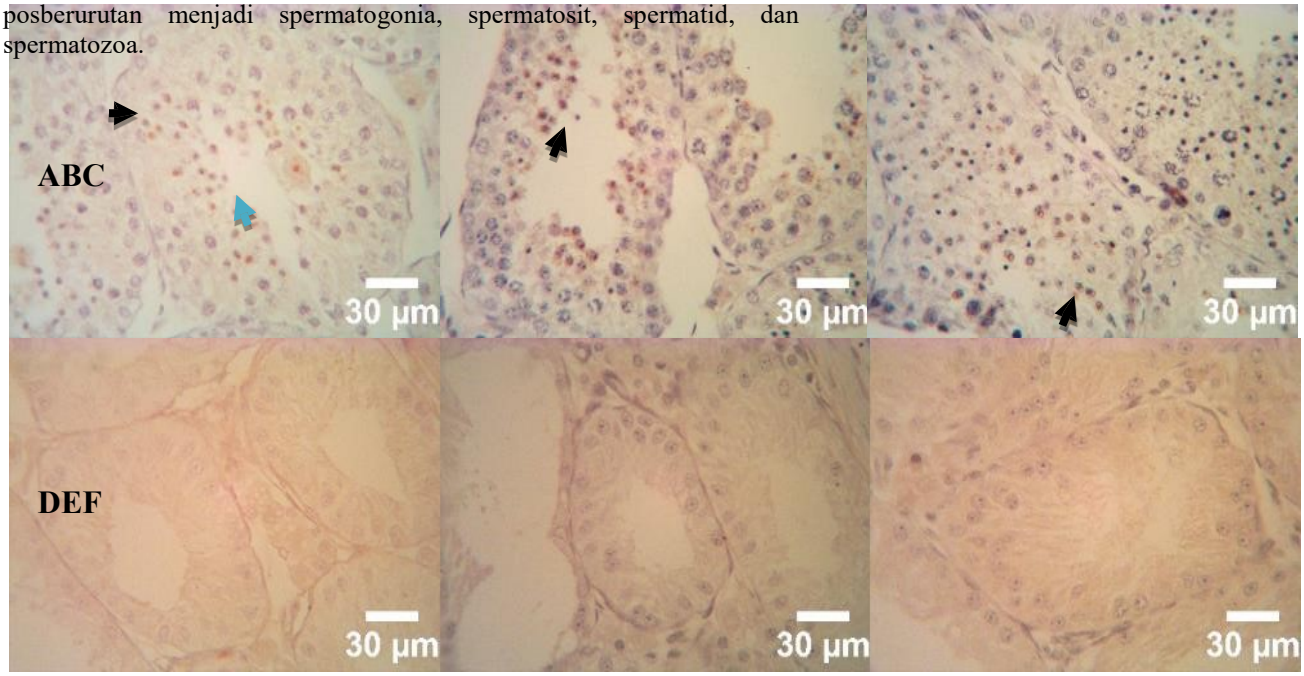
HASIL DAN DISKUSI

Lektin memainkan peran penting dalam proses yang terkait dengan pengenalan dan interaksi sel, sintesis dan transportasi protein, regulasi pembelahan sel, pembuahan, imunitas bawaan, dll.(De Schutter dan Van Damme, 2015; Feizi dan Haltiwanger, 2015). Lektin khusus untuk struktur karbohidrat tertentu. Beberapa lektin hanya dapat berinteraksi dengan residu manosa atau glukosa dan yang lainnya hanya dengan galaktosa. Ada lektin lain yang khusus untuk fucose, asam sialic, dan monosakarida lainnya(Kobayashi dkk., 2014; Nagdas et al., 2014).

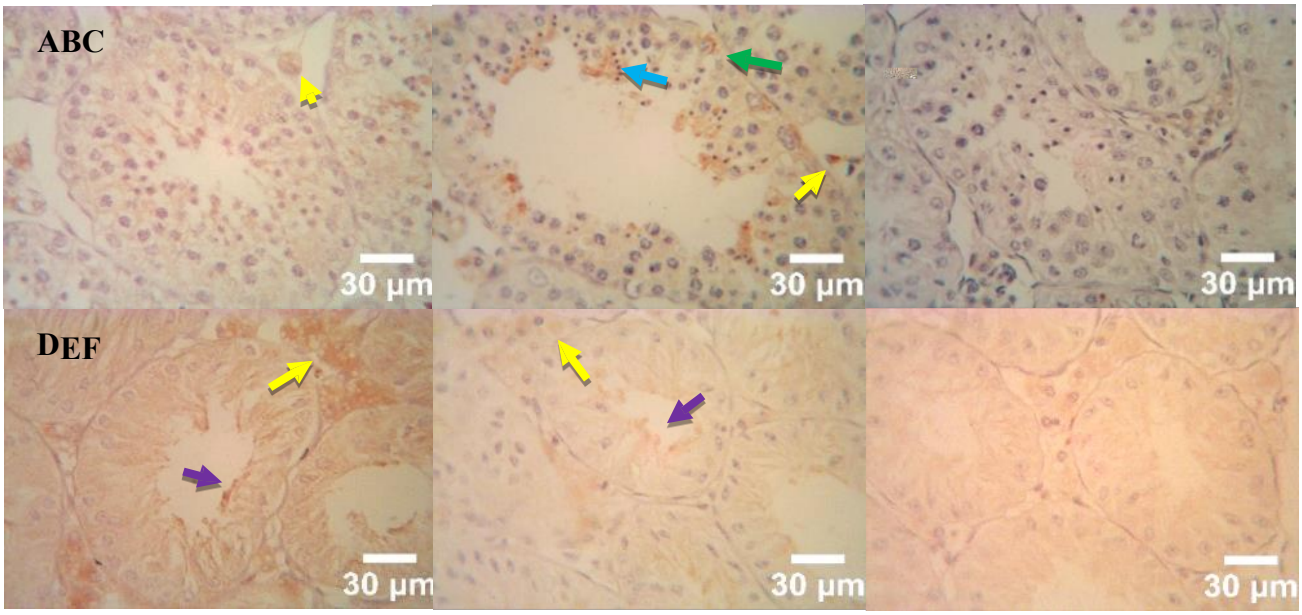
The Reaktivitas LCA pada testis landak Sunda dewasa terdeteksi kuat pada awal spermatid di kaput (Gambar 1A), corpus (Gambar 1B), dan cauda (Gambar 1C). Reaktivitas LCA terdeteksi pada spermatid akhir hanya di kaput (Gambar 1A). Reaktivitas LCA tidak terlihat pada spermatogonia, spermatosit primer, spermatid awal, spermatid akhir, sel Leydig dan sel Sertoli testis di kaput (Gambar 1D), korpus (Gambar1E), dan kauda (Gambar 1F) pada landak Sunda yang belum dewasa. BerdasarkanBarre et al. (2019), LCA khusus untuk mendeteksi residu gula alfa-D-manosa dan alfa-D-glukosa. Pewarnaan histokimia lektin LCA pada landak Sunda jantan dewasa yang terdeteksi pada spermatid awal dan spermatid akhir menunjukkan bahwa residu gula alfa-D-manosa dan alfa-D-glukosa diperlukan pada tahap awal hingga tahap akhir diferensiasi spermatid. Sedangkan pewarnaan histokimia lektin LCA yang tidak terdeteksi pada testis yang belum matang menunjukkan bahwa testis landak Sunda yang belum matang tidak memerlukan residu gula alfa-D-manosa dan alfa-D-glukosa. Penelitian pada tikus(Lee dan Damjanov, 1984)menunjukkanHasil yang berbeda di mana LCA bereaksi positif terhadap sel Sertoli, spermatogonia, spermatosit, spermatozoa, dan sel Leydig.

ThReaktivitas PHA-L pada landak Sunda dewasa terdeteksi lemah terhadap spermatogonia testis dalam korpus (Gambar 2B), dan negatif di caput (Gambar 2A) dan ekor (Gambar 2C). Pada spermatid akhir, reaktivitas PHA-L lemah dalam korpus (Gambar 2D). Reaktivitas PHA-L pada sel Leydig lemah pada kaput (Gambar 2A) dan korpus (Gambar 2C). Landak Sunda yang belum dewasa, PHA-L dalam sel Leydig menunjukkan reaktivitas positif di kaput (Gambar 2D), dan reaktivitas lemah dalam korpus (Gambar 2E). Sel Sertoli menunjukkan reaktivitas PHA-L yang lemah pada kaput dan korpus. BerdasarkanZhang dkk. (2014), PHA-L terikat N-acetylgalactosamine. Pewarnaan lektinhistokimia PHA-L yang terdeteksi pada sel Leydig, spermatogonia, dan spermatid akhir pada landak Sunda dewasa dan belum dewasa menunjukkan bahwa residu gula N-asetilgalaktosamin dibutuhkan dalam proses diferensiasi dan pematangan sel tersebut. BerdasarkanArya dan Vanhasttula (1985), kebutuhan glikokonjugat dalam porsi kecil sel Sertoli diduga berkaitan erat dengan

kemampuan agositik dan proses pembentukan badan sisa pada tahap akhir.SEBUAHmenurut Ahmed (2005), Sel Leydig merupakan sumber penting dari hormon androgen dan 90% dari hormon androgen dalam tubuh hewan diproduksi oleh testis. Residu gula N-acetylgalactosamine diperlukan untuk perkembangan sel Leydig yang optimal sehingga sel Leydig dapat memproduksi testosteron. Pewarnaan PHA-L pada tikus(Lee dan Damjanov, 1985) menunjukkaned bahwa PHA-L bereaksi
posberurutan menjadi spermatogonia, spermatosit, spermatid, dan spermatozoa.

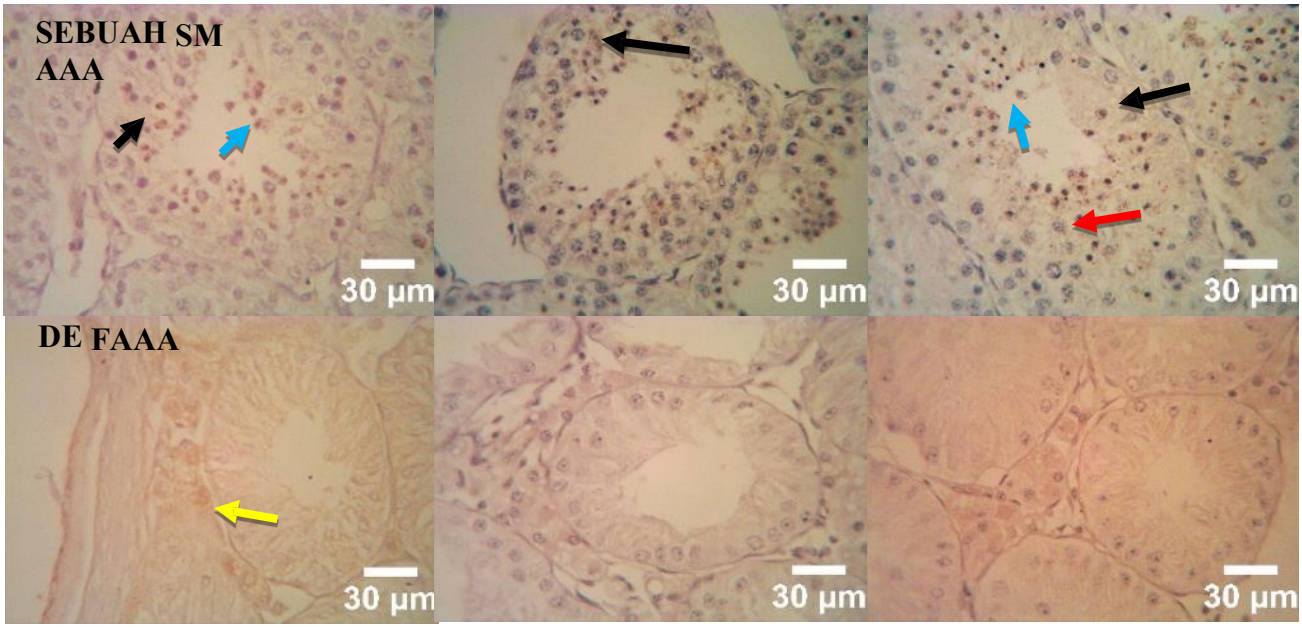


Gambar 1. Tmikrograf estikuler Hystrix javanica (pewarnaan histokimia lektin dengan LCA). A: reaktivitas LCA positif pada spermatid awal (panah hitam) dan spermatid akhir (panah biru) pada kaput testis landak Sunda dewasa. B: Reaktivitas LCA pada spermatid awal (panah hitam) dalam korpus testis landak Sunda dewasa. C: Reaktivitas LCA pada spermatid awal (panah hitam) pada kauda testis landak Sunda dewasa. Reaktivitas LCA negatif pada daerah kaput (D), korpus (E) dan cauda (F) testis landak Sunda yang belum matang.



Gambar 2.Tmikrograf estikuler Hystrix javanica (pewarnaan histokimia lektin dengan PHA-L). A: reaktivitas lektin positif pada sel Leydig (tanda panah kuning) testis landak Sunda dewasa di daerah caput. B: reaktivitas lektin positif pada spermatogonia (panah hijau), spermatid akhir (panah biru) dan sel Leydig (panah kuning) landak Sunda dewasa di regio korpus. C: reaktivitas lektin negatif di daerah ekor testis landak Sunda dewasa. D: Reaktivitas PHA-L terdeteksi pada sel Leydig (panah kuning) dan sitoplasma sel Sertoli (panah ungu) testis landak Sunda imatur. E: testis landak Sunda yang belum matang dalam korpus menunjukkan reaktivitas PHA-L positif pada Leydig (panah kuning) dan sitoplasma sel Sertoli (panah ungu). F: testis landak Sunda yang belum matang pada kauda menunjukkan reaktivitas PHA-L negatif.

Pewarnaan histokimia ektin dengan PSA untuk spermatosit primer landak Sunda dewasa menunjukkan reaktivitas minggu di daerah cauda (Gambar 3C). Reaktivitas PSA terdeteksi sedang pada spermatosit primer, spermatid awal di daerah kaput (Gambar 9A) dan lemah di korpus (Gambar 3B) dan kauda (Gambar 3C). Reaktivitas PSA terdeteksi sedang pada spermatid akhir di kaput (Gambar 3A), dan lemah di kauda (Gambar 3C). Pewarnaan histokimia lektin dengan PSA testis landak sunda muda menunjukkan reaktivitas yang lemah pada sel Leydig di kaput (Gambar 3D). BerdasarkanZhang et al. (2014), Lektin histokimia PSA berikatan dengan manosa. PSA bereaksi positif terhadap spermatosit primer, spermatid awal, dan spermatid akhir dari landak Sunda dewasa, dan bereaksi positif terhadap sel Leydig landak Sunda yang belum dewasa. BerdasarkanNovelina dkk. (2010), glikokonjugat berperan penting dalam berbagai proses metabolisme tubuh seperti regenerasi, diferensiasi sel, adhesi, dan komunikasi antar sel serta proses fungsional lainnya. Sel yang bereaksi positif terhadap PSA menunjukkan bahwa sel tersebut membutuhkan sisa gula mannose dalam perkembangannya. BerdasarkanWahyuni dkk. (2016), glikokonjugat yang terdeteksi dalam spermatid menunjukkan pentingnya glycoconjugate dalam spermatogenesis, terutama pada spermiogenesis.

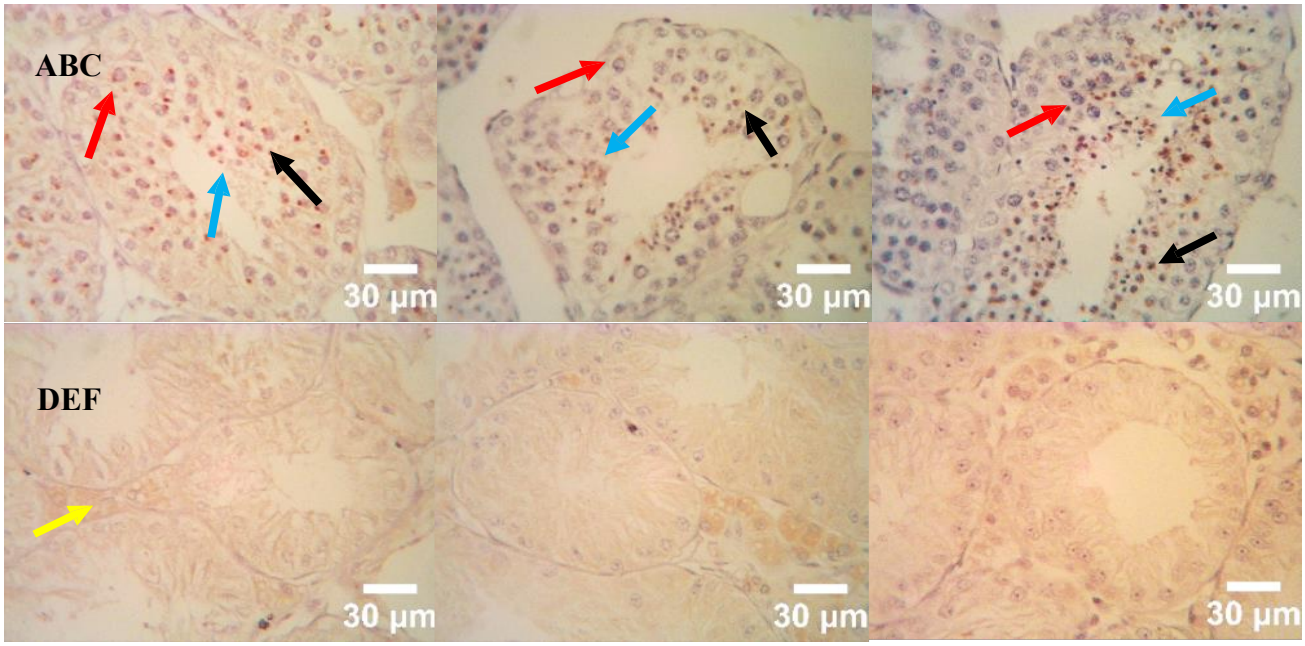


Gambar 3. Tmikrograf estikuler Hystrix javanica (pewarnaan histokimia lektin dengan PSA). A: testis landak sunda dewasa di daerah caput menunjukkan reaktivitas PSA pada spermatid awal (panah hitam) dan spermatid akhir (panah biru). B: testis landak sunda dewasa dalam korpus yang menunjukkan reaktivitas PSA pada spermatid awal (panah hitam). C: testis landak Sunda dewasa pada bagian ekor menunjukkan reaktivitas PSA pada spermatosit primer (panah merah), spermatid awal (panah hitam) dan spermatid akhir (panah biru). D: testis landak Sunda yang belum dewasa pada kaput menunjukkan reaktivitas PSA positif pada sel Leydig (tanda panah kuning), sedangkan pada korpus (E) dan kauda (F) negatif.

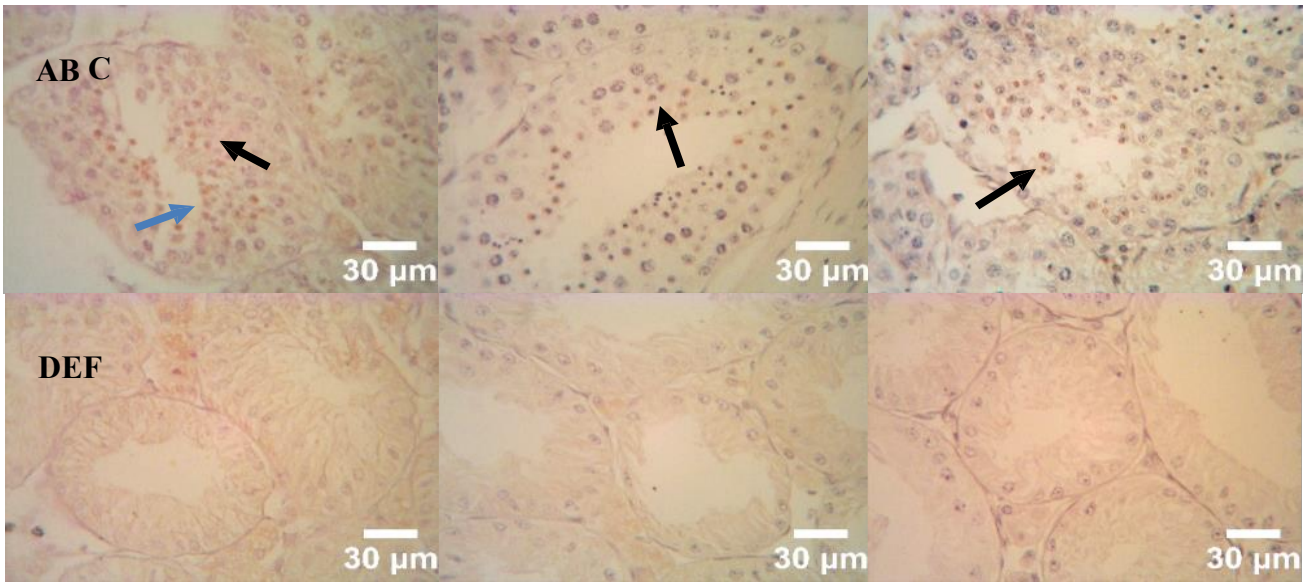
Pewarnaan lektin histokimia dengan SJA pada spermatid awal landak Sunda dewasa menunjukkan reaktivitas yang kuat pada kauda (Gambar 4C), reaksi cukup positif pada kaput (Gambar 4A), dan reaksi lemah pada korpus (Gambar 4B). Spermatid akhir menunjukkan reaktivitas SJA sedang di kaput (Gambar 4A). Sel Leydig landak Sunda yang belum dewasa menunjukkan reaktivitas SJA yang lemah di kaput (Gambar 4D). BerdasarkanZhang et al. (2014), SJA mengikat N-acetylgalactosamine. SJA menunjukkan reaksi positif pada spermatid awal dan spermatid akhir pada landak Sunda dewasa, dan sel Leydig landak Sunda yang belum dewasa. Sel yang bereaksi positif terhadap SJA menunjukkan bahwa sel tersebut membutuhkan N-acetylgalactosamine untuk perkembangannya. Pola positif ditemukan dari tahap awal hingga tahap akhir spermatid. Meskipun pada tahap akhir intensitas reaksi positif menurun, pola afinitas landak sunda positif hampir sama dengan pola afinitas positif kuda.(Verini-Supplizi dkk., 2000).

LPewarnaan histokimia ektin landak Sunda dewasa menunjukkan bahwa WGA bereaksi positif kuat pada spermatosit primer, spermatid awal dan spermatid akhir pada kaput (Gambar 5A) dan kauda (Gambar 5C), sedangkan pada korpus (Gambar 5B) positif lemah. Pada landak Sunda yang belum dewasa, WGA bereaksi lemah secara positif pada sel Leydig dalam korpus (Gambar 5E). BerdasarkanWahyuni dkk. (2016)WGA mengikat gula N-acetylglucosamine yang diperlukan untuk proses diferensiasi sel spermatosit dan spermatid. Reaktivitas WGA pada tikus terdeteksi pada spermatogonia, spermatosit, spermatozoa, sel Sertoli, sel Leydig, dan lamina propria.(Lee dan Damjanov, 1985; Shegedin dkk., 2017). Pada hamster Syria, reaktivasi WGA terdeteksi di spermatogonia(Hernández dkk. 2018).

LPewarnaan histokimia ektin dengan LCA, PHA-L, SJA, PSA, dan WGA menunjukkan adanya alpha-D-mannose dan alpha-D-glukosa, N-acetylgalactosamine, mannose, dan residu N-acetylglucosamine(Murakami dkk., 2014; Belicky dan Tkac, 2015)di testis yang belum matang dan matang.



Gambar 4. Mikrograf testis Hystrix javanica (pewarnaan histokimia lektin dengan SJA). A: Testis landak Sunda dewasa di daerah kaput menunjukkan reaktivitas SJA pada spermatosit primer (panah merah), spermatid awal (panah hitam) dan spermatid akhir (panah biru). B: testis landak Sunda dewasa dalam korpus menunjukkan reaktivitas SJA pada spermatosit primer (panah merah), spermatid awal (panah hitam). C: Testis landak Sunda dewasa di daerah ekor menunjukkan reaktivitas SJA pada spermatosit primer (panah merah), spermatid awal (panah hitam). D: testis yang belum matang pada kaput menunjukkan reaktivitas SJA pada sel Leydig (panah kuning), sedangkan pada korpus (E) dan kauda (F) menunjukkan reaktivitas SJA negatif.



Gambar 5. Mikrograf testis Hystrix javanica (pewarnaan lektinhistokimia dengan WGA). Testis landak Sunda dewasa di kaput (A) dan korpus (B) menunjukkan reaktivitas WGA positif pada spermatid awal (panah hitam) dan spermatid akhir (panah biru). C: testis landak sunda di daerah ekor menunjukkan reaktivitas WGA positif pada spermatid (panah hitam). Landak sunda yang belum dewasa pada bagian caput (D) dan cauda (F) menunjukkan reaktivitas WGA negatif, sedangkan pada daerah korpus. E: menunjukkan reaktivitas positif dalam sel Leydig.

KESIMPULAN

Kesimpulannya, penelitian ini menunjukkan bahwa pewarnaan histokimia lektin dengan LCA, PHA-L, SJA, PSA, dan WGA menunjukkan adanya alpha-D-mannose dan alpha-D-glukosa, N-acetylgalactosamine, mannose, dan N-acetylglucosamine. sisa gula pada testis landak sunda yang belum matang dan matang dengan intensitas lemah sampai sangat kuat. Pada testis yang belum matang, N-acetylgalactosamine dapat terlibat dalam perkembangan dan pematangan sel Leydig dan Sertoli, sedangkan pada testis yang matang, alpha-D-mannose dan alpha-D-glukosa, N-acetylgalactosamine, mannose, dan Residu N-acetylglucosamine berperan penting dalam proses pematangan spermatid awal hingga spermatid akhir.

DEKLARASI

Kontribusi penulis

Teguh Budipitojo mengembangkan konsep dan merancang eksperimen, menganalisis dan menginterpretasikan data. Irma Padeta mengumpulkan sampel jaringan dan memasukkannya ke dalam larutan Bouin, jaringan yang diproses untuk metode parafin-embedded. Beninda Ulima Yulianti mengembangkan konsep dan merancang eksperimen, memvisualisasikan reaktivitas lektin, dan menulis naskah. Dian Bekti Hadi Masithoh menulis naskah dan menganalisis datanya. Semua penulis membaca dan menyetujui naskah akhir.

Minat yang bersaing

The penulis belum menyatakan konflik kepentingan.

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REFERENSI

Ahmed AE (2005). Morphological, Glycohistochemical Studies on the Embryonic and Adult Bovine Testis. Ludwig-Maximilians-UniversitratMunchen. Munich. Available at: https://edoc.ub.uni-muenchen.de/3978/1/Abd-Elmaksoud_Ahmed.pdf

Arya M and Vanha-Perttula T (1985). Lectin-binding pattern of bull testis and epididymis. Journal of Andrology, 6: 230-242. DOI: https://doi.org/10.1002/j.1939-4640.1985.tb00839.x

Bacha Jr WJ and Bacha LM (2012). Color Atlas of Veterinary Histology. 3rd Edition. A Wolter Kluwer Company, New York, pp. 203-220. Available at: https://onlinelibrary.wiley.com/doi/abs/10.1111/avj.12035

Barre A, Bourne Y, Van Damme EJM and Rougé P (2019). Overview of the structure–function relationships of mannose-specific lectins from plants, algae and fungi. The International Journal of Molecular Sciences, 20(2): 254. DOI: https://doi.org/10.3390/ijms20020254

Belicky S and Tkac J (2015). Can glycoprofiling be helpful in detecting prostate cancer?. Chemické zvesti, 69(1): 90–111. DOI: https://doi.org/10.1515/chempap-2015-0052

De Schutter K and Van Damme EJ (2015). Protein-carbohydrate interactions as part of plant defense and animal immunity. Molecules, 20(5): 9029-9053. DOI: https://doi.org/10.3390/molecules20059029

Dias RDO, Machado LDS, Migliolo L and Franco OL (2015). Insights into animal and plant lectins with antimicrobial activities. Molecules, 20(1): 519-541. DOI: https://doi.org/10.3390/molecules20010519

Feizi TE and Haltiwanger RS (2015). Editorial overview: Carbohydrate-protein interactions and glycosylation: Glycan synthesis and recognition: finding the perfect partner in a sugar-coated life. Current Opinion in Structural Biology, 34:7-9. DOI: https://doi.org/10.1016/j.sbi.2015.10.005

Hernández JM, Seco-Rovira V, Beltrán-Frutos E, Ferrer C, Serrano-Sánchez MI and Pastor LM (2018). Lectin-binding pattern of glycoconjugates during spontaneoustesticular recrudescence in Syrian hamster (*Mesocricetus auratus*) after exposure to short photoperiod. Andrologia, 51(1): 13148. DOI: https://doi.org/10.1111/and.13148

Aplin K (2016). *Hystrix javanica*. IUCN Red List of Threatened Species. DOI: https://dx.doi.org/10.2305/IUCN.UK.2016-2.RLTS.T10752A22231749.en

Kobayashi Y, Tateno H, Ogawa H, Yamamoto K and Hirabayashi J (2014). Comprehensive List of Lectins: Origins, Natures, and Carbohydrate Specificities. In: Hirabayashi J (editor) Lectins. Methods and protocols. New York: Humana Press. DOI: https://10.1007/978-1-4939-1292-6_45

Lee MC and Damjanov I (1984). Anatomic distribution of lectin-binding sites in mouse testis and epididymis. Differentiation, 27: 74-81. DOI: https://doi.org/10.1111/j.1432-0436.1984.tb01410.x

Lee MC and Damjanov I (1985). Lectin binding sites on human sperm and spermatogenic cells. Anatomical Record, 212: 282-287. DOI: https://doi.org/10.1002/ar.1092120310

Li Y, Li N, Yu X, Huang K, Zheng T, Cheng X, Zeng S and Liu X (2018). Hematoxylin and eosin staining of intact tissues via delipidation and ultrasound. Scientific Report, 8: 12259. DOI: https://doi.org/10.1038/s41598-018-30755-5

Murakami Y, Hasegawa Y, Nagano K and Yoshimura F (2014). Characterization of wheat germ agglutinin lectin-reactive glycosylated OmpA-like proteins derived from *Porphyromonas gingivalis*. Infection and Immunity, 82(11): 4563-4571. DOI: https://doi.org/10.1128/IAI.02069-14

Nagdas SK, Buchanan T and Raychoudhury S (2014). Identification of peroxiredoxin-5 in bovine caudaepididymal sperm. Molecular and Cellular Biochemistry, 387:113–121. DOI: https://doi.org/10.1007/s11010-013-1876-3

Novelina S, Satryaningtijas A, Agungpriyono S, Setijanto H and Sigit K (2010). Morfologi dan Histokimia Kelenjar Mandibularis Walet Linchi (Collocalialinchi) Selama Satu Musim Berbiak dan Bersarang. Jurnal Kedokteran Hewan, 4: 192. Available at: https://pdfs.semanticscholar.org/c4f7/96e69915163d5084e0d0cce964f50b257057.pdf

Setchell BP (1986). The movement of fluids and substances in testis. Australian Journal of Biological Sciences, 39:193-207.

Shegedin A, Yashchenko A and Lutsyk A (2017). Modification of rat testis carbohydrate determinants in postnatal morphogenesis as detected by lectin probes. Journal of Cell Science and Therapy, 8:274.

Verini-Supplizi AG, Stradaioli G, Agioli O and Parillo F (2000). Localization of the lectin reactive sites in adult and prepubertal horse testes. Research in Veterinary Science, 69: 113-118.

Wahyuni S, Agungpriyono S, Adnyane IKM, Hamny H, Jalaluddin M, Gholib, Akmal M, Adam M, Aliza D and Siregar TN (2016). Studi Histokimia Lektin pada Sel-sel Spermatogenik Testis Muncak (Muntiacus muntjak muntjak). Jurnal Kedokteran Hewan, 10 (1): 82-85.

Zhang RY, Neu TR, Bellenberg S, Kuhllcke U, Sand W and Vera M (2014). Use of lectin to *in situ* visualize glycoconjugates of extracellular polymeric substances in acidophilic archaeal biofilms. Microbial Biotechnology, 8(3): 448-461. DOI: https://doi.org/10.1111/1751-7915.12188

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Asosiasi Polimorfisme Gen Transkripsi Positif Hipofisis *Factor-1* dengan Sifat Berat Badan di BC_1 Ayam Hibrida (*Gallus gallus gallus* Linnaeus, 1758) hasil Perkawinan Silang Betina F_1 Broiler dan Pelung Jantan

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ABSTRAK
Gen Positive Transcription Factor-1 pituitary sangat erat kaitannya dengan pertumbuhan dan produktivitas ayam. Penelitian ini dilakukan untuk mendeteksi Polimorfisme Nukleotida Tunggal pada gen Exon 6 Pituitary Positive Transcription Factor-1 dan hubungannya dengan pertumbuhan berat badan pada ayam hibrida backcross pertama. Prosedur penelitian meliputi persilangan ayam pedaging pertama berbiji betina dengan ayam pelung jantan untuk mendapatkan ayam silangan silang pertama, ayam umur hari menetas dipertahankan selama 49 hari, bobot badan ayam umur sehari diukur setiap tujuh hari, diisolasi DNA oleh Chelex. Metode 5%, gen Pituitary Positive Transcription Factor-1 diamplifikasi dengan PCR, pita DNA divisualisasikan menggunakan elektroforesis, dan produk PCR diurutkan menggunakan metode Sanger. Urutan DNA diselaraskan menggunakan perangkat lunak Clustal omega untuk mendapatkan Polimorfisme Nukleotida Tunggal. Analisis Polimorfisme Nukleotida Tunggal menggunakan uji korelasi Pearson antara bobot badan ayam umur 49 hari dengan titik polimorfisme. Hasil penelitian menunjukkan bahwa bobot badan ayam pedaging hibrida pertama lebih tinggi dibandingkan ayam pelung tetapi lebih rendah dari ayam pedaging filial pertama. Polimorfisme Nukleotida Tunggal tidak ditemukan pada gen ekson 6 Pituitari Positif Transkripsi Faktor-1 pada ayam hibrida backcross pertama. Hasil penelitian menunjukkan bahwa bobot badan ayam pedaging hibrida pertama lebih tinggi dibandingkan ayam pelung tetapi lebih rendah dari ayam pedaging filial pertama. Polimorfisme Nukleotida Tunggal tidak ditemukan pada gen ekson 6 Pituitari Positif Transkripsi Faktor-1 pada ayam hibrida backcross pertama. Hasil penelitian menunjukkan bahwa bobot badan ayam pedaging hibrida pertama lebih tinggi dibandingkan ayam pelung tetapi lebih rendah dari ayam pedaging filial pertama. Polimorfisme Nukleotida Tunggal tidak ditemukan pada gen ekson 6 Pituitari Positif Transkripsi Faktor-1 pada ayam hibrida backcross pertama.
Kata kuncis: Growth, ayam hibrida, gen PIT-1, SNP

PENDAHULUAN

Ayam kampung atau dikenal dengan 'ayam buras' (ayam non broiler) sangat digemari oleh masyarakat Indonesia, terutama di pedesaan. Ayam kampung digolongkan menjadi empat kelompok fungsional yaitu ayam kampung penghasil daging dan telur, ayam kicau, yang digunakan dalam upacara adat, aduan, dan ayam aduan (Hidayat dan Asmarasari, 2015). Berdasarkan (Zein dan Sulandari, 2009) suatu studi molekuler genetik, menginformasikan bahwa semua populasi ayam peliharaan berasal dari satu moyang (monophyletic) yaitu ayam hutan merah (Gallus gallus) yang berasal dari Asia Tenggara. Ayam lokal Indonesia dikembangkan melalui proses domestikasi dan dikenal dengan ayam kampung. Ayam kampung hasil persilangan antara ayam hutan Gallus bankiva dan Gallus varius yang tersebar di wilayah Indonesia khususnya di Pulau Jawa dan Nusa Tenggara. Ayam lokal atau sering dikenal dengan istilah 'ayam kampung' memiliki keunggulan pada kualitas daging dan telurnya, namun keunggulan tersebut tidak diikuti dengan kemampuan produktivitas yang baik pada daging dan telurnya (Zedi dan Sulandari, 2009).

Produktivitas ayam lokal relatif rendah, sebagai implikasi dari sistem pemeliharaan yang ekstensif. Ayam lokal Indonesia harus dipelihara secara optimal untuk mendukung industri perunggasan skala kecil sehingga menjadi solusi untuk memenuhi permintaan konsumsi pangan dalam negeri yang terus meningkat (Daryono et al., 2010). Upaya peningkatan produktivitas ayam lokal meliputi program seleksi dan kawin silang. BerdasarkanCheng(2010)Pemuliaan selektif bertujuan untuk menghasilkan ayam ras unggul dengan kualitas fenotipe yang disesuaikan dengan kebutuhan manusia. Program seleksi yang ditargetkan akan memberikan nilai ekonomi yang tinggi dalam penggunaan ayam lokal, yaitu dengan meningkatkan kualitas ayam lokal melalui program persilangan dan pemuliaan selektif karakter tertentu. Informasi dasar lainnya seperti karakteristik, asal usul, penampilan, dan produktivitas ayam lokal diperlukan untuk mengoptimalkan pemanfaatan ayam lokal di Indonesia. Informasi ini diharapkan dapat menjadikan ayam lokal Indonesia lebih dikenal, berkembang, dan dilestarikan, sehingga dapat dimanfaatkan secara berkelanjutan (SuLandari dkk., 2007). Oleh karena itu, diperlukan penelitian yang dapat mempelajari keragaman genetik dan mengidentifikasi gen yang bertanggung jawab terhadap pertumbuhan ayam hibrida.

Dengan kemajuan genetika molekuler, maka program seleksi dapat dilakukan lebih dini melalui analisis pada tingkat DNA. Gen Pituitary Positive Transcription Factor-1 (PIT-1) merupakan salah satu gen yang berkaitan erat dengan pertumbuhan dan produktivitas ayam (Miyai dkk., 2005). Seperti yang dinyatakan oleh Jiang et al.(2004) ekson 6 pada gen PIT-1 memiliki pengaruh yang signifikan kegembiraan untuk meningkatkan pertumbuhan bobot ayam. Tujuan penelitian ini adalah untuk mendapatkan ayam hibrida yang mewarisi karakteristik unggul dari kedua induk dengan karakteristik pertumbuhan yang baik menyerupai ayam broiler dan karakter fenotipik yang baik, daya tahan tubuh, kualitas daging dan telur yang baik menyerupai ayam lokal, serta peningkatan kualitas genetik melalui seleksi molekuler. Oleh karena itu, penelitian ini juga menganalisis hubungan polimorfisme ekson 6 gen PIT-1 dengan bobot badan ayam hibrida.

MATERIAL DAN METODE

Persetujuan etis

Prosedur dalam penelitian ini telah dilakukan dengan mengikuti pedoman komite etik Fakultas Kedokteran Hewan, Universitas Gadjah Mada.

Perkawinan silang ayam

Pada penelitian ini digunakan ayam pedaging hibrida pertama (BC1) hasil persilangan antara ayam broiler F1 betina dan ayam pelung jantan. Ayam Day Old (DOC) dipertahankan selama tujuh minggu dengan pencahayaan 24 jam menggunakan bola lampu 10 watt, suhu udara ± 30 ℃ dan kelembaban 40-50%, diberi pakan BR I (protein 21,00-23,00% dan energi). 3000 kkal / kg) buatan PT. Japfa Comfeed Indonesia Perusahaan Terbuka (Plc) ad libitum. 12 ekor induk terdiri dari 7 jantan dan 5 betina. Selanjutnya DOC dipelihara secara intensif selama 7 minggu di kandang khusus untuk meminimalkan pengaruh luar yang dapat mengganggu kesehatan, memudahkan pemantauan pertumbuhan, dan memudahkan pemberian makan ayam. Pengukuran bobot badan DOC setiap 7 hari bertujuan untuk melihat pertumbuhan DOC selama periode observasi selama 7 minggu. Pengukuran karakter kuantitatif dan observasi karakter kualitatif dilakukan pada hari terakhir observasi pada hari ke-49.

Isolasi DNA

Isolasi DNA dengan metode Chelex 5% dengan konsentrasi Chelex yang dimodifikasi sesuai dengan tahap optimasi. Sebanyak 10 µl darah ayam dimasukkan ke dalam tabung 1,5 mL ditambah dengan 1 ml buffer Tris-EDTA (TE). Kemudian dimasukkan ke dalam tabung microcentrifuge 1,5 mL, disentrifugasi dengan kecepatan 13.000rpm selama 3 menit. Supernatan dipindahkan ke tabung eppendorf baru, kemudian pelet ditambahkan 200µl larutan 5 persen Chelex, 18 µl dithiothreitol (DTT)
0.05 M, 2 µl proteinase K, kemudian campur berbagai sampel secara cepat selama 30 detik dengan vortex dan diinkubasi pada suhu 56 ℃ selama 2 jam, dan vortex setiap 15 menit. Kemudian diinkubasi pada 100 ℃ selama 8 menit, dan disentrifugasi pada 13.000 rpm selama 3 menit. Supernatan dipindahkan ke tabung microcentrifuge 1,5 ml, dan disimpan pada suhu -20 ℃ (Butler, 2009).

Amplifikasi DNA

Amplifikasi gen Pituitary Positive Transcription Factor-1 (PIT-1) dilakukan dengan PCR, dengan komposisi reaksi kit Bioline PCR sebanyak 12,5 µl, primer forward 5'-GGCACTTTGGAGAACAAAGC-3 'sebanyak 1,25 µl, 5'-CTCGTGGTGCTCCTTGATAA-3 'reverse primer sebanyak 1,25 µl, 5 µl sampel DNA, dan 5 µl ddH2O sehingga total volume 25 µl. Primer spesifik yang digunakan adalah MR5 (untuk ekson 6 dengan kode akses AJ236855) dari Gallus gallus (Nie et al., 2008). Program PCR yang digunakan adalah 95 denaturasi awal selama 5 menit, diikuti oleh 35 siklus denaturasi pada 95 selama 15 detik, anil pada 60 selama 60 detik, dan ekstensi pada 72 ℃ selama 60 detik, ekstensi ekstra pada 72 selama 10 menit (Van As dkk., 2000).

Persiapan agarose

Agarose ditimbang menurut konsentrasi agar (genom = 1%) (hasil PCR 1,8-2%). Selanjutnya dimasukkan ke dalam beaker glass dan ditambahkan Tris-borate-EDTA (TBE) sesuai volume ruang. Kemudian dimasukkan ke dalam oven, panaskan hingga larut (bening). Sebuah cetakan dipasang dan dipasang dengan sisir. Agar ditambahkan 2-3 µl flourosave, kemudian dituang ke dalam cetakan. Agarosa dibiarkan mengeras.

Elektroforesis

Elektroforator disiapkan. Agar dimasukkan ke dalam elektroforator Mupid-exU ™. Tris-borate-EDTA / TBE (pencelupan) ditambahkan sampai agar-agar tenggelam. Sampel dimasukkan ke dalam sumur. Elektroforator ditutup, dihidupkan, waktu diatur (20-30 menit = 100 volt, 1 jam = 50 volt) kemudian divisualisasikan di bawah sinar UV dengan sistem pencitraan gel AnalytikJena ™ dan didokumentasikan dengan GelDoc ™ Documentation System.

Pengurutan dengan metode Sanger

Produk PCR diurutkan dengan metode sekuens Sanger (Sanger et al., 1977) di Base Company pertama, Selangor, Malaysia.

Analisis data

Korelasi antara bobot ayam dianalisis menggunakan uji statistik program SPSS 16.0 one-way ANOVA dan metode LSD post hoc untuk menilai signifikansi antar strain ayam. Data sekuensing DNA dirangkai menggunakan program Gene Studio, penyelarasan beberapa sekuens menggunakan software Clustal Omega, dan uji korelasi Pearson antara bobot badan ayam dengan Single Nucleotide Polymorphism (SNP, Arnedo dkk., 2007).

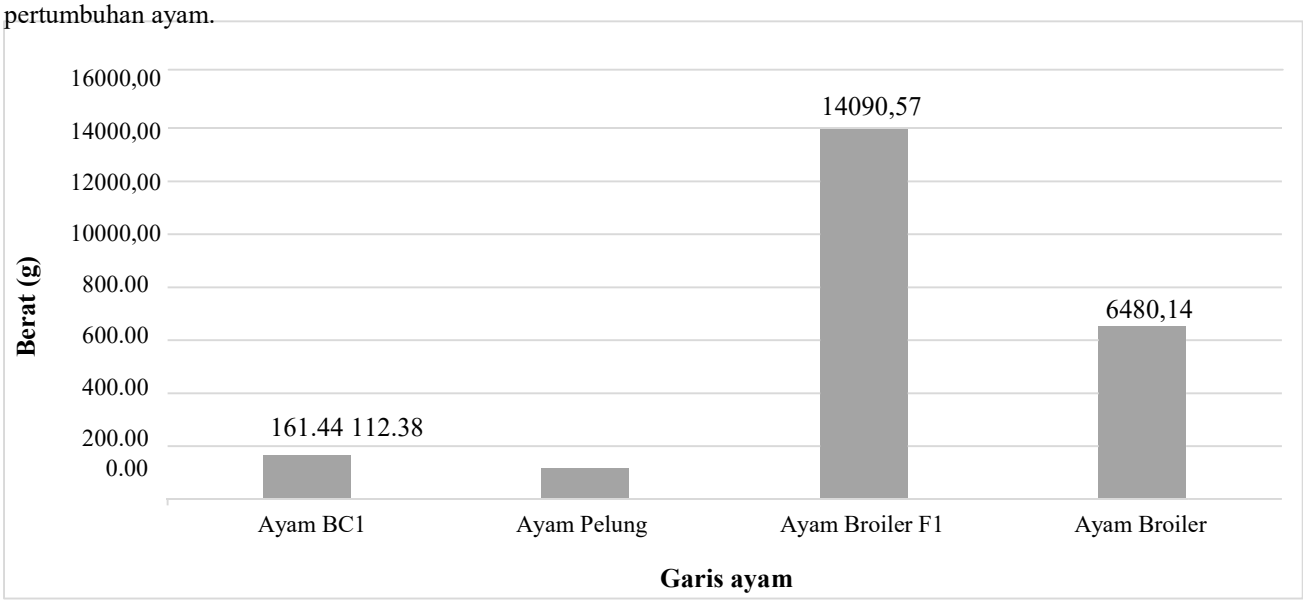
HASIL DAN PEMBAHASAN

Pertumbuhan ayam

Pada penelitian ini dilakukan persilangan antara ayam broiler F1 betina dan ayam pelung jantan pada ayam silangan pertama atau hibrida BC1. Perbandingan bobot ayam hibrida BC1, pelung, broiler, dan Ayam broiler F1 selama 7 minggu disajikan difigure 1.

Rata-rata bobot ayam dari yang terendah sampai yang tertinggi mulai dari ayam pelung, ayam hibrida BC1, ayam broiler F1, dan ayam broiler. Rata-rata bobot badan ayam broiler BC1 (161,44 gr) selama tujuh minggu menunjukkan hasil yang lebih rendah dibandingkan dengan bobot rata-rata ayam broiler F1 (648,14 g) (Roosdianto, 2010), dan ayam broiler (1409,57 gr) (Suryaman, 2010), tetapi lebih tinggi dari bobot rata-rata ayam pelung (112,38 gr). Hal ini didasarkan pada karakter yang diturunkan dari induknya, ayam hibrida BC1 mengusung karakter ayam broiler yang pertumbuhannya cepat, sehingga ayam hibrida BC1 memiliki bobot yang lebih tinggi dari ayam pelung. Pertumbuhan dan perkembangan ayam dipengaruhi oleh faktor-faktor tertentu, antara lain faktor intrinsik seperti genetika dan kelamin, serta faktor ekstrinsik seperti proses pembiakan ayam, faktor lingkungan, dan jenis pakan (Oktafiantari, 2016).

Signifikansi jenis ayam terhadap bobot ayam selama 7 minggu ditunjukkan pada tabel 1. Ayam hibrida BC1 memiliki laju pertumbuhan yang lebih tinggi dibandingkan dengan ayam pelung tetapi memiliki laju pertumbuhan yang lebih rendah dibandingkan ayam broiler dan ayam F1.Tmampu 1diperoleh perbedaan yang signifikan karena nilai signifikansi 0,00 lebih kecil dari standar deviasi 0,05. Artinya jenis-jenis ayam mempengaruhi bobot ayam. Pertumbuhan ayam ras BC1 berada di antara garis pertumbuhan ayam pelung dan ayam broiler karena ayam hibrida BC1 memiliki kedua garis keturunan. Oleh karena itu, penting untuk mengetahui lebih jauh penyebab perbedaan pertumbuhan ayam tersebut, dengan mengkaji polimorfisme gen ekson 6 PIT-1 yang telah diakui sebagai salah satu penanda genetik pertumbuhan ayam.



Gambar 1. Perbandingan bobot badan rata-rata ayam broiler BC1, Pelung, Broiler dan F1 selama tujuh minggu

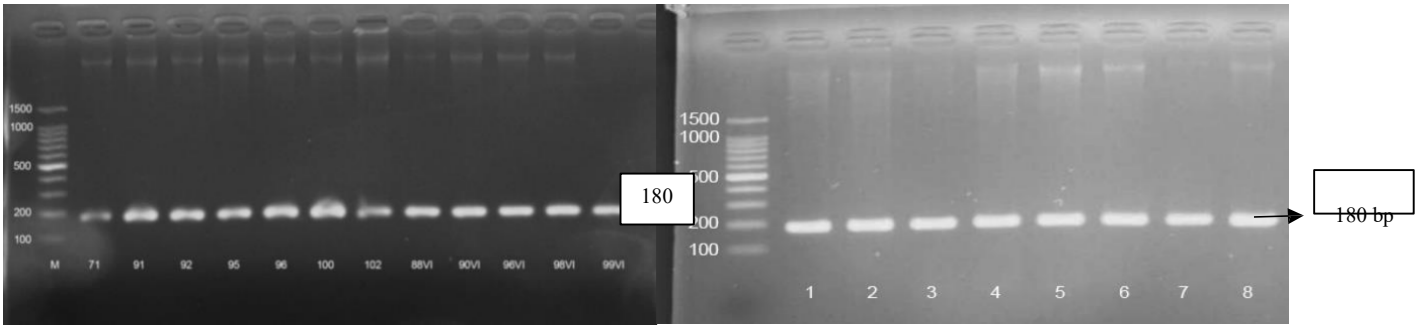
Table 1. Perbedaan bobot ayam pada ayam BC1, Pelung, Broiler dan F1 Broiler selama tujuh minggu

Line Ayam	Umur (minggu)						
	1	2	3	4	5	6	7
Hybrid SM_1	480,67 ± 6,18^{b}	650,33 ± 9,64^{b}	950,17 ± 24,37^{b}	122± 4306^{b}	156.4± 43,79^{b}	230.92± 45.49^{b}	419.08± 100.6^{b}
Broiler	194.0± 0,00^{d}	461,00 ± 0,00^{d}	842,00 ± 0,00^{d}	1309 ± 0,00^{d}	1817± 0,00^{d}	2347± 0,00^{d}	2897± 0,00^{d}
Pelung	320,33 ± 2,52^{a}	440,33 ± 11,93^{a}	570,33 ± 17,21^{a}	840,00 ± 27,22^{a}	124.3 ± 30.10^{a}	185,67 ± 420,19^{a}	258,67 ± 540,09^{a}
F_1broiler	940,30 ± 0,00^{c}	230,00 ± 0,00^{c}	387,00 ± 0,00^{c}	583,70 ± 0,00^{c}	833± 0,00^{c}	1100,3 ± 0,00^{c}	1308,70± 0,00^{c}

Polimorfisme gen faktor-1 transkripsi hipofisis positif

Seleksi molekuler dilakukan sebagai cara untuk meningkatkan kualitas genetik. Penelitian ini bertujuan untuk mendeteksi adanya polimorfisme gen PIT-1 terhadap pertumbuhan bobot ayam hibrida. Gen Pituitary Positive Transcription Factor-1 (PIT-1, POU1F1, atau GHF1) pada ayam terletak pada kromosom 1 dengan panjang 14 kb sebagai penanda genetik yang digunakan untuk membantu dalam seleksi awal berdasarkan hubungan antar penanda dan sifat kuantitatif yang diharapkan (Yamada dkk., 1993). Gen PIT-1 merupakan salah satu gen yang sangat erat kaitannya dengan pertumbuhan dan produktivitas ayam karena gen PIT-1 mengontrol ekspresi gen pengkode hormon pertumbuhan dan hormon prolaktin (Miyai dkk., 2005). Oleh karena itu, dapat dinyatakan bahwa gen PIT-1 merupakan kandidat gen yang memiliki prospek untuk digunakan sebagai penanda genetik dalam program seleksi ayam lokal. Elektroforesis dilakukan untuk mengetahui hasil amplifikasi fragmen DNA dengan PCR. Hasil elektroforesis dapat dilihat pada gambar 2 dan gambar 3.

Berdasarkan visualisasi gen ekson 6 PIT-1, 12 sampel BC1 memiliki panjang nukleotida 180 bp (Gambar 2a). Pada hasil PCR gen ekson 6 PIT-1 (Gambar 2b)Sampel 1-4 adalah ayam broiler yang memiliki panjang nukleotida 180 bp dan sampel 5-8 adalah ayam pelung yang juga memiliki panjang nukleotida 180 bp. Hasil amplifikasi DNA menunjukkan fragmen yang baik yang ditunjukkan dengan munculnya pita DNA yang tebal dan bening, kemudian dari hasil amplifikasi dengan PCR dilakukan proses sekuensing untuk menentukan sekuens nukleotida gen. Penjajaran ekson 6 LUBANG-1 gen ditampilkan diMeja 2.



(a) (b)

Gambar 2. Hasil amplifikasi gen Exon 6 Pituitary Positive Transcription Factor-1 (180 bp) dengan PCR (a) hybrid BAyam C1 (b) 1, 2, 3, 4: Ayam broiler; 5, 6, 7,8: Ayam pelung

Tabel 2. Polimorfisme nukleotida tunggal gen faktor-1 transkripsi positif hipofisis ekson 6

No. Sample	Polimorfisme gen PIT-1 Exon 6		Haplotype	Bobot ayam pada hari ke-49 (g)
	Substitusi	Substitusi		
AJ236855	A	T	Reference	-
Hybrid BC_1 1	A	T	Reference	680.00
Hybrid BC_1 2	A	T	Reference	490.00
Hybrid BC_1 3	A	T	Reference	493.00
Hybrid BC_1 4	A	T	Reference	471.00
Hybrid BC_1 5	A	T	Reference	369.00
Hybrid BC_1 6	A	T	Reference	414.00
Hybrid BC_1 7	A	T	Reference	352.00
Hybrid BC_1 8	A	T	Reference	348.00
Hybrid BC_1 9	A	T	Reference	368.00
Hybrid $BC_1$10	A	T	Reference	338.00
Hybrid BC_1 11	A	T	Reference	349.00
Hybrid BC_1 12	A	T	Reference	357.00
Broiler 1	G	A	1	-
Broiler 2	G	A	1	-
Broiler 3	G	A	1	-
Broiler 4	G	A	1	-
Pelung 1	G	A	1	321.00
Pelung 2	G	A	1	224.00
Pelung 3	G	A	1	231.00

Catatans: *A: Adenin; G: Guanin; T: Timin

Berdasarkan Tabel 2, Gen ekson 6 PIT-1 pada ayam hibrida BC1 memiliki struktur nukleotida yang sama dengan acuan (AJ236855), sehingga urutan nukleotida tersebut tidak membentuk haplotipe baru. Namun ayam pelung dan ayam broiler memiliki 2 SNP yang terletak di wilayah pengkodean. SNPs terdiri dari 2 titik substitusi termasuk Adenin ke Guanin dan Timin ke Adenin. Ayam pelung dan ayam broiler membentuk urutan nukleotida yang sama sehingga dari 2 SNP tersebut akan membentuk 1 haplotipe yang sama. Tabel 3 merupakan hasil uji korelasi Pearson yang digunakan untuk mengetahui korelasi antara bobot ayam dengan titik polimorfisme. Hasil penelitian menunjukkan bahwa dua titik polimorfisme adalah substitusi A928G dan substitusi T929. Substitusi A928G terdiri dari genotipe GG (fenotipe mutan) pada ayam pelung hari ke-49 dan bobot rata-rata 258,7 g, dan genotipe AA (fenotipe tipe liar) pada ayam hibrida BC1 dengan bobot rata-rata 419,08 g. Untuk titik polimorfisme kedua, T929A terdiri dari genotipe TT (fenotipe mutan) pada ayam pelung dengan bobot rata-rata 49 hari untuk 258,67 g dan genotipe AA (fenotipe tipe liar) pada ayam hibrida BC1 memiliki bobot ayam rata-rata 419,08 g. Frekuensi genotipe pada titik substitusi A928G dan titik substitusi T929A memiliki nilai yang sama yaitu 0,5. Koefisien korelasi di kedua titik adalah -0,588. Titik substitusi A928G dan titik substitusi T929A memiliki nilai signifikansi lebih kecil dari Untuk titik polimorfisme kedua, T929A terdiri dari genotipe TT (fenotipe mutan) pada ayam pelung dengan bobot rata-rata 49 hari untuk 258,67 g dan genotipe AA (fenotipe tipe liar) pada ayam hibrida BC1 memiliki bobot ayam rata-rata 419,08 g. Frekuensi genotipe pada titik substitusi A928G dan titik substitusi T929A memiliki nilai yang sama yaitu 0,5. Koefisien korelasi di kedua titik adalah -0,588. Titik substitusi A928G dan titik substitusi T929A memiliki nilai signifikansi lebih kecil dari Untuk titik polimorfisme kedua, T929A terdiri dari genotipe TT (fenotipe mutan) pada ayam pelung dengan bobot rata-rata 49 hari untuk 258,67 g dan genotipe AA (fenotipe tipe liar) pada ayam hibrida BC1 memiliki bobot ayam rata-rata 419,08 g. Frekuensi genotipe pada titik substitusi A928G dan titik substitusi T929A memiliki nilai yang sama yaitu 0,5. Koefisien korelasi di kedua titik adalah -0,588. Titik substitusi A928G dan titik substitusi T929A memiliki nilai signifikansi lebih kecil dari Koefisien korelasi di kedua titik adalah -0,588. Titik substitusi A928G dan titik substitusi T929A memiliki nilai signifikansi lebih kecil dari Koefisien korelasi di kedua titik adalah -0,588. Titik substitusi A928G dan titik substitusi T929A memiliki nilai signifikansi lebih kecil dari 00,05, yaitu 0,021. Berdasarkan tabel 3 dapat dijelaskan fenotipe mutan pada kedua titik yang mempengaruhi penurunan bobot ayam. Sehingga dapat disimpulkan bahwa hubungan titik polimorfisme dengan bobot ayam berkorelasi sedang secara signifikan negatif. Padahal pada penelitian sebelumnya yang dilakukan olehJiang et al.(2004) bahwa pada gen MR5 atau ekson 6 PIT-1 terdapat SNP yang berhubungan secara bermakna dengan karakter fenotipik pertumbuhan ayam. Terjadi delesi pada nukleotida C yang menyebabkan perubahan susunan asam amino setelah titik mutasi yang disebabkan oleh mutasi frameshift. Akibat mutasi frameshift, terjadi perubahan struktur protein yang mengakibatkan terjadinya kesalahan fungsi protein, atau penurunan pembentukan protein.

Table 3. Hasil uji korelasi polimorfisme gen Pituitary Positive Transcription Factor-1 terhadap rerata bobot ayam hari ke-49

Polimorfisme	**A928G Substitusi**		**T929SSA Substitusi**	
Genotipe	GG (mutan)	AA (tipe liar)	AA (mutan)	TT (tipe liar)
Genotipe frekuensi	0.5	0.5	0.5	0.5
Sayaberat ayam pada hari ke-49 (g)	258.67	419.08	258.67	419.08
Correlkoefisien ation (r)	-0,588		-0,588	
Signifilevel tidak bisa	0,021 (P <0,05)		0,021 (P <0,05)	
Menipuklusi	Signifitidak bisa dengan korelasi negatif sedang		Signifitidak bisa dengan korelasi negatif sedang	

KESIMPULA
N

Kesimpulan penelitian menunjukkan bahwa ayam broiler hibrida pertama hasil persilangan antara ayam broiler F1 betina dan ayam pelung jantan bobot badan lebih rendah dengan rata-rata bobot badan pada minggu ke-7 sebesar 419,08 g dibandingkan ayam broiler F1, tetapi lebih tinggi dari ayam pelung. . Tidak ditemukan polimorfisme gen Exon 6 Pituitary Positive Transcription Factor-1 pada ayam hibrida backcross pertama hasil persilangan antara ayam broiler F1 betina dan ayam Pelung jantan.

DEKLARASI

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REFERENSI

Arnedo M, Taffé P, Sahli R, Furrer H, Hirschel B, Elzi L, Weber R, Vernazza P, Bernasconi E Darioli R et al. (2007). Contribution of 20 single nucleotide polymorphisms of 13 genes to dyslipidemia associated with antiretroviral therapy. Pharmacogenet Genom,17:755-764. DOI: https://www.doi.org/10.1097/FPC.0b013e32814db8b7.

Butler JM (2009). DNA extraction from forensic samples using Chelex. Cold Spring Harbor Protocols, 4: 6. DOI:https://www.doi.org/10.1101/pdb.prot5229.

Cheng HW (2010). Breeding of tomorrow's chickens to improve wellbeing. Poultry Science, 89: 805-813. DOI:https://www.doi.org/10.3382/ps.2009-00361.

Daryono BS, Roosdianto I and Saragih HTS (2010). Phenotypical characters in hybrids chicken of crossbreeds between pelung and cemani. Jurnal Veteriner, 11 (4): 257-263. Available at: https://ojs.unud.ac.id/index.php/jvet/article/view/3460/2494.

Hidayat C and Asmarasari SA (2015). Native chicken production in Indonesia: a review. Indonesian Journal of Animal Science, 17 (1): 1-11. DOI: https://www.doi.org/10.25077/jpi.17.1.1-11.2015.

Jiang R, Li J, Qu L, Li H and Yang N (2004). A new single nucleotide polymorphism in the chicken pituitary-specific transcription factor (POU1F1) gene associated with growth rate. Animal Genetics, 35: 344-346. DOI: https://www.doi.org/10.1111/j.1365-2052.2004.01164.x.

Miyai S, Yoshimura S, Iwasaki Y, Takekoshi S, Lloyd RV and Osamura R (2005). Induction of GH, PRL, and TSH beta mRNA by transfection of PIT-1 in a human pituitary adenoma-derived cell line. Cell Tissue,322:269-277. DOI: https://www.doi.org/10.1007/s00441-005-0033-z.

Nie Q, Meixia F, Liang X, Min Z, Zhangmin L, Zipin L, Guohuang W, Wensen B, Canjian L, Wei Z et al. (2008). The PIT-1 gene polymorphisms were associated with chicken growth traits. Genetics, 9: 20. DOI: https://www.doi.org/ 10.1186/1471-2156-9-20.

Oktafiantari R (2016). Ghrelin gene polymorphisms encoding growth in chicken [(*Gallus gallus gallus* (Linnaeus, 1758)] second generation of backcross derived crosses between ♀ Pelung with ♂ the first generation of Backcross. Thesis. Faculty of Biology Universitas Gadjah Mada, Indonesia, p. 30.

Roosdianto I (2010). Inheritance of Phenotypic Characters of chickens (F_1) results of crosses of chicken (*Gallus gallus domesticus* Linnaeus, 1758) Pelung with Broiler. Thesis. Faculty of Biology Universitas Gadjah Mada, Indonesia, pp. 24-48.

Sanger F, Nicklen S and Coulson AR (1977). DNA sequencing with chainterminating inhibitors. Proceedings of the National

Academy of Sciences of the United States of America, 74 (12): 5463-5467. DOI: https://www.doi.org/10.1073/pnas.74.12.5463.

Sulandari S, Zein MSA, Payanti S, Sartika T, Astuti M, Widyastuti T, Sujana E, Darana S, Setiawan I and Garnida D (2007). Indonesian Local Chicken Biodiversity Resources: Benefits and Potential. Biology Research Center. Bogor, LIPI, Indonesia.

Suryaman (2010). The Comparison of morphometry of native chicken, pelung chicken and first breed chicken (F_1) result from crossbreeding between Pelung and native chickens age 5-12 Weeks. Thesis. Faculty of Animal Science, IPB University, Bogor, Indonesia. Available at: http://repository.ipb.ac.id/handle/123456789/12721.

Van As P, Buys N, Onagbesan OM and Decuypere E (2000). Complementary DNA cloning and ontogenic expression of pituitary-specific transcription factor of chickens (*Gallus domesticus*) from the pituitary gland. General and Comparative Endocrinology, 120: 127–136. DOI: https://www.doi.org/10.1006/gcen.2000.7529.

Yamada S, Hata J and Yamashita S (1993). Molecular cloning of fish Pit-1 cDNA and its functional binding to promoter of gene expressed in the pituitary. Journal of Biological Chemistry, 268(32): 24361-24366. Available at: https://www.jbc.org/content/268/32/24361.long.

Zein MSA and Sulandari S (2009). Pedigree investigation of Indonesian chicken by sequences hypervariable-1 d-loop mitochondrial dna. Jurnal Veteriner, 10(1): 41-49. Available at: http://ojs.unud.ac.id/index.php/jvet/article/view/3345.

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Trace Elements Profiles of Pregnant Camels (*Camilus dromedaries*), Fetus, and Amniotic Fluid at Birth and their Associations with Calf Birth Weight

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ABSTRACT

Amniotic fluid is a dynamic complex mixture that carries components contributing to the regulation of fetal development. The present study aimed to measure the levels of trace elements, such as Fe, Zn, Cu, Mg, Se, and Mn in amniotic fluid, maternal serum, and venous umbilical cord serum at delivery. The study further investigated the relationships among levels of elements in amniotic fluid (AF), maternal serum (MS), and venous umbilical cord serum (VUCS) in order to assess the possibility of monitoring abnormal fetal growth. Blood samples were obtained from 30 pregnant female camels at delivery and the corresponding VUCS and AF were examined. The Fe, Zn, Cu, Mg, Se, and Mn were analyzed on the atomic absorption spectrophotometer. Concentrations of trace elements, Fe, Zn, Cu, Mg, Se, and Mn in VUCS were significantly higher, compared to MS or AF. The trace elements, Fe, Zn, Cu, Mg, Se, and Mn were present in significantly lower concentrations of AF than in MS or VUCS. Neonatal birth weight did not correlate with MS levels of the trace elements. However, neonatal weight correlated positively with venous cord serum Fe, Se, and Zn levels. There was a scarcity of correlation between maternal and fetus trace elements in the pregnant camels. In conclusion, AF could even be the result of simple filtration of maternal blood. Evaluation of selected trace element levels in MS did not appear to be useful within the assessment of fetus growth. The findings of this study indicated an active transport for Fe, Zn, Cu, Mg, Se, and Mn between pregnant camels and fetus.

Keywords: Calf birth weight, Dromedary camel, Placental barriers, Pregnancy, Trace element

INTRODUCTION

Newborn calves of dromedary camels show weakness and low birth weight in a high proportion, leading to economic loss. Calf birth weight was very variable, and depended on the gender of the fetus, breed, parity (Freetly et al., 2000; Nagy and Juhász, 2019) and the nutritional state of the mother (Zachara et al., 1986), especially in the late pregnancy period that fetal requirements increase for energy, protein and minerals. Usual fetal development depends on suitable store of trace elements, including iron (Fe), zinc (Zn), and copper (Cu), magnesium (Mg), and selenium (Se) and manganese (Nandakumaran et al., 2016). The viable role of trace minerals in fetal development and their growth was recently suggested as well as their relationship to calf birth weight (Graham et al., 1994). Deficiency of trace minerals such as Fe and Zn can retard the fetal growth (Mitchell et al., 1998). Likewise, Se (Black, 2001; Mitchell et al., 1998) and Cu deficiency (Mills and Davies, 1979) were implicated as possible factors that could impair fetal development. Lower levels of the trace minerals such as Cu, Fe, and Zn were stated in aborted fetus, suggesting a possible role for these minerals in fetal growth and development (Graham et al., 1994). Pregnancy placed a considerable burden on the homeostasis of trace elements in mammals (Black, 2001), whereby the physiology of pregnant animals, and the requirements of the growing fetus changed (Faye and Bengoumi, 1994). There is evidence that camels were susceptible to trace element disorders similar to those of other ruminants (Faye et al., 1992). There were several reports of clinical mineral defects in camels (Faye et al., 1992; Faye and Bengoumi, 1994; Zong-Ping et al., 1994; Chuka Ozegbe, 2005), and their prevalence and importance were likely to be misjudged as signs of subclinical deficiency may not be detected over a long period of time.

In all mammalian species, amniotic fluid (AF) builds up early, and then decreases with the growth of the embryo (Suliburska et al., 2016). The role of the trace elements contained in AF is not clear. It was suggested that this fluid could be an important source of fetal nutrition (Abdelrahman and Kincaid, 1993). Each fetus was completely dependent on its mother via the placenta for the supply of trace elements (Perveen et al., 2002). The

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sensitivity of the fetus to the absorption of trace elements was not only strongly influenced by the uptake by the mother, the stage of pregnancy and the placental transport, but also by the inherent ability of the fetal organs to accumulate reserves (Black, 2001). The transport of trace elements from the pregnant animal to the fetus varied during pregnancy, and it gradually increased during this period of time (Tibary and Anouassi, 1997). Some trace elements were transported across the placenta in an active process, while others appeared to be transported passively (Romeu et al., 1986). Camels have an epitheliochorial type of placenta in which the fetal membranes do not invade the endometrial layer of the uterus (Tibary and Anouassi, 1997). The mechanisms involved in transplacental transport of trace elements from the pregnant camel to the fetus are still not well known. In order to more fully understand the transfer of trace elements from dam to fetus; concentrations of the trace elements in maternal, venous umbilical cord serum and amniotic fluid were investigated. Although some data exist for a number of species, including rabbits (Kriesten Schmidtmann et al., 1986), rats (Romeu Alemany et al., 1986), mice (McArdle and Erlich, 1991), sheep (Langlands Bowles et al., 1982), cattle (Van Wouwe et al., 1991) and humans (Hurley, 1976; Zhou et al., 2019), references were few for camels. The purpose of present study was to determine the element concentrations of Fe, Zn, Cu, Mg, Se and Mn in maternal, fetal cord blood and amniotic fluid at birth. Moreover, correlations between elements in fetal cord serum, amniotic fluid and maternal blood were established. Present study aimed to be a contribution for a better understanding of the mechanism of trace element transport from the pregnant dam to the fetus, and of the effect of certain elements on birth weight.

MATERIALS AND METHODS

Ethical approval

All institutional and national guidelines for the care and use of animals were followed according to the guidelines approved All procedures involving the care and the use of the animals were approved by the ethics committee of the faculty of veterinary medicine, institutional animal care and use Committee, Aswan University, Egypt.

Animals and study area

The present study was carried out on 30 pregnant camels (camels' dromedaries) during September 2016 to February 2018 in a private farm in Daraw village, Aswan province, Egypt. Camels were free from brucellosis and tuberculosis (with average age: 7 to 10 years; weight: 450 to 550 kg), which were vaccinated against Camel pox, Brucella and Rift Valley Fever. All the animals were raised under the semi-intensive system in which they were fed with barely and alfalfa hay with very limited grazing, and with common salt supplements, and had free access to drinking water. The camels were housed in an open yard. The pregnant camels were selected in consultation with cameleer who recorded their mating history, and the pregnancy was confirmed by rectal palpation.

Sampling

Ten milliliters of blood was collected from each pregnant camel immediately after delivery via jugular vein and from the umbilical cord vein of newborn while the placenta was still not separated. The umbilical cord vein contained a serum which was enriched with all necessary nutrients from the maternal blood within the placenta to supply the fetus. The blood samples were allowed to clot, and centrifuged at 3000 round per minute (rpm) for 15 minutes; the serum was separated and stored at – 20 °C for further analysis. During the first stage of labor, the amino-chorionic sac (transparent, a vascular amnion) appeared within the vulva with the fetus parts were visible within the water bag, a ten- milliliter syringe fitted with a twenty-gauge needle was wont to collect a sample of amniotic fluid which was turbid, yellowish and watery by penetrating the amnion, taking care to avoid contamination from blood or allantois fluid. Following rupture of the amnion, a sample of cord blood was obtained from umbilical cord vein. Calf birth weights were measured before colostrum was taken. All the collected blood serum and amniotic fluid samples were analyzed for Fe, Zn, Cu, Mg, Se and Mn by using an atomic absorption spectrophotometer (Shimadzu, Model AA-6601, Japan).

Statistical analyses

All obtained data were analyzed using Statistical Package for the Social Sciences (SPSS) version 25 (Armonk, NY: IBM Corp). Differences in the element contents in maternal, venous umbilical cord serum and amniotic fluid were statistically analyzed with Analysis of variance (ANOVA). Pearson's correlation was used to examine the relationships between each of trace elements and calf birth weight. Likewise, correlation of trace element levels among maternal, venous umbilical cord serum and amniotic fluid were analyzed. Mean values in the same row with different letters were statistically significant, and the highest values were represented with the letter (a). Statistical significance was declared at the $p \leq 0.05$ level and the data were presented as the mean ± Standard Error (SE).

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RESULTS

Trace element contents in maternal, venous umbilical cord serum and amniotic fluid

Iron

As shown in table 1, the mean levels of iron were 0.66 ± 0.12 parts per million (ppm) in maternal serum, 0.37± 0.09 ppm in amniotic fluid and 2.98 ± 0.66 ppm in venous umbilical cord serum, respectively. The venous umbilical cord serum iron levels were significantly higher than those in maternal serum ($P < 0.05$) and amniotic fluid; and, the maternal serum iron levels were also significantly higher than those in amniotic fluid ($P < 0.05$).

Zinc

Zinc levels in the maternal serum, venous umbilical cord, and amniotic fluid were 0.78 ± 0.10 ppm, 1.08 ± 0.29 ppm and, 0.53 ± 0.09 ppm respectively. The venous umbilical cord had a significant higher concentration of zinc than maternal serum and amniotic fluid ($P < 0.05$). Beside, zinc levels were also significantly higher in the maternal serum than in the amniotic fluid ($P < 0.05$).

Copper

The mean copper levels in maternal and cord serum and amniotic fluid were 0.75 ± 0.08 ppm, 0.91 ± 0.14 ppm and 0.61 ± 0.09 ppm; respectively. There was a significant difference between copper levels in maternal, cord and amniotic fluid ($P < 0.05$).

Magnesium

The mean levels of magnesium in maternal and venous umbilical cord serum and amniotic fluid were 7.21 ± 0.75 ppm, 8.42 ± 0.46 ppm and 6.78 ± 0.44 ppm respectively. The mean magnesium levels in both maternal and venous umbilical cord serum were significantly higher than those in amniotic fluid ($P < 0.05$).

Selenium

As shown in table 1, the mean levels of selenium were1.06 ± 0.24 ppm, 0.79 ± 0.15ppm and 0.66 ± 0.06 ppm in venous umbilical cord serum, maternal serum and amniotic fluid, respectively. The content of selenium in amniotic fluid was significantly lower than that in both maternal and venous cord serum ($P < 0.05$); and maternal serum selenium levels were also significantly lower than those in venous umbilical cord serum ($P < 0.05$).

Manganese

The mean manganese levels in maternal serum, amniotic fluid and venous umbilical cord serum were 0.27 ± 0.15ppm, 0.18 ± 0.04 ppm and 0.52 ± 0.13ppm respectively (Table 1). Mean manganese levels in maternal serum were significantly lower than that in venous umbilical cord serum ($P < 0.05$). Besides, magnesium levels were also significantly higher in the maternal serum than in the amniotic fluid ($P < 0.05$).

The correlation between maternal serum and amniotic fluid trace element contents

Several elements in AF were effectively influenced by those in maternal serum (Table 2). Concentrations of Fe, Cu, Mg, Se and Mn in AF were positively correlated with those in maternal serum (r: 0.09, 0.08, 0.01, 0.41 and 0.12), respectively. In contrast, there was a negative correlation of Zn contents between AF and maternal serum (MS) with r: - 0.19.

The correlation between maternal and venous umbilical cord serum trace element contents

Maternal serum concentrations of Fe, Cu and se were negatively correlated with those in venous umbilical cord serum (VUCS) (r: - 0.04, - 0.11 and - 0.07), respectively, but there were positive correlations of Zn, Mg and Mn contents between MS and VUCS with r: 0.01, 0.07 and 0.08 respectively (Table 3).

The relationship of trace elements levels in maternal serum, Venus umbilical cord serum and amniotic fluid with calf birth weight

The mean birth weight of the camels was 25.5±0.08 kg. Pearson correlation showed a significant positive correlation ($P < 0.05$) amongst VUCS levels of Fe, Zn and Se and calf birth weight (Table 4). Fe level in the AF showed a significant negative correlation (table 5) with calf birth weight ($P < 0.05$). No significant correlation ($P > 0.05$) could be shown between calf birth weights and levels of trace elements in maternal serum samples (Table 6).

Table 1. Element levels in maternal sera, venous umbilical cord sera and amniotic fluid of camels at parturition

Parameter (ppm)	Maternal serum (n=30)	Amniotic fluid (n=30)	Cord serum (n=30)
Fe	0.66 ± 0.12^{b}	0.37 ± 0.09^{c}	2.98 ± 0.66^{a}
Zn	0.78 ± 0.10^{b}	0.53 ± 0.09^{c}	1.08 ± 0.29^{a}
Cu	0.75 ± 0.08^{b}	0.61 ± 0.09^{c}	0.91 ± 0.14^{a}
Mg	7.21 ± 0.75^{b}	6.78 ± 0.44^{c}	8.42 ± 0.46^{a}
Se	0.79 ± 0.15^{b}	0.66 ± 0.06^{c}	1.06 ± 0.24^{a}
Mn	0.27 ± 0.15^{b}	0.18 ± 0.04^{c}	0.52 ± 0.13^{a}

Data are expressed as mean values ± Standard error (SE); the number of studied samples in each fluid is shown in parentheses. ^{a,b,c} Mean± SE in the same row with different superscripts are significantly ($P<0.05$) different. n: the number of studied samples.

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Table 2. Correlation between concentrations of trace elements in maternal sera versus amniotic fluid of camels at parturition

Parameter (ppm)	Pearson correlation Coefficient (r)	p Significant correlation at p= 0.05
Fe	0.09	0.65
Zn	-0.19	0.31
Cu	0.08	0.69
Mg	0.01	0.97
Se	0.41	0.03
Mn	0.12	0.53

Table 3. Correlation between concentrations of trace elements in maternal sera versus venous umbilical cord sera of camels at parturition

Parameter (ppm)	Pearson correlation Coefficient (r)	Significant correlation at p= 0.05
Fe	-0.04	0.84
Zn	0.01	0.94
Cu	-0.11	0.55
Mg	0.07	0.70
Se	-0.07	0.71
Mn	0.08	0.60

Table 4. Relationship between trace elements concentrations in maternal serum and calf birth weight

Elements (ppm)	Pearson correlation Coefficient (r)	Significant correlation at p= 0.05
Fe	-0.23	0.23
Zn	0.11	0.57
Cu	-0.09	0.76
Mg	-0.15	0.43
Se	0.11	0.56
Mn	-0.02	0.92

Table 5. Relationship between trace elements concentrations in amniotic fluid and calf birth weight

Parameter (ppm)	Pearson correlation Coefficient (r)	Significant correlation at p= 0.05
Fe	-0.43	0.02
Zn	0.07	0.70
Cu	0.12	0.58
Mg	0.19	0.32
Se	-0.35	0.87
Mn	0.15	0.44

Table 6. Relationship between trace elements concentrations in venous umbilical cord sera and calf birth weight

Parameters (ppm)	Pearson correlation Coefficient (r)	Significant correlation at p= 0.05
Fe	0.37	0.04
Zn	0.37	0.04
Cu	-0.24	0.21
Mg	-0.11	0.56
Se	0.48	0.01
Mn	0.24	0.20

DISCUSSION

To the best of knowledge, the current study is first study applied This was often the primary study to characterize the trace elements profiles in MS, VUCS and AF of pregnant dromedary camel , and to investigate the correlations between the concentration of the elements levels in MS, VUCS and AF, and calf birth weight. whether the correlations between element levels in MS, VUCS and AF exists or not. There were two main reasons to study the physiological relationships between blood trace elements profiles in the dams and the body weight of their newborn. First, a dam that calves a heavy calf had a greater risk of dystocia. Second, a calf which was too weak at delivery may have more problems of vitality. For good intrauterine development, the fetus needs a sufficient amount of nutrients and trace elements that can only be obtained from the mother's blood via the placenta (Rossipal et al., 2000). In the present study, the function of placenta as a fetal-maternal barrier in the transfer of some minerals from dam to fetus during gestation was well investigated. In this study, the significant

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higher concentration of Zn and Mn in VUCS than in MS was proved. Present study was in agreement with previous ones comparing the levels of metal elements in maternal and cord blood (Baig et al., 2003; Nandakumaran, 2016; Zhou et al., 2019) in human. It was supposed that Zn and Mn may cross the placenta via active transport, and the fetus exerts specific demand for Zn and Mn. However, other clarifications for the higher Mn concentrations in cord blood was also suggested, such as lower or restricted removal of Mn by fetus or inability of the fetus to use Mn (Widdowson et al., 1974).

The demand for trace elements increased rapidly during gestation, and this may result in a decline in maternal and/or fetal stores (Al-Saleh et al., 2004). In the present study, venous umbilical cord serum Fe, Cu, Se and Mg concentrations were significantly higher than those in the corresponding dam serum.

Iron was the most vital element within the blood which contributes to hemoglobin composition, and was additionally essential for the successful development of the fetus. In the present study, levels of Fe in VUCS were higher than those in MS. This finding was in accordance with that reported previously in human (Rallis and Papasteriadis, 1987) and in ovine (Gooneratne and Christensen, 1989), and indicated active transport of this element across camel placenta. This trend of changes during gestation in camel appeared too compatible with the observations in the pregnant cattle, where a decrease in placental transfer of Fe appeared to occur in the last third of pregnancy (Richards, 1999).

There was constant increase in Cu deposition throughout the fetal period and, therefore, an increasing demand for Cu by the fetus (Eltohamy et al., 1986). In this study, the level of Cu in VUCS was higher than that in MS, whereas another study showed inverse results (Zhou et al., 2019).This interaction implied that fetus has a capacity to sequester maternal Cu, even when the dam is Cu deficient (Graham et al., 1994). According to Seboussi et al. (2010) and Eltohamy et al. (1986), Cu concentration in serum decreased at the end of pregnancy due to active transfer from hepatic storage of the dam to its fetus. A significant correlation between Se and Cu was observed in camels receiving a selenium supplementation (Koller et al., 1984).

In the present study, the levels of Se in VUCS were higher than that in MS. This may be due to that selenium level which may readily crosses the camel placenta as observed in bovine (Wooten et al., 1996) and human (Baig et al., 2003). As shown in the present study, Mg levels in VUCS were significantly higher than that in MS, which was in accordance with the recent study in human (Nandakumaran et al., 2016). The results showed that the concentration of trace elements in AF was found to be lower than MS. These data suggested that trace elements in AF may be derived from blood. The role of trace elements contained in AF was not clear. Because AF was swallowed by the fetus, it was suggested that this fluid may be an important source of certain trace elements for fetal nutrition (Wooten et al., 1996).

In the current study, we did not observe a correlation between the concentration of the various elements under investigation in the maternal serum and the birth weight of the calf. Maternal serum levels of the various elements studied did not correlate positively or negatively with calf birth weight. Birth weight of the calf was one of the basic issues to judge pregnant camel management, and to expect the possibility of newborn thickness or mortality. According to various earlier studies (Barhat et al., 1979; Al Mutairi, 2000; Bissa, 2002; Nagy and Juhász, 2019), birth weight of camel calves varied from 19 to52 kg. The calf birth weight in the present study ranged between 18 and 45 kg, and the absence of a correlation of trace element levels with calf weight led to assume that the levels of these elements in MS were not useful values for the assessment of fetal weight. However, Fe levels in the AF were found to correlate negatively with calf birth weight while levels of other elements did not reveal any significant correlation. Interestingly, Fe, Zn and Se levels in the VUCS were found to have a positive correlation with calf birth weight. Amongst the variation factors, breed, parity and weight of the pregnant dam, sire, and the year and month of birth were reported to effect calf birth weight significantly (Barhat et al., 1979; Al Mutairi, 2000; Bissa, 2002; Nagy and Juhász, 2019). To the best of knowledge, no study was demonstrated an association between trace elements concentrations and camel calf birth weight. In cow, Graham et al. (1994) reported that fetal size increased as fetal Cu increased, and was less than or equal to maternal Cu. Gooneratne and Christensen (1989) showed that neither maternal nor fetal Mn were correlated with fetal size. Clearly, further studies are necessary to examine the effects of these trace elements on calf birth weight.

Several elements in AF and VUCS were effectively influenced by those in MS (Table 2 and 3). For the VUCS, the results showed a positive correlation between concentrations of Zn, Mg and Mn in VUCS and those in MS. In contrast, there were negative correlations of Fe, Cu and Se contents between VUCS and MS. On the other hand, concentrations of Fe, Cu, Mg, Se and Mn in AF were positively correlated with those in MS, while, a negative correlation of Zn contents between AF and MS was reported. There was no significant correlation between all minerals in MS and VUCS or AF. Unfortunately, there were no many previous studies reported regarding this aspect in camel. Mitchell et al. (1998) reported a positive correlation among maternal and fetal Cu, maternal and fetal Mn, and maternal and fetal Zn. In contrast, there was a lack of correlation between maternal

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and fetal Fe in cow. A positive correlation between maternal and fetal trace elements suggested fetal dependence on the dam for its supply of nutrients, including trace elements. A lack of correlation suggested fetal independence. Mechanisms regulating interactions between nutrients were still poorly described Mechanisms regulating retention, excretion or interactions between nutrients at sites of cell transport or storage proteins were needed further investigations, but studies such as presented here can direct future research toward biochemical descriptions of nutrient interactions.

CONCLUSION

The present results indicated a lively placenta transport of Fe, Zn, Mg, Mn, Cu and Se appear to be exchanged actively between dam and fetus. Fe, Zn, Cu, Mg, Se and Mn exchanged passively between dam and amniotic fluid. Evaluation of Fe, Zn, Cu, Mg, Se and Mn in maternal serum did not appear to be useful within the assessment of fetal growth**.**

DECLARATION

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Competing interests

The authors declare that they have no conflict of interest exists.

Author`s contribution

Walaa M. Essawi collected the samples, designed the experiment, performed laboratory analyses and participated in the preparation of the manuscript. Hagar F. Gouda contributed to data analysis and prepared the manuscript (writing and revision). All authors approved the final version of manuscript before publication.

REFERENCES

Abdelrahman M and Kincaid R (1993). Deposition of copper, manganese, zinc, and selenium in bovine fetal tissue at different stages of gestation. Journal of dairy science. 76(11): 3588-3593. DOI: https://doi.org/10.3168/jds.S00220302(93)77698-5.

Al-Saleh E, Nandakumaran M, Al-Shammari M, Al-Falah F and Al-Harouny A (2004). Assessment of maternal-fetal status of some essential trace elements in pregnant women in late gestation: relationship with birth weight and placental weight. Journal of Maternal-Fetal and Neonatal Medicine, 16(1): 9-14. DOI: https://doi.org/10.1080/14767050412331283157.

Al Mutairi SE (2000). Evaluation of Saudi camel calves' performance under an improved management system. Review of breeding and veterinary medicine in tropical countries (Revue d'élevage et de médecine vétérinaire des pays tropicaux), 53(2): 219-222. DOI:http://dx.doi.org/10.19182/remvt.9757.

Baig S, Hasnain N and Ud-Din Q (2003). Studies on Zn, Cu, Mg, Ca and phosphorus in maternal and cord blood. Journal of Pakistan Medical Association, 53(9): Sep 2003. Available at: https://www.jpma.org.pk/article-details/263.

Barhat N, Chowdhary M and Gupta A (1979). Note on relationship among gestation length, birth weight, placental weight and intrauterine development index in Bikaneri camel. Indian journal of animal research, 13(2):115-117. Available at: https://arccjournals.com.

Bissa UK (2002). Selectivity, Longevity and Productivity in Indian Camels (Camelus dromedarius), Rajasthan University of Veterinary and Animal Sciences, Bikaner, Available at: http://krishikosh.egranth.ac.in/handle/1/5810136975.

Black RE (2001). Micronutrients in pregnancy. British Journal of Nutrition, 85(2):193-197. DOI: https://doi.org/10.1079/bjn2000314.

Chuka Ozegbe P (2005). Comparative biochemical assessment of the amniotic fluid and maternal plasma of pregnant rabbits, Veterinary Archive, 75(5): 431-437. Available at: https://hrcak.srce.hr/31916.

Eltohamy M, Salama A and Yousef A (1986). Blood constituents in relation to the reproductive state in she-camel (Camelus dromedarius). Contributions to Tropical Agriculture and Veterinary Medicine, 24(4): 425-430. Available at: https://europepmc.org/article/med/3566702.

Faye B and Bengoumi M (1994) Trace-elements status in camels. Biological trace element research, 41(1-2): 1-11. DOI: https://doi.org/ 10.1007/bf02917213.

Faye B, Saint-Martin G, Cherrier R and Ruffa A (1992). The influence of high dietary protein, energy and mineral intake on deficient young camel (*Camelus dromedarius*)—II. Changes in mineral status. Comparative Biochemistry and Physiology Part A: Physiology, 102(2): 417-424. DOI: https://doi.org/10.1016/0300-9629(92)90155-j.

Freetly H, Ferrell C and Jenkins T (2000). Timing of realimentation of mature cows that were feed-restricted during pregnancy influences calf birth weights and growth rates. Journal of Animal Science, 78(11): 2790-2796. DOI: https://doi.org/10.2527/2000.78112790x.

To cite this paper: Essawi WM and Gouda HF (2020). Trace Elements Profiles of Pregnant Camels (*Camilus dromedaries*), Fetus and Amniotic Fluid at Birth and their Association with Calf Birth Weight. *World Vet. J.,* 10 (3): 312-319. DOI: https://dx.doi.org/10.36380/scil.2020.wvj40

Gooneratne S and Christensen D (1989). A survey of maternal and fetal tissue zinc, iron, manganese and selenium concentrations in bovine. Canadian Journal of Animal Science, 69(1): 151-159. DOI: https://doi.org/10.4141/cjas89-018.

Graham TW, Thurmond MC, Mohr FC, Holmberg CA, Anderson ML and Keen CL (1994). Relationships between maternal and fetal liver copper, iron, manganese, and zinc concentrations and fetal development in California Holstein dairy cows. Journal of Veterinary Diagnostic Investigation, 6(1): 77-87. DOI: https://doi.org/10.1177/104063879400600114.

Hurley LS (1976). Perinatal effects of trace element deficiencies. Trace elements in human health and disease, 2: 301-314. Available at: https://www.elsevier.com/en-xm.

Koller L, Whitbeck G and South P (1984). transplacental transfer and colostral concentrations of selenium in beef cattle. American journal of veterinary research, 45(12): 2507-2510. Available at: https://europepmc.org/article/med/6524746.

Kriesten K, Schmidtmann Wand Murawski U (1986). Iron and copper concentrations in the maternal and fetal serum, placenta and amniotic fluid during the reproductive stadium as well as in the milk of rabbits. Comparative biochemistry and physiology. Comparative physiology, 83(2): 291-296. DOI: https://doi.org/ 10.1016/0300-9629(86)90576-1.

Langlands J, Bowles J, Donald D, Smith AJ, Paull DR and Davies HI (1982). Deposition of copper, manganese, selenium and zinc in the ovine fetus and associated tissues. Australian journal of agricultural research, 33(3): 591-605. DOI: https://doi.org/10.1071/AR9820591.

McArdle H and Erlich R (1991). Copper uptake and transfer to the mouse fetus during pregnancy. The Journal of nutrition, 121(2): 208-214. DOI: https://doi.org/10.1093/jn/121.2.208.

Mills C and Davies N (1979). perinatal changes in the absorption of trace elements. Development of mammalian absorptive processes. Ciba Foundafion Series, 70: 247-266. DOI: https://doi.org/10.1002/9780470720530.ch14.

Mitchell J, Nicol F and Beckett G (1998). Selenoprotein expression and brain development in preweanling selenium-and iodine-deficient rats. Journal of Molecular Endocrinology, 20(2): 203-210. DOI: https://doi.org/10.1677/jme.0.0200203.

Nagy P and Juhász J (2019). Pregnancy and parturition in dromedary camels I. Factors affecting gestation length, calf birth weight and timing of delivery. Theriogenology, 134: 24-33. DOI: https://doi.org/10.1016/j.theriogenology.2019.05.017.

Nandakumaran M, Al-Sannan B, Al-Sarraf H and Al-Shammari M (2016). Maternal–fetal transport kinetics of manganese in perfused human placental lobule in vitro. The Journal of Maternal-Fetal and Neonatal Medicine, 29(2): 274-278. Available at: https://www.tandfonline.com/doi/full/10.3109/14767058.2014.998193.

Perveen S, Altaf W, Vohra N, Bautista ML, Harper RG and Wapnir RA (2002). Effect of gestational age on cord blood plasma copper, zinc, magnesium and albumin. Early human development, 69(1-2): 15-23. DO: https://doi.org/10.1016/S0378-3782(02)00024-5

Rallis T and Papasteriadis A (1987). A study on iron and copper concentrations of foetal liver in sheep. Journal of Veterinary Medicine Series A, 34(1-10): 582-584. DOI: https://doi.org/10.1111/j.1439-0442.1987.tb00318.x.

Richards MP (1999). Zinc, copper, and iron metabolism during porcine fetal development. Biological trace element research. 69(1): 27-44. DOI. https://doi.org/10.1007/BF02783913.

Romeu A, Alemany M and Arola L (1986). Net transfer of essential metals from mother to fetus in the second half of pregnancy in the rat. Neonatology, 49(4): 204-210. DOI. https://doi.org/10.1159/000242532.

Rossipal E, Krachler M, Li F and Micetic-Turk D (2000) Investigation of the transport of trace elements across barriers in humans: studies of placental and mammary transfer. Acta Paediatrica, 89(10): 1190-1195. DOI: https://doi.org/ 10.1080/080352500750027556.

Seboussi R, Faye B, Alhadrami G, Askar M, Ibrahim W, Mahjoub B, Hassan K, Moustafa T and Elkhouly A (2010). Selenium distribution in camel blood and organs after different level of dietary selenium supplementation. Biological trace element research, 133(1): 34-50. DOI. https://doi.org/10.1007/s12011-009-8410-1.

Sulibursk a J, Kocyłowski R, Komorowicz I, Grzesiak M, Bogdański P and Barałkiewicz D (2016). Concentrations of mineral in amniotic fluid and their relations to selected maternal and fetal parameters. Biological trace element research, 172(1): 37-45. DOI: https://doi.org/10.1007/s12011-015-0557-3.

Tibary A and Anouassi A (1997) Theriogenology in Camelidae: Anatomy, Physiology, Pathology and Artificial Breeding. Available at: https://openlibrary.org/books/OL17569021M.

Van Wouwe JP, Hoogenkamp S and Van den Hamer CJ (1991). Zinc and copper of fetal organs during the second trimester of pregnancy. Biological trace element research, 29(3): 175. DOI: https://doi.org/10.1007/BF03032675.

Wapnir R, Gyasi I, Harper R, Moyse J and Teichberg S (1996). Placental copper transport in the rat. II: Effect of maternal copper deficiency. Placenta, 17(7): 479-486. DOI: https://doi.org/10.1016/S0143-4004(96)90030-2.

Widdowson EM, Dauncey J and Shaw J (1974). Trace elements in fetal and early postnatal development. Proceedings of the Nutrition Society, 33(3): 275-284. DOI: https://doi.org/10.1079/PNS19740050.

Wooten L, Shulze RA, Lancey RW, Lietzow M and Linder MC (1996). Ceruloplasmin is found in milk and amniotic fluid and may have a nutritional role. The Journal of Nutritional Biochemistry, 7(11): 632-639. DOI: https://doi.org/10.1016/S0955-2863(96)00127-1.

Zachara BA, Wąsowicz W, Gromadzińska J, Skłodowska M and Krasomski G (1986). Glutathione peroxidase activity, selenium, and lipid peroxide concentrations in blood from a healthy polish population. Biological trace element research, 10(3): 175-187. DOI:https://doi.org/10.1007/BF02795616.

Zhou C, Zhang R, Cai X, Xiao R and Yu H (2019). Trace elements profiles of maternal blood, umbilical cord blood, and placenta in Beijing, China. The Journal of Maternal-Fetal and Neonatal Medicine, 32(11): 1755-1761. Available at: https://www.tandfonline.com/doi/abs/10.1080/14767058.2017.1416602.

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Zong-Ping L, Zhuo M and You-Jia Z (1994). Studies on the relationship between sway disease of Bactrian camels and copper status in Gansu province. Veterinary research communications, 18(4): 251-260. DOI:https://doi.org/10.1007/BF01839191.

To cite this paper: Essawi WM and Gouda HF (2020). Trace Elements Profiles of Pregnant Camels (*Camilus dromedaries*), Fetus and Amniotic Fluid at Birth and their Association with Calf Birth Weight. *World Vet. J.,* 10 (3): 312-319. DOI: https://dx.doi.org/10.36380/scil.2020.wvj40

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Effects of Curcumin Supplementation on Viability and Antioxidant Capacity of Buffalo Granulosa Cells under *In Vitro* Culture Conditions

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ABSTRACT

The current study was conducted to investigate the possible protective effect of curcumin supplementation on buffalo granulosa cells (GCs) under *in vitro* culture condition. Buffalo ovaries were collected from local abattoir in physiological saline solution and transported directly to laboratory. Follicular fluid containing GCs and cumulus-oocyte-complexes were aspirated from antral follicles with diameter 2-8 mm. The collected GCs were seeded (Approximately 375,000 viable cells) in an 8-well culture plate containing tissue culture medium-199 (TCM-199) and kept at 37 °C in a humidified atmosphere of 5% CO_2. The curcumin was supplemented to TCM media at levels of 1, 2.5, 5 and 10 µM for 24 and 48 h at 37 °C or kept without treatment as control group. The viability of cells was determined using the trypan blue test. Intracellular reactive oxygen species (ROS) level was assessed by measuring the fluorescent intensity of 6-carboxy-2′,7′-dichlorodihydro fluorescein diacetate (H_2DCFDA). In addition, mitochondrial activity of GCs was determined. The results of the present study indicated that the viability of GCs under culture conditions was significantly decreased in groups treated with 1, 2.5, 5 and 10 µM curcumin (86.0%, 86.26%, 83.0% and 74.0%, respectively) compared to control group (93.60 %). The two groups of granulosa cells cultured with 2.5 and 5 µM curcumin recorded greater level of mitochondrial activity than the groups cultured with 1 µM and 10 µM curcumin. Moreover, there was a significant increase in ROS level in group cultured with 10 µM curcumin, compared to control and other experimental groups. The enzyme activity of catalase (CAT), superoxide dismutase (SOD), glutathione (GSH) and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) was increased after treating *in vitro* cultured granulosa cells with 5 µM of curcumin. However, the enzymatic activity of CAT, SOD, GSH and DPPH was declined significantly 48 h post-curcumin treatment. In conclusion, supplementation of curcumin at low concentration (2.5 µM) for 24 h to *in vitro* cultured GCs improved intracellular metabolic activity and antioxidant protective system, whereas it could not sustain this action for 48 h. Moreover, supplementation of curcumin at high concentration and for long duration may negatively affect viability of GCs under *in vitro* culture condition via induction of oxidative stress.

Key words: Antioxidant, Buffalo, Granulosa cells, Oxidative stress, Viability.

INTRODUCTION

Oxidative stress, mediated by oxygen-derived free radicals (also known as reactive oxygen species, ROS) is a frequent state affecting nearly all living organisms because of suboptimal environmental conditions. In homeostatic situation, there is stability between the production of ROS and scavenging power of cells through the cellular antioxidant system (Panieri et al., 2016). Nevertheless, when the production of ROS overcomes the cellular antioxidant capability, it perhaps contributes to a problem referred to oxidative stress (Agarwal et al., 2005). The ROS level could be elevated endogenously during many physiological and reproductive procedures including ovulation (Agarwal et al., 2005; Gupta et al., 2010). Moreover, the use of oxygen as a respiratory substrate was reported to produce oxidative stress throughout the aerobic metabolic process and energy production (Frisard and Ravussin, 2006). Furthermore, other endogenous sources (mitochondria, inflammatory cell activations, plasma membrane nicotinamide adenine dinucleotide phosphate, oxidase, lysosomes, and peroxisomes) may affect the production of ROS in mammalian cells (Klaunig et al., 2009).

Incidence of oxidative stress mediated by ROS was found to be having a negative effect on female reproductive system and finally causes infertility (Agarwal et al., 2012). Ovarian granulosa cells (GCs), the major cellular constituent in a follicle, have two vital functions in female reproduction: steroid production and defend the oocyte throughout ovulation (Yada et al., 1999; Sohel et al., 2013; Cinar and Sohel, 2015). At the end of follicular growth, GCs in the dominant follicle are differentiated into luteal cells by an ovulatory luteinizing hormone (LH) surge (Duffy and Stouffer,

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2003). This method is crucial for successful ovulation and formation of corpus luteum to keep the pregnancy. On the other hand, all through ovulation after the pre-ovulatory rise of LH, inflammatory cells particularly neutrophils and macrophages are vastly hired to produce ROS to facilitate follicular rupture and the release of the oocyte (Shkolnik et al., 2011), indicating exposure of GCs to some sort of oxidative stress during ovulation. In addition to an endogenous source, environmental sources of ROS could make the situation more complex.

One of the plants efficiently used in folk medicine is Curcuma *longa Linn* (Hatcher et al., 2008). In this herb, curcumin component has the highest proportion (Aggarwal et al., 2007). Curcumin is a yellow polyphenol compound found in turmeric (Esatbeyoglu et al., 2012), and its chemical structure is *1, 7-bis (4-hydroxy-3- methoxyphenyl)-1, 6-eptadiene-3, 5-dione* (Nadkarni, 2007; Kádasi et al., 2012). It has proven to be a highly effective anti-carcinogenic, antiviral, antioxidant (Steward et al., 2008; Correa et al., 2013; Tapia et al., 2013), and anti-inflammatory substance in human and animal models (Epstein et al., 2010; Sung et al., 2012).

Curcumin acts as an antioxidant since it scavenges reactive oxygen and nitrogen species (Barzegar and Moosavi-Movahedi, 2011; Trujillo et al., 2013 ; Mohebbati et al. 2017) and induces cytoprotective enzymes such as glutathione-Stransferase (GST), γ-glutamyl cysteine ligase (γ-GCL) and heme oxygenase-1 (HO-1) (Dinkova-Kostova et al., 2008; Reyes-Fermín et al., 2012). It is able to scavenge hydrogen peroxide, peroxyl radicals, superoxide anion, hydroxyl radicals, singlet oxygen, nitric oxide, and peroxynitrite anion (Trujillo et al., 2013). It has been revealed that curcumin causes endogenous antioxidant defense systems by modulating transcription factors such as nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (Tapia et al., 2012; Liu et al. 2016; Zhang et al., 2019a; Zhang et al., 2019b; Zhu et al., 2020), activator protein-1 (AP-1), and nuclear factor kappa B (NFκB) (Pinkus et al., 1996). Therefore, the aim of this study was to investigate the effects of curcumin supplementation to *in vitro* culture media of buffalo GCs on their viability and enzymatic defense system.

MATERIALS AND METHODS

Experimental groups

A primary culture of GCs was used as a basic technique to study the effects of curcumin supplementation on buffalo GCs cultured *in vitro* in TCM-199 medium. Primary cultures of GCs were grown in six groups. The groups were divided as the following: group 1: untreated (control), group 2: untreated control and add dimethyl sulfoxide (DMSO) (it is the dissolving solution for curcumin), group 3: treated only with 1 µM curcumin, group 4: treated only with 2.5 µM curcumin, group 5: treated only with 5 µM curcumin and group 6: treated only with 10 µM curcumin. The recovered cells were grown until they reached up to 40–50% confluency before being allocated into the different treatment groups. A minimum of 10 ovaries were used in each biological replicate. Three biological replicates of GCs were used for each experimental assay done in this study.

Collection of ovaries and granulosa cells

Granulosa cells were collected and cultured according to the procedure described by Sohel et al. (2017). A total of 120 buffalo ovaries were obtained from a local slaughterhouse, and transported in 0.9% saline solution at 37 °C within 2 h of collection. A minimum of 10 ovaries were used in each replicate in order to obtain a sufficient number of GCs for different assays. Ovaries were washed twice with 0.9% saline solution and then washed once with 70% ethanol. The follicular contents (follicular fluid containing GCs and cumulus-oocyte complexes) were aspirated from antral follicles of 2–8 mm in diameter by an 18-gauge needle attached to a 5-mL syringe and placed in a 50-mL sterile falcon tube containing 10-mL TCM-199 medium (Sigma- Aldrich, M5017, Steinheim, Germany). After collection, tubes were left for 15 min at 37 °C to allow the oocyte-cumulus complexes and cellular debris to settle at the bottom of the tube. The upper liquid containing GCs was then collected in a 15-mL falcon tube, and centrifuged at 1800 rpm for 5 min to obtain the GCs. The collected GCs were washed with 5 ml of phosphate buffer saline (PBS) that is free from calcium magnesium by repeat pipetting followed by centrifugation at 1500 rpm for 10 min. Finally, 3 ml of trypsin was added and the tube incubated at 37 °C for 3 min, then 5 ml of TCM-199 was added to inactivate trypsin by repeat pipetting of GCs followed by centrifugation at 1500 rpm for 10 min.

In vitro culture and treatment of granulosa cells

Approximately 375,000 viable cells were seeded in an 8-well culture plate (Corning Incorporated, Kennebunk, ME, USA), *in vitro* culture in medium containing TCM-199 medium (Sigma-Aldrich, D6046, Steinheim, Germany) supplemented with 10% fetal bovine serum (vol/vol), penicillin (100 U/mL) and streptomycin (100 µg/mL) (Sigma Aldrich, P4333, Steinheim, Germany) and kept at 37 °C in a humidified atmosphere of 5% CO_2 until reached 40–50% confluency. The curcumin was added to the TCM-199 medium at the following levels (Control, DMSO, 1 µM, 2.5 µM, 5 µM and 10 µM) for 48 h with change the medium once after 24 h.

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Cell morphology and viability

After treatment, GCs from different treatment groups were observed using inverted microscopy for confluency and changes in morphology. The viability of cells was determined using the trypan blue exclusion test as described by Strober (2015) with some modifications. Briefly, after the treatment, both adherent and floating cells from each treatment group were collected and resuspended in one mL of *in vitro* culture medium. Following that, 100 µL of cell suspension and 100 µL of 0.4% trypan blue were mixed into a micro-centrifuge tube and incubated for 1–2 min at room temperature. Ten microliters of cell mixture/ trypan blue were applied to the hemocytometer and placed under a microscope (Inverted Microscope, Leica DMI 3000B, Wentzler, Germany) at magnification of 20X for counting live and dead cells. GC viability was calculated as a percentage of viable cells from total cell count.

Cytotoxicity assay

In the present study, the neutral red uptake assay supplies a quantitative estimation of how many feasible cells in culture. It is one of the most applied cytotoxicity tests with many biomedical and environmental applications. It is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in the lysosomes. The granulosa cells were seeded in 96-well tissue culture plates and were treated for the appropriate period. The plates were incubated for 2 h with a medium containing neutral red. The cells were subsequently rinsed with media, the dye was removed properly and the absorbance was read using a spectrophotometer. Once the cells have been treated, the assay can be completed in <3 h (Repetto et al., 2008).

Intracellular reactive oxygen species detection

Intracellular ROS accumulation in different treatments and control group was assessed by 6-carboxy-2′, 7′-dichlorodihydro fluorescein diacetate (H_2DCFDA, Sigma-Aldrich, USA) according to the protocol described by Sohel et al. (2017). The GCs from each group were incubated with 400 µL of 15 µM H_2DCFDA for 20 min in the dark at 37 °C. Cells were then washed twice with PBS and images were immediately captured with a Nikon Eclipse Ti-S microscope (Nikon Instruments Inc., Tokyo, Japan) using a green-fluorescence filter at excitation/emission: ~492–495/517–527 nm and images were acquired by NIS Elements software. For quantitative analysis, the mean fluorescence intensity of five non-overlapping fields in each well was measured using Image J software (Rueden et al., 2017). Data are presented as mean ± standard deviation.

Mitochondrial activity

Mitochondrial activity of buffalo GCs was determined using MitoTracker Red CMXRos (M7512, Invitrogen, Karlsruhe, Germany) according to the previous published protocol (Prastowo et al., 2017) with small modifications. The GCs from each group were incubated with 15 µL of 200-nM MitoTracker red dye for 45 min, followed by two washings with PBS and were then fixed overnight at 4 °C with 4% formaldehyde. The mitochondrial activity of GC samples was visualized under a laser scanning confocal microscope (LSM 710; Carl Zeiss, Germany) using specific excitation lasers at 579– 599 nm. A constant level of laser gain (master gain = 700), pinhole (1 µm) and pixel size (1024 × 1024) were applied during image acquisition aim to allow image fluorescence signal comparison. Resulted images were then processed using ZEN 2011 software (Carl Zeiss, Germany). For quantitative analysis, the mean fluorescence intensity of five non-overlapping fields in each well was measured using Image J software. Data are presented as mean ± SD.

Enzyme activity

1, 1-Diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) radical scavenging assay

The ability of different extracts to act as hydrogen donors was measured by DPPH radical scavenger activity. The assay was carried out according to method of Blois (1958). The DPPH, a stable free radical, contains an odd electron, which is responsible for the absorbance at 515-517 nm and for a visible deep purple color. When DPPH accepts an electron from an antioxidant compound, it is reduced to 1,1-diphenyl-2-picrylhydrazine (decolorized non-radical, DPPH2).

Determination of superoxide dismutase (SOD) activity

SOD activity was assayed in the liver tissue by the method of Marklund and Marklund,(1974) at 420 nm for 1 min on a Shimadzu UV-2450 spectrophotometer (Shimadzu, Kyoto, Japan). Activity was expressed as the amount of enzyme that inhibits the autoxidation of pyrogallol by 50%, which is equal to 1 U/mg protein.

Determination of catalase (CAT) activity

The activity of CAT was measured by spectrophotometric method based on the decomposition of H_2O_2 as described by Aebi (1984).

Determination the profile of GSSG and GSH by HPLC

The thiols compounds of oxidized and reduced glutathione were detected by HPLC using the method of Jayatilleke and Shaw (1993). Glutathione (oxidized and reduced) reference standard purchased from Sigma-Aldrich Chemical Co (G4376, USA). The powder of glutathione was dissolved in 75% methanol in a stock of 1 mg/ml and diluted before application to HPLC. The HPLC system of Agilent (Santa Clara, USA) consisted of quaternary pump, a column oven,

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Rheodine injector and 20µl loop, UV variable wavelength detector. The report and chromatogram taken from Chemstation program purchased from Agilent. Synerji RP Max column 3.9 at wavelength 210 nm with flow rate 2ml/min was used. Pot. Phosphate buffer - acetonitrile at pH 2.7 was used as an isocratic mobile phase.

Determination of adenosine tri-phosphate content in media by HPLC

The detection of adenosine tri-phosphate (ATP) by HPLC was done according to the method of Teerlink et al. (1993)

Statistical analysis

A minimum of three biological replicates was used in each experiment per each essay (number of each replicate = 3n and total number of replicates = 72n). Statistical differences of means were compared between different experimental groups and were analyzed by applying one-way ANOVA, followed by Duncan's multiple range test that was used to detect differences among means. Differences in values of means were considered significant at $P \leq 0.05$. The General Linear Model (GLM) procedure on SAS Software (SAS, 2004) was used for statistical analysis. Data are supposed to be normally distributed and were expressed as mean ± SD of three biological replicates.

The parameters were analyzed according to the following model:

$Y_{ijk} = \mu + A_i + e_{ij.}$

Y_{ijk} = the measured trait.

μ = Overall means.

A_i = The Effect of different levels of curcumin.

e_{ij} = Experimental error.

RESULTS

Viability

The viability of *in vitro* cultured granulosa cells (figure 3) was significantly decreased ($p \leq 0.05$) on groups treated with DMSO (88.0±1.6%), 1 µM curcumin (86.0±1.6%)**,** 2.5 µM curcumin (86.26±1.6%)**,** 5 µM curcumin (83.0±1.6%) and 10 µM curcumin (74.0±1.6%) compared to control group (93.60±1.6 %).

Mitochondrial activity

There were no significant differences in mitochondrial activity of granulosa cells cultured with DMSO, 2.5 µM curcumin and control group (Figures 2 and 4). In addition, there was no significant difference in the activity of mitochondria between the group cultured with 2.5 µM curcumin and 5 µM curcumin. Moreover, the two groups cultured with 1 µM curcumin and 10 µM curcumin did not show differences on mitochondrial activity. However, the two groups of granulosa cells cultured with 2.5 µM and 5 µM curcumin recorded higher level of mitochondrial activity than the groups cultured with 1 µM and 10 µM curcumin.

Reactive oxygen species (ROS) level

There were no significant differences on ROS level of granulosa cells cultured with DMSO, 1 µM and 2.5µM curcumin group (Figures 1 and 5). However, there was a significant ($p \leq 0.05$) increase in ROS level in group cultured with 10µM curcumin compared to control and other treatments. In addition, the group of granulose cells cultured with 5µM curcumin recorded higher level of ROS than the groups cultured with 1µM, 2.5µM and 10µM curcumin.

Activity of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) in in vitro cultured granulosa cells

There were no significant differences on DPPH radical scavenger activity of granulose cells cultured with DMSO, 2.5µM after 48 h, 5µM after 48 h, 10µM after 48 hand 1µM after 24 h cultured of curcumin (Figure 6). In addition, there was no significant difference between granulose cells cultured with 1µM after 48 h, 10 µM after 24 h and 2.5 µM after 24 h cultured of curcumin. In addition, there was a significant difference between the granulose cells cultured as a control group and 5µM curcumin group for 24 h. However, the two groups of granulose cells cultured with 5µM curcumin for 24 hours and 1 µM curcumin for 48 h recorded high level of DPPH radical scavenger activity in compared with other groups.

Activity of superoxide dismutase (SOD) in in vitro cultured granulosa

There were significant differences on SOD activity between the granulose cells cultured in control group and experimental groups supplemented with curcumin (Figure 7). The maximum activity of SOD was recorded in granulose cells cultured with 5 µM curcumin for 24 h compared to control group that had the lowest activity of this enzyme. In addition, there were no significant differences on SOD activity of granulose cells cultured with DMSO, 1µM curcumin for 24 h, 2.5 µM, 5 µM, and 10 µM curcumin for 48 h. In addition, too no significant difference of granulose cells cultured 2.5 µM curcumin for 24 h, 5 µM curcumin for 24 h and 1 µM curcumin for 48 h, As noted the group of granulose cells cultured with 5 µM curcumin for 24 h recorded higher level of SOD activity than other experimental groups.

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Activity of catalase in in vitro cultured granulosa cells

There were significant differences on CAT activity between the granulose cells cultured as a control group, DMSO and 5 µM curcumin groups for 24 h (Figure 8). In addition, no significant differences on CAT activity of granulose cells cultured DMSO, 1 µM curcumin for 24 h, 2.5 µM curcumin for 24 h, 10 µM curcumin for 24 h, 1 µM, 2.5 µM, 5 µM, and 10 µM curcumin for 48 h. As noted, the group of granulose cells cultured with 5 µM curcumin for 24 h recorded higher level of SOD activity than the others groups.

Activity of glutathione in in vitro cultured granulosa cells

There were significant differences ($P < 0.05$) on GSH activity between the granulose cells cultured control group, DMSO and 5 µM curcumin for 24 h (Figure 9). As noted, the group of granulose cells cultured with 5 µM curcumin for 24 h recorded higher level of SOD activity than the others groups.

Activity of oxidized glutathione in in vitro cultured granulosa cells

There was a significant ($P < 0.05$) decline in GSSG level on groups treated with curcumin at concentration of 5 µM and 10 µM after 24 h of culture in addition the same trend was observed in granulosa cells treated with curcumin at concentration of 1 µM after 48 h compared to all experimental groups (Figure 10). However, the highest level of this enzyme was recorded in control group.

Intracellular adenosine triphosphate content in in vitro cultured granulosa cells

There were significant differences ($P < 0.05$) on CAT activity between the granulose cells cultured in control group, DMSO and 5 µM curcumin for 24 h (Figure 11). The content of ATP was increased gradually and significantly ($P < 0.05$) in ascending pattern in GCs and reached the maximum profile after 24 H in the group cultured with 5 µM. After that, the profile of ATP was decreased ($P < 0.05$) in GCs cultured with curcumin at concentration of 5 and 10 µM for 48 h. The lowest profile of ATP was recorded in control group.

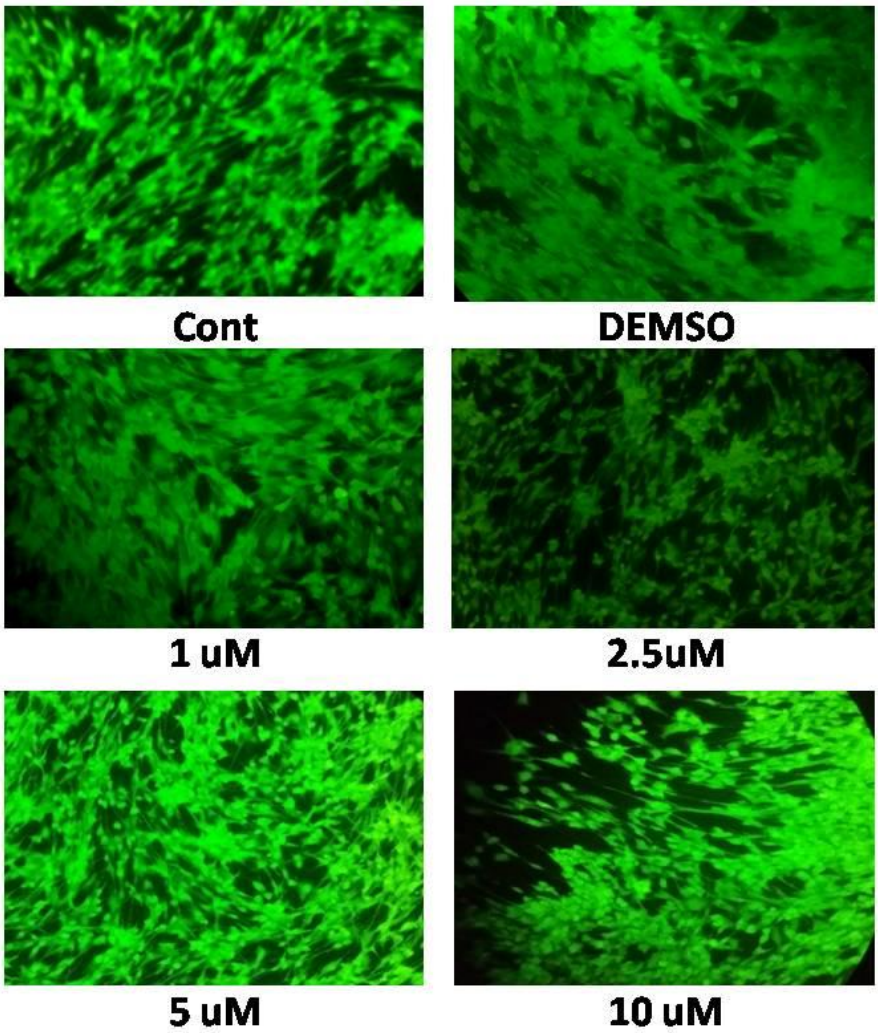

Figure 1. Image of *in vitro* cultured granulosa cells stained with H_2DCFDA measuring the level of reactive oxygen species (ROS) after supplementation with different concentrations of curcumin (1, 2.5, 5 and 10 µM) after 24h. The image was taken by inverted microscope (Leica DMI 3000B, Wentzler, Germany) at magnification of 20X.

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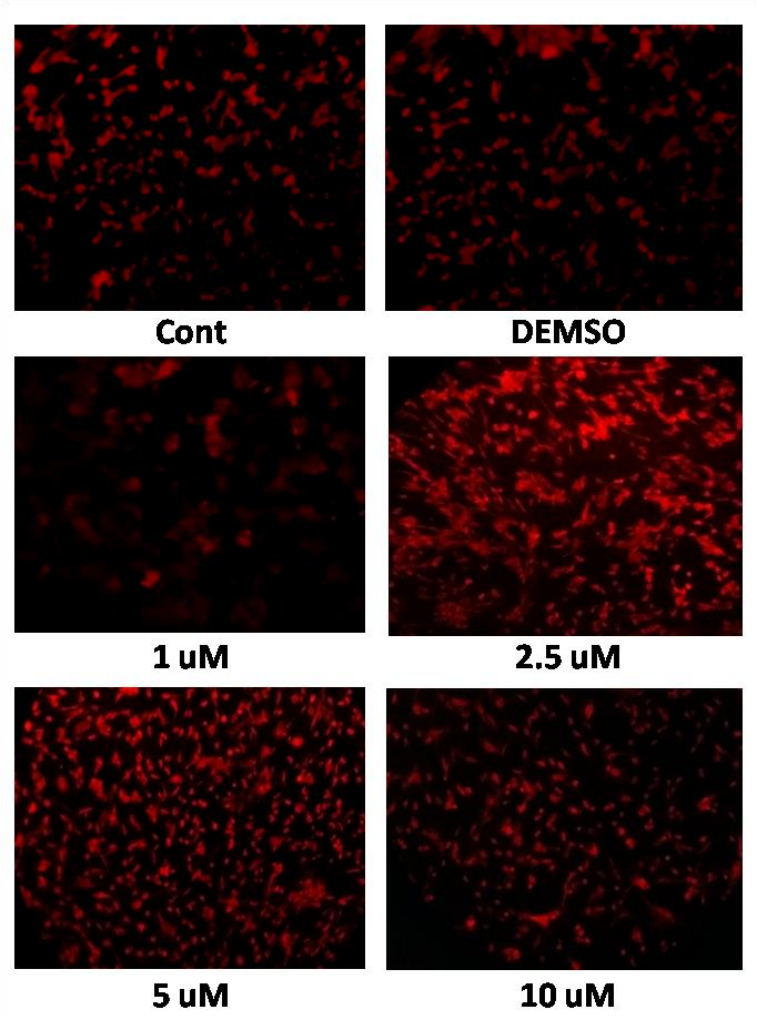

Figure 2. Image of *in vitro* cultured granulosa cells stained with mitotraker red measuring mitochondrial activity after supplementation with different concentrations of curcumin (1, 2.5, 5 and 10 µM) after 24h. The image was taken by inverted microscope (Leica DMI 3000B, Wentzler, Germany) a magnification of 20X.

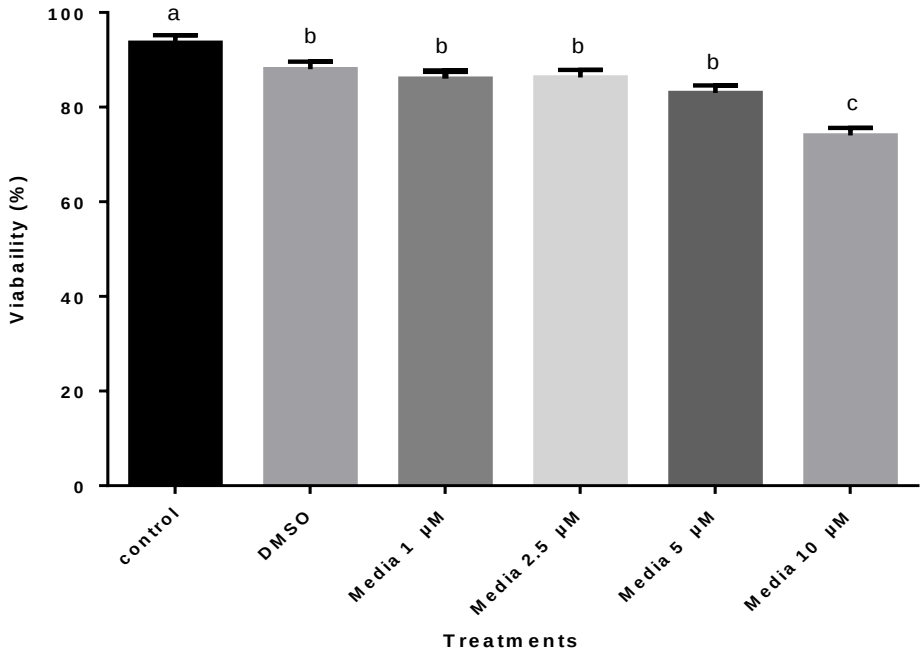

Figure 3. Viability of *in vitro* cultured granulosa cells treated with different concentrations of curcumin (1, 2.5, 5 and 10 µM) after 24h.

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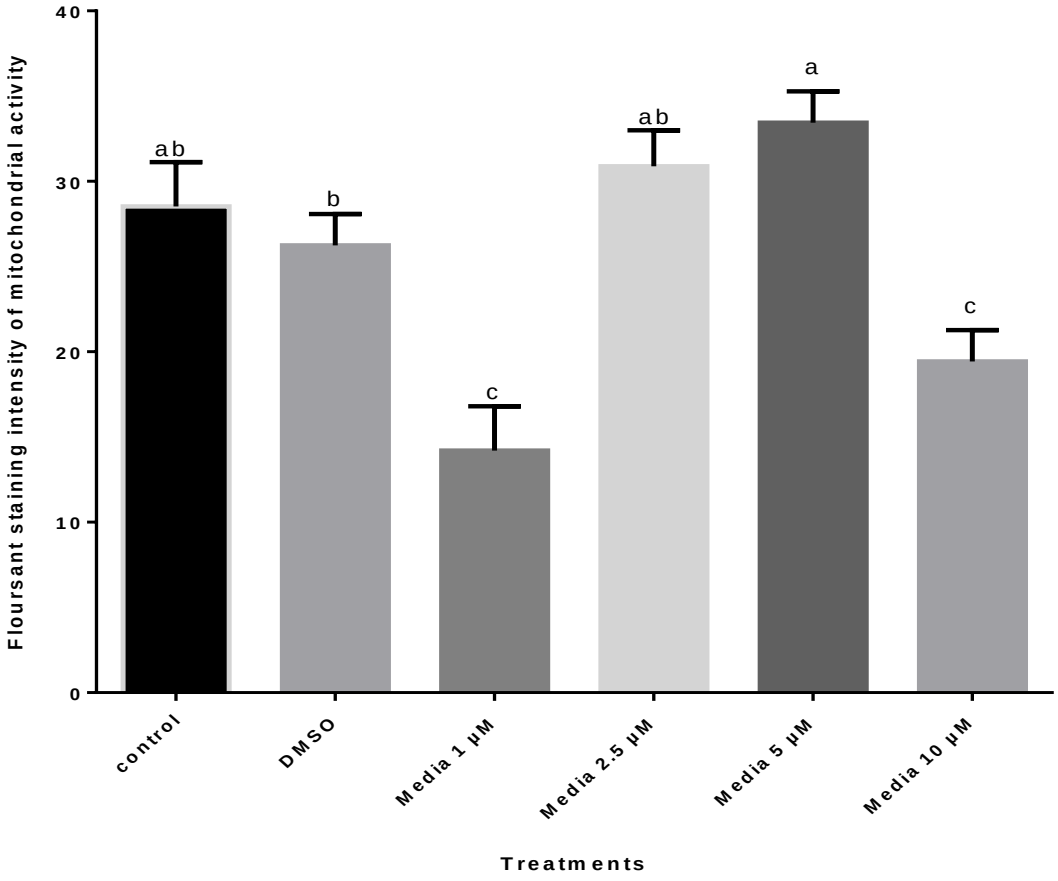

Figure 4. Mitochondrial activity of i*n vitro* cultured granulosa cells treated with different concentrations of curcumin (1, 2.5, 5 and 10 µM) after 24h.

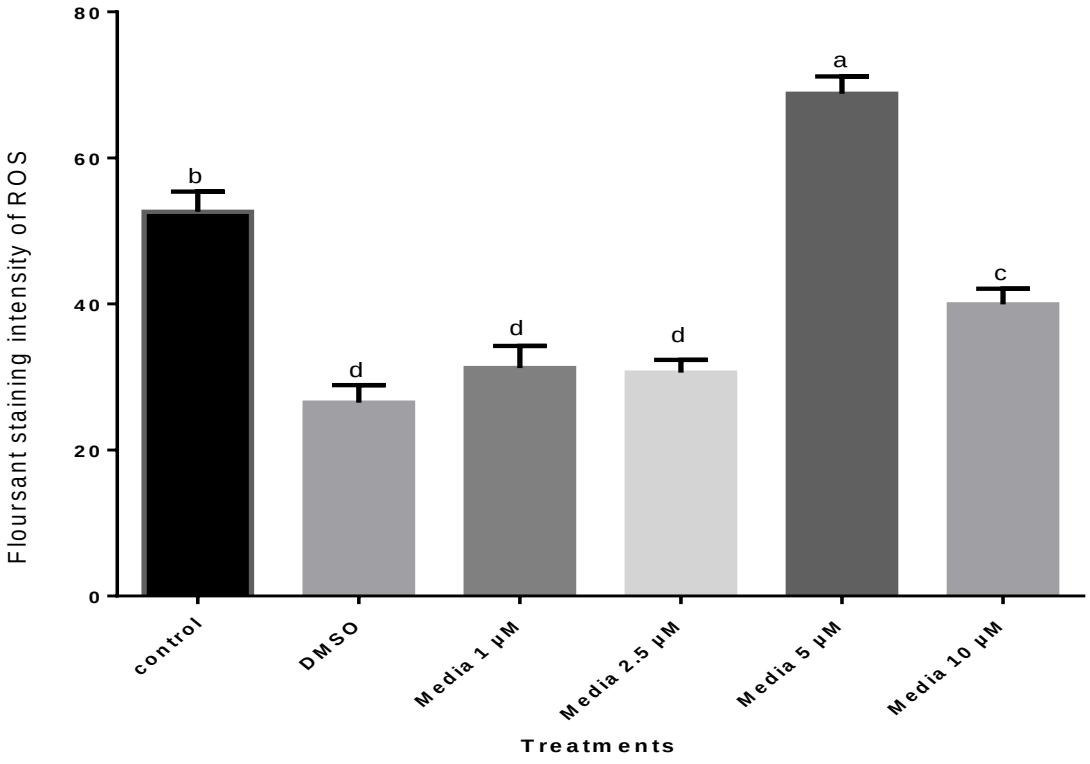

Figure 5. Intracellualr reactive oxygen species level of *in vitro* cultured granulosa cells treated with different concentrations of curcumin (1, 2.5, 5 and 10 µM) after 24h.

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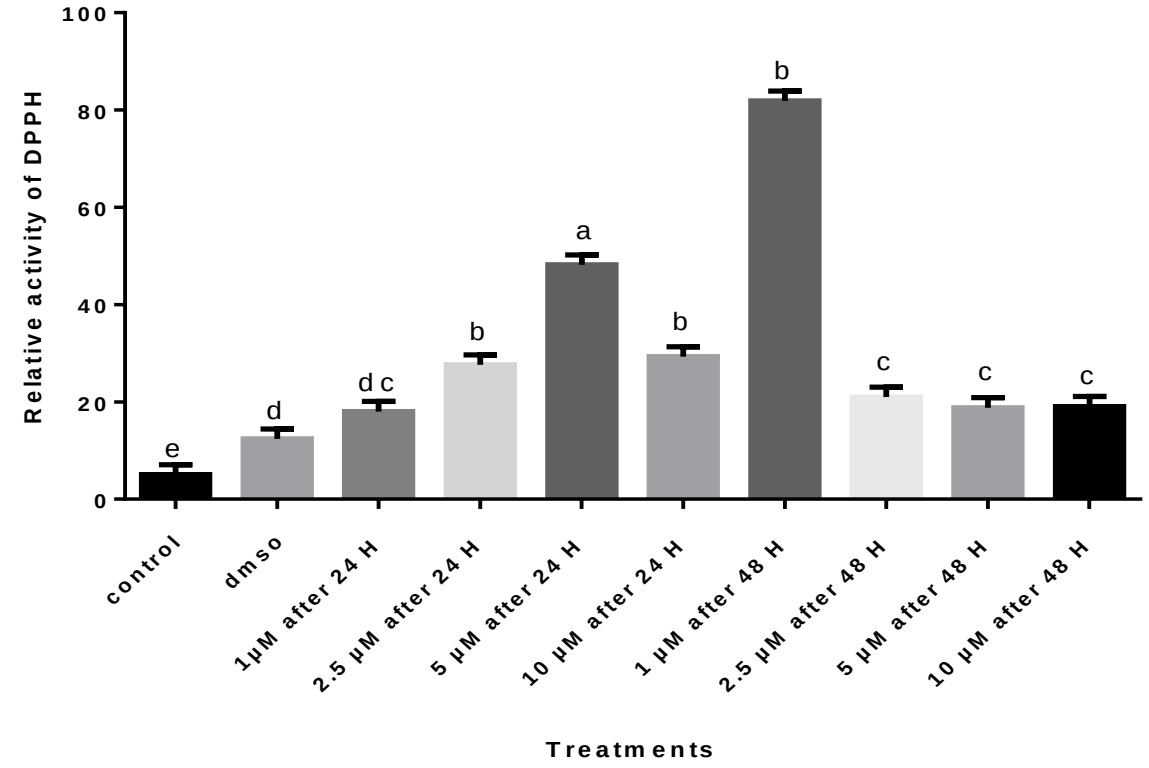

Figure 6. Enzymatic activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) in *in vitro* cultured granulosa cells treated with different concentrations of curcumin (1, 2.5, 5 and 10 µM) after 24h and 48h.

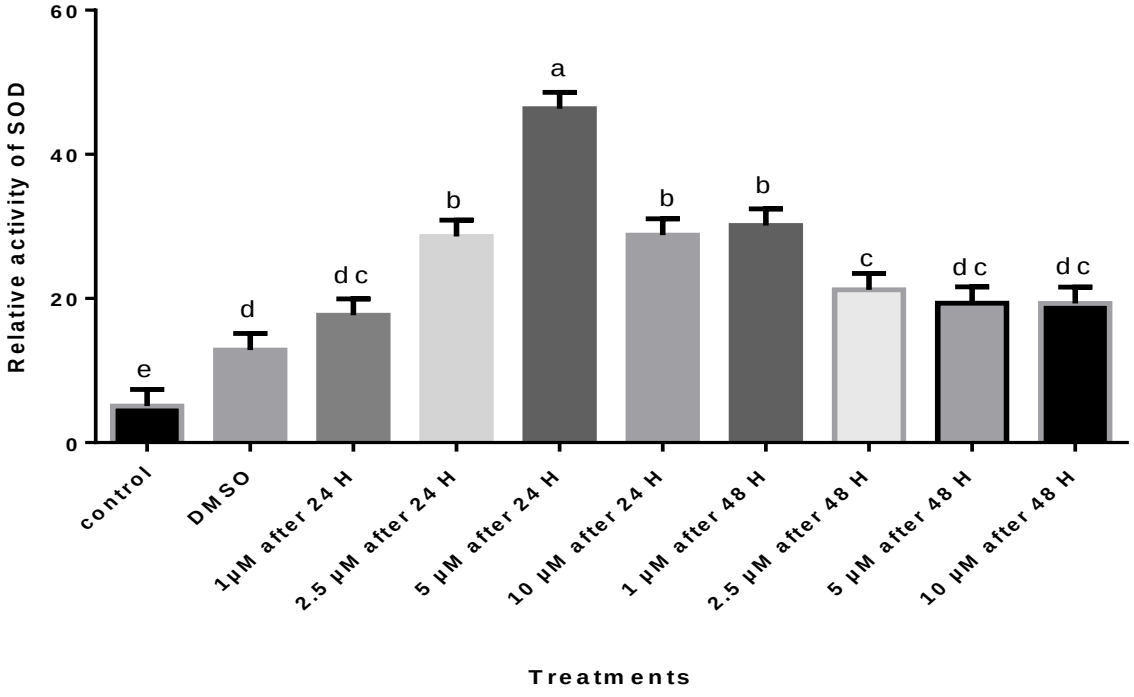

Figure 7. Enzymatic activity of superoxide dismutase (SOD) in *in vitro* cultured granulosa cells treated with different concentrations of curcumin (1, 2.5, 5 and 10 µM) after 24h and 48h.

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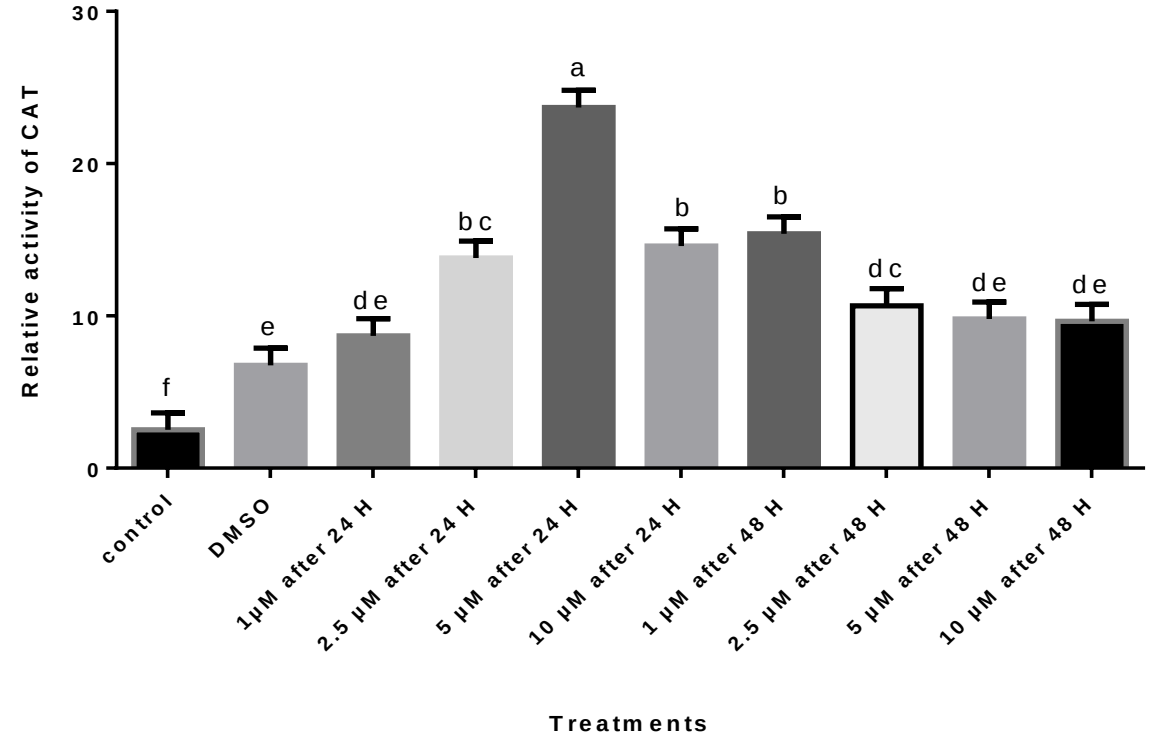

Figure 8. Enzymatic activity of catalase (CAT) in *in vitro* cultured granulosa cells treated with different concentrations of curcumin (1, 2.5, 5 and 10 µM) after 24h and 48h.

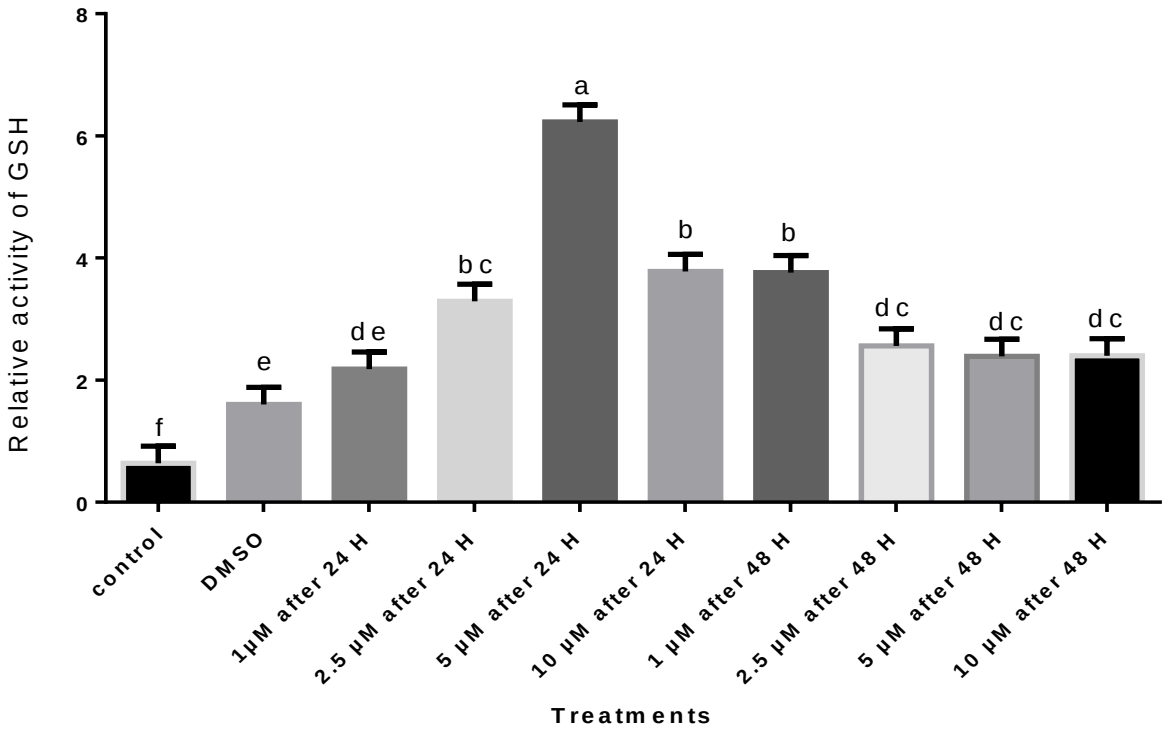

Figure 9. Enzymatic activity of glutatione (GSH) in *in vitro* cultured granulosa cells treated with different concentrations of curcumin (1, 2.5, 5 and 10 µM) after 24h and 48h.

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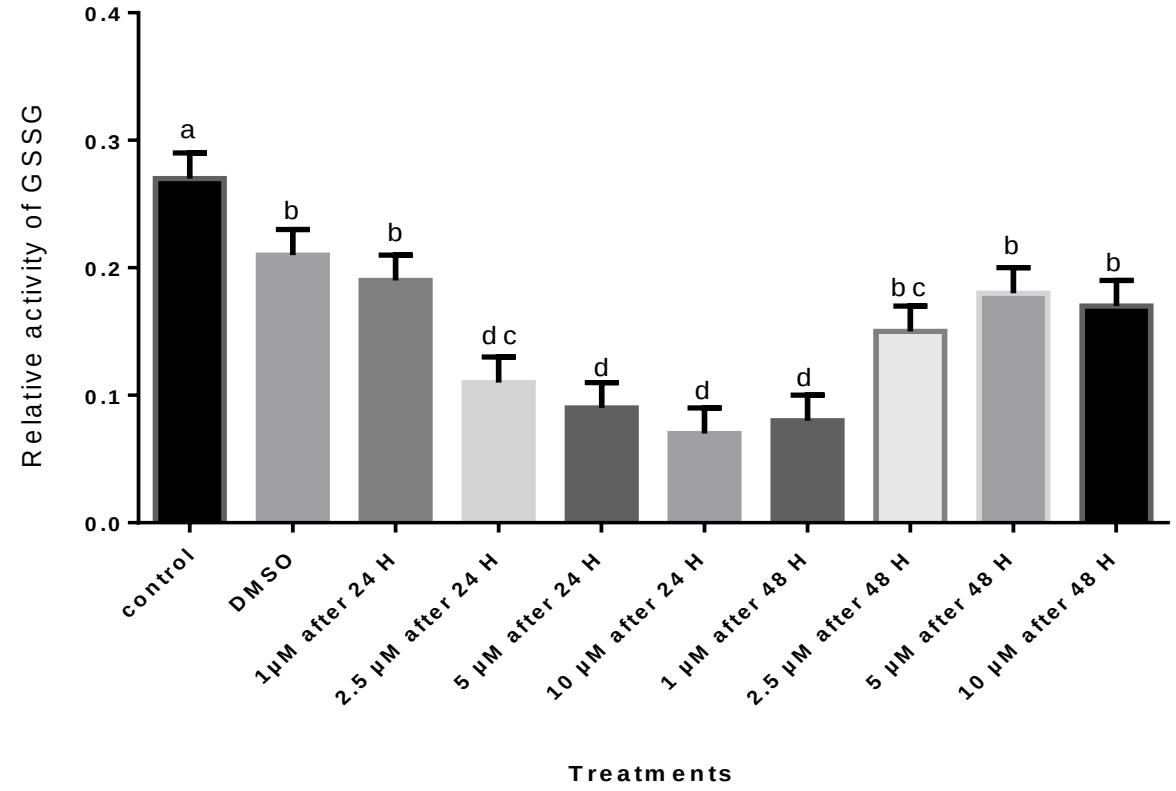

Figure 10. Enzymatic activity of oxidized glutatione (GSSG) in *in vitro* cultured granulosa cells treated with different concentrations of curcumin (1, 2.5, 5 and 10 µM) after 24h and 48h.

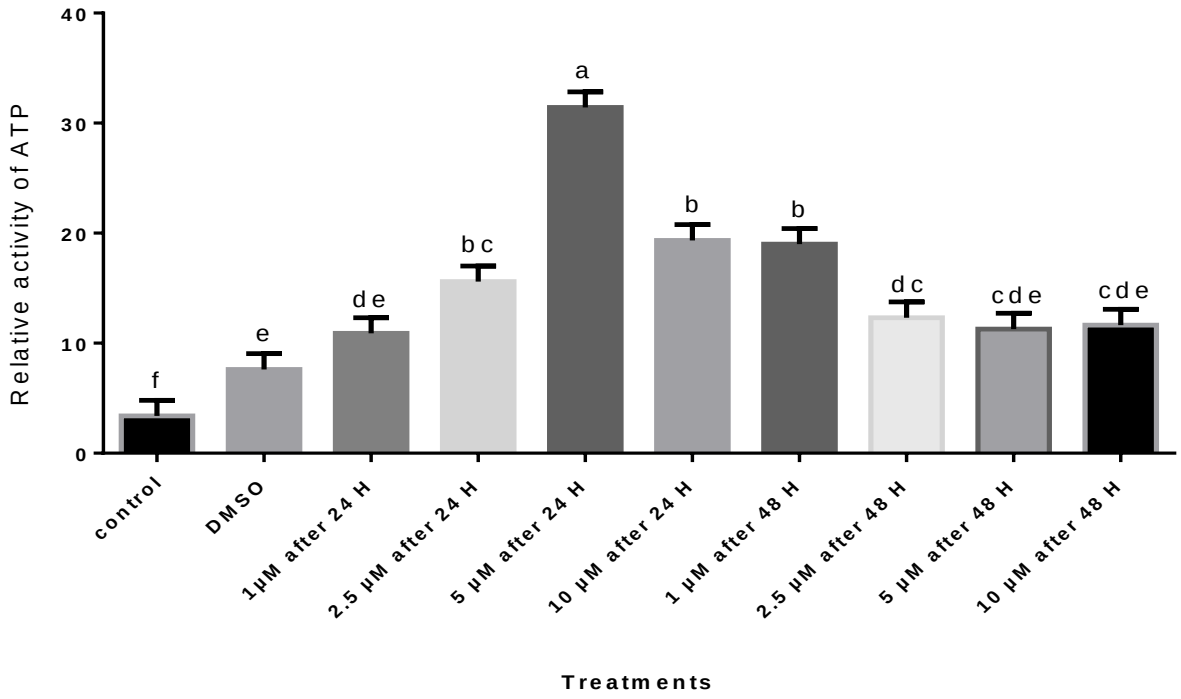

Figure 11. Intracellular adenosine triphosphate (ATP) content in *in vitro* cultured granulosa cells treated with different concentrations of curcumin (1, 2.5, 5 and 10 µM) for 24h and 48h.

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DISCUSSION

Granulosa cells represent ovarian somatic cells that are in direct contact with oocytes. The GCs supports the oocyte via secretory activity, protective and nutritive effects. Therefore, GCs play a major role in acquisition of oocyte development potential and ovulation process (Buccione et al., 1990; Joyce et al., 2001; Su et al., 2009). However, these cells are also affected by oxidative stress that could be induced by ROS produced either by normal metabolic activity or as a result of *in vitro* culture conditions (Aggarwal et al., 2005). The induction of oxidative stress could subsequently lead to apoptosis of livings cells (Al Dhaheri et al., 2014).

In the current study, the culture medium was supplemented with different concentrations of curcumin to alleviate the excessive ROS accumulated in GCs under *in vitro* culture condition. Results of the present study indicated reduction of granulosa cells viability cultured on groups treated with DMSO (88.0%), 1 µM curcumin (86.0%), 2.5 µM curcumin (86.26%), 5 µM curcumin (83.0%) and 10 µM curcumin (74.0%) compared to control group (93.60 %). In accordance with our results, Kádasi et al. (2012 and 2017) reported reduction in growth of *in vitro* culture swine granulosa cells after curcumin supplementation with 10 and 100 $\mu g.mL^{-1}$ compared to control and 1 $\mu g.mL^{-1}$. In addition, it was reported that curcumin down-regulated proliferation of colon cancer cells (Hanif et al., 1997). This negative effect of curcumin in cultured cells is exerted through apoptosis induction (Bhaumik et al., 1999; Liduan et al., 2004; Voznesens' ka et al., 2010). On contrast, Aktas et al. (2012) have shown a positive proliferative effect of curcumin on mice ovarian follicular cells by preventing apoptosis. Indeed, the variation in curcumin effect observed in our study and other investigations could be explained by type of cells under investigations, conditions of culture, dose and duration of treatment (Kádasi et al., 2012 and 2017).

The reduced viability of granulosa cells was coupled with increased level of ROS in groups treated with 5 µM of curcumin compared to other experimental groups in the current study. Although, curcumin is a well-known antioxidant (Mantzorou et al., 2018) that is used for reducing incidence of oxidative stress (Santos-Parker et al., 2017). However, high concentration of curcumin could induce cell death (Raza et al., 2008). Nevertheless, when the production of ROS overcomes the cellular antioxidant capability, this may lead to a problem referred to as oxidative stress (Agarwal et al., 2005). The ROS level could be elevated endogenously during many physiological procedures including ovulation (Agarwal et al., 2005). However, during *in vitro* cell culture, the ROS could be elevated to the level that cause oxidative stress (Rizzo et al., 2012; Castro et al., 2014; Hatami et al., 2014). Interestingly, our results indicated that the level of ROS and mitochondrial activity were elevated on granulosa cells cultured with 5 µM of curcumin which was linked with reduced viability of this group, confirming harmful side effects of increasing level of this compound during cell culture. Oxidative stress that occurred due to supplementation of exogenous oxidants has induced apoptosis in different types of mammalian cells, including hepatocytes (Haidara et al., 2002), epithelial cells (Jungas et al., 2002), and fibroblasts (Ran et al., 2004). On the other side, curcumin has maintained the mitochondrial respiratory function as well as redox status of PC12 cell line without influencing ROS and viability of cells (Raza et al., 2008). This in accordance with our results that demonstrated increased mitochondrial activity and ATP content in GCs supplemented with curcumin for 24 h during *in vitro* culture however, that was coupled with reduced cellular viability.

Several intracellular enzymes comprise the defense systems of mammalian cells. For example, SOD, GPX1 and CAT, GSSG and DPPH are contributing to scavenging capacity of cells to reduce the harmful effects of oxidative stress induced by ROS (Qin et al., 2015). In the current study, the enzyme activity of CAT, SOD, GSH and DPPH was increased after treating cultured granulosa cells with 5 µM of curcumin however all these enzymes were declined significantly reduced after 48 h. A recent study done by Qin et al., (2015) demonstrated a protective effect of curcumin on alleviating oxidative stress of porcine granulosa cells by rescuing the activity of antioxidant enzymes. However, the present study indicated that although curcumin increase the level of different antioxidant enzymes after 24 h of *in vitro* cell culture but it could not sustain this biological action after 48 h and cell viability was reduced due to increased ROS level.

CONCLUSION

The present findings indicated negative effect of *in vitro* culture on granulosa cell viability and redox status. Antioxidant compound namely curcumin increased the negative effect of *in vitro* culture when added at higher concentration (10 µM). However, low concentration (2.5 µM) of curcumin could maintain metabolic activity as well as defense system by up-regulation of antioxidant enzymes for short duration.

DECLARATIONS

Author's contributions

All authors have contributed to Lab work, the experimental design, writing and revision of the manuscript.

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Competing interests
All authors declare no competing interests that might interfere with the data provided in the current manuscript.

Consent to publish
All the authors approved and agreed to publish the manuscript.

REFERENCES

Aebi H (1984). Catalase in vitro, In: methods in enzymology. Elsevier, pp. 121-126.

Agarwal A, Aponte-Mellado A, Premkumar BJ, Shaman A and Gupta S (2012). The effects of oxidative stress on female reproduction: a review. Reproductive Biology and Endocrinology, 10 (1): 49. DOI: http://dx.doi.org/10.1186/1477-7827-10-49.

Agarwal A, Gupta S and Sharma RK (2005). Role of oxidative stress in female reproduction. Reproductive Biology and Endocrinology, 3 (1): 28. DOI: http://dx.doi.org/10.1186/1477-7827-3-28.

Aggarwal BB, Kumar A, Aggarwal MS and Shishodia S (2005). Curcumin derived from turmeric (Curcuma longa): a spice for all seasons. Phytopharmaceuticals in Cancer Chemoprevention, 23: 351-387. Available at: http://www.sciepub.com/reference/38129

Aggarwal BB ,Sundaram C, Malani N and Ichikawa H (2007). Curcumin: the Indian solid gold, In: The molecular targets and therapeutic uses of curcumin in health and disease. Springer, pp. 1-75. DOI: http://dx.doi.org/10.1007/978-0-387-46401-5_1

Aktas C, Kanter M and Kocak Z (2012). Antiapoptotic and proliferative activity of curcumin on ovarian follicles in mice exposed to whole body ionizing radiation. Toxicology and Industrial Health, 28 (9): 852-863. DOI: http://dx.doi.org/10.1177/0748233711425080

Al Dhaheri Y, Attoub S, Ramadan G, Arafat K, Bajbouj K, Karuvantevida N , AbuQamar S, Eid A and Iratni R (2014). Carnosol induces ROS-mediated beclin1-independent autophagy and apoptosis in triple negative breast cancer. PLoS One, 9 (10): e109630. DOI: http://dx.doi.org/10.1371/journal.pone.0109630.

Barzegar A and Moosavi-Movahedi AA (2011). Intracellular ROS protection efficiency and free radical-scavenging activity of curcumin, PLoS One, 6 (10): e26012. DOI: http://dx.doi.org/10.1371/journal.pone.0026012.

Bhaumik S, Anjum R, Rangaraj N, Pardhasaradhi BV and Khar A (1999). Curcumin mediated apoptosis in AK-5 tumor cells involves the production of reactive oxygen intermediates. Federation of European Biochemical Societies, 456 (2): 311-314. DOI: http://dx.doi.org/10.1016/s0014-5793(99)00969-2.

Blois MS (1958). Antioxidant determinations by the use of a stable free radical. Nature, 181 (4617): 1199-1200. DOI: http://dx.doi.org/10.1371/journal.pone.0026012

Buccione R, Vanderhyden B, Caron PJ and Eppig JJ (1990). FSH-induced expansion of the mouse cumulus oophorus in vitro is dependent upon a specific factor(s) secreted by the oocyte. Developmental Biology, 138 (1): 16-25. DOI: http://dx.doi.org/10.1016/0012-1606(90)90172-f.

Castro SV, Carvalho AA, Silva CM, Santos FW, Campello CC, de Figueiredo JR and Rodrigues AP (2014). Frozen and fresh ovarian tissue require different culture media to promote in vitro development of bovine preantral follicles. Biopreservation and Biobanking, 12 (5): 317-324. DOI: http://dx.doi.org/10.1089/bio.2014.0020.

Cinar MU and Sohel MMH (2015). Advancement in Molecular Genetics to understand the molecular reproduction of Livestock – follicular development. Research in Agriculture Livestock and Fisheries, 1 (1): 47-60. DOI: http://dx.doi.org/10.3329/ralf.v1i1.22355.

Correa F, Buelna-Chontal M, Hernandez-Resendiz S, Garcia-Nino WR, Roldan FJ ,Soto V, Silva-Palacios A, Amador A, Pedraza-Chaverri J, Tapia E and Zazueta C (2013). Curcumin maintains cardiac and mitochondrial function in chronic kidney disease. Free Radical Biology and Medicine, 61: 119-129. DOI: http://dx.doi.org/10.1016/j.freeradbiomed.2013.03.017.

Dinkova-Kostova AT and Talalay P (2008). Direct and indirect antioxidant properties of inducers of cytoprotective proteins. Molecular Nutrition Food Research, 52(S1): S128-S138. DOI: https://doi.org/10.1002/mnfr.200700195.

Duffy DM and Stouffer RL (2003). Luteinizing hormone acts directly at granulosa cells to stimulate periovulatory processes: modulation of luteinizing hormone effects by prostaglandins. Endocrine, 22 (3): 249-256. DOI: http://dx.doi.org/10.1385/endo:22:3:249.

Epstein J ,Sanderson IR and Macdonald TT (2010). Curcumin as a therapeutic agent: the evidence from in vitro, animal and human studies. British Journal of Nutrition, 103 (11): 1545-1557. DOI: http://dx.doi.org/10.1017/S0007114509993667.

Esatbeyoglu T, Huebbe P, Ernst IM, Chin D, Wagner AE and Rimbach G (2012). Curcumin--from molecule to biological function. Angewandte Chemmie International Edition, 51 (22): 5308-5332. DOI: http://dx.doi.org/10.1002/anie.201107724.

Frisard M and Ravussin E (2006). Energy metabolism and oxidative stress: impact on the metabolic syndrome and the aging process. Endocrine, 29 (1): 27-32. DOI: http://dx.doi.org/10.1385/ENDO:29:1:27.

Haidara K, Morel I, Abalea V, Gascon Barre M and Denizeau F (2002). Mechanism of tert-butylhydroperoxide induced apoptosis in rat hepatocytes: involvement of mitochondria and endoplasmic reticulum. Biochimica et Biophysica Acta (BBA) . Molecular Cell Research, 1542(1-3): 173-185. DOI: http://dx.doi.org/10.1016/s0167-4889(01)00178-1.

Hanif R, Qiao L, Shiff SJ and Rigas B (1997). Curcumin, a natural plant phenolic food additive, inhibits cell proliferation and induces cell cycle changes in colon adenocarcinoma cell lines by a prostaglandin-independent pathway. Journal of Laboratory and Clinical Medicine, 130 (6): 576-584. DOI: http://dx.doi.org/10.1016/s0022-2143(97)90107-4.

Hatami S, Zavareh S, Salehnia M, Lashkarbolouki T and Karimi I (2014). Comparison of oxidative status of mouse pre-antral follicles derived from vitrified whole ovarian tissue and vitrified pre-antral follicles in the presence of alpha lipoic acid. Journal of Obstetrics and Gynaecology Research, 40 (6): 1680-1688. DOI: http://dx.doi.org/10.1111/jog.12394.

To cite this paper: Ghanem N, Amin A, Saeed AM, Abdelhamid ShM, El-Sayed A, Farid OA, Dessouki ShM and Faheem MS (2020). Effects of Curcumin Supplementation on Viability and Antioxidant Capacity of Buffalo Granulosa Cells under *In Vitro* Culture Conditions. *World Vet. J.*, 10 (2): 146-159. DOI: https://dx.doi.org/10.36380/scil.2020.wvj19

Hatcher H, Planalp R, Cho J, Torti FM, and Torti SV (2008). Curcumin: from ancient medicine to current clinical trials. Cellular and Molecular Life Sciences, 65(11): 1631–1652. https://doi.org/10.1007/s00018-008-7452-4.

Jayatilleke E and Shaw S (1993). A high-performance liquid chromatographic assay for reduced and oxidized glutathione in biological samples. Analytical Biochemistry, 214 (2): 452-457. DOI: http://dx.doi.org/10.1006/abio.1993.1522.

Joyce IM, Pendola FL, O'Brien M and Eppig JJ (2001). Regulation of prostaglandin-endoperoxide synthase 2 messenger ribonucleic acid expression in mouse granulosa cells during ovulation. Endocrinology, 142 (7): 3187-3197. DOI: http://dx.doi.org/10.1210/endo.142.7.8268.

Jungas T, Motta I, Duffieux F, Fanen P, Stoven V and Ojcius DM (2002). Glutathione levels and BAX activation during apoptosis due to oxidative stress in cells expressing wild-type and mutant cystic fibrosis transmembrane conductance regulator. The Journal of Biological Chemistry, 277 (31): 27912-27918. DOI: http://dx.doi.org/10.1074/jbc.M110288200.

Kádasi A, Maruniakova N, Stochmalova A, Bauer M, Grossmann R, Harrath AH, Kolesarova A and Sirotkin AV (2017). Direct effect of curcumin on porcine ovarian cell functions. Animal Reproduction Science, 182: 77-83. DOI: http://dx.doi.org/10.1016/j.anireprosci.2017.05.001.

Kádasi A, Sirotkin AV, Maruniaková N, Kolesárová A, Bulla J and Grossmann R (2012). The effect of curcumin on secretory activity, proliferation and apoptosis of the porcine ovarian granulosa cells. The Journal of Microbiology, Biotechnology and Food Sciences, 2(1): 349. DOI: http://dx.doi.org/10.1016/j.anireprosci.2017.05.001.

Klaunig JE, Kamendulis LM and Hocevar BA (2010). Oxidative stress and oxidative damage in carcinogenesis. Toxicologic Pathology, 38 (1): 96-109. DOI: http://dx.doi.org/10.1177/0192623309356453.

Liu G, Xiang T, Wu QF and Wang WX (2016). Curcumin suppresses the proliferation of gastric cancer cells by downregulating H19. Oncology Letters, 12(6): 5156-5162. DOI: http://dx.doi.org/10.3892/ol.2016.5354.

Mantzorou M, Pavlidou E, Vasios G,Tsagalioti E and Giaginis C (2018). Effects of curcumin consumption on human chronic diseases: A narrative review of the most recent clinical data. Phytotherapy Research, 32 (6): 957-975. DOI: http://dx.doi.org/10.1002/ptr.6037.

Marklund S and Marklund G (1974). Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. European Journal of Biochemistry, 47 (3): 469-474. DOI: http://dx.doi.org/10.1111/j.1432-1033.1974.tb03714.x.

Mohebbati R, Anaeigoudari A, Khazdair MR (2017). The effects of Curcuma longa and curcumin on reproductive systems. Endocrine Regulations, 51(4): 220-228. DOI: http://dx.doi.org/10.1515/enr-2017-0024

Nadkarni K (2007). Indian Materia Medica, Ed. Bombay Popular Mumbai, 1: 691-694. Available at: https://trove.nla.gov.au/work/18643838?q&versionId=21887031.

Panieri E and Santoro M (2016). ROS homeostasis and metabolism: a dangerous liason in cancer cells. Cell Death & Disease 7, e2253. DOI: https://doi.org/10.1038/cddis.2016.105

Pinkus R ,Weiner LM and Daniel V (1996). Role of oxidants and antioxidants in the induction of AP-1, NF-kappaB, and glutathione S-transferase gene expression. The Journal of Biological Chemistry, 271 (23): 13422-13429. DOI: http://dx.doi.org/10.1074/jbc.271.23.13422.

Prastowo S, Amin A, Rings F, Held E, Wondim DS, Gad A, Neuhoff C, Tholen E, Looft C, Schellander K and Tesfaye D (2016). Fateful triad of reactive oxygen species, mitochondrial dysfunction and lipid accumulation is associated with expression outline of the AMP-activated protein kinase pathway in bovine blastocysts. Reproduction, Fertility and Development, 29 (5): 890-905. DOI: http://dx.doi.org/10.1071/RD15319.

Qin X, Cao M, Lai F, Yang F, Ge W, Zhang X, Cheng S, Sun X, Qin G, Shen W and Li L (2015). Oxidative stress induced by zearalenone in porcine granulosa cells and its rescue by curcumin in vitro. PLoS One, 10 (6): e0127551. DOI: http://dx.doi.org/10.1371/journal.pone.0127551.

Ran Q, Liang H, Gu M, Qi W, Walter CA, Roberts LJ, Herman B, Richardson A and Van Remmen H (2004). Transgenic mice overexpressing glutathione peroxidase 4 are protected against oxidative stress-induced apoptosis. The journal of Biological Chemistry, 279 (53): 55137-55146. DOI: http://dx.doi.org/10.1074/jbc.M410387200.

Raza H, John A, Brown EM, Benedict S and Kambal A (2008). Alterations in mitochondrial respiratory functions, redox metabolism and apoptosis by oxidant 4-hydroxynonenal and antioxidants curcumin and melatonin in PC12 cells. Toxicology and Applied Pharmacology, 226 (2): 161-168. DOI: http://dx.doi.org/10.1016/j.taap.2007.09.002.

Repetto G, Del Peso A and Zurita JL (2008). Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nature Protocols, 3 (7): 1125-1131. DOI: http://dx.doi.org/10.1038/nprot.2008.75.

Reyes-Fermin LM, Gonzalez-Reyes S, Tarco-Alvarez NG, Hernandez-Nava M, Orozco-Ibarra M and Pedraza-Chaverri J (2012). Neuroprotective effect of alpha-mangostin and curcumin against iodoacetate-induced cell death. Nutritional Neuroscience, 15 (5): 34-41. DOI: http://dx.doi.org/10.1179/1476830512Y.0000000011.

Rueden CT, Schindelin J, Hiner MC, DeZonia BE, Walter AE, Arena ET and Eliceiri KW (2017). ImageJ2: ImageJ for the next generation of scientific image data. BMC Bioinformatics, 18(1): 529. doi:10.1186/s12859-017-1934-z

Santos-Parker JR, Strahler TR, Bassett CJ, Bispham NZ, Chonchol MB and Seals DR (2017). Curcumin supplementation improves vascular endothelial function in healthy middle-aged and older adults by increasing nitric oxide bioavailability and reducing oxidative stress. Aging (Albany NY), 9 (1): 187-208. DOI: http://dx.doi.org/10.18632/aging.101149.

Shkolnik K, Tadmor A, Ben-Dor S, Nevo N, Galiani D and Dekel N (2011). Reactive oxygen species are indispensable in ovulation. Proceedings of the National Academy of Sciences, 108 (4): 1462-1467. DOI: http://dx.doi.org/10.1073/pnas.1017213108.

Sohel MM, Hoelker M, Noferesti SS, Salilew-Wondim D, Tholen E, Looft C , Rings F, Uddin MJ, Spencer TE, Schellander K and Tesfaye D (2013). Exosomal and Non-Exosomal Transport of Extra-Cellular microRNAs in Follicular Fluid: Implications for Bovine Oocyte Developmental Competence. PLoS One, 8 (11): e78505. DOI: http://dx.doi.org/10.1371/journal.pone.0078505.

Sohel MMH, Konca Y, Akyuz B, Arslan K, Sariozkan S and Cinar MU (2017). Concentration dependent antioxidative and apoptotic effects of sulforaphane on bovine granulosa cells in vitro. Theriogenology, 97:17-26. DOI: 10.1016/j.theriogenology.2017.04.015.

Steward WP and Gescher AJ (2008). Curcumin in cancer management: recent results of analogue design and clinical studies and desirable future research. Molecular Nutrition & Food research, 52 (9): 1005-1009. DOI: http://dx.doi.org/10.1002/mnfr.200700148.

Strober W (2015). Trypan blue exclusion test of cell viability. Current protocols in immunology, 111:A3.B.1-A3.B.3. DOI: http://dx.doi.org/10.1002/0471142735.ima03bs111.

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Su YQ, Sugiura K and Eppig JJ (2009). Mouse oocyte control of granulosa cell development and function: paracrine regulation of cumulus cell metabolism, In: Seminars in Reproductive Medicine, 27 (1): 32-42. DOI: https://doi.org/10.1055/s-0028-1108008

Sung B, Prasad S, Yadav VR and Aggarwal BB (2012). Cancer cell signaling pathways targeted by spice-derived nutraceuticals. Nutrition and cancer, 64 (2): 173-197. DOI: http://dx.doi.org/10.1080/01635581.2012.630551.

Tapia E, Soto V, Ortiz-Vega K M, Zarco-Marquez G, Molina-Jijon E, Cristobal-Garcia M, Santamaria J, Garcia-Nino WR, Correa F, Zazueta C and Pedraza-Chaverri J (2012). Curcumin induces Nrf2 nuclear translocation and prevents glomerular hypertension, hyperfiltration, oxidant stress, and the decrease in antioxidant enzymes in 5/6 nephrectomized rats. Oxidative medicine and cellular longevity, 2012: 269039. DOI: http://dx.doi.org/10.1155/2012/269039.

Tapia E, Zatarain-Barron ZL, Hernandez-Pando R, Zarco-Marquez G, Molina-Jijon E, Cristobal-Garcia M, Santamaria J and Pedraza-Chaverri J (2013). Curcumin reverses glomerular hemodynamic alterations and oxidant stress in 5/6 nephrectomized rats. Phytomedicine, 20 (3-4): 359-366. DOI: http://dx.doi.org/10.1016/j.phymed.2012.11.014.

Teerlink T, Hennekes M, Bussemaker J and Groeneveld J (1993). Simultaneous determination of creatine compounds and adenine nucleotides in myocardial tissue by high-performance liquid chromatography. Analytical Biochemistry, 214 (1): 278-283. DOI: http://dx.doi.org/10.1006/abio.1993.1488.

Trujillo J, Chirino YI, Molina-Jijon E, Anderica-Romero AC, Tapia E and Pedraza-Chaverri J (2013). Renoprotective effect of the antioxidant curcumin: Recent findings. Redox Biology, 1 (1): 448-456. DOI: http://dx.doi.org/10.1016/j.redox.2013.09.003.

Voznesens'ka T, Bryzhina TM, Sukhina VS, Makohon NV and Aleksieieva IM (2010). Effect of NF-kappaB activation inhibitor curcumin on the oogenesis and follicular cell death in immune ovarian failure in mice. Fiziolohichnyi Zhurnal, 56 (4): 96-101. DOI: http://dx.doi.org/10.15407/fz56.04.096.

Yada H, Hosokawa K, Tajima K, Hasegawa Y and Kotsuji F (1999). Role of ovarian theca and granulosa cell interaction in hormone productionand cell growth during the bovine follicular maturation process. Biology of Reproduction, 61 (6): 1480-1486. DOI: http://dx.doi.org/10.1095/biolreprod61.6.1480.

Zheng L, Tong Q and Wu C (2004). Growth-inhibitory effects of curcumin on ovary cancer cells and its mechanisms. Journal of Huazhong University of Science and Technology, 24 (1): 55-58. DOI: http://dx.doi.org/10.1007/bf02830706.

Zhang Y, Huang Y, Deng H and Huang X (2019a). Curcumin attenuates oxidative stress in RAW264.7 cells by increasing the activity of antioxidant enzymes and activating the Nrf2-Keap1 pathway. PLoS One, 14(5): e0216711. DOI: http://dx.doi.org/10.1371/journal.pone.0216711.

Zhang Y, Lin XY, Zhang JH, Xie ZL, Deng H, Huang YF and Huang XH (2019b). Apoptosis of mouse myeloma cells induced by curcumin via the Notch3-p53 signaling axis. Oncology Letters, 17(1):127-134. DOI:http://dx.doi.org/ 10.3892/ol.2018.9591.

Zhu G, Shen Q, Jiang H, Ji O, Zhu L and Zhang L (2020). Curcumin inhibited the growth and invasion of human monocytic leukaemia SHI-1 cells in vivo by altering MAPK and MMP signalling. Pharmacy Biology, 58(1):25-34. DOI: http://dx.doi.org/10.1080/13880209.

To cite this paper: Ghanem N, Amin A, Saeed AM, Abdelhamid ShM, El-Sayed A, Farid OA, Dessouki ShM and Faheem MS (2020). Effects of Curcumin Supplementation on Viability and Antioxidant Capacity of Buffalo Granulosa Cells under *In Vitro* Culture Conditions. *World Vet. J.*, 10 (2): 146-159. DOI: https://dx.doi.org/10.36380/scil.2020.wvj19

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In Vitro Investigation of the Antibacterial Effect of Silver Nanoparticles on ESBL-producing *E. coli* and *Klebsiella* spp. Isolated from Pet Animals

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ABSTRACT

Despite the presence of modern antibacterial drugs, bacterial infections are still a major threatening problem due to the enormous increase in multi-drug-resistant bacteria. Nanoparticles have been extensively used as an applicable and safe alternative to antibiotics. The present study aimed to explore the inhibitory effect of silver nanoparticles on Extended Spectrum Beta lactamase (ESBL) producing *E. coli* and *Klebsiella* spp. in vitro as well as their effect on the expression of antibiotic resistance genes. Different samples (i.e., wound swabs, Fecal swabs, and urine samples) were collected from dogs and cats. Phenotypic and molecular identification, antibiotic susceptibility testing, and double-disk synergy test were carried out for the identification of ESBL producing *E. coli* and *Klebsiella* spp. Silver nanoparticles were tested for their in vitro antibacterial potential and there were reports of their minimum inhibitory concentration and minimum bactericidal concentration. Moreover, the effect of silver nanoparticles on the expression of antibiotic resistance genes (i.e., *blaTEM, blaSHV,* and *blaCTX*) was assessed as well as their effect on the structural integrity of the bacterial cells using Scanning Electron Microscope (SEM). Results revealed that 23 isolates (19.16%) (*E. coli*=17, *Klebsiella* spp.=6) were confirmed as ESBL producing. Silver nanoparticles indicated a promising antibacterial effect where the minimum inhibitory concentration of AgNPs for ESBL producing *E. coli* was measured as 0.31 mg/ml, and 0.62 mg/ml for ESBL-producing *Klebsiella* spp., while the minimum bactericidal concentration of ESBL-producing *E. coli* and *Klebsiella* spp. was reported as 0.15 mg/ml and 0.3 mg/ml, respectively. Consequently, the expression of antibiotic resistance genes was downregulated in both bacteria species and there was a noticeable toxic effect of AgNPs on *E. coli* and *Klebsiella* spp. cells which was investigated using SEM. It can be concluded that silver nanoparticles have a promising antibacterial activity and could be considered an applicable alternative for the control of ESBL producing bacteria.

Keywords: *E. coli*, ESBLs, *Klebsiella* spp., Pets, Silver nanoparticles

INTRODUCTION

In the current decade, the search for antibiotic alternatives has become one of the most quintessential issues due to the massive expansion of antibiotic resistance. The usage of silver nanoparticles has been widely recognized due to their known bactericidal as well as bacteriostatic actions against different types of bacterial and fungal pathogens (Saeb et al., 2014). Silver nanoparticles (AgNPs) have a broad-spectrum antimicrobial effect due to their large surface area providing the chance for better contact with microbes (Li et al., 2010). Furthermore, silver nanoparticles have a lower propensity to induce microbial resistance than other antimicrobial agents (Ansari et al., 2014). It is also worth mentioning that these nanoparticles have a non-toxic effect on the human at low concentrations (Bindu et al., 2015). They are characterized by their powerful antioxidant and antibacterial effect because of bioactive molecules on the exterior surface of silver nanoparticles (Keshari, 2020).

Extended Spectrum β-Lactamase producing bacteria (ESBL) are types of bacteria that show resistance to several types of antibiotics through the hydrolysis of the b-lactam ring of antibiotics (Kizilca et al., 2012) and they can transfer resistance to penicillins, third-generation cephalosporins, and monobactams (Ejaz et al., 2011). Moreover, most of them are not inhibited by other non-b-lactam antibiotics since the resistance encoding genes of other antibiotic classes can also be carried by the plasmids containing the ESBL-encoding genes (Alyamani et al., 2017; Fan et al., 2014). *Escherichia coli* and *Klebsiella* spp. remain the major ESBL-producing microorganisms isolated worldwide. They are considered the most emerging Extended-spectrum β-lactamase (ESBL) with a serious effect on the community (Devrim et al., 2011). The CTX-M-type enzymes are the largest ESBL groups spreading globally, followed by TEM and SHV groups (Sukmawinata et al, 2020). Researchers from different countries have successfully isolated and identified ESBL-producing bacteria from different types of samples, including isolates from human in Bahrain (Shahid et al., 2014), different samples (e.g., fecal swabs, animal feeds, water, and excreta) from different animals (e.g, dogs, cats, sheep,

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goat, chickens, turkey, ducks, and human, (Okapara et al., 2018), isolates of urine samples from dogs and cats in USA and Switzerland (Thungrat et al., 2015; Zogg et al., 2018), also isolates of fecal swabs from dogs and cats in Newzeland (Karkaba et al., 2019). Nanoparticles are known mainly by their direct action on the bacterial cell wall which is different from other antibiotic resistance mechanisms and needs no penetration of bacterial cells (Wang et al., 2019). Subsequently, they are less predisposed to develop resistance, compare to antibiotics. AgNps have proved a good antibacterial and antioxidant activity against *E. coli* and *Klebsiella pneumoniae* isolates (Khan et al., 2020). Moreover, it was demonstrated that AgNPs have a powerful toxic action on ampicillin resistant *Klebsiella Pneumoniae* genes and bacterial proteins as well as bacterial membrane damage and oxidative stress (Hamida et al., 2020). Therefore, this study aimed to explore the inhibitory effect of silver nanoparticles on ESBL-producing *E. coli* and *Klebsiella* spp. in vitro as well as their effect on the expression of resistance genes. In doing so, the phenotypic and molecular identification of ESBL producing *E. coli* and *Klebsiella* spp. was carried on different samples collected from dogs and cats living in various places in Egypt. The bactericidal activity of Ag nanoparticles was assessed via different microbiological and molecular techniques.

MATERIALS AND METHODS

Ethical approval

The study was conducted according to ethical guidelines approved by the Faculty of Veterinary Medicine, Cairo University. There were no experiments applied to human participants.

Samples collection and preparation

A total of 120 samples were collected from diseased dogs (n=55) and cats (n=65). All samples were collected according to the guidelines of the Institutional Animal Care and Use Committee at Cairo University and approved by Vet-CU-IACUC (Vet CU 16072020198), Cairo, Egypt. Written consent was obtained from the animal owners after they were informed on the use of their animal samples in the study. Samples included fecal swabs (n=61), wound swabs (n=17), and urine samples (n=42). Samples were collected from Al-Shaab Veterinary Hospital, Surgery and Medicine Departments at Faculty of Veterinary medicine, Cairo University as well as animal laboratories in Cairo and Giza from March to December 2019. Samples were collected from animals suffering from gastrointestinal tract disturbances, and acute or chronic cystitis. In addition, the wounded animals had certain medical conditions and could provide no appropriate response to the prescribed antibiotics (Huber et al.,2013). All investigated samples were collected under aseptic conditions and safety precautions. Samples were directly inoculated into 9 ml of sterile physiological saline (Okapara et al., 2018)**.** Urine samples were collected from each case via catheter and urine was collected from the distal part under aseptic conditions by the collection of the midstream urine sample (Cystocentesis) as reported by Huber et al. (2013). Samples were appropriately labelled and transported without delay to the laboratory and processed immediately.

Phenotypic characterization and Antibiogram testing for *E. coli* and *Klebsiella* spp. isolates

Wound and fecal swabs were inoculated onto MacConkey agar (Oxoid) supplemented with ampicillin (100 mg/L; Mac-AMP100, Oxoid) according to Okapara et al. (2018). Urine samples were centrifuged, and the sediment was inoculated directly on MacConkey agar (Oxoid). All inoculated plates were incubated at 37˚c for 18-24 hours and examined for bacterial growth. Both lactose fermenter colonies and late lactose fermenter colonies were selected for further examinations. The purified isolates were finally confirmed biochemically with citrate, oxidase, indole, catalase, Voges Proskauer, methyl red, urease, and triple sugar iron (TSI) tests according to Cruickshank et al. (1975). All isolates were tested for their susceptibility to different antimicrobial drugs and antibiotics (Table 1). The antimicrobial susceptibility test was performed using the disc diffusion method (Kibry-Bauer method) on Muller-Hinton agar plates (Oxoid) and the interpretation was performed based on CLSI (2018).

Doubled-disc synergy test

ESBL production was identified using Double Disk Synergy Test (DDST) according to Iqbal et al. (2017). Three antibiotics were used for DDST ceftriaxone (30µg), Amoxicillin-clavulanic acid (20/10µg), and ceftazidime (30µg, Oxoid). Discs were placed at a distance of 1.5cm. ESBL positive organism are showing development of the inhibition zone towards the clavulanate disc at 37°C after 24-hours-incubation.

Molecular characterization of ESBL-genes in *E. coli* and *Klebsiella* spp. isolates

Extraction of DNA was performed using QIAmp DNA Mini Kit instructions (QIAGEN, Germany). Specific primers were used for the amplification of *blaTEM*, *blaSHV*, and *blaCTX* genes (Table 2). The preparation of the PCR Master Mix was performed according to Emerald Amp GT PCR Master Mix (Takara). The reaction mixture consisted of 12.5μl Emerald Amp GT PCR Master Mix (2x premix), 4.5 μl PCR grade water, 1μl of each primer in the concentration

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of (20 pmol), 6μl of Template DNA, and leading to a total of 25μl. The cycling condition was like that used in (Hasman et al., 2005). The ladder was mixed gently by pipetting up and down, and 6 µl of the required ladder was directly loaded. The PCR products were resolved by electrophoresis on an agarose gel according to Sambrook et al. (1989) with some modification.

Table 1. Antibiotics used in antimicrobial susceptibility test and their resistance pattern against *E. coli* and *Klebsiella* spp. isolates

Antibiotic	Disc content	Resistance pattern of *E. Coli*			Resistance pattern of *Klebsiella* spp.		
		Sensitive (%)	Intermediate (%)	Resistant (%)	Sensitive (%)	Intermediate (%)	Resistant (%)
Aminoglycosides							
Amikacin	30 µg	89	5	6	86	6	8
Gentamycin	10 µg	53	18	29	72	0	28
Streptomycin	10 µg	47	0	53	28	20	52
Kanamycin	10 µg	31	13	56	60	12	28
Cephalosporins (1st generation)							
Cephalexin	30 µg	8	0	92	18	6	76
Cephalosporins (3rd generation)							
Cefotaxime	30 µg	9	4	87	52	0	48
Ceftazidime	30 µg	19	0	81	36	4	60
Ceftriaxone	30 µg	23	6	71	38	22	40
Miscellaneous antibiotics							
Chloramphenicol	10 µg	49	27	34	45	31	24
Nitrofurinations	300 µg	69	13	18	58	24	28
Other *β lactam*							
Aztreonam	30 µg	41	6	53	66	10	24
Penicillins							
Ampicillin	10 µg	0	0	100	0	0	100
Amoxycillin	(20/10) µg	37	0	63	32	8	60
Clavulanic acid							
Quinolones							
Ciprofloxacin	5 µg	71	11	18	88	0	12
Nalidixic acid	30 µg	68	3	29	92	0	8
Tetracyclines							
Tetracycline	30 µg	34	6	60	42	22	36

Table 2. Oligonucleotide primers and probes used in PCR and SYBR Green real-time PCR

Gene	Primer sequence (5'-3')	Amplification size	Reference
bla_{TEM}	ATCAGCAATAAACCAGC	516 bp	Colom et al. (2003)
	CCCCGAAGAACGTTTTC		
bla_{SHV}	AGGATTGACTGCCTTTTTG	392 bp	
	ATTTGCTGATTTCGCTCG		
blaCTX	ATG TGC AGY ACC AGT AAR GTK ATG GC	593 bp	Archambault et al. (2006)
	TGG GTR AAR TAR GTS ACC AGA AYC AGC GG		
gyrA (Klebsiella spp.*)*	CGC GTA CTA TAC GCC ATG AAC GTA	-	Brisse and Verhoef (2001)
	ACC GTT GAT CAC TTC GGT CAG G		
16S rRNA (E. coli)	GCTGACGAGTGGCGGACGGG	-	Tivendale et al. (2004)
	TAGGAGTCTGGACCGTGTCT		

In vitro assessment of the antibacterial effect of Ag NPs suspension

Silver nanoparticles powder was purchased from the National Research Center, Egypt. The dispersion process was done using an ultrasonic processor (Cole-Parmer instruments, Illinois U.S.A.). For nanofluid preparation, 10 mg of Ag NPs of average size 58 nm were dispersed in 1ml of sterile Muller Hinton broth and sonicated for 5 minutes at 20000 HZ frequency from 3 to 5 times to avoid aggregation (Tayel et al., 2010). Pure colonies from both *E. coli* and *Klebsiella* spp.

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isolates were picked up and suspended in Muller Hinton broth. Suspensions were adjusted to match McFarland standard 0.5 (1.5×10^8 CFU /ml) to be ready for antimicrobial testing. The tests were adapted according to CLSI (2018). The minimum inhibitory concentration (MIC) of AgNPs was determined in a sterile 96-well microtiter plate. The 100 µL of sterile muller Hinton broth was pipetted into the well No.1 through No.12 of the column. In the next step, 100 µL of the Ag NPs suspension (10mg/ml) were added into well No.1 of column (A), to reach a total volume of 200 µL with a concentration of (5 mg/ml). Two-fold serial dilution was applied starting with the previously mentioned concentration. Later, 100 µL of the *E. coli* suspension (McFarland 0.5) was added to the wells of the column, and then the initial concentration changed to 2.5 mg/ml. Well No.11 served as a negative growth (sterility control) containing Ag NPs suspension plus sterile broth only while well No.12 was served as a positive bacterial control containing broth plus the bacterial inoculum only. These steps were repeated with *Klebsiella* spp. in another microtiter plate. The microtiter plates were incubated at 37 °C for 24 hours in a shaker incubator. The lowest concentration of Ag NPs in the series inhibiting the growth of the bacteria in vitro was taken as the MIC. For MBC determination, 50 µL from each well was spread on MacConkey agar plates and incubated at 37°C for a further 48 hours. Growth-free plates validated that the used concentration inhibited bacterial growth. These tests were performed in triplicate.

Assessment of the inhibitory effect of AgNPs on the expression of resistance genes in *E. coli* and *Klebsiella* spp. using SYBR Green RT- PCR

The effect of sub-MIC dose (1/2) of AgNPs on the expression of *blaTEM*, *blaSHV*, and *blaCTX* genes was studied in the presence of 16s rRNA for *E. coli* and gyrA for *Klebsiella* spp. as housekeeping genes (Brisse and Verhoef, 2001;Tivendal et al., 2004). The RT-PCR procedure was performed in Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Egypt. Extraction of RNA was performed according to RNeasy Mini Kit instructions (QIAGEN, Germany, GmbH). Oligonucleotide primers and probes used in SYBR Green real-time PCR are shown in Table 2. PCR Master Mix QuantiTect SYBR Green PCR Kit was used. The reaction mixture consisted of 12.5μl 2x QuantiTect SYBR Green PCR Master Mix, 0.25μl Revert Aid Reverse Transcriptase (ThermoFisher, 200 U/µL), 0.5μl of each primer (20 pmol), 8.25μl RNase Free Water, 3μl Template RNA, leading to a total of 25μl. The cycling conditions were performed according to previous studies (Brisse and Verhoef, 2001; Colom et al., 2003; Tivendale et al., 2004; Archambault et al., 2006)**.** Amplification curves and CT values were determined by the strata gene MX3005P software. To estimate the variation of gene expression of the different samples, the CT of each sample was compared with that of the control group according to the " ΔΔCt method CT" stated by Yuan et al. (2006) and samples were tested in triplicates. The dissociation curves of different samples were compared to exclude false-positive results.

Evaluation of morphological changes in *E. coli* and *Klebsiella* spp. upon their interaction with silver nanoparticles

Samples included untreated samples (control) and AgNPs treated *E. coli* and *Klebsiella* spp. colonies. The samples were fixed by glutaraldehyde 2.5% and dehydrated by the serial dilution of ethanol with agitation using an automatic tissue processor (Leica EM TP, Leica Microsystems: Austria). In the next step, they were dried using CO_2 critical point drier (Model: Audosamdri-815, Tousimis; Rockville, Maryland, USA). The samples were coated by a gold sputter coater (SPI-Module, USA). They were examined by Scanning electron microscopy (Model: JSM- 5500 LV; JEOL Ltd –Japan) using a high vacuum mode at the Regional Center of Mycology and Biotechnology, Cairo, Egypt.

Statistical analysis

Statistical analysis was performed using R-programme. One-way ANOVA was run to evaluate the statistical significance between the control and treated samples. *P-value* less than 0.05 was considered statistically significant.

RESULTS

Phenotypic identification of *E. coli* and *Klebsiella* spp. isolates

Out of 120 samples, *E. coli* and *Klebsiella* spp. were detected in 62 (51.6%) and 25 (20.8%) cases, respectively. On MacConkey agar, *E. coli* appeared as medium-sized, smooth, round, lactose-fermenting colonies, pink to red with bile salt precipitate surrounding the colonies. On the other hand, *Klebsiella* spp. was observed as medium-sized, pink, lactose- fermenting, round, shiny, and mucoid colonies. Microscopic examination of Gram-stained pure colonies of *E. coli* and *klebsiella* spp. isolates were gram-negative rod-shaped bacteria. Isolates were confirmed biochemically. *E. coli* was negative in urease, oxidase, Voges Proskauer (vp), citrate tests while positive for catalase, methyl red (MR), Indole tests and A/A with gas production and negative H_2S production for TSI test. *Klebsiella* spp. was positive in urease, oxidase, Voges Proskauer, catalase and citrate test and A/A with gas production and negative indole, Methyl red, and H_2S production for TSI test.

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Antimicrobial susceptibility testing

Using different groups of antibiotics (Table 1), the antibiogram was assessed for *E. coli* and *Klebsiella* spp. isolates. Ampicillin showed the highest percentage for resistance in both *E. coli* and *Klebsiella* spp. as shown in the resistance pattern (Figure 1). The most prominent groups in resistance pattern were Cephalosporins first and third generation represented by Cephalexin (92%, 76%), Cefotaxime (87%, 48%), Ceftazidime (81%, 60%), Ceftriaxone (71%, 40%) for *E. coli* and *Klebsiella* spp., respectively. Moreover, Amoxicillin Clavulanic acid indicated 63% resistance for *E. coli* isolates and 60% for *Klebsiella* spp. The overall result of the antibiogram revealed that about 29 isolates (*E. coli* =20, *Klebsiella* spp.=9) were suspected to be ESBL producing isolates.

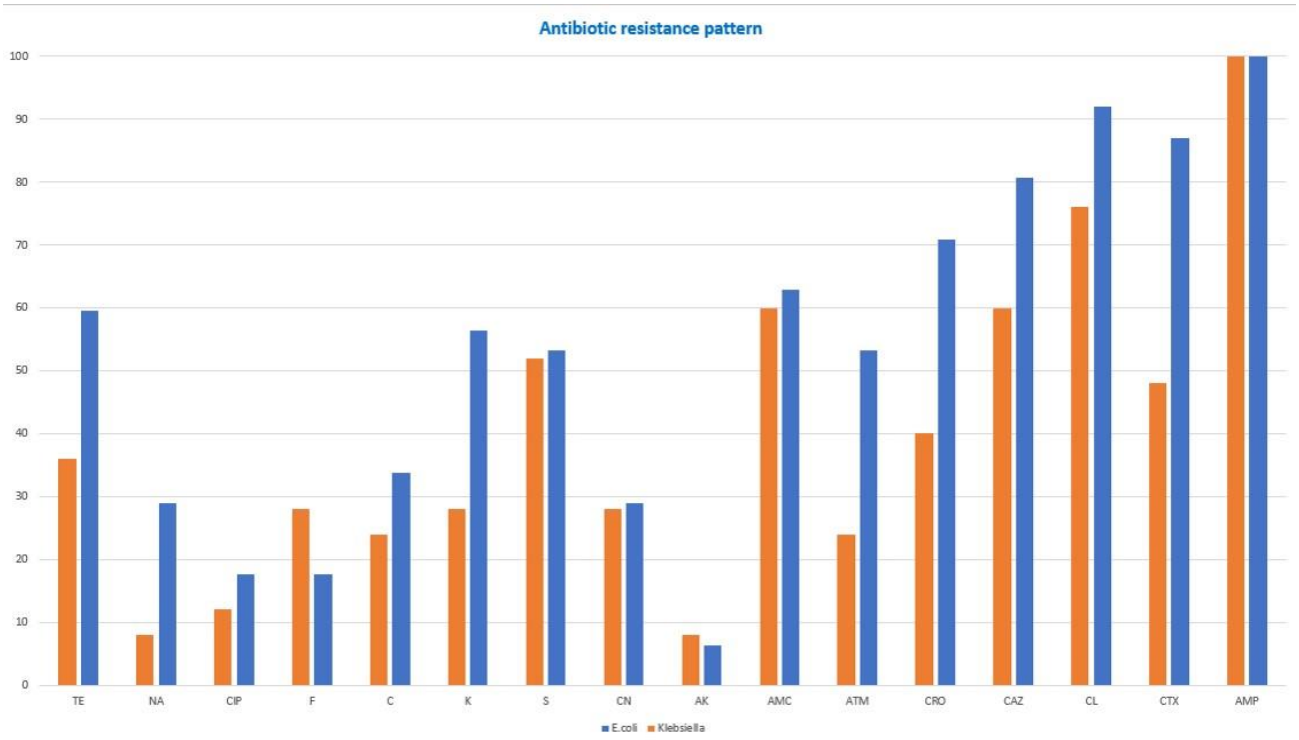

Figure 1. Antibiotic resistance pattern of the used antibiotics against *E. coli* and *Klebsiella* spp. isolates

Double-disc synergy test method

Detection of suspected isolates by DDST revealed that 23 isolates (*E. coli*=17, *Klebsiella*=6) were ESBL producing isolates. The number of isolated ESBL producing *E. coli* and *Klebsiella* spp. for each type of sample is demonstrated in Table 3.

Molecular detection of ESBL -encoding genes

PCR screening of genes encoding ESBL revealed the presence of *blaCTX*, *blaSHV*, and *blaTEM* genes in all the tested isolates except one *Klebsiella* spp. isolate which did not harbor *blaCTX* gene (Figure 2).

In vitro evaluation of the antibacterial effect of Ag NPs

Scanning Electron Microscopy of AgNPs (Figure 3) revealed that Ag NPs were spherical with the average size of the 58 nm. The MIC of AgNPs tested for ESBL-producing *E. coli* was 0.31 mg/ml, and 0.62 mg/ml for ESBL-producing *Klebsiella* spp. The minimum bactericidal concentration (MBC) of ESBL-producing *E. coli* and *Klebsiella* spp. was 0.15 mg/ml and 0.3 mg/ml, respectively.

Effect of sub MIC concentration of AgNPs on the expression of resistance genes.

Expression of *blaTEM*, *blaSHV*, and *blaCTX* genes was downregulated with sub-MIC doses of AgNPs (150 µg/ml for *E. coli* and 310 µg/ml for *Klebsiella* spp.), compared to untreated sample as shown in (Figure 4).

Effect of silver nanoparticles on the integrity of cells

The SEM images of untreated (control) cells showed healthy cells with clear unpenetrated cell membranes while treated cells showed cell membrane damage due to the adherence of AgNPs with the bacterial cell membrane and penetration into the cells causing cell death (Figure 5).

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Table 3. Prevalence of ESBL producers from dogs and cats in Egypt

Origin	No. of samples with ESBL producer (%)		
	EC	KS	Total
Wound swabs (n=17)	-	2 (11.7)	2 (11.7%)
Fecal swabs (n=61)	10 (16.4%)	1 (1.6%)	11 (18%)
Urine samples (n=42)	7 (16.7%)	3 (7.1%)	10 (23.8%)
Total (n=120)	17 (14.16%)	6 (5%)	23 (19.6%)

EC: Escherichia coli; KS: *Klebsiella* spp.

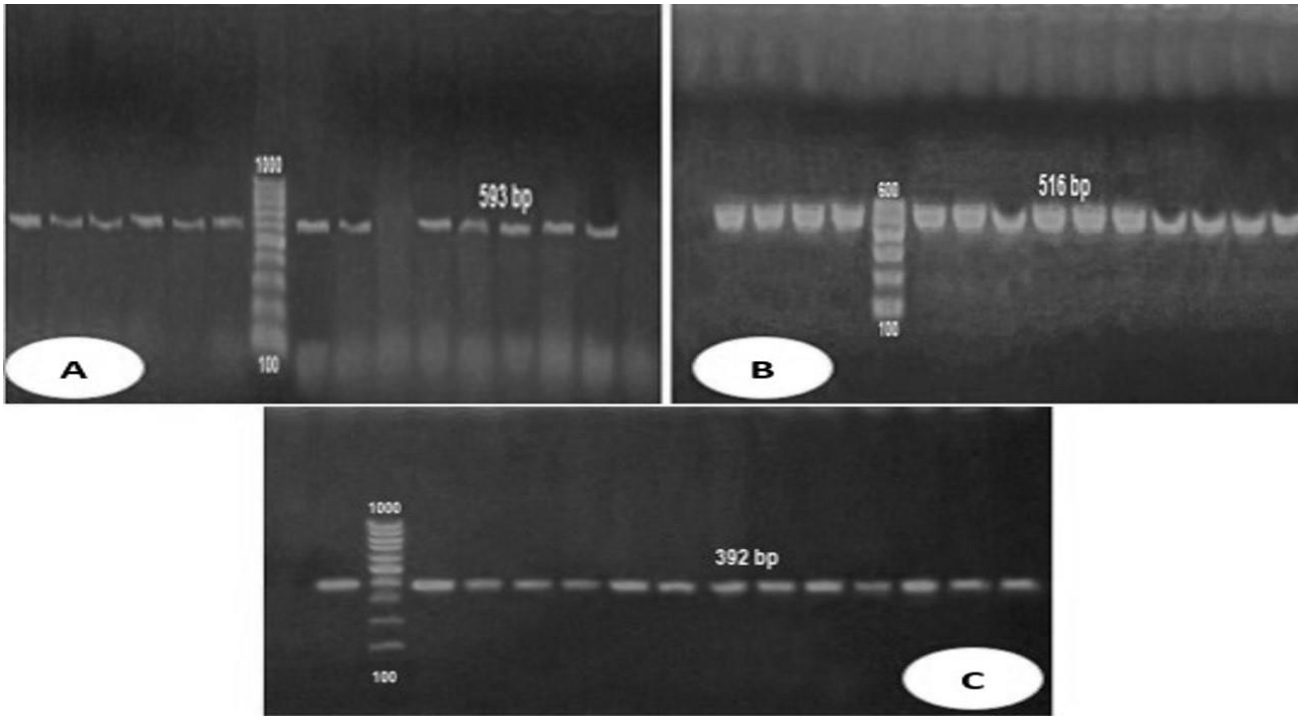

Figure 2. Agarose gel electrophoresis of A- *blaCTX* gene (Amplicon size593 bp), B- *blaTEM* gene (Amplicon size 516 bp), C-*blaSHV* gene (Amplicon size 392 bp) Ladder [Gelpilot100 bp plus ladder (Qiagen, 100-1500 bp)]

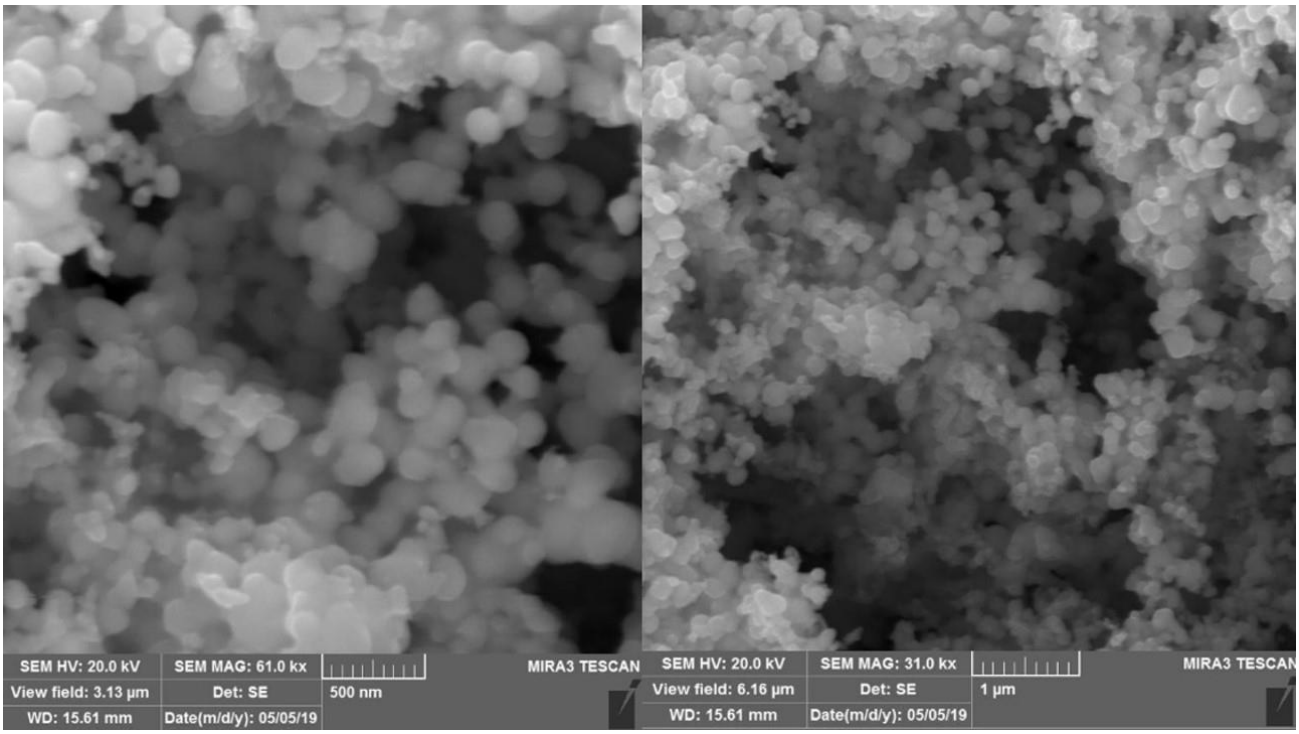

Figure 3. SEM of AgNPs revealed that Ag NPs are spherical in shape and the average size is 58 nm.

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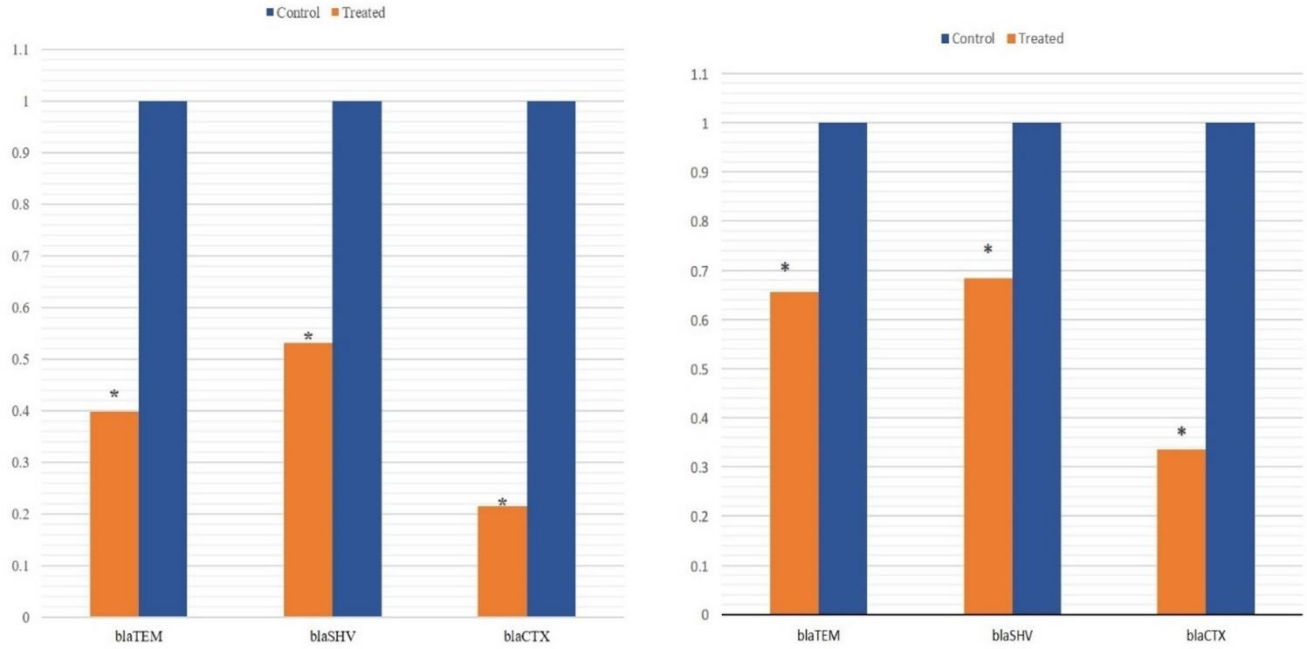

Figure 4. Effect of Silver nanoparticles on the antibiotic resistance genes expression in Left *E. coli*, Right *Klebsiella* spp. Stars indicate significant difference between control and treated samples for each gene.

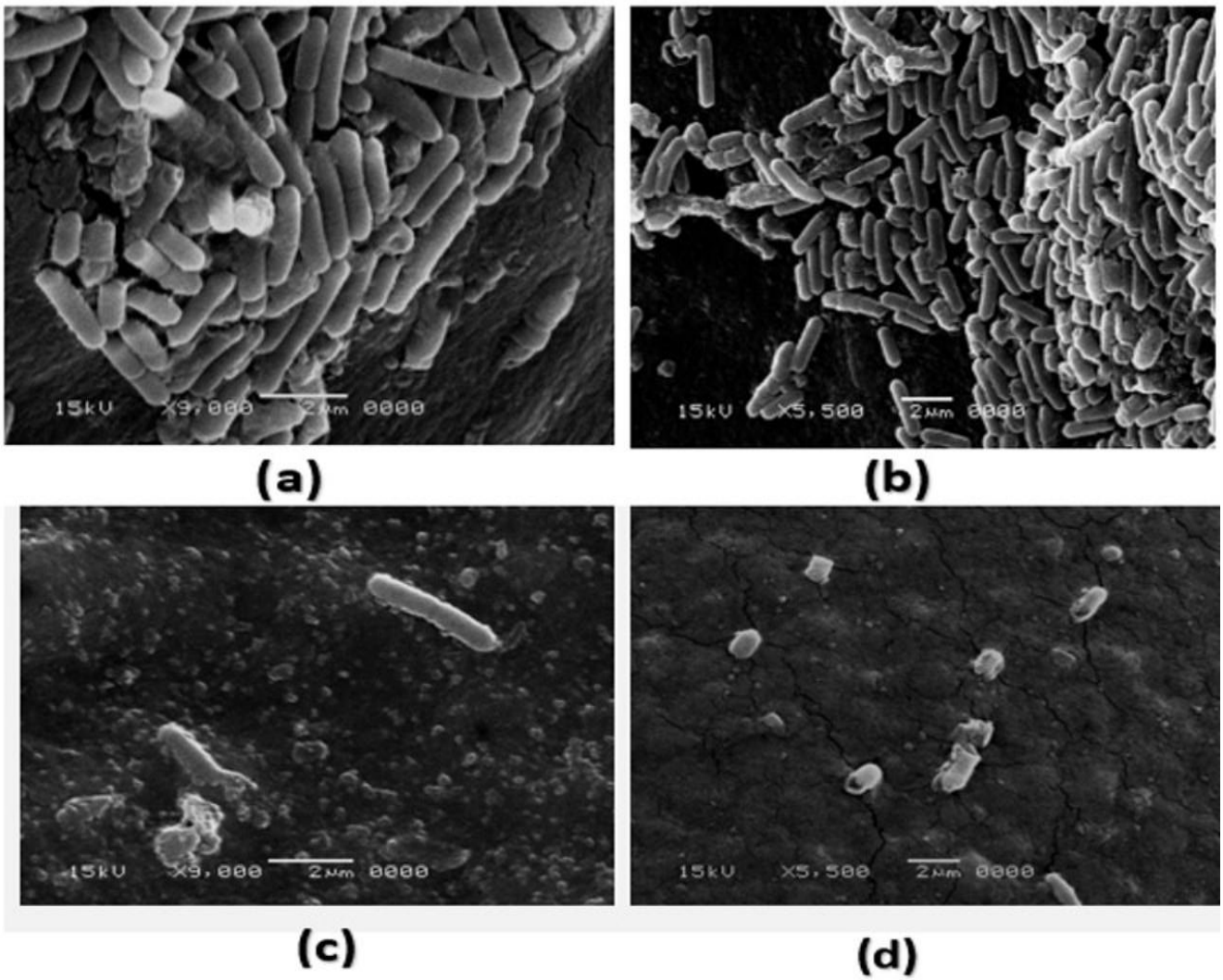

Figure 5. Scanning Election microscopic pictures of AgNPs treated and untreated *E. Coli* and *Klebsiella* spp. The control cells showed normal, clear and unpenetrated cell membrane (**a, b**). AgNps treated cells showed cell membrane penetration and perforation of cells leading to excretion of cell metabolites and cell death (**c, d**)

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DISCUSSION

Despite the presence of various modern antimicrobial agents and antibacterial drugs, bacterial infections are still a major threatening problem due to the enormous increase in multi-drug-resistant bacteria. Mobile genetic elements, such as plasmids, are capable of transferring antimicrobial resistance determinant elements among different bacterial populations and play an important role in the epidemiology of antimicrobial resistance (Carattoli, 2013). The extensive misuse of antibiotics has become a leading cause of the emergence of several hazards to public health, such as superbugs, which resist all the current drugs (Khameneh et al., 2016). The clinical effectiveness of beta-lactams has been diminished owing to the massive increase in resistant bacteria and prolonged patient recovery (Denisuik et al., 2013; Mathers et al., 2015). Extended-spectrum beta-lactamases (ESBLs) in pet animals is a threatening issue which has emerged worldwide. Since their emergence, ESBLs have most often been found in *Escherichia coli* and *Klebsiella pneumonia* (Bonnet 2004; Livermore et al. 2006; Mathers et al. 2015). In the present study, the prevalence of ESBL producing *Escherichia coli* and *Klebsiella* spp. was investigated in different samples obtained from pet animals. MacConkey agar (Oxoid) supplemented by Ampicillin was used for the isolation of ESBL-producing isolates which facilitate screening of the isolates rather than Macconkey supplemented with Cephalosporine as reported by Okapara et al. (2018). ESBL-producing *E. coli* and *Klebsiella* spp. were detected in 23 out of the 120 samples of the current study representing 19.6 % of the total samples. In previous studies, ESBL- producing *K.pneumoniae* were recorded as 41% (Okapara et al., 2018) and 7.5% (Liu et al., 2017). ESBL-producing isolated *E. coli* was detected in 17 (14.16%) samples, 7 from urine, and the other 10 from fecal samples. The obtained results of the current study were indicative of higher percentages, compared to those of ESBL isolated *E. coli* obtained from dogs and cats in Switzerland 8% (Huber et al., 2013) and New Zealand 6.4%, (Karkaba et al., 2019) and lower than those obtained from pet animals in Switzerland 54.7%, (Zogg et al., 2018) and from dairy farms in Germany 75.6%, (Odenthal et al., 2016)**.** Three genes (i.e., *TEM*, *SHV*, and *CTX-M*) are the most predominant in ESBL-producing bacteria (Paterson and Bonomo, 2005). The *blaSHV* and *blaTEM* were detected in all the isolates while *blaCTX-M* was found in 92.3%. Huber et al. (2013) investigated ESBL genes of ESBL producing *E. coli* isolates where *blaCTX-M* was found in 100% of isolates and *blaTEM* in 87.5%. Searching new effective bactericidal alternatives has become an urgent issue for combatting drug resistance. Silver nanoparticles have been established as a promising approach as an alternative for antimicrobial agents in the treatment of several medicinal problems (Beyth et al., 2015; Hassanen and Ragab, 2020)**.** They have attracted great concerted attention and have been broadly used in a variety of applications as antibacterial/antifungal agents in a diverse range of products, including air sanitizer sprays, pillows, respirators, wet wipes, detergents, soaps, shampoos, toothpaste, air filters, coatings of refrigerators, vacuum cleaners, washing machines, food storage containers, cellular phones (Sun et al., 2001). Moreover, they do not cause high level of toxicity in human as well as they have broad-spectrum antibacterial actions (Chandran et al., 2006).

In the present study, AgNPs were tested against ESBL-producing *E. coli* and *Klebsiella* spp. isolates in vitro by the detection of MIC and MBC. Manikprabhu and Lingappa (2014) determined the antibacterial effect of AgNPs with size (28-50nm) against ESBL producing *E. coli* where MIC and MBC were found to be (in the range of 0.11 and 0.22 mg/ml**)**. Moreover, biogenic nanosilver of (20-70nm) was used against ESBL-producing *k. pneumoniae* and *E. coli*, where the recorded MIC and MBC were 1.4µg and 2µg, respectively (Subashini et al., 2014). This indicated that AgNPS had a good bacteriostatic effect according to MIC and good bactericidal effect according to MBC on ESBL-producing *E. coli* and *Klebsiella* spp. As reported, the small size of nanoparticles potentiates the antibacterial effect on microorganisms (Smekalova et al., 2016). Also, AgNPs had the same effect against ESBL- and non ESBL- producing bacteria (Ansari et al., 2014).

It should be noted that it is not easy to compare the obtained results of the antibacterial effect of AgNPs in the current research with those of previous studies since different researchers employed different methods to study the antibacterial effect of AgNPs against different types of bacteria. Besides, the effect of AgNPs against microorganisms was influenced by the size, shape, stability, and concentration of AgNPs (Bandyopadhyay et al., 2018). We found that the concentration of AgNPs used to inhibit or kill microorganisms differed from one another as MIC and MBC values of AgNPs against *E. coli* were lower than those of *Klebsiella* spp.

In the current study, SYBR Green RT-PCR was used to investigate the influence of silver nanoparticles against ESBL-producing *E. coli* and *Klebsiella* spp. resistance genes (*blaCTX-M*, *blaTEM*, and *blaSHV*). The sub-MIC dose (150µg) of silver nanoparticles of average size 58 nm was tested against *E. coli* and showed the downregulation of *blaCTX-M*, *blaTEM*, and *blaSHV* genes with fold change about 0.21, 0.39, 0.53, respectively, for *E. coli*. Furthermore, the sub-MIC dose (310µg) of silver nanoparticles was tested against *Klebsiella* spp. and indicated the downregulation of genes expression with 0.33, 0.65, 0.68 for *blaCTX-M*, *blaTEM*, and *blaSHV* resistance genes, respectively. These results indicated that AgNPs could effectively influence the gene expression of *E. coli* (*blaCTX-M*, *blaTEM*, and *blaSHV*) genes more than that of *Klebsiella* spp, which would subsequently reflect in their resistance pattern.

In the current study, the morphological changes in *E. coli* and *Klebsiella* spp. cells were evaluated before and after the treatment with silver nanoparticles using SEM. The SEM observations in treated cells confirmed cell membrane

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damage due to the adherence of AgNPs with bacterial cell membranes and penetration into the cells causing cell death. On the other hand, untreated cells indicated healthy cells with a clear unpenetrated cell membrane. The bactericidal effect of silver nanoparticles is still of unknown mechanism. Many studies suggest that their binding to the bacterial cell membrane may disrupt cell permeability (Kvítek et al., 2008) while other studies propose that the bactericidal effect did not only caused by contact with cell membrane but also because of penetration into the bacterial cell leading to the inactivation of DNA replication and causing cell death (Morones et al., 2005).

CONCLUSION

In the present study, the antibacterial effect of silver nanoparticles was investigated in vitro against Extended Spectrum Beta lactamase producing *E. coli* and *Klebsiella* spp. The findings revealed that using silver nanoparticles as an alternative to antimicrobial agents had an obvious effect on minimum inhibitory concentration, minimum bactericidal concentration , Bacterial cell wall integrity as well as genetically on the expression of antibiotic resistance genes. This trial is very encouraging for the control of antibiotic-resistant bacteria.

DECLARATIONS

Authors' contributions

Omnia A Khalil, Mona I Enbaawy, Eman Ragab, Hossam Mahmoud, and Taher Salah designed the plan of work, supervised the experiment, and revised the manuscript. Eman Ragab is the corresponding author and responsible for English editing, statistical analysis, and formatting the manuscript.

Competing interests

The authors declare no conflicts of interest.

Consent to publish

Written consent was obtained from the owners of the animals after they were informed on the use of their animal samples in the study

REFERENCES

Alyamani EJ, Khiyami AM, Booq RY, Majrashi MA, Bahwerth FS, and Rechkina E (2017). The occurrence of ESBL-producing *Escherichia coli* carrying aminoglycoside resistance genes in urinary tract infections in Saudi Arabia. Annals of Clinical Microbiology and Antimicrobials, 16(1): 1. DOI: https://www.doi.org/10.1186/s12941-016-0177-6

Ansari MA, Khan HM, Khan AA, Cameotra SS, Saquib Q, and Musarrat J (2014). Interaction of A l2 O 3 nanoparticles with E scherichia coli and their cell envelope biomolecules. Journal of applied microbiology, 116(4): 772-783. DOI: https://www.doi.org/10.1111/jam.12423

Archambault M, Petrov P, Hendriksen RS, Asseva G, Bangtrakulnonth A, Hasman H, and Aarestrup FM (2006). Molecular characterization and occurrence of extended-spectrum beta-lactamase resistance genes among Salmonella enterica serovar Corvallis Thailand, Bulgaria, and Denmark. Microbial Drug Resistance, 12(3): 192-198. DOI: https://www.doi.org/10.1089/mdr.2006.12.192

Bandyopadhyay S, Banerjee J, Bhattacharyya D, Samanta I, Mahanti A, Dutta TK, Ghosh S, Nanda PK, Dandapat P and Bandyopadhyay S (2018). Genomic identity of fluoroquinolone-resistant bla CTX-M-15-Type ESBL and pMAmpC β-lactamase producing *Klebsiella pneumoniae* from buffalo milk, India. Microbial Drug Resistance, 1: 24(9): 1345-1353. DOI: https://doi.org/10.1089/mdr.2017.0368

Beyth N, Houri-Haddad Y, Domb A, Khan W, and Hazan R (2015). Alternative antimicrobial approach: nano-antimicrobial materials. *Evidence-Based Complementary* and *Alternative Medicine*, 2015: 246012. DOI: http://dx.doi.org/10.1155/2015/246012

Bindu D, Mythili S, Inthira KS, palanisamy R, Lingesh KT, and Thandapani S (2015). New Generation of Silver Nanoparticles against Extended Spectrum Beta Lactamase Producing Organisms. International Journal of Drug Development & Research, 7(3): 001-003. Available at: https://www.ijddr.in/drug-development/new-generation-of-silver-nanoparticles-against-extended-spectrum-betalactamase-producing-organisms.pdf

Brisse S, and Verhoef J (2001). Phylogenetic diversity of *Klebsiella pneumonia* and *Klebsiella oxytoca* clinical isolates revealed by randomly amplified polymorphic DNA, gyrA and parC gene sequencing and automated ribotyping. *International Journal* of Systematic and Evolutionary *Microbiology*, 51: 915-924.
DOI:: https://www.doi.org/10.1099/00207713-51-3-915

Carattoli A. Plasmids and the spread of resistance(2013) . International Journal of Medical Microbiology 1;303(6-7):298-304. DOI : https://doi.org/10.1016/j.ijmm.2013.02.001

Chandran SP, Chaudhary M, Pasricha R, Ahmad A, and Sastry M (2006). Synthesis of gold nanotriangles and silver nanoparticles using Aloe vera plant extract. Biotechnology Progress, 22: 577-583. DOI: https://www.doi.org/10.1021/bp0501423

Clinical and Laboratory Standards Institute (CLSI) (2018). Performance standards for antimicrobial susceptibility testing. 27th Informational Supplement Document M100- S27, CLSI, Wayne, 37(1). Available at :https://clsi.org/media/1469/m100s27_sample.pdf

Colom K, PèrezJ, Alonso R, Fernández-Aranguiz A, Lariño E, and Cisterna R (2003). Simple and reliable multiplex PCR assay for detection of blaTEM,blaSHV and blaOXA-1 genes in Enterobacteriaceae. FEMS Microbiology Letters, 223: 147-151. DOI:https://www.doi.org/10.1016/S0378-1097(03)00306-9

Cruickshank R, Duguid RP, Marmion BP, and Swain RH (1975). Medical Microbiology.New York: Churchill Livingstone, Available at: https://agris.fao.org/agris-search/search.do?recordID=XF2015010062

Denisuik AJ, Lagacé-Wiens PR, Pitout JD, Mulvey MR, Simner PJ, Tailor F, Karlowsky JA, Hoban DJ, Adam HJ, and Zhanel GG (2013), Canadian Antimicrobial Resistance Alliance (CARA). Molecular epidemiology of extended-spectrum β-lactamase-, AmpC β-lactamase-and

carbapenemase-producing *Escherichia coli* and *Klebsiella pneumoniae* isolated from Canadian hospitals over a 5 year period: CANWARD 2007–11. Journal of Antimicrobial Chemotherapy, 1: 68(suppl_1): 57-65. DOI: https://doi.org/10.1093/jac/dkt027

Devrim I, Gulfidan G, Gunay I, Agin H, Güven B, Yilmazer MM, and Dizdarer C (2011). Comparison of in vitro activity of ertapenemwith other carbapenems against extended- spectrum beta-lactamase-producing Escherichia coli and Kleibsella species isolated in a tertiary children's hospital. *Expert Opinion* on *Pharmacotherapy*, 12: 845–849. DOI: https://www.doi.org/10.1517/14656566.2011.559460

Ejaz H, Zafa A, Mahmood S, and Javed MM (2011). Urinary tract infections caused by extended spectrum β-lactamase (ESBL) producing *Escherichia coli* and *Klebsiella pneumoniae*. African Journal of Biotechnology, 10(73): 16661-16666. DOI https://www.doi.org/10.5897/AJB11.2449

Fan NC, Chen HH , Chen CL Ou LS, Lin TY, Tsai MH and Chiu CH (2014). Rise of community-onset urinary tract infection caused by extended-spectrum β-lactamase-producing *Escherichia coli* in children. Journal of microbiology,Immunology and Infection 47(5): 399-405. DOI: https://www.doi.org/10.1016/j.jmii.2013.05.006

Hamida RS, Ali MA, Goda DA, Khalil MI, and Redhwan A (2020). Cytotoxic effect of green silver nanoparticles against ampicillin resistant *Klebsiella pneumoniae*. Royal Socitey of Chemistry Advances, 10: 21136-21146. DOI: https://www.doi.org/10.1039/D0RA03580G

Hasman H, Mevius D, Veldman K, Olesen I and Aarestrup FM (2005). β-Lactamases among extended-spectrum β-lactamase (ESBL)-resistant Salmonella from poultry, poultry products and human patients in The Netherlands. Journal of Antimicrobial Chemotherapy. 1;56(1):115-21. Doi: https://doi.org/10.1093/jac/dki190

Hassanen EI, and Ragab E (2020). In Vivo and In Vitro Assessments of the Antibacterial Potential of Chitosan-Silver Nanocomposite Against Methicillin-Resistant Staphylococcus aureus–Induced Infection in Rats. Biological Trace Element Research, Apr 18: 1-4. DOI: https: www.doi.org/10.1007/s12011-020-02143-6

Huber H, Zweifel C, Wittenbrink MM, and Stephan R (2013). ESBL-producing uropathogenic Escherichia coli isolated from dogs and cats in Switzerland.Veterinary Microbiology, 162: 992-996. DOI: https://www.doi.org/10.1016/j.vetmic.2012.10.029.

Iqbal R, Ikram N, Shoaib M, Asad MJ, Tahir R, Mehmood, Niazi A, Asghar A, Bushra Ishfaq and Faiza Naseer (2017). Phenotypic cofirmatory disc diffusion test (PCDDT), double disc synergy test (DDST), E-test OS diagnostic tool for detection of extended spectrum beta lactamase (ESBL) producing Uropathogens. Journal of Applied Biotechnology and Bioengineering, 3(3): 344–349 DOI: https://www.doi.org/10.15406/jabb.2017.03.00068

Karkaba A, Hillb K, Benschop J, Pleydellc E, and Grinberg A (2019). Carriage and population genetics of extended spectrum β-lactamaseproducing Escherichia coli in cats and dogs in New Zealand.Veterinary Microbiology, 233: 61-67. DOI: https://www.doi.org/10.1016/j.vetmic.2019.04.015.

Keshari AK, Srivastava R, Singh P, Yadav B, and Nath G (2020). Antioxidant and antibacterial activity of silver nanoparticles synthesized by Cestrum nocturnum. Journal of Ayurveda and Integrative Medicine, 11(1): 37-44. DOI: https://www.doi.org/10.1016/j.jaim.2017.11.003

Khameneh B, Diab R, Ghazvini K, and Fazly Bazzaz BS (2016). Breakthroughs in bacterial resistance mechanisms and the potential ways to combat them. Microbial Pathogenesis, 95: 32–42. DOI: https://www.doi.org/10.1016/j.micpath.2016.02.009

Khan T, Yasmin A, and Townley HE (2020). An evaluation of the activity of biologically synthesized silver nanoparticles against bacteria, fungi and mammalian cell lines. Colloids and surfaces B: Biointerfaces, 194: 111156. DOI: https://www.doi.org/10.1016/j.colsurfb.2020.111156

Kizilca O, Siraneci R, and Yilmaz A (2012). Risk factors for community-acquired urinary tract infection caused by ESBL-producing bacteria in children. Pediatrics *International*, 54(6): 858-862. DOI: https://www.doi.org/10.1111/j.1442-200X.2012.03709.x

Kvítek L, Panáček A, Soukupova J, Kolář M, Večeřová R, Prucek R, Holecova M, and Zbořil R (2008). Effect of surfactants and polymers on stability and antibacterial activity of silver nanoparticles (NPs). The Journal of Physical Chemistry C, 17: 112(15): 5825-5834. DOI: https://www.doi.org/10.1021/jp711616v

Li WR, Xie XB, Shi QS, Zeng HY, OU-Yang YS, and Chen Y (2010). Antibacterial activity and mechanism of silver nanoparticles on Escherichia coli. *Applied Microbiology* and *Biotechnology*, 85: 1115-1122. DOI 10.1007/s00253-009-2159-5

Liu Y, Yang Y, Chen Y, and Xia Z (2017). Antimicrobial resistance profiles and genotypes of extended-spectrum b-lactamase- and AmpC b-lactamase-producing *Klebsiella pneumoniae* isolated from dogs in Beijing, China. Journal of Global Antimicrobial Resistance, 10: 219–222. DOI: https://www.doi.org/10.1016/j.jgar.2017.06.006

Livermore DM, Hope R, Fagan EJ, Warner M, Woodford N, and Potz N (2006). Activity of temocillin against prevalent ESBL-and AmpC-producing Enterobacteriaceae from south-east England. Journal of Antimicrobial Chemotherapy. 1:57(5):1012-4. DOI : https://doi.org/10.1093/jac/dkl043

Manikprabhu D, and Lingappa K (2014). Synthesis of silver nanoparticles using the Streptomycces Coelicolor Klmp33 pigment: An antibacterial agent against extended-spectrum beta-lactamase (ESBL) producing Escherichia Coli. Materials Science and Engineering C, 45: 434-437. DOI: https://www.doi.org/10.1016/j.msec.2014.09.034

Mathers AJ, Peirano G, and Pitout JD (2015). The role of epidemic resistance plasmids and international high-risk clones in the spread of multidrug-resistant Enterobacteriaceae. Clinical microbiology reviews. 28(3):565-591. DOI: https://www.doi.org/10.1128/CMR.00116-14

Morones JR, Elechiguerra JL, Camacho A, Holt K, Kouri JB, Ramírez JT, and Yacaman MJ (2005). The bactericidal effect of silver nanoparticles. Nanotechnology, Aug 26; 16(10): 2346. DOI: https://www.doi.org/10.1088/0957-4484/16/10/059

Odenthal S, Akineden Ö and Usleber E (2016). Extended-spectrum β-lactamase producing Enterobacteriaceae in bulk tank milk from German dairy farms. International Journal of Food Microbiology, 238: 72–78. DOI: https://www.doi.org/10.1016/j.ijfoodmicro.2016.08.036

Okapara EO, Ojo EO, Awoyomi OJ, Dipeolu MA, Oyekunle MA, and Schwarz S (2018). Antimicrobial usage and presence of extended-spectrum- β-lactamase-producing Enterobactereaceae in animal-rearing households of selected rural and peri-urban communities.Veterinary Microbiology, 218: 31-39. DOI: https://www.doi.org/10.1016/j.vetmic.2018.03.013

Paterson DL and Bonomo RA (2005). Extended_spectrumbeta_lactamases: a clinical update. Clinical Microbiology Review,18: 657-686. DOI: https://www.doi.org/10.1128/CMR.18.4.657-686.2005

Saeb ATM, Alshammari AS, Al-Brahim H, and Al-Rubeaan KA (2014). Production of silver nanoparticles with strong and stable antimicrobial activity against highly pathogenic and multidrug resistant bacteria. Scientific World Journal, 1: 704-708. DOI: https://www.doi.org/10.1155/2014/704708

Sambrook J, Fritsch EF, Maniatis T, and Sambrook-Fritsch-Maniatis (1989). Molecular Cloning: A Laboratory Manual ;[prepared for Use in the CSH Courses on the Molecular Cloning of Eukaryotic Genes]. Cold Spring Harbor Laboratory Press, Available at: https://www.cshlpress.com/pdf/sample/2013/MC4/MC4FM.pdf

Shahid M, Al-Mahmeed A, Murtadha MM, Qareeballa A, Eltahir MA, Tabbara KS, Ismaeel AY, Dar FK, Giha HA and Bindayna KM (2014). Characterization of cephalosporin-resistant clinical Enterobacteriaceae for CTX-M ESBLs in Bahrain. Asian Pacific Journal of Tropical Medicine, 7(1): S212-S216. DOI:https://www.doi.org/10.1016/s1995-7645(14)60234-0

Smekalova M, Aragon V, Panacek A, Prucek R, Zboril R and Kvitek L (2016). Enhanced antibacterial effect of antibiotics in combination with silver nanoparticles against animal pathogens. The Veterinary Journal, 209: 174-179. DOI: https://www.doi.org/10.1016/j.tvjl.2015.10.032

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Subashini J, Khanna VG, and Kannabiran K (2014). Anti-ESBL activity of silver nanoparticles biosynthesized using soil Streptomyces species *Bioprocess* and *Biosystems Engineering*, 37: 999–1006. DOI: https://www.doi.org/10.1007/s00449-013-1070-8

Sukmawinata E, Uemura R, Sato W, Thu Htun M, and Sueyoshi M (2020) . Multidrug-Resistant ESBL/AmpC-Producing *Klebsiella pneumoniae* Isolated from Healthy Thoroughbred Racehorses in Japan. Animals 10(3):369. DOI: https://doi.org/10.3390/ani10030369

Sun YP, Atorngitjawat P, and Meziani MJ (2001). Preparation of silver nanoparticles via rapid expansion of water in carbon dioxide micro-emulsiuon into reductant solution. Langmuir 17: 5707-5710. DOI: https:// www.doi.org/10.1021/la0103057

Tayel AA, El-Tras WF, Moussa S, El-Baz AF, Mahrous H, Salem MF, and Brimer L (2010). Antibacterial action of zinc oxide nanoparticles against foodborne pathogens. Journal of Food Safety, 31: 211-218. DOI: https://www.doi.org/10.1111/j.1745-4565.2010.00287.x

Thungrat K, price SB, Carpeter DM, and Boothe DM (2015). Antimicrobial susceptibility patterns of clinical Escherichia coli isolates from dogs and cats in the united states:january 2008 through january 2013.Veterinary Microbiology. 179(3-4) 287-295. DOI: https://www.doi.org/10.1016/j.vetmic.2015.06.012.

Tivendale KA, Allen JL,Ginns CA, Crabb BS, and Browning GF (2004). Association of iss and iucA, but not tsh, with plasmid- mediated virulence of avian pathogenic Escherichia coli. Infection and Immunity, 72(11): 6554-6560. DOI: https://www.doi.org/10.1128/IAI.72.11.6554-6560.2004

Wang Y, Hu Y, Cao J, Bi Y, Lv N, Liu F, Liang S, Shi Y,Jiao X, Gao GF, et al. (2019). Antibiotic resistance gene reservoir in live poultry markets. Journal of infection 78(6): 11-53. DOI: https://www.doi.org/10.1016/j.jinf.2019.03.012

Yuan JS, Reed A,Chen F, and Stewart CN (2006). Statistical analysis of real-time PCR data. BMC Bioinformatics, 7-85. Available at: https://bmcbioinformatics.biomedcentral.com/articles/10.1186/1471-2105-7-

Zogg AL, Zurfluha K, Schmittb S, Nüesch-Inderbinena M, and Stephana R (2018). Antimicrobial resistance, multilocus sequence types and virulence profiles of ESBL producing and non-ESBL producing uropathogenic Escherichia coli isolated from cats and dogs in Switzerland.Veterinary Microbiology, 216: 79-84. DOI: https://www.doi.org/10.1016/j.vetmic.2018.02.011.

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Microbiological Studies on Naturally Present Bacteria in Camel and Buffalo Milk

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ABSTRACT

The aim of current study was to isolate and identify naturally occurring probiotic *Lactobacillus* species in buffalo milk, camel milk, and camel urine to investigate their susceptibility to antibiotics and their antibacterial activity against pathogenic bacteria. A total number of seven samples which included three milk samples from buffalo, three milk samples from camel, and one urine sample from camel were collected and used in this study. The samples were cultured, and 18 isolated strains were identified by using 16S rRNA multiplex Polymerase Chain Reaction analysis, which was performed following DNA extraction from the isolated bacteria. Buffalo and camel milk were different in their *Lactobacilli* content. All *Lactobacilli* strains that were found in both camel milk and camel urine, were also found in buffalo milk, *Lactobacilli* strains in camel milk and urine were generally more resistant to the antibiotic. *Lactobacilli* isolated from buffalo milk, camel milk, and also camel urine presented variable degrees of antibacterial activity against pathogenic bacteria. Further studies should be conducted with more samples to gain more information in the field of antibacterial activity of probiotic *lactobacilli* and to understand the mechanisms of their activity. Hopefully, they can be used as natural alternatives instead of synthetic antibiotics.

Keywords: Antibacterial, Antibiotics, *Lactobacillus*, Probiotics

INTRODUCTION

Antibiotic resistance is considered as a global health crisis threatening the lives of both humans and animals. Many clinically isolated pathogenic bacteria are becoming increasingly resistant to antibiotics and disinfectants which make infection of these bacteria difficult to treat. During their evolution, bacteria have been developing several sophisticated mechanisms of antibiotic resistance to all types of antibiotics with no exception (Davies and Davies, 2010). The growing threat of antibiotic resistance necessitates the employment of creative approaches towards the discovery of novel alternatives to antibiotics. The use of probiotics is one of the options that is being discussed by the medical community to be used as an alternative to antibiotics (Brunel and Guery, 2017).

Probiotics are living microorganisms which confer health benefits to the host upon their administration in suitable amounts (FAO/WHO, 2011). The beneficial balance of the intestinal microbiota is one of the health-promoting properties that can be presented by probiotic microorganisms. Probiotics have been prescribed for patients with gastrointestinal disease and complaints (Williams et al., 2010). There is a set of cumulative evidence that supports the use of probiotics, both in food products and supplements to provide protection against infectious diseases including respiratory infections (Hao et al., 2011; Ozen et al., 2015). *Lactobacilli*, *Enterococci*, and *Bifidobacteria* are families of Lactic Acid Bacteria (LAB) and they constitute the most frequently used strains of probiotics (Fijan, 2014). The LAB constitute a diverse group of microorganisms that are naturally present in human diet and in both gastrointestinal and urogenital tract of animals (Ruiz Rodriguez et al., 2019) The main objective of the current study was to isolate and identify naturally occurring probiotic *Lactobacilli* in buffalo milk as well as camel milk and urine to investigate their susceptibility to antibiotics as well as their antibacterial activity against representative pathogenic bacterial strains of both Gram-positive and Gram-negative bacteria to assess their potential use as natural alternatives to synthetic antibiotics.

MATERIALS AND METHODS

Ethical approval

Institutional Animal Ethics Committee, local laws and regulations were considered in performing our experiment. All procedures involving the use of the animals were approved by the ethics committee of National Research Centre, Egypt.

Sample collection

A total number of seven samples including three milk samples from three different buffalos, three milk samples from three different camels and one urine sample from a separate camel were collected during the summer of 2016 from

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private, individually owned healthy animals in Giza governorate, Egypt. The samples were collected under aseptic conditions in sterile containers and stored on ice. *Lactobacillus* spp. was isolated from the collected samples by using MRS medium as a selective medium. An amount of 1 ml of each of the milk samples as well as 1 ml of the urine sample was dissolved in 100 ml of MRS broth (pH 6.5) and incubated at 37 °C for 24 h in aerobic condition. The initial cultures were subcultured for seven times at 37 °C under low pH (pH 4.5) and anaerobic condition in the presence of 10% CO_2 to eliminate unwanted bacteria. Single colonies were selected and streaked onto MRS agar media at pH 4.8 to obtain pure colonies. Finally, single pure colonies of *Lactobacillus* were selected for further characterization and identification (Shokryazdan et al., 2014).

Characterization of isolated bacteria

The isolated bacteria were evaluated by different biochemical and molecular tests including Gram stain and Catalase test as well as bacterial morphology. The isolate bacteria were identified as *Lactobacilli* based on being Gram-positive, Catalase-negative and having rod-shape under light microscope. The *Lactobacilli* identification of isolated bacteria were further confirmed by using 16S rRNA multiplex polymerase chain reaction (PCR) analysis.

Gram staining

A prepared smear of 24 h cultured bacteria was heat fixed on a slide. Gram staining based on standard technique was then performed and then slides were observed under light microscope (Bergey et al., 1994).

Catalase test

Fresh liquid cultures which contained overnight grown cultures from selected single colonies were used for Catalase test. An amount of 3% hydrogen peroxide solution was dropped onto 1 ml of the culture. The formation of gas bubbles was considered as positive Catalase test and these samples were neglected while the other samples with negative Catalase test were selected since *Lactobacilli* are known to be Catalase-negative.

Molecular identification of probiotic strains

The DNA was extracted from the isolated bacteria and *Lactobacillus* strains were confirmed by using 16S rRNA multiplex PCR analysis (Kwon et al., 2004). The reaction mixture (25 µl) contained 12.5 µl of PCR Master Mix, 5 µl primer mixture comprising 50 pmol of each primer, 4.5 µl of water, and 3 µl of DNA template. PCR amplification was performed in Applied Biosystem 2720 thermal cycler, and DNA fragments were amplified as follows. Initial heating at 94 °C for 2 min, 35 cycles consisting of denaturation at 94 °C for 20 sec, annealing at 51 °C for 40 sec, extension at 68 °C for 30 sec, and final extension step in 7 min at 68 °C. The PCR products were separated on 1.5% agarose gel by electrophoresis and analyzed by RedSafe Nucleic Acid Staining Solution (Intron Biotechnology, Korea).

Antibiotic susceptibility of *Lactobacilli*

A wide panel of 14 antibiotic disks was tested against 7 mixed cultures of probiotic *Lactobacilli* isolated from both buffalo and camel samples (Figures 2-8). Antibiotic susceptibility test was performed by using the disk-diffusion method with some modifications (ISO, 2010). *Lactobacilli* activated cultures were swabbed on MRS agar plates instead of Muller Hinton Agar plates. Fourteen different antibiotic disks were used for the susceptibility test including Trimethoprim/sulfamethoxazole (SXT-25), Ofloxacin (OFX5), Cefuroxime (CXM-30), Amoxicillin with clavulanic acid (AmC-30), Cefotaxime (CTX-30), Cefaclor (CEC-30), Rifampicin (RD-5), Erythromycin (E-15), Vancomycin (Va-30), Amikacin (AK-30), Ampicillin with sulbactam (SAM-20), Cefadroxil (CFR-30), Azithromycin (AZM-15), and Doxycycline (DO-30). All plates were incubated for 24 h at 37^{o}C and inhibition zones were measured.

Antibacterial activity of *Lactobacilli*

The ability of the seven mixed cultured of isolated probiotic *Lactobacilli* to inhibit the growth of pathogenic bacteria was investigated against nine pathogenic standard strains of both Gram-positive and Gram-negative bacteria (Figures 9-15). Gram-positive strains were represented by *Staphylococcus aureus* (ATCC 26923), *Staphylococcus aureus* (ATCC 29213), *Staphylococcus epidermidis* (ATCC 12228), *Streptococcus pneumoniae* (ATCC 29619), and *Enterococcus faecalis* ATCC (29212). Gram- negative strains were represented by *Pseudomonas aeruginosa* ATCC (27853), *Escherichia coli* ATCC (25922), *Escherichia coli* ATCC (10536), and *Klebsiella pneumoniae* ATCC (700603). Antibacterial activity of probiotic *Lactobacilli* was tested by using Agar-well diffusion method with some modifications (Bauer et al., 1966; Sgouras et al., 2004). Wells of 7 mm diameter were made on Muller-Hinton agar plates. Each plate was swabbed with the respective test pathogen. From each probiotic *Lactobacillus* strain which previously incubated under anaerobic conditions for 24 h at 37^{o}C, 70 µl of MRS liquid culture were placed in the respective wells. After 24 h of incubation at 37 ^{o}C, the inhibition zones were measured and recorded in cm.

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Statistical analysis

The *in vitro* antibacterial activity was conducted in triplicate. All the data were then subjected to SPSS Version 21 (IBM, New York, US). Statistical analysis was performed using two-way ANOVA followed by Duncan's Multiple Range Test to determine significant difference. The given values represented mean ± Standard Deviation (SD). A probability value $P<0.05$ was taken as significant difference (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Lactobacilli isolated from buffalo milk, camel milk, and camel urine were subjected to characterization and identification by using different biochemical and molecular identification methods. A total number of seven samples were collected including three milk samples and one urine sample from camel and three milk samples from buffalo. *Lactobacilli* were isolated by growing the bacterial contents of the samples on MRS medium as selective medium. The bacterial colonies were initially identified as *Lactobacilli* based on being Gram-positive and Catalase-negative as well as being rod-shaped under the microscope. Mixed colonies of each sample in MRS broth medium were used to extract DNA for molecular identification using 16S rRNA multiplex PCR analysis. The mixed colonies of each sample were also used to test antibiotic susceptibility and antibacterial activity of the isolated strains.

Multiplex PCR analysis

The results from 16S rRNA multiplex PCR analysis have been demonstrated in figure 1. A total number of 18 isolated bacteria from buffalo milk, camel milk, and camel urine were identified as *Lactobacilli*. *Lactobacillus* species were identified based on the size of the PCR product (Kwon et al., 2004). The results indicated that buffalo and camel milk were different in their *Lactobacilli* content. There were also differences in *Lactobacilli* content of different milk samples collected from the same species. The results indicated the presence of *L. casei*, *L. acidophilus*, *L. rhamnosus*, *L. plantarum*, *L. gasseri* and *L. delbrueckii* in buffalo milk samples. Meanwhile, both camel milk and camel urine samples expressed the presence of *L. casei*, *L. acidophilus* and *L. plantarum*.

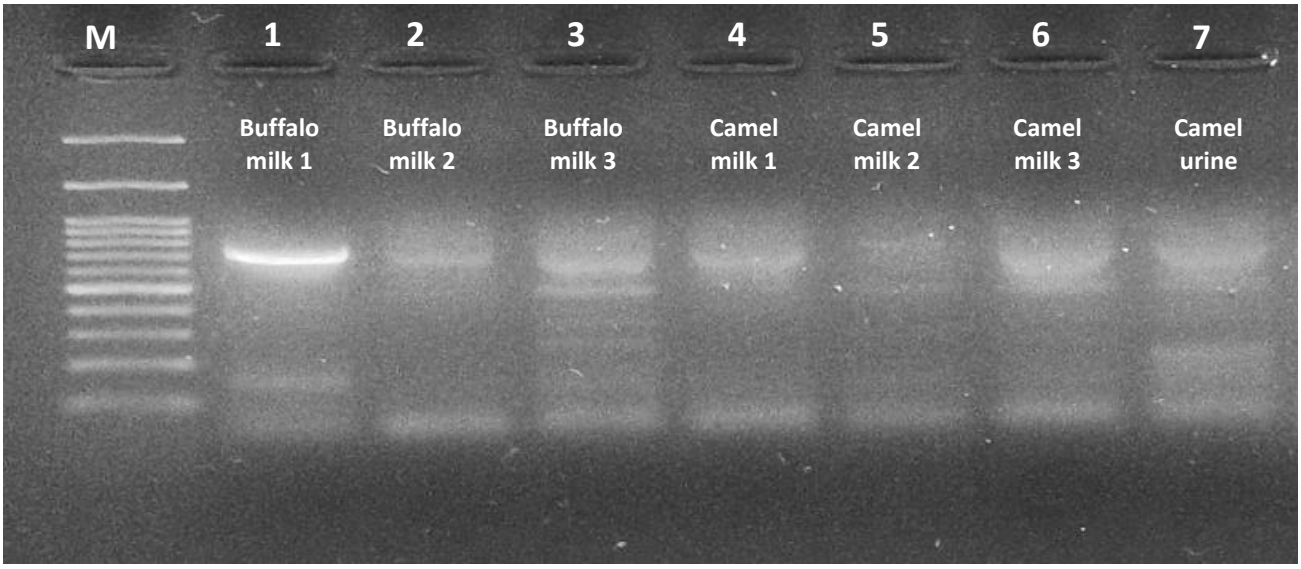

Figure 1. Agarose gel electrophoreses of PCR products from multiplex PCR assays. Multiplex PCR assays were performed with a mixture of seven species-specific or group-specific primers for L. acidophilus, *L. bulgaricus* (same as *L. delbrueckii* subsp. *bulgaricus*), *L. casei*-group *L. gasseri*, *L. plantarum*, *L. reuteri* and *L. rhamnosus* and two bacterial conserved primers. Lanes 1–7 designate the PCR product from each genomic DNA extracted from single or mixed cell suspension isolated from representative host used as PCR template. Lane 1: *L. casei*, *L. delbrueckii*; lane 2: *L. casei*; lane 3: *L. casei*, *L. acidophilus*, *L. rhamnosus*, *L. plantarum* and *L. gasseri*; lane 4: *L. casei*; lane 5: *L. plantarum*; Lane 6: *L. plantarum*; lane 7: *L. plantarum*; lane M: 100 bp-DNA ladder.

Antibiotic susceptibility of *Lactobacilli*

Antibiotic susceptibility of *Lactobacillus* strains was tested by using a panel of 14 antibiotics (Figures 2-8). It was clear that all samples had extremely significant resistant ($p < 0.0001$) to Cefadroxil (CFR-30) with inhibition zones of 0.0 cm. Cefaclor (CEC-30) exclusively did not present any inhibition to the growth of one of buffalo milk samples (buffalo milk 2) and all camel milk and urine samples. While Vancomycin (Va-30) did not cause any inhibition to only one of buffalo milk samples (buffalo milk 1). The rest of antibiotics exclusively presented no inhibition to camel samples which included Cefuroxime (CXM-30), Cefotaxime (CTX-30), Erythromycin (E-15), Ampicillin with sulbactam (SAM-20), and Azithromycin (AZM-15). Furthermore, camel urine sample was the only one to be totally resistant to (AmC-30)

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with 0.0 cm growth inhibition. On the other hand, all samples were sensitive to Trimethoprim/sulfamethoxazole (SXT-25), Ofloxacin (OFX5), Rifampicin (RD-5) and Doxycycline (DO-30) with varying degrees of inhibition.

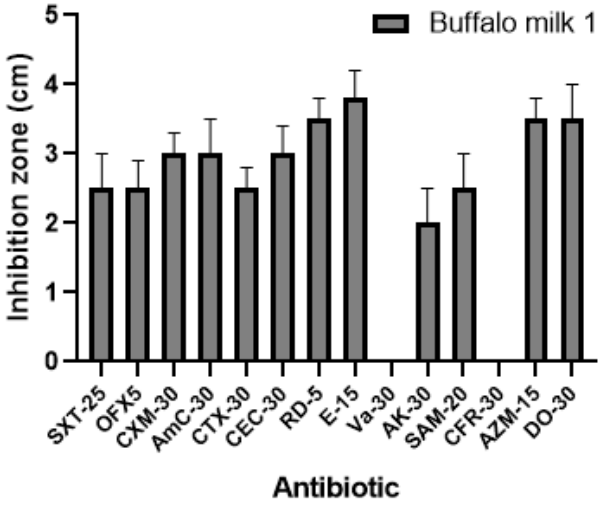

Figure 2

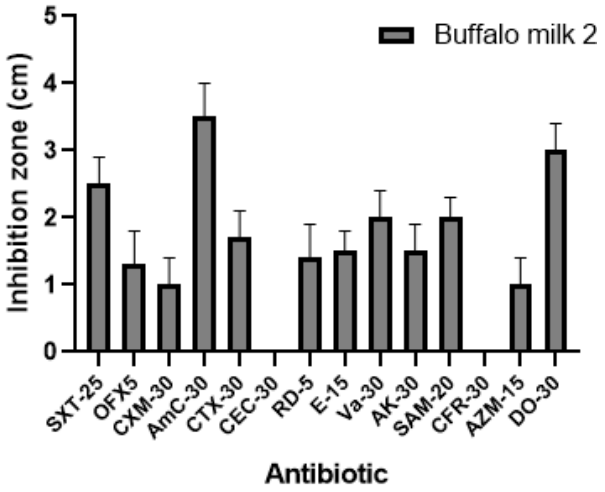

Figure 3

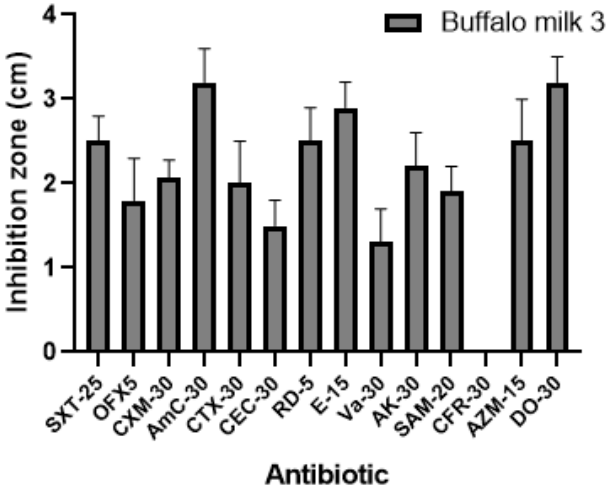

Figure 4

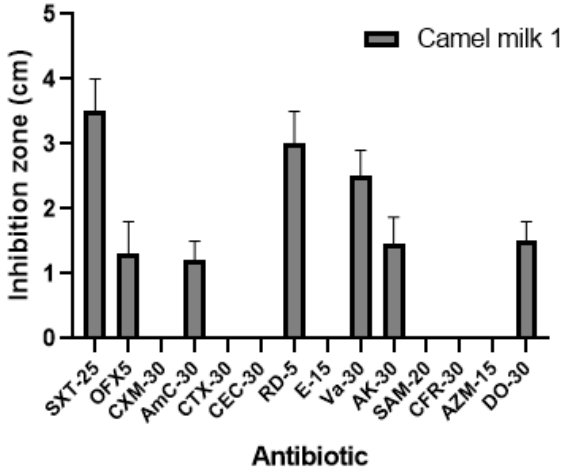

Figure 5

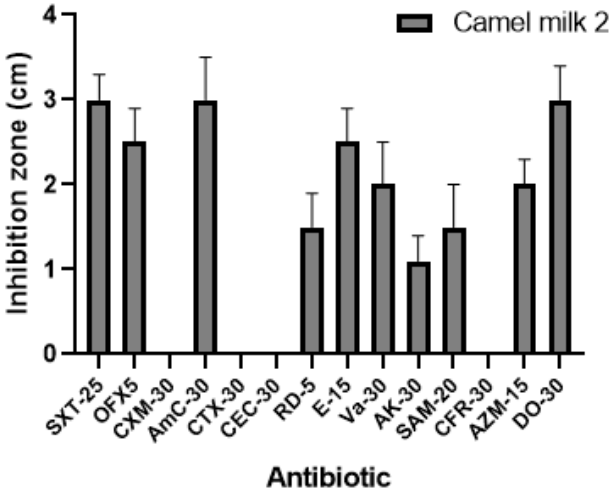

Figure 6

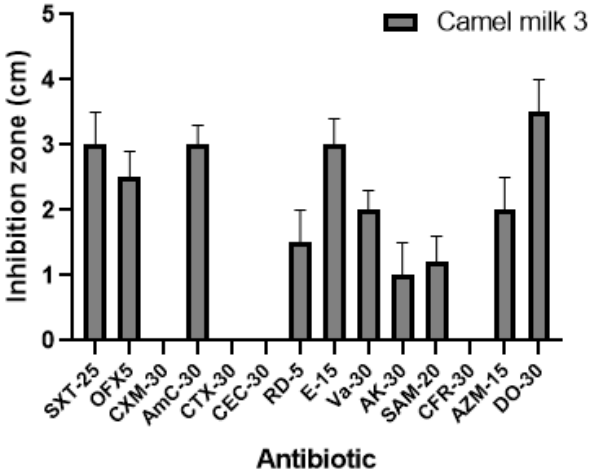

Figure 7

Camel urine

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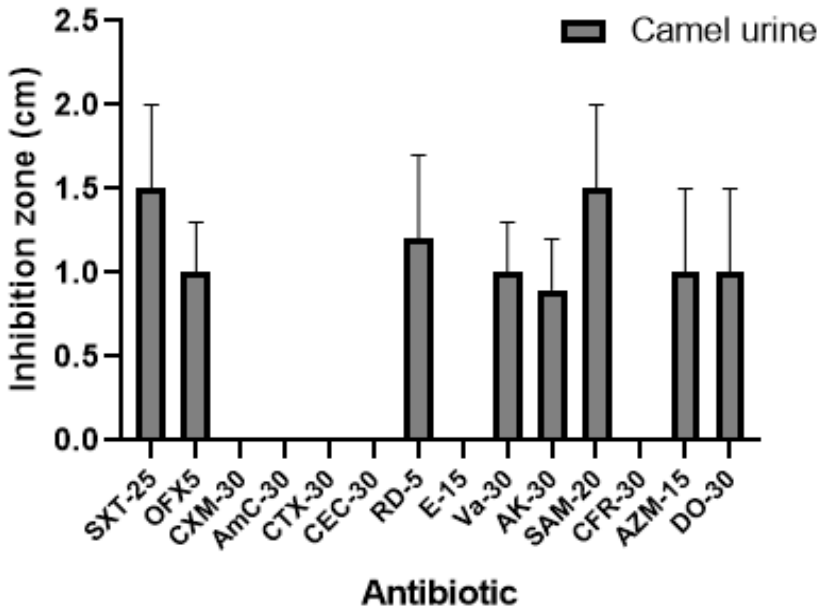

Figure 8

Figures 2-8. Antibiotic susceptibility of the probiotic *Lactobacilli* isolated from buffalo milk, camel milk or camel urine. Fourteen antibiotic disks were used for the susceptibility test including Trimethoprim/sulfamethoxazole (SXT-25), Ofloxacin (OFX5), Cefuroxime (CXM-30), Amoxicillin with clavulanic acid (AmC-30), Cefotaxime (CTX-30), Cefaclor (CEC-30), Rifampicin (RD-5), Erythromycin (E-15), Vancomycin (Va-30), Amikacin (AK-30), Ampicillin with ctam (SAM-20), Cefadroxil (CFR-30), Azithromycin (AZM-15), and Doxycycline (DO-30).

Antibacterial activity of *Lactobacilli*

The antibacterial activity of the isolated probiotic *Lactobacilli* was investigated against nine pathogenic standard strains of both Gram- positive and Gram- negative bacteria (Figures 9-15). The results indicated that the antibacterial activity of *Lactobacilli* which were isolated from camel urine was in general extremely significant lower ($p < 0.0001$) than the antibacterial activity of *Lactobacilli* from both buffalo milk and camel milk against all tested bacterial strains. The antibacterial effect of buffalo milk three was significantly higher ($P = 0.0045$) than all other samples against *E. coli* 25922 while the antibacterial activity of camel milk two was significantly higher against *S. aureus* 29213 ($P = 0.0014$), *S. pneumonia* 29619 ($P = 0.0014$) and *E. faecalis* 29212 ($P = 0.0014$) when compared to its effect against *E. coli* 10536.

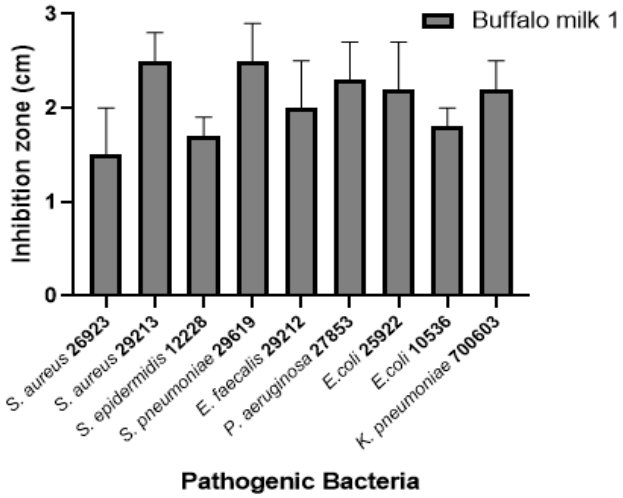

Figure 9

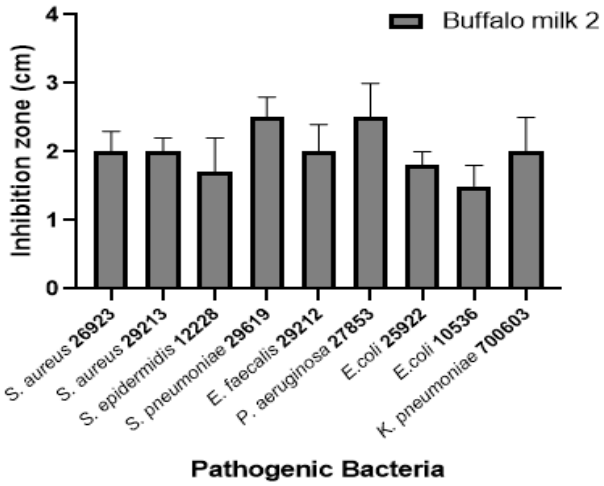

Figure 10

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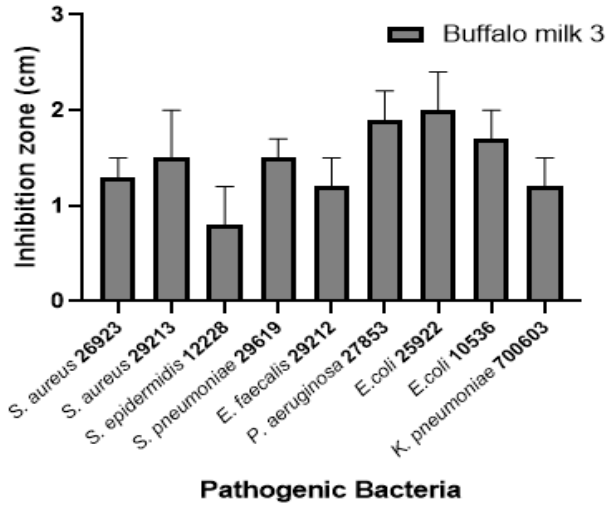

Figure 11

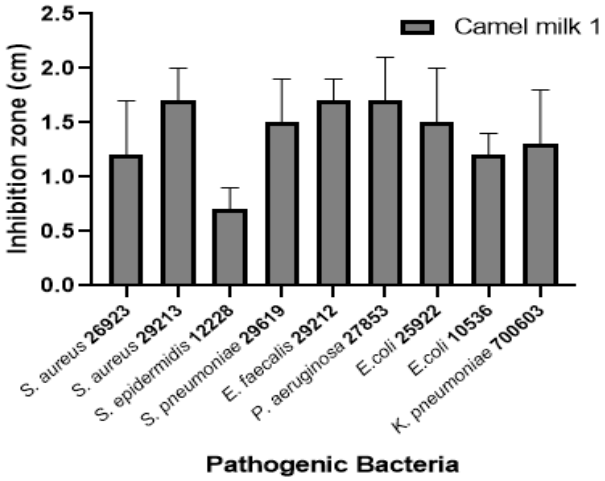

Figure 12

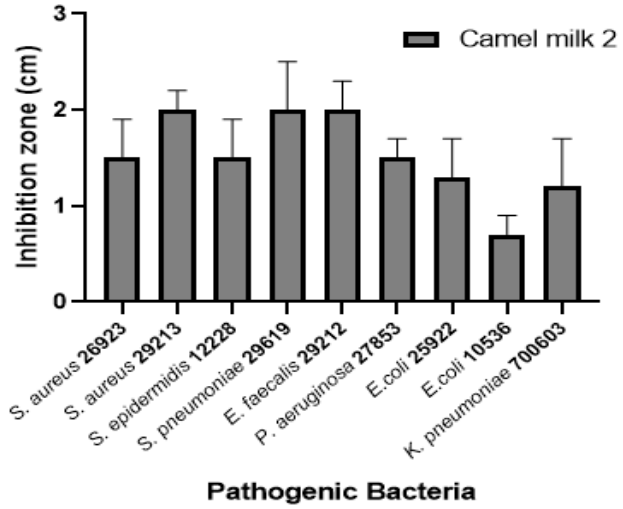

Figure 13

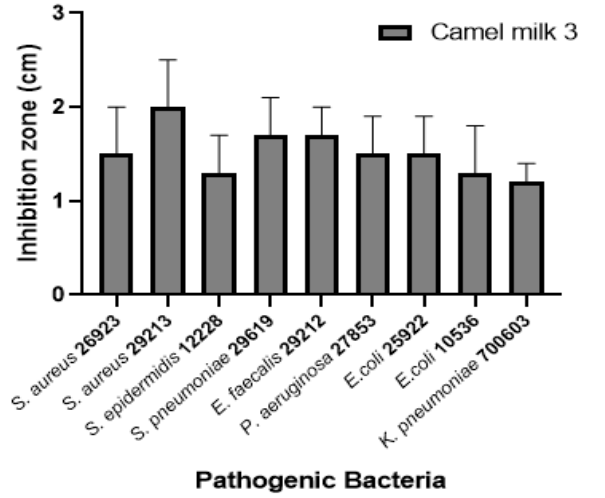

Figure 14

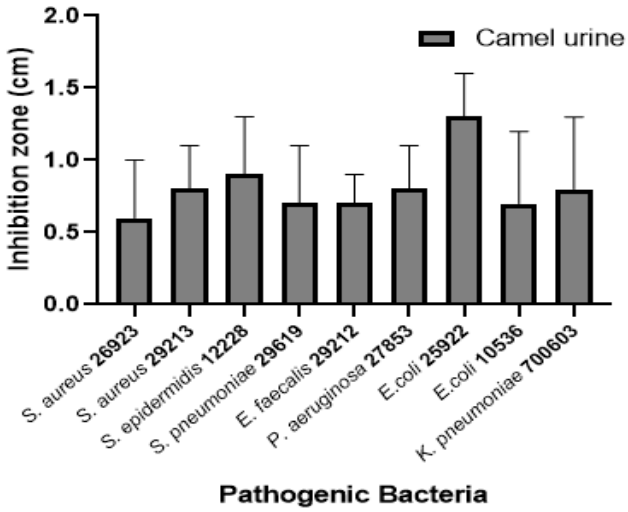

Figure 2

Figures 9-15: The antibacterial activity of the probiotic *Lactobacilli* isolated from buffalo milk, camel milk or camel urine. The antibacterial activity was investigated against both Gram-positive and Gram- negative bacteria using Agar-well diffusion method. Gram-positive strains were represented by *S. aureus* (ATCC 26923), *S. aureus* (ATCC 29213), *S. epidermidis* (ATCC 12228), *S. pneumoniae* (ATCC 29619), and *E. faecalis* ATCC (29212). Gram- negative strains were represented by *P. aeruginosa* ATCC (27853), *E. coli* ATCC (25922), *E. coli* ATCC (10536), and *K. pneumoniae* ATCC (700603).

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DISCUSSION

Probiotic bacteria have been recognized for their beneficial health effects in humans and animals. Their consumption in traditional food was associated with an extended life span and protection against diseases (Kechagia et al., 2013). The mechanisms of their beneficial effects include the protection against infectious disease either by direct competition with pathogenic microorganisms or by the modulation of the immune system and improving the digestion and reduction of metabolic disorders (Azad et al., 2018; Ghosh et al., 2019; Yousefi et al., 2019).

The main source of probiotics is fermented food including fermented milk, cheese and other dairy products. Probiotics are also isolated from both human and animal gastrointestinal tract. Furthermore, probiotic strains have been isolated from non-dairy fermented substrates including meat and fruits. Surprisingly, probiotic strains are also present in both human and animal milk which are originally expected to be sterile (McGuire and McGuire, 2015). These findings are consistent with the findings that breast-fed infants are less affected by gastrointestinal infections and have fewer allergies than formula-fed infants (Fontana et al., 2013). The same is also true about urine which had been thought to be sterile but after the development of sequencing techniques it was found that urine is colonized by normal flora including *Lactobacillus* and *Streptococcus* (Akgul and Karakan, 2018).

The diversity of probiotic *Lactobacilli* which isolated from different animal species has been documented (Abdou et al., 2018;Abdou et al., 2019). This diversity is the result of several factors including nutrition, infections, antibiotics, stress and various disease conditions. The variety of probiotic strains causes different types of benefits for the host.

It was clear from present findings that *Lactobacilli* strains isolated from both camel milk and camel urine were more resistant to the effect of antibiotics than *Lactobacilli* isolated from buffalo milk. This could be useful for restoring the gut microbiota after antibiotic treatment (Gueimonde et al., 2013). Although all *Lactobacilli* strains found in both camel milk and camel urine were also found in buffalo milk, the first two presented more resistance in general to antibiotic. This could be due to the acquisition of plasmids from other bacteria (Gueimonde et al., 2013). Camel milk and urine have been used in traditional medicine for several years to treat many diseases (Hu et al., 2017). In spite of the popularity of buffalo and cow milk and their preference among general public, camel milk is a very important replacement in arid and semi-arid areas where buffalo and cow milk are lacking. The camel milk investigation for bacterial content found it to be rich in LAB (Bin Masalam et al., 2018). In current study buffalo and camel milk were different in their *Lactobacilli* content. This difference might be due to the difference in milk composition (Yoganandi et al., 2014), which may allow the growth of different *lactobacillus* strains. *Lactobacillus plantarum* was isolated from camel milk and it is one of the frequently isolated LAB from raw camel milk (Khedid et al., 2009; Edalati et al., 2019).

Probiotic *Lactobacilli* have the potential to be used as natural alternatives to currently used synthetic antibiotics due to their antagonistic activity against various pathogenic bacteria (Prabhurajeshwar and Chandrakanth, 2017). In current study, it has been indicated that *Lactobacilli* isolated from buffalo milk, camel milk as well as camel urine presented variable degrees of antibacterial activity against pathogenic bacteria. Although present data indicated that isolated *Lactobacilli* from camel urine had the least antibacterial activity when compared to both buffalo and camel milk, the antibacterial, antifungal and antiviral activity of both camel milk and urine were reported previously (Al-Bashan, 2011; Hu et al., 2017). One of the reasons for the least antibacterial activity of camel urine could be using only one sample of it. The antibacterial activities of camel milk and urine in general may be partly due to the presence of different probiotic *Lactobacilli* including *Lactobacillus plantarum* and *Lactobacillus casei* which had been found earlier to represent promising antimicrobial activity (Bin Masalam et al., 2018).

CONCLUSION

The present study indicated the variability in contents of *lactobacillus* strains which isolated from buffalo milk, camel milk, and camel urine. Although some strains were similar among these samples, they presented different susceptibility to antibiotics and had different antibacterial activity against pathogenic bacteria. Further studies should be conducted with more samples to gain more information in the field of antibacterial activity of probiotic *lactobacilli* and to understand the mechanisms of their activity. Hopefully, they will be used as natural alternatives instead of synthetic antibiotics.

DECLARATIONS

Authors' contributions

Amr M. Abdou participated in the molecular identification of probiotic strains, performed the statistical analysis and drafted the manuscript. Riham H. Hedia participated in characterization of isolated bacteria, molecular identification of probiotic strains and antibiotic susceptibility of *Lactobacilli*. Shimaa T. Omara participated in characterization of isolated bacteria, molecular identification of probiotic strains, and antibacterial activity of *Lactobacilli*. Mai M. Kandil

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participated in sample collection and participated in the molecular identification of probiotic strains. M. A. Bakry participated in sample collection and characterization of isolated bacteria. Mohammad M. Effat proposed the idea of current study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declared that they had no competing interests.

REFERENCE

Abdou AM, Fouad EA, Alam SS, and Hakim AS (2019). Isolation and Identification of Probiotic Lactobacilli from Non-ruminant Animals. International Journal of Veterinary Science, 8(4): 349-354. Available at: http://www.ijvets.com/.../349-354.pdf

Abdou AM, Hedia RH, Omara ST, Mahmoud MAE, Kandil MM, and Bakry MA (2018). Interspecies comparison of probiotics isolated from different animals. Veterinary World, 11: 227-230. DOI: https://doi.org/10.14202/vetworld.2018.227-230

Akgul T, and Karakan T (2018). The role of probiotics in women with recurrent urinary tract infections. Turkish Journal of Urology, 44: 377-383. DOI: https://doi.org/10.5152/tud.2018.48742

Al-Bashan MM (2011). In vitro Assessment of the Antimicrobial Activity and Biochemical Properties of Camel's Urine Against Some Human Pathogenic Microbes. Middle-East Journal of Scientific Research, **7**: 947-958. Available at: https://bengreenfieldfitness.com/wp-content/uploads/2017/02/In-vitro-Assessment-of-the-Antimicrobial-Activity-and-Biochemical-Properties-of-camels-urine.pdf

Azad MAK, Sarker M, and Wan D (2018). Immunomodulatory Effects of Probiotics on Cytokine Profiles. BioMed Research International, 2018: 8063647. DOI: https://doi.org/10.1155/2018/8063647

Bauer AW, Kirby WM, Sherris JC, and Turck M (1966). Antibiotic susceptibility testing by a standardized single disk method. American Journal of Clinical Pathology, 45(4): 493-496. DOI: https://doi.org/10.1093/ajcp/45.4_ts.493

Bergey DHJ, John GH, Noel RK, and Peter HAS (1994). Bergey's Manual of Determinative Bacteriology, 9 th ed. Avaliable at:

https://books.google.com.eg/books?id=jtMLzaa5ONcC&printsec=frontcover&hl=ar&source=gbs_ge_summary_r&cad=0#v=onepage&q&f=false

Bin Masalam MS, Bahieldin A, Alharbi MG, Al-Masaudi S, Al-Jaouni SK, Harakeh SM, and Al-Hindi RR (2018). Isolation, Molecular Characterization and Probiotic Potential of Lactic Acid Bacteria in Saudi Raw and Fermented Milk. Evidence-Based Complementary and Alternative Medicine, 2018, 7970463. DOI: https://doi.org/10.1155/2018/7970463

Brunel AS, and Guery B (2017). Multidrug resistant (or antimicrobial-resistant) pathogens - alternatives to new antibiotics? Swiss Medical Weekly, 147: w14553. DOI: https://doi.org/10.4414/smw.2017.14553

Davies J, and Davies D (2010). Origins and evolution of antibiotic resistance. Microbiology and Molecular Biology Reviews, 74: 417-433. DOI: https://doi.org/10.1128/MMBR.00016-10

Edalati E, Saneei B, Alizadeh M, Hosseini SS, Zahedi Bialvaei A, and Taheri K (2019). Isolation of probiotic bacteria from raw camel's milk and their antagonistic effects on two bacteria causing food poisoning. New Microbes and New Infections, 27: 64-68. DOI: https://doi.org/10.1016/j.nmni.2018.11.008

FAO/WHO (2011). ICMR-DBT guidelines for evaluation of probiotics in food. Indian Journal of Medical Research, 134(1): 22-25. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3171912/

Fijan S (2014). Microorganisms with claimed probiotic properties: an overview of recent literature. Int International Journal of Environmental Research and Public Health, 11(5): 4745-4767. https://doi.org/10.3390/ijerph110504745

Fontana L, Bermudez-Brito M, Plaza-Diaz J, Munoz-Quezada S, and Gil A (2013). Sources, isolation, characterisation and evaluation of probiotics. British Journal of Nutrition, 109 Suppl 2: S35-50. DOI: https://doi.org/10.1017/S0007114512004011

Ghosh T, Beniwal A, Semwal A, and Navani NK (2019). Mechanistic Insights Into Probiotic Properties of Lactic Acid Bacteria Associated With Ethnic Fermented Dairy Products. Frontiers in Microbiology, 10: 502. DOI: https://doi.org/10.3389/fmicb.2019.00502

Gueimonde M, Sánchez B, G de Los Reyes-Gavilán C, and Margolles A (2013). Antibiotic resistance in probiotic bacteria. Frontiers in microbiology, *4*, 202. https://doi.org/10.3389/fmicb.2013.00202

Hao Q, Lu Z, Dong BR, Huang CQ, and Wu T (2011). Probiotics for preventing acute upper respiratory tract infections. Cochrane Database of Systematic Reviews, CD006895. DOI: https://doi.org/10.1002/14651858.CD006895.pub2

Hu Z, Chang X, Pan Q, Gu K, and Okechukwu PN (2017). Gastroprotective and Ulcer Healing Effects of Camel Milk and Urine in HCl/EtOH, Non-steroidal Anti-inflammatory Drugs (Indomethacin), and Water-Restraint Stress-induced Ulcer in Rats. Pharmacognosy Magazine, 13: 559-565. DOI: https://doi.org/10.4103/pm.pm_135_17

International Organization for Standardization (ISO) (2010). Milk and milk products—determination of the minimal inhibitory concentration (MIC) of antibiotics applicable to bifidobacteria and non-enterococcal lactic acid bacteria (LAB). ISO 10932/IDF 233 standard. Available at: https://www.iso.org/standard/46434.html

Kechagia M, Basoulis D, Konstantopoulou S, Dimitriadi D, Gyftopoulou K, Skarmoutsou N, and Fakiri EM (2013). Health benefits of probiotics: a review. ISRN Nutrition, Article ID: 481651. DOI: https://doi.org/10.5402/2013/481651

Khedid K, Faid M, Mokhtari A, Soulaymani A, and Zinedine A (2009). Characterization of lactic acid bacteria isolated from the one humped camel milk produced in Morocco. Microbiological Research, 164: 81-91. DOI: https://doi.org/10.1016/j.micres.2006.10.008

Kwon HS, Yang EH, Yeon SW, Kang BH, and Kim TY (2004). Rapid identification of probiotic Lactobacillus species by multiplex PCR using species-specific primers based on the region extending from 16S rRNA through 23S rRNA. FEMS Microbiology Letters, 239: 267-275. DOI: https://doi.org/10.1016/j.femsle.2004.08.049

McGuire MK, and McGuire MA (2015). Human milk: mother nature's prototypical probiotic food? Adv Nutr. Jan 15; 6(1): 112-123. DOI: https://doi.org/10.3945/an.114.007435.

Ozen M, Kocabas Sandal G, and Dinleyici EC (2015). Probiotics for the prevention of pediatric upper respiratory tract infections: a systematic review. Expert Opinion on Biological Therapy, 15: 9-20. DOI: https://doi.org/10.1517/14712598.2015.980233

Prabhurajeshwar C, and Chandrakanth RK (2017). Probiotic potential of Lactobacilli with antagonistic activity against pathogenic strains: An in vitro validation for the production of inhibitory substances. Biomedical journal, 40(5): 270–283. DOI: https://doi.org/10.1016/j.bj.2017.06.008

Ruiz Rodriguez LG, Mohamed F, Bleckwedel J, Medina R, De Vuyst L, Hebert EM, and Mozzi F (2019). Diversity and Functional Properties of Lactic Acid Bacteria Isolated From Wild Fruits and Flowers Present in Northern Argentina. Frontiers in Microbiology, 10: 1091. DOI: https://doi.org/10.3389/fmicb.2019.01091

Sgouras D, Maragkoudakis P, Petraki K, Martinez-Gonzalez B, Eriotou E, Michopoulos S, Kalantzopoulos G, Tsakalidou E, and Mentis A (2004). In vitro and in vivo inhibition of Helicobacter pylori by Lactobacillus casei strain Shirota. Applied and Environmental Microbiology, 70(1): 518-526. DOI: https://doi.org/10.1128/aem.70.1.518-526.2004

Shokryazdan P, Sieo CC, Kalavathy R, Liang JB, Alitheen NB, Faseleh Jahromi M, and Ho YW (2014). Probiotic potential of Lactobacillus strains with antimicrobial activity against some human pathogenic strains. BioMed Research International, 927268. DOI: https://doi.org/10.1155/2014/927268

Steel RGD, and Torrie JH (1980). Principles and Procedures of Statistics. 2nd ed. McGraw Hill Book Company, New York.

Williams MD, Ha CY, and Ciorba MA (2010). Probiotics as therapy in gastroenterology: a study of physician opinions and recommendations. Journal of Clinical Gastroenterology, 44: 631-636. DOI: https://doi.org/10.1097/MCG.0b013e3181d47f5b

Yoganandi J, Mehta BM, Wadhwani KN, Darji VB, and Aparnathi KD (2014). Evaluation and Comparison of Camel Milk with Cow Milk and Buffalo Milk for Gross Composition. Journal of Camel Practice and Research, 21(2): 259-265. DOI: https://doi.org/10.5958/2277-8934.2014.00046.0

Yousefi B, Eslami M, Ghasemian A, Kokhaei P, Salek Farrokhi A, and Darabi N (2019). Probiotics importance and their immunomodulatory properties. Journal of Cellular Physiology, 234 (6): 8008-8018. DOI: https://doi.org/10.1002/jcp.27559

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Cross Protectivity of Yolk Immunoglobulin Anti-Hemagglutinin Protein of High Pathogenic Avian Influenza A subtypes H5N1 Administered on Chicken Infected by High Pathogenic Avian Influenza A subtypes H5N1

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ABSTRACT

Yolk Immunoglobulin (IgY) against Avian Influenza (AI) is commonly used as immunotherapy and immunodiagnostic techniques. Application of IgY mixed in drinking water is known effective to inhibit AI replication. The effectivity of IgY anti-Hemagglutinin Protein (anti-HA) of High Pathogenic Avian Influenza (HPAI) clade 2.1 (A/Chicken/Blitar/2003) was tested against infection of High Pathogenic Avian Influenza clade 2.3.2 (A/Duck/Sidoarjo/2012). The inhibiting activity was observed through Immunohistochemistry. Sixty chickens were infected with 10^5 EID_{50}/ml of HPAI clade 2.3.2 (A/Duck/Sidoarjo/2012). Yolk Immunoglobulin with different amounts (0 µg, 100 µg, 200 µg and 400 µg) were administered at three different times which were 24 hours before infection, at the time of infection, and 24 hours after infection. The observation was conducted for 7 days. During post infection observation, death chickens were managed for immunohistochemistry assay to observe the present of virion and IgY sialic acid 2,3-alfa galactosa (SA α 2,3 gal) blocking activity in septa alveoli. By the end of observation all chickens were euthanized for immunohistochemistry assay. The result showed that anti-HA IgY obtained from HPAI clade 2.1 could protecting infection of HPAI clade 2.3.2. According to immunohistochemistry assay, the administration of IgY can neutralize the infecting virus marked by the number of virions observed in septa alveoli of the lungs. Regarding the assay, the dose of 200 µg and 400 µg of IgY applied 24 hours before the infection, can reduce clinical signs and mortality of infected chicken (80-100%). The best dose of the IgY to protect them from infection of clade 2.3.2 (A/Duck/Sidoarjo/2012) was 400 µg administered 24 hours before infection. It could be concluded that administration of IgY anti-Haemaglutinin Protein (anti-HA) of High Pathogenic Avian Influenza (HPAI) clade 2.1 (A/Chicken/Blitar/2003) could protect chickens against the infection of HPAI clade 2.3.2 (A/Duck/Sidoarjo/2012), even though they belong different clades. The protection rate was 80-100%. Further research should be done to discover the cross-protectivity of IgY as preventive method against HPAI outbreak.

Key words: Avian influenza virus, IgY anti-HA, Immunotherapy, Productivity.

INTRODUCTION

Avian Influenza (AI) is commonly known as fowl plaque which is a disease caused by infection of Influenza A virus which belongs to the family Orthomyxoviridae. This disease is susceptible for many species of birds (Bouma et al., 2009; Webby and Webster, 2003; De Jong et al., 1997). According to the genotype, It is classified into 16 Haemagglutinin and 9 Neuraminidase subtypes (Bergervoet et al., 2019). According to virulence, It is classified into two groups which are Low Pathogenic Avian Influenza (LPAI) and High Pathogenic Avian Influenza (HPAI) (OIE, 2016). Both LPAI and HPAI are originated from H5 and H7 subtypes (Bouma et al., 2009; Webby and Webster, 2003; De Jong et al., 1997), and it has become attention-getting to international trade community since HPAI causing a great loss by the outbreak, and the LPAI causing annual problem and has potency to mutate into HPAI (MacLachlan et al., 2016). It has become endemic in many countries such as Indonesia (Daniel et al., 2012).

Prevention has been already conducted such as routine vaccination and biosecurity management but annual outbreak remained ongoing. Poultry farm companies in Indonesia have conducting vaccination more than 400 million doses since 2004 (Bouma et al., 2009). AI is an enveloped segmented single-stranded negative sense RNA virus. Under electronic microscopes it is seen on pleomorphic, spherical, or velamentous forms. Its virion consists of 10-14.6 kb genome divided into eight segments arranged on helical-symmetrically order. It has seven structural proteins such as Haemagglutinin protein (HA), Neuraminidase protein (N), two Matrix proteins (M1 and M2), and three Polymerase proteins (PB1, PB2, and PA). HA and N are enveloped protein lining on the membrane form spikes that has important roles on pathogenicity, classification and neutralization of the virus (MacLachlan et al., 2016; Knipe and Howley, 2013). Specific antibody is usually used as a diagnostic rule or as a prevention for specific diseases. The antibody obtains from animals needs a good production procedure regarding to animal welfare instructions (Hau and Hendriksen, 2005). Antibody obtained from Yolk Immunoglobulin (IgY) is homolog to Immunoglobulin G (IgG) obtained from mammals.

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Recently the application of IgY obtained from eggs as immunotherapy is rising because the concentration of immunoglobulin is higher compared to IgG obtained from mammals. One of the privileges of obtaining immunoglobulin from chickens is that chickens have high sensitivity of antigen exposure, thus immune response and IgY production are persistent (Hau and Hendriksen, 2005).

AI virus transfers through the airway or orally, then Haemagglutinin protein of the virus bounds to the receptors of sialic acid alfa 2 and 3- galactosa (SA α 2,3 gal) proteins. This binding triggers the fusion of the virus into cells (Knipe and Howley, 2013). This binding could be failed if specific antibody against HA protein block the process. HA antibody obtained from Yolk Immunoglobulin might have the potency to block this process to prevent AI infection in chickens. This research was conducted to know the effectivity of anti-HA from HPAI clade 2.1 (A/Chicken/Blitar/2003) against infection of HPAI clade 2.3.2 (A/Duck/Sidoarjo/2012).

MATERIALS AND METHODS

Sixty chickens (21 days-old) were used in this experiment. They were divided into three groups randomly. Each group consisted of four subgroups of treatments which were consisted of five chickens respectively. IgY was obtained by infecting HA protein of HPAI clade 2.1 (A/Chicken/Blitar/2003) into Specific Pathogen Free (SPF) layer chicken. IgY was extracted from the eggs, and stored at -20°C (Narat, 2003). Yolk Immunoglobulin was given to each major group at three different times. IgY was administered 24 hours before infection, 24 hours after infection, and at the time of infection on Group I, II, and III respectively. The amounts of IgY given on each sub groups were 0 µg, 100 µg, 200 µg, and 400 µg respectively. Each of dose was diluted on distilled water till the total volume was one ml. The research was conducted at Biosecurity Level-2 (BSL-2) of Faculty of Veterinary Medicine, Airlangga University Indonesia. The temperature was set at 16 ^{o}C.

The chickens were infected with 10^5 EID_{50}/ml dose of the antigen (A/Duck/Sidoarjo/2012). The observation has been started at the time of infection continuing for 7 days. During this period, all death chickens were recorded, then prepared for immunohistochemistry assay for determining the degree of cell destruction and sialic acid 2,3-alfa galactosa (SA α 2,3 gal) blocking activity from anti-HA on cell tropism according to the antigen (virion detection) and antibody detection (anti-anti HA). By the end of observation, the rest of living chickens were euthanized through cervical dislocation method. Samples for immunohistochemistry were collected from the lung. The lung was dipped in 10% formalin buffer, then processed to make slides (Damayanti et al., 2004). Before the immunohistochemistry procedures applied to the slides, they were prepared for deparaffinization to wear the wax off. After cleaning up the slide, 250 µl of primer antibody (anti H5N1 and anti-anti HA) that had been diluted (1:1600) was added to the slides, then it was incubated for 60 minutes. Then activity of peroxidase was blocked by adding three drops of hydrogen peroxidase (H_2O_2), then it was incubated for 20 minutes. The slides were then rinsed using PBS for three times. Moreover, anti-rabbit conjugate labelled with Biotin-Streptavidin was added followed by DAB substrate. After that the slides were rinsed, they were dipped on Haematoxilyn for two minutes. They were transferred into Scott solution, then incubated for 2 minutes. They were rinsed, and then covered by cover glass. Positive result marked by the present of brown color on the slide (Damayanti et al., 2004). Obtained data was analyzed using ANOVA (Analysis of Variance) on Statistical Programs for Social Scientific (SPSS) program. The possible results were analyzed according to the Least Significance Different (LSD) analysis (Kusriningrum, 2012).

Ethical approval

The arrangement of this research had been approved by the ethics commission of experimental animals of Faculty of Veterinary Medicine Airlangga University, Indonesia.

RESULTS AND DISCUSSION

Observation has been conducted for seven days after the infection. It revealed that each treatment showed different effects according to mortality rate; the presence of virion captured on septa alveoli, and the presence of IgY in septa alveoli of chickens. In group I, chickens which were not treated by anti-HA were death on day 2 until day 3 after infection of (A/Duck/Sidoarjo/2012) (Diagram 1). In contrary, chickens treated with anti-HA (with amounts of 100µg, 200µg, and 400µg) showed healthy condition, and no clinical signs were present. Even though clinical signs were absence, one of the chickens administered with 100µg of antibody died on the second day after the infection while others remained intact until the end of the observation period (Diagram 1). It could be concluded that administration of anti-HA 24 hours before the infection could give 80-100% of protectivity (Table 1).

Group II which were treated with anti-HA at the same time of infection showed different results. Administration of anti-HA has protected the chickens from mortality only on day 1 after being infected. On the second day of infection, the

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mortality of chickens could be seen on each subgroup; primely on the subgroup not treated by anti-HA. The subgroup which were not treated with anti-AH (0 µg) has started the mortality prior to the subgroup treated with 100 µg of anti-HA. Mortality still could be seen even on the subgroup treated by 400 µg anti-HA (Diagram 2). According to the protection rate, administration of anti-HA at the same time of infection could gave 40-80% of protection (Table 1).

While administration of anti-HA on Group III has completely protected the chickens from mortality only on day 1 after the infection, Mortality occurred on all subgroups even on the one administrated with 400 µg of anti-HA. The mortality rate was significantly around 60% of the group population (Diagram 3). This rate is the largest among other treated groups. By the end of the observation, only subgroups of chickens treated with 200 µg and 400 µg of anti-HA have survived, while all chickens in other subgroups were death. It could be concluded that anti-HA given 24 hours after being infected gave a protection of 40% (Table 1). Regarding the dose of anti-HA, administrations of 200 µg and 400 µg of anti-HA are more protective than 100 µg of anti-HA. They could give protection around 40-100%. Administration of both doses 24 hours before the infection indicated a protection lasting longer than other times of administration (Diagrams 1-3). All data were collected, then processed into ANOVA analysis. According to the ANOVA analyses, the results showed a significant difference ($p<0.05$) (Table 2). Thus, it was processed into LSD analysis. The results of analyses revealed that the administration of anti-HA is influenced by the time of administration and the doses. Administration of anti-HA 24 hours before infection could give aa appropriate protection more and last longer than the other administration times. This discovery was supported by the result of immune-histochemistry (IHC) assay. According to IHC results, there was an absence of AI virus in septa intra-alveola from chickens treated with 400µg anti-HA on Group I. It was marked by the absence of dark-brown colour like formation observed on IHC slides (Figure 1). In contrast, the presences of AI virus observed on the chickens treated with anti-HA with 200µg and 100µg of anti-HA on Group I. The presence of AI virus also has been observed on the chickens which were not treated with anti-HA antibody (Figure 1). Administration of anti-HA in Group II and Group III seemed that they could not neutralize the virus as good as Group I, thus the number of virions have increased on both groups (Figure 1). The presents of virion inside the septa-alveoli of lungs could disturb respiration of infected chickens (OIE, 2016). Regarding the effective dose of protection, 400 µg of anti-HA gave best protection among others. In poultry, AI virus enters the host body through respiratory system and orally, then attaches to receptor sialic acid alfa 2,3- galactosa (SA α 2,3 gal) protein which laid on mucosal epithelium located on both respiratory and gastrointestinal tracts (Costahurtado et al., 2014; Webby and Webster, 2003). The transmission commonly occurs through contaminated water source, ingestion of contaminated feed and nasal discharge (Achenbach and Bowen, 2011).

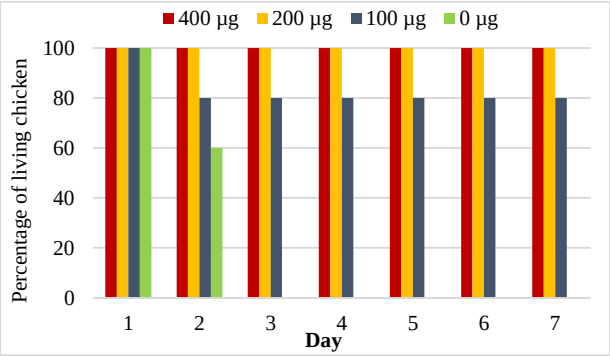

Diagram 1. Percentage of living chicken after administration of anti-HA 24 hours before infection

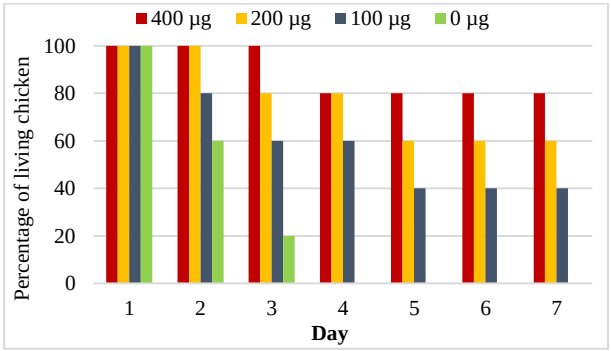

Diagram 2. Percentage of living chicken after administration of anti-HA at infection time

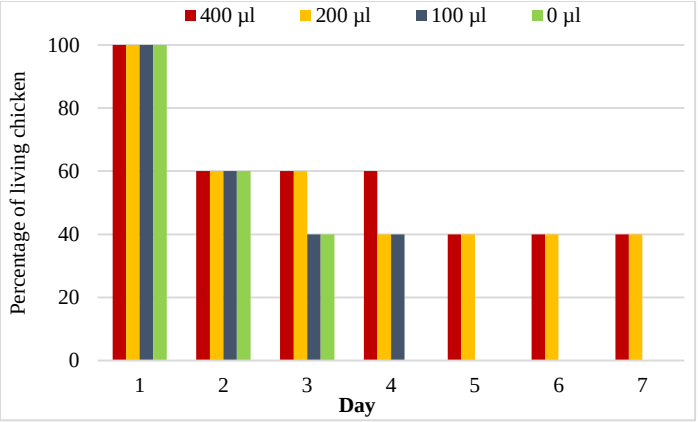

Diagram 3. Percentage of living chicken after administration of anti-HA 24 hours after infection

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Table 1. Protectivity rate of anti-HA antibody obtained from egg yolk (IgY).

	Application of Antibody Anti-HA (IgY)		
Dose	**24 hours before infection (%)**	**0 hours before infection (%)**	**24 hours after infection (%)**
0 µg/head	0	0	0
100 µg/head	80	40	0
200 µg/head	100	60	40
400 µg/head	100	80	40

%: means protectivity rate

Table 2. The amount of IgY and the time administration influence the protectivity of chickens

Amount	**Time of apllicatipn of anti-HA**	**(Mean ± SD)**
400	D-1	$4,8^{a} \pm 0,83$
	D-0	$10^{b} \pm 1,41$
	D+1	$23,2^{c} \pm 1,09$
200	D-1	$12,8^{d} \pm 1,09$
	D-0	$18,8^{e} \pm 1,09$
	D+1	$35,2^{f} \pm 1,09$
100	D-1	$30,8^{g} \pm 1,09$
	D-0	$35,6^{f} \pm 0,89$
	D+1	$41,2^{h} \pm 1,09$
0	D-1	$47,6^{i} \pm 0,89$
	D-0	$47,2^{i} \pm 1,09$
	D+1	$47,6^{i} \pm 0,89$

Different superscript on the same column showing significant different ($p < 0.05$). D-1: 24 h before infection, D-0: at the time of infection, D+1: 24 hours after infection.

In this research anti-HA obtained from egg yolk called IgY was used which is equivalent to mammalian Immunoglobulin G (IgG), since it is distinguished as the ancestor. As the IgY is equivalent to mammalian IgG, it has similar functions as the main humoral immune-system to eradicate antigens (Agrawal et al., 2016). IgY is frequently used as substitution from mammalian antibody because the production process is more respecting animal welfare. Moreover, it is easier to be done and the amount of immunoglobulin obtained is larger among small-sized animals (Narat, 2003; Ko and Ahn, 2007; Wen et al., 2012; Agrawal et al., 2016). Its capability to binding and target specifity is higher than mammalian Immunoglobulin G (IgG) which makes it has potential as therapeutic therapy for respiratory infections (Abbas et.al., 2018). Application of IgY is noticed capable to prevent bacterial and viral infections (Narat, 2003; Ko and Ahn, 2007; Wen et al., 2012; Agrawal et al., 2016). It could be applied in human too which gives many advantages (Pereira et.al., 2019; Constantin et. al., 2020). According to the IHC of anti-HA detection, anti-HA administered orally could be seen on septa alveoli of lungs as brown color (Figure 2). Immunotherapy given orally is capable to block receptors on the epithelium of mucosa on digestion system. It is directly transported through all over the body absorbed by intestine to capillaries, then transported to portal vein in liver and then vena cava in heart (Rahimi et al., 2007). Administration of anti-HA 24 hours before being infected suggested that it could compete binding of the virus to receptor SA α 2,3 gal protein. Administration of anti-HA obtained from horses given to the mouse intraperitoneal before infection can give 100% protection (Lu et al., 2006).

This research used anti-AH obtained HPAI clade 2.1 (A/Chicken/Blitar/2003) against the infection of HPAI clade 2.3.2 (A/Duck/Sidoarjo/2012). Both viruses belong to different clades, different hosts and different time isolations. According to the results, even both viruses are different; the cross-protection was observed. This finding extents the fact that cross-reactivity among clades of H5 subtypes occurred (Dharmayanti et al., 2017; Ducatez et al., 2011). This evidence is not only occurred among H5 subtypes. Cross-reactivity also occurred among H7 subtypes to H3 and H4 subtypes. It is noticed that cross-reactivity between H7 and H3 is stronger than H7 and H4 (Guo et al., 2016). Cross-reactivity among subspecies in same family not only occurs in AI. It also occurs in Newcastle Disease (Aldous et al., 2016). The distinct point is that cross-reactivity in Newcastle Disease seems stronger than AI. Both viruses are single stranded negative sense RNA virus. RNA viruses are easy to mutate because their polymerase enzymes lack of proof-reading. Among them, the mutation rate of AI is higher because its genome arranged on some segments leading to antigenic shift and antigenic drift (MacLachlan et al., 2016).

This finding reveals the possibility of anti-HA hyper-immune serum application on AI prevention. Routine vaccination as one of the main prevention methods could be possibly optimized by application of anti-HA serum orally. Further researches need to be done since this research is conducted in controllable and variables environment.

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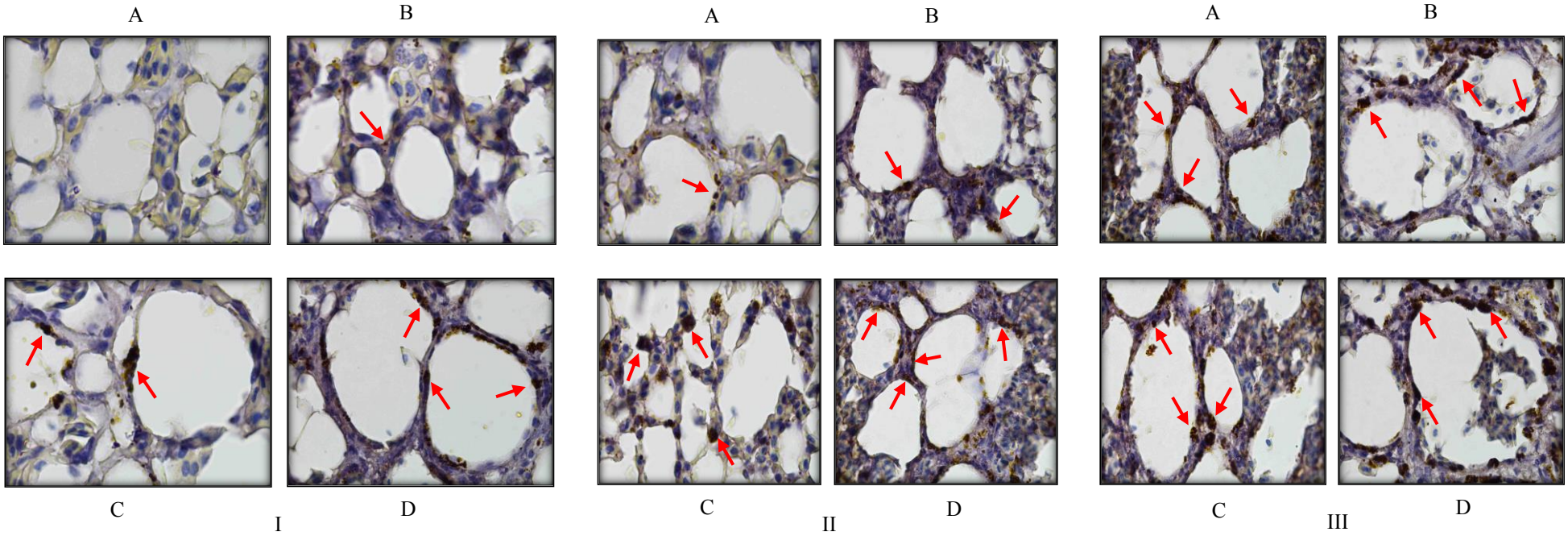

Figure 1. Immunohisochemistry of chicken lungs. Arrows indicate the presence of Avian Influenza Virus on septa alveoli. I; chicken administered with anti-HA IgY 24 hours before infection. II; chicken administered with anti-HA IgY 24 hours after infection. III; chicken administered with anti-HA IgY 24 at time of infection. A; Amount of administered IgY is 400 µg. B; Amount of administered IgY is 200 µg. C; Amount of administered IgY is 100 µg. D; Amount of administered IgY is 0µg.

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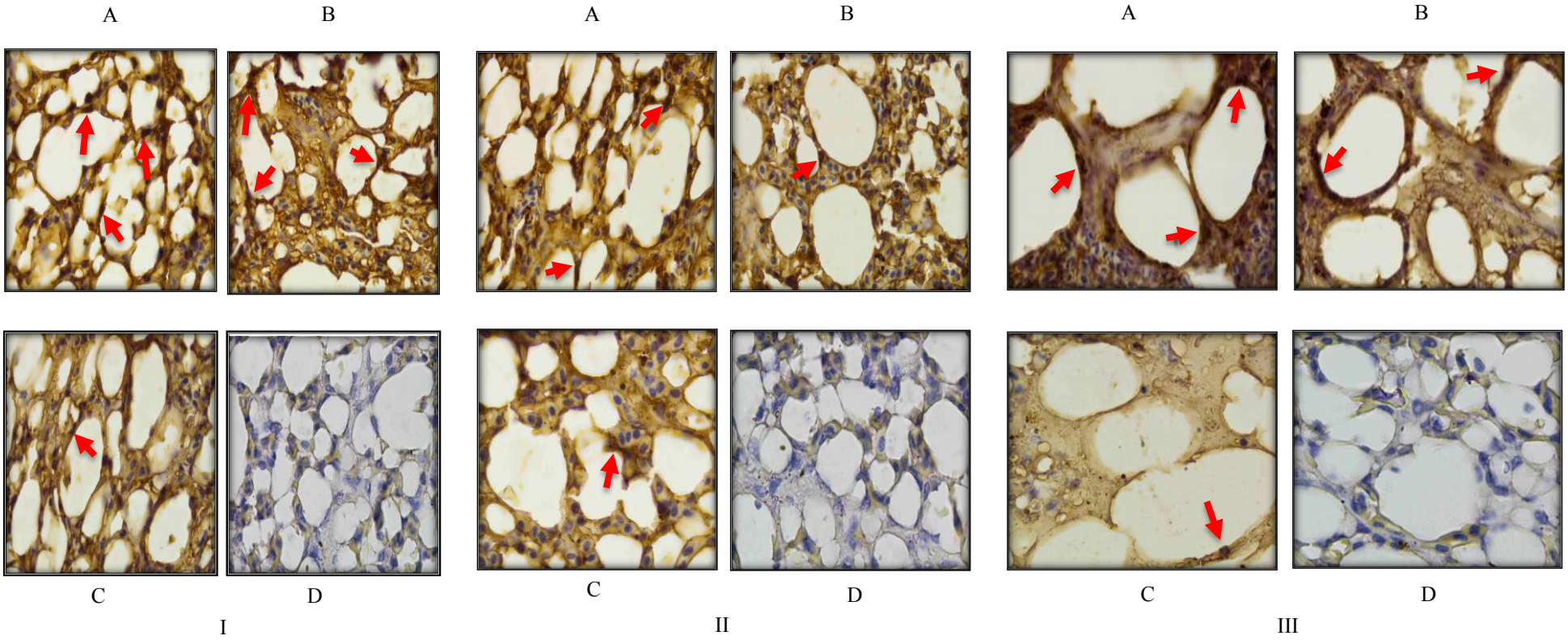

Figure 2. Immunohisochemistry of chicken lungs. Arrows indicate the blocking activity of anti-HA IgY on Avian Influenza Virus on septa alveoli. I; chicken administered with anti-HA IgY 24 hours before infection. II; chicken administered with anti-HA IgY 24 hours after infection. III; chicken administered with anti-HA IgY 24 at time of infection. A; Amount of administered IgY is 400 µg. B; Amount of administered IgY is 0µg.

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CONCLUSION

It can be concluded that application of anti-HA obtained High Pathogenic Avian Influenza (HPAI) clade 2.1 (A/Chicken/Blitar/2003) could give a protection from infection of HPAI clade 2.3.2 (A/Duck/Sidoarjo/2012), although they were originated from different clades. The protection rate was 80-100% applied 24 hours before infection.

DECLARATIONS

Authors' contribution

Suwarno contributed on data analysis and the write up of the manuscript. I also contribute on the production of IgY and formulating the dose of administered IgY and processing the sample on Immunohistochemistry assay. Rahaju Ernawati and Nanik Sianita Widjaya contributed on data analysis and the write up of the manuscript and calculating the dose of EID_{50}/ml and conducting the challenge test. All authors read and approved the final draft of manuscript.

Competing interests

The authors have not declared any conflict of interests.

Consent of publish

All the authors agree to publish this manuscript in World`s Veterinary Journal.

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REFERENCES

World Organization for Animal Health (OIE) (2016). Avian influenza (infection with avian influenza viruses). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, pp. 1–23. Available at: https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.03.04_AI.pdf

Achenbach JE and Bowen RA (2011).Transmission of avian influenza a viruses among species in an artificial barnyard. PLoS ONE, 6(3):1-7. DOI: https://doi.org/10.1371/journal.pone.0017643

Agrawal R Hirpurkar SD Sannat C and Gupta AK (2016). Comparative study on immunoglobulin Y transfer from breeding hens to egg yolk and progeny chicks in different breeds of poultry. Veterinary World, 9(4): 425–431. DOI: https://dx.doi.org/10.14202%2Fvetworld.2016.425-431

Aldous EW and Alexander DJ (2016). Detection and differentiation of newcastle disease virus (avian paramyxovirus type 1).Avian Pathology,30:117-128/ DOI: https://doi.org/10.1080/03079450120044515

Bergervoet SA, Pritz-Verschuren SBE, Gonzales JL, Bossers A, Poen MJ, Dutta J, Khan Z, Kriti D, Van Bakel H, Bouwstra R et al. (2019).Circulation of low pathogenic avian influenza (LPAI) viruses in wild birds and poultry in the Netherlands 2006–2016. Scientific Reports, 9(1):1–12. DOI: https://doi.org/10.1038/s41598-019-50170-8

Bouma A Claassen I Natih K Klinkenberg D Donnelly CA Koch G and Boven MV (2009). Estimation of transmission parameters of H5N1 avian influenza virus in chickens. PLoS Pathogens, 5(1):1-13. DOI: https://doi.org/10.1371/journal.ppat.1000281

Costa-hurtado M, Afonso CL, Miller PJ, Spackman E, Kapczynski DR, Swayne DE, Shepherd E, Smith D, Zsak A and Pantin-Jackwood M (2014). Virus interference between H7N2 low pathogenic avian influenza virus and lentogenic newcastle disease virus in experimental co-infections in chickens and turkeys. Veterinary Research, 45(1): 1–11. DOI: https://dx.doi.org/10.1186%2F1297-9716-45-1

Wright PF Neumann G and Kawaoka Y (2013). Orthomyxoviruses. In Knipe DM and Howley PM (Editor in Chief), Fields Virology 6th Edition, Philadelphia, PA 19103, pp. 1186-1243.

Guo L, Wang D, Zhou H, Wu C, Gao X, Xiao Y, Ren L, Paranhos-Baccalà G, ShuY, Jin Q and Wang J (2016). Cross-reactivity between avian influenza A (H7N9) virus and divergent H7 subtypic- and heterosubtypic influenza A viruses. Scientific Reports, 6: 1-8. DOI: https://dx.doi.org/10.1038%2Fsrep22045

Hau J and Hendriksen CFM (2005). Refinement of polyclonal antibody production by combining oral immunization of chickens with harvest of antibodies from the egg yolk. ILAR Journal, 46(3): 294–299. DOI: https://dx.doi.org/10.1093/ilar.46.3.294.

Indriani R, Dharmayanti NLPI and Martindah E (2017). Cross - reaction of duck and chicken sera against avian influenza H5N1 virus clades 2.1.3 and 2.3.2 antigens by hemagglutination inhibition test. Jurnal Biologi Indonesia, 13(2): 325–329. DOI: https://dx.doi.org/10.14203/jbi.v13i2.3407

de Jong JC, Claas ECJ, Osterhaus ADME, Webster RG and Lim WL (1997). A pandemic warning?.Nature, 389: 554-555. DOI: https://dx.doi.org/10.1038%2F39218

Ko KY and Ahn DU (2007).Preparation of immunoglobulin Y from egg yolk using ammonium sulfate precipitation and ion exchange chromatography. Poultry Science, 86(2): 400–407. DOI: https://doi.org/10.1093/ps/86.2.400

Lu J, Guo Z, Pan X, Wanf G, Zhang D, Li Y, Tan B, Ouyang L and Yu X (2006). Passive immunotherapy for influenza A H5N1 virus infection with equine hyperimmune globulin F(ab′1)2 in mice. Respiratory Research, 7: 1–7. DOI: https://doi.org/10.1186/1465-9921-7-43

Narat M (2003). Production of Antibodies in Chickens.Food Technology and Biotechnology, 41(3): 259–267.

Rahimi S, Zahra MS, Salehi TZ, Torshizi MAK and Grimes JL (2007). Prevention of Salmonella infection in poultry by specific egg-derived antibody. International Journal of Poultry Science, 6(4): 230–235. DOI: http://dx.doi.org/10.3923/ijps.2007.230.235

Webby RJ and Webster RG (2003). Are we ready for pandemic influenza?. Science, 302(5650): 1519–1522. DOI: https://doi.org/10.1126/science.1090350

Wen J, Zhao S, He D, Yang Y, Li Y and Zhu S (2012). Preparation and characterization of egg yolk immunoglobulin Y specific to influenza B virus. Antiviral Research, 93(1): 154–159. DOI: https://doi.org/10.1016/j.antiviral.2011.11.005

Abbas AT, El-Kafrawy SA, Sohrab SS and Azhar EIA (2019). IgY antibodies for the immunoprophylaxis and therapy of respiratory infections. Human Vaccines and Immunotherapeutics, 15(1): 264-275. DOI: https://doi.org/10.1080/21645515.2018.1514224

Constantin C, Neagu M, Supeanu TD, Chiurciu V and Spandidos DA (2020). IgY-turning the psge toward passive immunization in COVID-19 infection (Review). EXPERIMENTAL AND THERAPEUTIC MEDICINE, 20:151-158. DOI: https://doi.org/10.3892/etm.2020.8704

Pereira EPV, van Tillburg MF, Florean EOPT and Guedes MIF (2019). Egg yolk antibodies (IgY) and their applications in human and veterinary health:review. International Immunopharmacology, 73: 293-303. DOI: https://doi.org/10.1016/j.intimp.2019.05.015

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Distribution Profile and Function of Carbohydrate Residues in Testes of Immature and Mature Sunda Porcupine (*Hystrix javanica*)

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ABSTRACT

The population of Sunda porcupine (*Hystrix javanica*) declines each year since it is rarely found in nature. The present study aimed to obtain information about the distribution of carbohydrate residues contained in immature and mature of Sunda porcupine's testes and to discuss its relevant functions. This study used six testes obtained from four immature and two mature Sunda porcupine originated from Ngawi Regency, East Java Province, Indonesia. Testis tissues were stained with hematoxylin and eosin and lectin histochemistry of *Lens culinaris* agglutinin (LCA), *Phaseolus vulgaris* leucoagglutinin (PHA-L), Pisum sativum agglutinin (PSA), Sophora japonica agglutinin (SJA), and *Wheat germ* agglutinin (WGA). Data were analyzed with descriptive and semi-quantitative method. Lectin histochemical staining with LCA, PHA-L, SJA, PSA, and WGA indicated the presence of alpha-D-mannose and alpha-D-glucose, N-acetylgalactosamine, mannose, and N-acetylglucosamine residues in the immature and mature testes with weak to very strong intensity. In the immature testes of Sunda porcupine, there was positive reactivity with PHA-L for Leydig and Sertoli cells, N-acetylgalactosamine may play an important role in the development and maturation of Leydig and Sertoli cells. Mature testes showed a strong positive reaction to the LCA, SJA, PSA, and WGA which indicated the significant roles of alpha-D-mannose and alpha-D-glucose, N-acetylgalactosamine, mannose, and N-acetylglucosamine residues on the maturation process of early spermatid to the late spermatid. These results can be used as basic data to be implemented in the conservation efforts of Sunda porcupine.

Key words: Carbohydrate residue, Lectin, Spermatogenesis, Sunda porcupine, Testes

INTRODUCTION

Hystrix javanica is an Indonesian endemic porcupine, commonly called the Sunda porcupine. Taxonomy of Sunda porcupine is: kingdom Animalia, phylum Chordata, class Mammalian, order Rodentia, family Hystricidae, genus *Hystrix* and species *H. javanica*. Based on the International Union for Conservation of Nature (IUCN), Sunda porcupine is classified in least-concern category which means that is still relatively spread, abundant and no major threat to the existence of Sunda porcupine. The Convention on International Trade in Endangered Species (CITES) which regulates the trade of endangered species of wildlife and plants, includes the Sunda porcupine in the appendix III list which means it has not been considered endangered category (Aplin, 2016).

Testes are reproductive organs that amount to one pair. The testes are in the scrotum and enveloped by tunica albuginea. The seminiferous tubules are present in the testes bounded by complex epithelium of spermatogenic cells and Sertoli cells. Spermatogenic cells produce spermatozoa (Bacha Jr and Bacha, 2012). The Sertoli cell is located in the terminal segment of the seminiferous tubule having functions as fluid transport and secretory activity as well as phagocytosis and intracytoplasmic spermatozoa degradation (Ahmed, 2005). The Sertoli cell has a pale or triangular nucleus that is a high cell extending from the basement membrane to the tubular lumen (Bacha Jr and Bacha, 2012). Interstitial tissue fills the space between seminiferous tubules with blood vessels, lymph vessels, and nerves of the testicular parenchyma (Setchell, 1986). Leydig cells are a very important source of androgens. More than 90% of the androgens in the organism are produced in testes (Ahmed, 2005).

Lectins can be defined as non-immune carbohydrate-binding proteins that can agglutinate and or form the precipitates of glycoconjugate. Glycoconjugates play a role in cell differentiation, cell maturation, cell recognition, cell adhesion, and cell interactions. The distribution of glycoconjugates in animal tissues can be investigated using histochemical lectin staining (Dias et al., 2015).

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Information on testicular biology of Sunda porcupine has not been previously reported. Limited data on the reproductive biology of Sunda porcupine was an important reason for this study. This information can be used to support Sunda porcupine breeding efforts in Indonesia. The aim of this study was to provide Information on carbohydrate residues of immature and mature testes of Sunda porcupine to determine the role of glycoconjugate in the spermatogenesis process.

MATERIALS AND METHODS

This study used the testes from the male Sunda porcupine (two adults and four immature) originated from Ngawi, East Java, Indonesia. Testes samples were trimmed by dividing three parts of the testis according to the location of epididymis which is caput, corpus, and cauda.

Hematoxylin and eosin staining

Conventional Hematoxylin and Eosin (H&E) staining, which has been used by histologists for more than 100 years, is the gold standard of histology structure (Li et al., 2018). The first stage of H&E staining was deparaffinization. The slides were dipped into the Harris Hematoxylin solution then dipped into the eosin solution. The next step was dehydration. The clearing process was done by inserting the slides into xylene. The mounting process was done by closing the tissue using glass decks and Entellan as an adhesive.

Lectin histochemical staining

The first step of lectin histochemical staining was deparaffinization with xylene and rehydration with ethanol. The slides were incubated in 3% H_2O_2 solution in methanol as endogenous peroxide inhibitor for 30 min and wash with PBS, then background sniper was applied to block non-specific proteins for 30 min. Fifteen microliter solution of *Lens culinaris* agglutinin (LCA), wheat germ agglutinin (WGA), *Sophora japonica* agglutinin (SJA), *Phaseolus vulgaris* leucoagglutinin (PHA-L), *Pissum sativum* agglutinin (PSA) and PBS was dropped as a negative control, then incubated overnight in 4^0 C. The positive reaction of lectins was visualized by diaminobenzidine (DAB) substrate, followed by Hematoxylin Harris solution for counterstain. Then slides were dehydrated with ethanol, cleared with xylene and mounted. The semi-quantitative analysis as a parameter was based on the brown color visualized in the cells with five criteria as negative (-), weak (+), moderate (++), strong (+++), and very strong (++++).

RESULTS AND DISCUSSION

Lectins play an important role in the processes associated with the recognition and interaction of cells, protein synthesis and transport, cell division regulation, fertilization, innate immunity, etc. (De Schutter and Van Damme, 2015; Feizi and Haltiwanger, 2015). Lectins are specific to certain carbohydrate structures. Some lectins can interact only with mannose or glucose residues and others only with galactose. There are other lectins specific to fucose, sialic acid, and other monosaccharides (Kobayashi et al., 2014; Nagdas et al., 2014).

The LCA reactivities on mature Sunda porcupine testicles detected strong on the early spermatids in caput (Figure 1A), corpus (Figure 1B), and cauda (Figure 1C). LCA reactivities detected on late spermatids only in caput (Figure 1A). The LCA reactivity was not shown in spermatogonia, primary spermatocytes, early spermatids, late spermatids, Leydig cells and testicular Sertoli cells in the caput (Figure 1D), corpus (Figure1E), and cauda (Figure 1F) in immature Sunda porcupine. According to Barre et al. (2019), LCA is specific to detect alpha-D-mannose and alpha-D-glucose sugar residues. Lectin histochemical staining of LCA in mature male Sunda porcupine detected in early spermatid and late spermatid shows that alpha-D-mannose and alpha-D-glucose sugar residues are needed at the early stages until the final stage of spermatid differentiation. While, lectin histochemical staining of LCA not detected in immature testicles indicates that the immature Sunda porcupine testicles did not require alpha-D-mannose and alpha-D-glucose sugar residues. Research on mice (Lee and Damjanov, 1984) showed different results in which LCA reacted positively to Sertoli cells, spermatogonia, spermatocytes, spermatozoa, and Leydig cells.

The PHA-L reactivity on mature Sunda porcupine was detected weak to testicular spermatogonia in corpus (Figure 2B), and negative in caput (Figure 2A) and caudal (Figure 2C). In late spermatid, PHA-L reactivities were weak in corpus (Figure 2D). Reactivity of PHA-L in Leydig cells was weak in caput (Figure 2A) and corpus (Figure 2C). Immature Sunda porcupine, PHA-L in Leydig cells showed positive reactivity in caput (Figure 2D), and weak reactivity in corpus (Figure 2E). Sertoli cells showed weak PHA-L reactivity in caput and corpus. According to Zhang et al. (2014), PHA-L bound N-acetylgalactosamine. Lectinhistochemical staining PHA-L detected in Leydig cells, spermatogonia, and late spermatids on mature and immature Sunda porcupine shows that N-acetylgalactosamine sugar residues are required in the process of differentiation and maturation of these cells. According to Arya and Vanha-perttula (1985), the need for glycoconjugate in small portions of Sertoli cells is thought to be closely related to the

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phagocytic ability and the process of forming residual bodies in the final stages. According to Ahmed (2005), Leydig cells are an important source of androgen hormones and 90% of the androgen hormones in the animal body are produced by the testes. N-acetylgalactosamine sugar residue is required for optimal development of Leydig cell so that Leydig cells can produce testosterone. PHA-L staining in mice (Lee and Damjanov, 1985) showed that PHA-L reacted positively to spermatogonia, spermatocytes, spermatids, and spermatozoa.

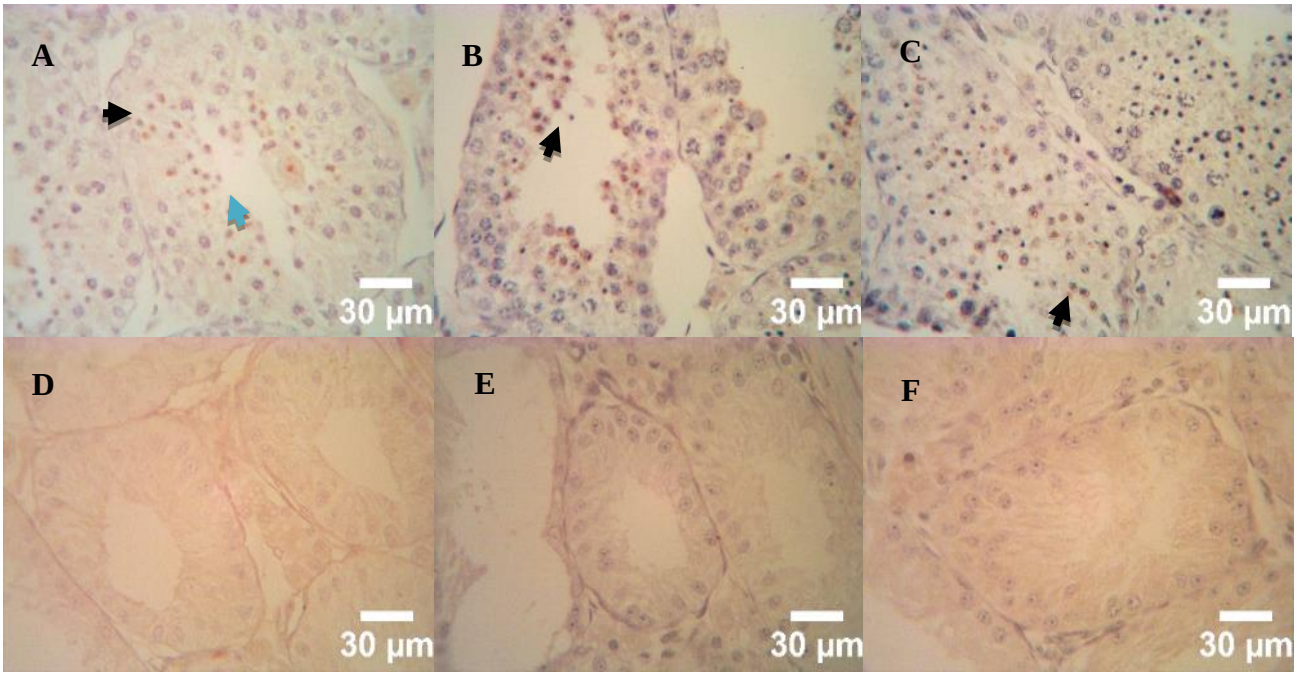

Figure 1. Testicular micrographs of *Hystrix javanica* (lectin histochemical staining with LCA). **A:** positive LCA reactivity in the early spermatid (black arrow) and the late spermatid (blue arrow) in caput of the mature Sunda porcupine testicle. **B:** LCA reactivity in the early spermatid (black arrow) in corpus of the mature Sunda porcupine testicle. **C:** LCA reactivity in the early spermatid (black arrow) in cauda of the mature Sunda porcupine testicle. Negative LCA-reactivity in caput (**D**), corpus (**E**) and cauda (**F**) regions of immature Sunda porcupine's testicle.

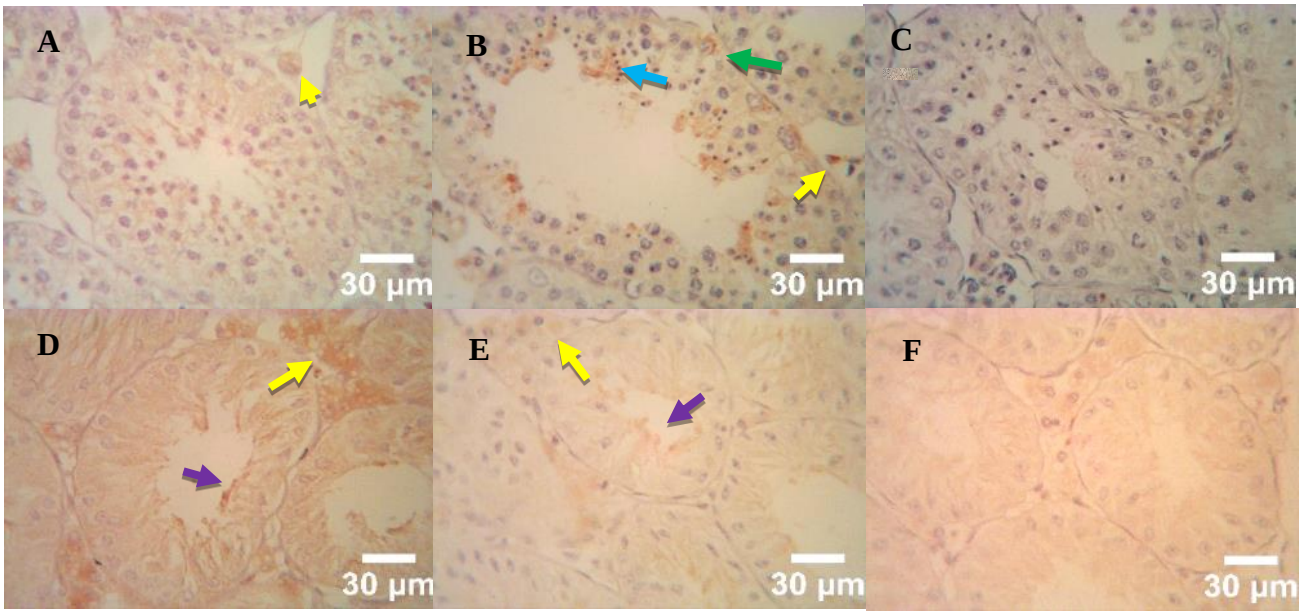

Figure 2. Testicular micrographs of *Hystrix javanica* (lectin histochemical staining with PHA-L). **A:** positive lectin reactivity in Leydig cells (yellow arrow) of testicle of mature Sunda porcupine in caput region. **B:** positive lectin reactivity in spermatogonia (green arrow), late spermatid (blue arrow) and Leydig cell (yellow arrow) of mature Sunda porcupine in corpus region. **C:** negative lectin reactivity in caudal region of mature Sunda porcupine testicle. **D:** PHA-L reactivities detected in Leydig cells (yellow arrow) and Sertoli cell cytoplasm (purple arrow) of immature Sunda porcupine testicle. **E:** an immature Sunda porcupine testicle in the corpus show positive PHA-L reactivities in the Leydig (yellow arrow) and Sertoli cell cytoplasm (purple arrow). **F:** the immature Sunda porcupine testicle in cauda showing negative PHA-L reactivity.

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Lectin histochemical staining with PSA for primary spermatocytes of mature Sunda porcupine showed week reactivity in cauda region (Figure 3C). PSA reactivity was detected moderate in primary spermatocyte, early spermatid in caput region (Figure 9A) and weak in corpus (Figure 3B) and cauda (Figure 3C). PSA reactivity was detected moderate in late spermatid in caput (Figure 3A), and weak in cauda (Figure 3C). Lectin histochemical staining with PSA of testis of immature Sunda porcupine showed weak reactivity in Leydig cells in caput (Figure 3D). According to Zhang et al. (2014), lectin histochemical PSA binds to mannose. PSA reacted positively to primary spermatocytes, early spermatids, and late spermatids of mature Sunda porcupine, and reacted positively to Leydig cells of immature Sunda porcupine. According to Novelina et al. (2010), glycoconjugate plays an important role in various metabolic processes of the body such as regeneration, cell differentiation, adhesion, and intercellular communication as well as other functional processes. Cells that react positively to PSA indicate that the cell requires a residual sugar mannose in its development. According to Wahyuni et al. (2016), the detectable glycoconjugate in spermatids indicates the importance of glycoconjugate in spermatogenesis, especially in spermiogenesis.

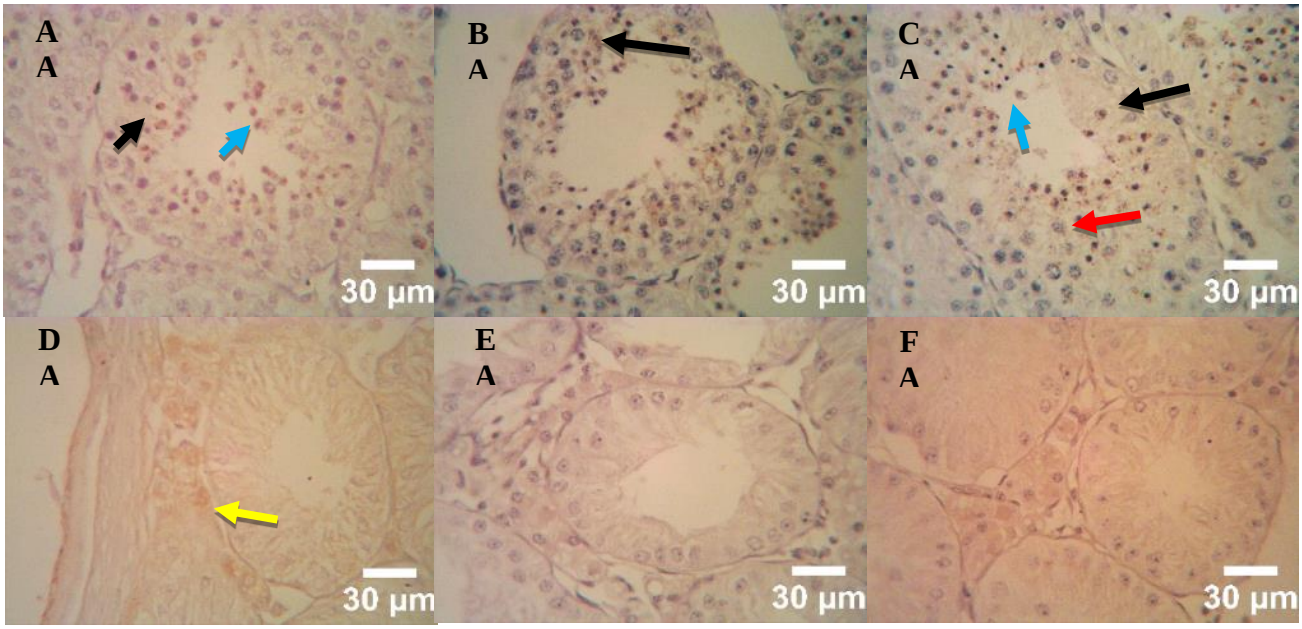

Figure 3. Testicular micrographs of *Hystrix javanica* (lectin histochemical staining with PSA). **A:** the testicles of mature Sunda porcupine in caput region showing PSA reactivities in the early spermatid (black arrow) and late spermatid (blue arrow). **B:** mature Sunda porcupine testicle in the corpus showing PSA reactivities in early spermatid (black arrow). **C:** testis of mature Sunda porcupine in caudal part showing PSA reactivities in primary spermatocyte (red arrow), early spermatid (black arrow) and the late spermatid (blue arrow). **D:** the immature Sunda porcupine testicle in caput showing positive PSA-reactivity in Leydig cell (yellow arrow), whereas the negative reactivity in corpus (**E**) and cauda (**F**)

Histochemical lectin staining with SJA in early spermatids of mature Sunda porcupine showed strong reactivity in cauda (Figure 4C), moderately positive reactions in caput (Figure 4A), and weak reactions in corpus (Figure 4B). The late spermatid showed moderate SJA reactivity in caput (Figure 4A). The immature Sunda porcupine's Leydig cells showed weak SJA reactivity in caput (Figure 4D). According to Zhang et al. (2014), SJA binds N-acetylgalactosamine. SJA showed positive reactions in the early spermatids and late spermatids of mature Sunda porcupine, and immature Sunda porcupine's Leydig cells. Cells that react positively to SJA show that the cell requires N-acetylgalactosamine for its development. The positive pattern was found from the early stage to the late stage of the spermatid. Although in the final stages, the intensity of the positive reaction decreased, the positive affinity pattern of Sunda porcupine was similar to that of the horse (Verini-Supplizi et al., 2000).

Lectin histochemical staining of mature Sunda porcupine showed that WGA reacted positively strong in primary spermatocytes, early spermatids and late spermatids in caput (Figure 5A) and cauda (Figure 5C), whereas in corpus (Figure 5B) was positively weak. In immature Sunda porcupine, WGA reacted positively weak on Leydig cells in the corpus (Figure 5E). According to Wahyuni et al. (2016), WGA binds with N-acetylglucosamine sugar that is necessary for differentiation process of spermatocyte and spermatid cell. WGA reactivity in rats was detected in spermatogonia, spermatocytes, spermatozoa, Sertoli cells, Leydig cells, and lamina propria (Lee and Damjanov, 1985; Shegedin et al., 2017). In Syrian hamster, WGA reactivities were detected in spermatogonia (Hernández et al.2018).

Lectin histochemical staining with LCA, PHA-L, SJA, PSA, and WGA indicated the presence of alpha-D-mannose and alpha-D-glucose, N-acetylgalactosamine, mannose, and N-acetylglucosamine residues (Murakami et al., 2014; Belicky and Tkac, 2015) in the immature and mature testes.

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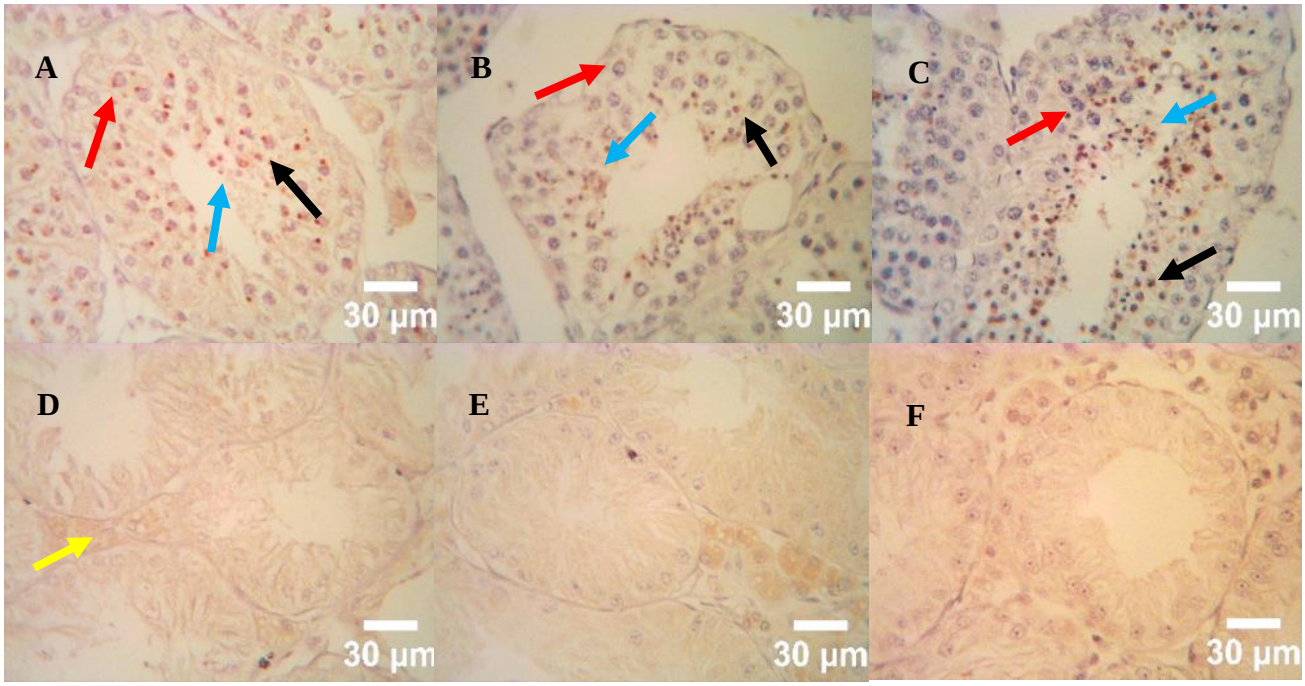

Figure 4. Testicular micrographs of *Hystrix javanica* (lectin histochemical staining with SJA). **A:** testicles of mature Sunda porcupine in caput region showing SJA reactivities in primary spermatocyte (red arrow), early spermatid (black arrow) and the late spermatid (blue arrow). **B:** mature Sunda porcupine testicle in the corpus show SJA reactivities in primary spermatocyte (red arrow), early spermatid (black arrow). **C:** testicle of mature Sunda porcupine in caudal regions show SJA reactivities in primary spermatocyte (red arrow), early spermatid (black arrow). **D:** immature testicle in caput show SJA reactivities in Leydig cells (yellow arrow), while show negative SJA-reactivity in corpus (**E**) and cauda (**F**).

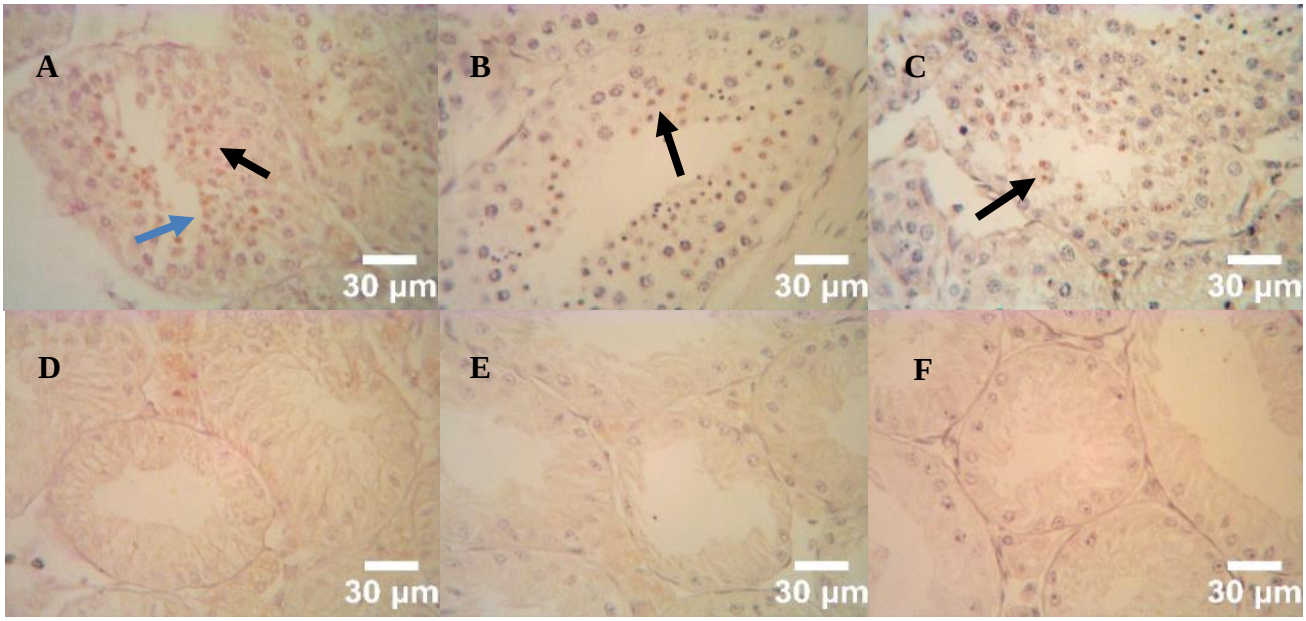

Figure 5. Testicular micrographs of *Hystrix javanica* (lectinhistochemical staining with WGA). The mature Sunda porcupine testicles in caput (**A**) and corpus (**B**) show positive WGA reactivities in early spermatid (black arrow) and the late spermatid (blue arrow). **C:** the testicle of Sunda porcupine in caudal regions show positive WGA reactivity in spermatids (black arrows). The immature Sunda porcupine in caput part (**D)** and cauda (**F**) show negative WGA reactivity, while in corpus region. **E:** show positive reactivity in the Leydig cells.

CONCLUSION

In conclusion, the present study showed that lectin histochemical staining with LCA, PHA-L, SJA, PSA, and WGA indicated the presence of alpha-D-mannose and alpha-D-glucose, N-acetylgalactosamine, mannose, and N-acetylglucosamine sugar residues in the immature and mature testes of Sunda porcupine with weak to very strong intensity. In the immature testes, N-acetylgalactosamine may involve in the development and maturation of Leydig and Sertoli cells, whereas in the mature testes, alpha-D-mannose and alpha-D-glucose, N-acetylgalactosamine, mannose, and

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N-acetylglucosamine residues play important roles in the maturation process of early spermatid to the late spermatid.

DECLARATIONS

Authors' contribution

Teguh Budipitojo developed the concepts and designed the experiments, analyzed and interpreted the data. Irma Padeta collected tissue samples and fixed them in Bouin's solution, processed tissues for paraffin-embedded method. Beninda Ulima Yulianti developed the concepts and designed the experiments, visualized lectin reactivity and wrote the manuscript. Dian Bekti Hadi Masithoh wrote the manuscript and analyzed data. All authors read and approved the final manuscript.

Competing interests

The authors have not declared any conflict of interest.

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REFERENCES

Ahmed AE (2005). Morphological, Glycohistochemical Studies on the Embryonic and Adult Bovine Testis. Ludwig-Maximilians-UniversitratMunchen. Munich. Available at: https://edoc.ub.uni-muenchen.de/3978/1/Abd-Elmaksoud_Ahmed.pdf

Arya M and Vanha-Perttula T (1985). Lectin-binding pattern of bull testis and epididymis. Journal of Andrology, 6: 230-242. DOI: https://doi.org/10.1002/j.1939-4640.1985.tb00839.x

Bacha Jr WJ and Bacha LM (2012). Color Atlas of Veterinary Histology. 3rd Edition. A Wolter Kluwer Company, New York, pp. 203-220. Available at: https://onlinelibrary.wiley.com/doi/abs/10.1111/avj.12035

Barre A, Bourne Y, Van Damme EJM and Rougé P (2019). Overview of the structure–function relationships of mannose-specific lectins from plants, algae and fungi. The International Journal of Molecular Sciences, 20(2): 254. DOI: https://doi.org/10.3390/ijms20020254

Belicky S and Tkac J (2015). Can glycoprofiling be helpful in detecting prostate cancer?. Chemické zvesti, 69(1): 90–111. DOI: https://doi.org/10.1515/chempap-2015-0052

De Schutter K and Van Damme EJ (2015). Protein-carbohydrate interactions as part of plant defense and animal immunity. Molecules, 20(5): 9029-9053. DOI: https://doi.org/10.3390/molecules20059029

Dias RDO, Machado LDS, Migliolo L and Franco OL (2015). Insights into animal and plant lectins with antimicrobial activities. Molecules, 20(1): 519-541. DOI: https://doi.org/10.3390/molecules20010519

Feizi TE and Haltiwanger RS (2015). Editorial overview: Carbohydrate-protein interactions and glycosylation: Glycan synthesis and recognition: finding the perfect partner in a sugar-coated life. Current Opinion in Structural Biology, 34:7-9. DOI: https://doi.org/10.1016/j.sbi.2015.10.005

Hernández JM, Seco-Rovira V, Beltrán-Frutos E, Ferrer C, Serrano-Sánchez MI and Pastor LM (2018). Lectin-binding pattern of glycoconjugates during spontaneoustesticular recrudescence in Syrian hamster (*Mesocricetus auratus*) after exposure to short photoperiod. Andrologia, 51(1): 13148. DOI: https://doi.org/10.1111/and.13148

Aplin K (2016). *Hystrix javanica*. IUCN Red List of Threatened Species. DOI: https://dx.doi.org/10.2305/IUCN.UK.2016-2.RLTS.T10752A22231749.en

Kobayashi Y, Tateno H, Ogawa H, Yamamoto K and Hirabayashi J (2014). Comprehensive List of Lectins: Origins, Natures, and Carbohydrate Specificities. In: Hirabayashi J (editor) Lectins. Methods and protocols. New York: Humana Press. DOI: https://10.1007/978-1-4939-1292-6_45

Lee MC and Damjanov I (1984). Anatomic distribution of lectin-binding sites in mouse testis and epididymis. Differentiation, 27: 74-81. DOI: https://doi.org/10.1111/j.1432-0436.1984.tb01410.x

Lee MC and Damjanov I (1985). Lectin binding sites on human sperm and spermatogenic cells. Anatomical Record, 212: 282-287. DOI: https://doi.org/10.1002/ar.1092120310

Li Y, Li N, Yu X, Huang K, Zheng T, Cheng X, Zeng S and Liu X (2018). Hematoxylin and eosin staining of intact tissues via delipidation and ultrasound. Scientific Report, 8: 12259. DOI: https://doi.org/10.1038/s41598-018-30755-5

Murakami Y, Hasegawa Y, Nagano K and Yoshimura F (2014). Characterization of wheat germ agglutinin lectin-reactive glycosylated OmpA-like proteins derived from *Porphyromonas gingivalis*. Infection and Immunity, 82(11): 4563-4571. DOI: https://doi.org/10.1128/IAI.02069-14

Nagdas SK, Buchanan T and Raychoudhury S (2014). Identification of peroxiredoxin-5 in bovine caudaepididymal sperm. Molecular and Cellular Biochemistry, 387:113–121. DOI: https://doi.org/10.1007/s11010-013-1876-3

Novelina S, Satryaningtijas A, Agungpriyono S, Setijanto H and Sigit K (2010). Morfologi dan Histokimia Kelenjar Mandibularis Walet Linchi (Collocalialinchi) Selama Satu Musim Berbiak dan Bersarang. Jurnal Kedokteran Hewan, 4: 192. Available at: https://pdfs.semanticscholar.org/c4f7/96e69915163d5084e0d0cce964f50b257057.pdf

Setchell BP (1986). The movement of fluids and substances in testis. Australian Journal of Biological Sciences, 39:193-207.

Shegedin A, Yashchenko A and Lutsyk A (2017). Modification of rat testis carbohydrate determinants in postnatal morphogenesis as detected by lectin probes. Journal of Cell Science and Therapy, 8:274.

Verini-Supplizi AG, Stradaioli G, Agioli O and Parillo F (2000). Localization of the lectin reactive sites in adult and prepubertal horse testes. Research in Veterinary Science, 69: 113-118.

Wahyuni S, Agungpriyono S, Adnyane IKM, Hamny H, Jalaluddin M, Gholib, Akmal M, Adam M, Aliza D and Siregar TN (2016). Studi Histokimia Lektin pada Sel-sel Spermatogenik Testis Muncak (Muntiacus muntjak muntjak). Jurnal Kedokteran Hewan, 10 (1): 82-85.

Zhang RY, Neu TR, Bellenberg S, Kuhllcke U, Sand W and Vera M (2014). Use of lectin to *in situ* visualize glycoconjugates of extracellular polymeric substances in acidophilic archaeal biofilms. Microbial Biotechnology, 8(3): 448-461. DOI: https://doi.org/10.1111/1751-7915.12188

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Polymorphism Association of *Pituitary Positive Transcription Factor-1* Gene with Body Weight Traits in BC_1 Hybrid Chicken (*Gallus gallus gallus* Linnaeus, 1758) from Cross Breeding between Female F_1 Broiler and Male Pelung

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ABSTRACT

Pituitary Positive Transcription Factor-1 gene is closely related to chicken growth and productivity. This research was conducted to detect Single Nucleotide Polymorphism in the exon 6 Pituitary Positive Transcription Factor-1 gene and its association with the bodyweight growth in the first backcross hybrid chicken. Procedures of the research included crossbreeding female first filial broiler chicken with male Pelung chicken to obtain first backcross hybrid chicken, Day Old chick hatched were maintained during 49 days, the bodyweight on the Day-Old chick measured every seven days, DNA was isolated by Chelex 5% method, Pituitary Positive Transcription Factor-1 gene was amplified by PCR, DNA band was visualized utilizing electrophoresis, and the PCR product was sequenced using Sanger method. The DNA sequence was aligned using Clustal omega software to gain Single Nucleotide Polymorphism. The Single Nucleotide Polymorphism was analyzed using the Pearson correlation test between chicken body weights of 49-days-old chickens with the polymorphism points. The conclusion indicated that the bodyweight of the first backcross hybrid chicken was higher than the Pelung chicken but lower than the first filial broiler chicken. Single Nucleotide Polymorphism was not found on the exon 6 Pituitary Positive Transcription Factor-1 gene in the first backcross hybrid chicken.

Keywords: Growth, Hybrid chickens, PIT-1 gene, SNP

INTRODUCTION

Indonesian native chickens or known as '*ayam buras'* (non-broiler chickens) are very popular by Indonesians, especially in rural areas. Indonesian native chickens are classified into four functional groups such as meat and egg producer, singing chicken, used in traditional ceremonies, fancy, and fighting cock (Hidayat and Asmarasari, 2015). According to (Zein and Sulandari, 2009) a genetic molecular study, informed that all domesticated chicken populations came from one ancestor (monophyletic), namely red jungle fowl (*Gallus gallus*) originated from Southeast Asia. Indonesian local chickens were developed through a process of domestication and known as native chickens. Native chickens were the result of a cross between jungle fowl *Gallus bankiva* and *Gallus varius* scattered in the territory of Indonesia, especially in Java and Nusa Tenggara. Local chickens or often known as '*ayam kampung'* have superiority in the quality of their meat and egg, but this superiority is not followed by good productivity capability in meat and egg (Zein and Sulandari, 2009).

The productivity of local chickens is relatively low, as an implication of the extensive system of maintenance. Indonesian local chickens must be maintained optimally to support the small-scale poultry industry so that it becomes a solution to fulfill the increasing demand for domestic food consumption (Daryono et al., 2010). The efforts to improve the productivity of local chickens include selection and crossbreeding programs. According to Cheng (2010), selective breeding is aimed to produce a superior chicken breed with adjusted phenotype quality according to human needs. The targeted selection program will provide a high economic mean in the use of local chickens, namely by improving the quality of local chickens through the crossing and selective breeding programs of specific characters. Other basic information such as specific characteristics, origin, performance, and productivity of local chickens are needed to optimize the utilization of local chickens in Indonesia. This information is expected to make Indonesian local chickens better known, developed, and preserved, so that they can be used sustainably (Sulandari et al., 2007). Therefore, we need research that can study genetic diversities and identify genes responsible for the growth of hybrid chickens.

With the progress of molecular genetics, the selection program can be carried out earlier through analysis at the DNA level. Pituitary Positive Transcription Factor-1 (PIT-1) gene is the one gene that is closely related to chicken growth and productivity (Miyai et al., 2005). As stated by Jiang et al. (2004) exon 6 in PIT-1 gene has a significant

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relationship to improving the growth of chicken weight. The purpose of this study was to obtain hybrid chickens that inherited superior characteristics from both broodstocks with good growth characteristics resembling broiler chickens and good phenotypic characters, body resistance, good quality of meat and eggs resembling local chickens, and genetic quality improvement through molecular selection. Therefore, this study also analyzed the relationship between exon 6 polymorphisms of the PIT-1 gene with hybrid chicken body weight.

MATERIALS AND METHODS

Ethical approval

The procedure in this research has been conducted following the guidelines of the ethical committee of the Faculty of Veterinary Medicine, Universitas Gadjah Mada.

Chicken cross breeding

In this study, the first backcross (BC_1) hybrid chickens were used as a result of crossing between female F_1 broiler chicken and male Pelung chicken. The Day Old Chick (DOC) was maintained for seven weeks with lighting 24 hours using 10 watts light bulb, air temperature ±30℃ and 40-50% humidity, feed by BR I (protein 21,00-23,00% and energy 3000 kcal/kg) made by PT. Japfa Comfeed Indonesia Public Listed Company (Plc) *ad libitum*. 12 broods were consisting of 7 males and 5 females. Furthermore, DOC was raised intensively for 7 weeks in special cages to minimize outside influences that can interfere with health, facilitating growth monitoring, and facilitating chickens' feeding. The DOC body weight measurement every 7 days was aimed to observe DOC growth during the observation period for 7 weeks. Quantitative character measurements and qualitative character observations were carried out on the last day of observation on the 49th day.

DNA isolation

DNA isolation with Chelex 5% method with the modified concentration of Chelex according to the optimization phase. A total of 10 µl of chicken blood was put into a 1.5 mL tube, added with 1 ml of Tris-EDTA (TE) buffer. Then put into a 1.5 mL microcentrifuge tube, centrifuged at a speed of 13,000rpm for 3min. The supernatant was transferred to the new eppendorf tube, then the pellet was added with 200µl of 5 percent Chelex solution, 18 µl of dithiothreitol (DTT) 0.05 M, 2 µl of proteinase K, then mix various samples rapidly 30s with vortex and incubated at 56℃ for 2 h, and vortex in every 15 min. Then incubated at 100℃ for 8 min, and centrifuged at 13,000 rpm for 3 min. The supernatant was transferred to a 1.5 ml microcentrifuge tube, and stored at -20℃ (Butler, 2009).

DNA amplification

The amplification of Pituitary Positive Transcription Factor-1 (PIT-1) gene was carried out by PCR, with the reaction composition of Bioline PCR kit as much as 12.5 µl, 5'-GGCACTTTGGAGAACAAAGC-3' forward primer as much as 1.25 µl, 5'-CTCGTGGTGCTCCTTGATAA-3' reverse primer as much as 1.25 µl, 5 µl of DNA samples, and 5 µl of ddH2O so that the total volume was 25 µl. The specific primer used was MR5 (for exon 6 with access code AJ236855) from *Gallus gallus* (Nie et al., 2008). The used PCR program was 95℃ initial denaturation for 5 min, followed by 35 denaturation cycles at 95℃ for 15s, annealing at 60℃ for 60 s, and extension at 72℃ for 60 s, extra extension at 72℃ for 10 min (Van As et al., 2000).

Agarose preparation

Agarose was weighed according to agar concentration (genome =1%) (PCR yield 1.8-2%). Next, it was put in a beaker glass and added with Tris-borate-EDTA (TBE) according to the chamber volume. Then it was put in the oven, heat until it dissolves (clear). A mold was set and installed with the comb. The agar was added with 2-3 µl of flourosave, then poured into the mold. The agarose was left to solidify.

Electrophoresis

Electrophorator was prepared. Agar was inserted into the electrophorator Mupid-exU™. Tris-borate-EDTA/TBE (immersion) was added until the agar was sinked. The sample was inserted into the well. Electrophorator was closed, turn on, time was set (20-30 min =100 volts, 1h =50 volts) then visualized under UV light by AnalytikJena™ gel imaging system and documented with GelDoc™ Documentation System.

Sequencing with Sanger method

The PCR product was sequenced by the Sanger sequencing method (Sanger et al., 1977) in first Base Company, Selangor, Malaysia.

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Data analysis

The correlation between chicken weights was analyzed using SPSS 16.0 one-way ANOVA program statistical test and post hoc LSD method to assess the significance between chicken strains. The data of DNA sequencing were assembled using the Gene Studio program, multiple sequences were alignment using Clustal Omega software, and Pearson correlation test between chicken body weight with Single Nucleotide Polymorphism (SNP, Arnedo et al., 2007).

RESULTS AND DISCUSSION

Chicken growth

In this study, crossings between female F_1 broiler chickens and male Pelung chickens were carried out and resulted in the first backcross or BC_1 hybrid chickens. The comparison of the weight of BC_1 hybrid chicken, Pelung, broiler, and F_1 broiler chicken for 7 weeks is presented in figure 1.

The average weight of chickens from the lowest to the highest starting from Pelung chicken, BC_1 hybrid chicken, F_1 broiler chicken, and broiler chicken. The average body weight of BC_1 hybrid chickens (161.44 gr) for the seven weeks showed lower results compared to the average weight of F_1 broiler chickens (648.14 g) (Roosdianto, 2010), and broiler chickens (1409.57 gr) (Suryaman, 2010), but higher than the average weight of Pelung chickens (112.38 gr). This was based on the inherited character of the broodstocks, the BC_1 hybrid chickens carried the character of broiler chickens which was rapid growth, and thus BC_1 hybrid chickens had a higher weight than Pelung chickens. The growth and development of chickens were influenced by certain factors, including intrinsic factors such as genetics and sex, and extrinsic factors such as the process of chicken breeding, environmental factors, and types of feed (Oktafiantari, 2016).

The significance of the chicken types to the chicken weights for 7 weeks is shown in table 1. The BC_1 hybrid chicken has a higher growth rate compared to Pelung chicken but has a lower growth rate than broiler chicken and F_1 chicken. Table 1, a significant difference is obtained because the significance value of 0.00 is less than the standard deviation of 0.05. Thus, it means that the types of chicken affect chicken weight. The BC_1 hybrid chicken growth is between the Pelung and broiler Chicken growth lines because BC_1 hybrid chickens have both bloodlines. Therefore, it is important to further investigate the causes of these differences in chicken growth, by assessing the polymorphism of the exon 6 PIT-1 gene which has been recognized as one of the genetic markers for chicken growth.

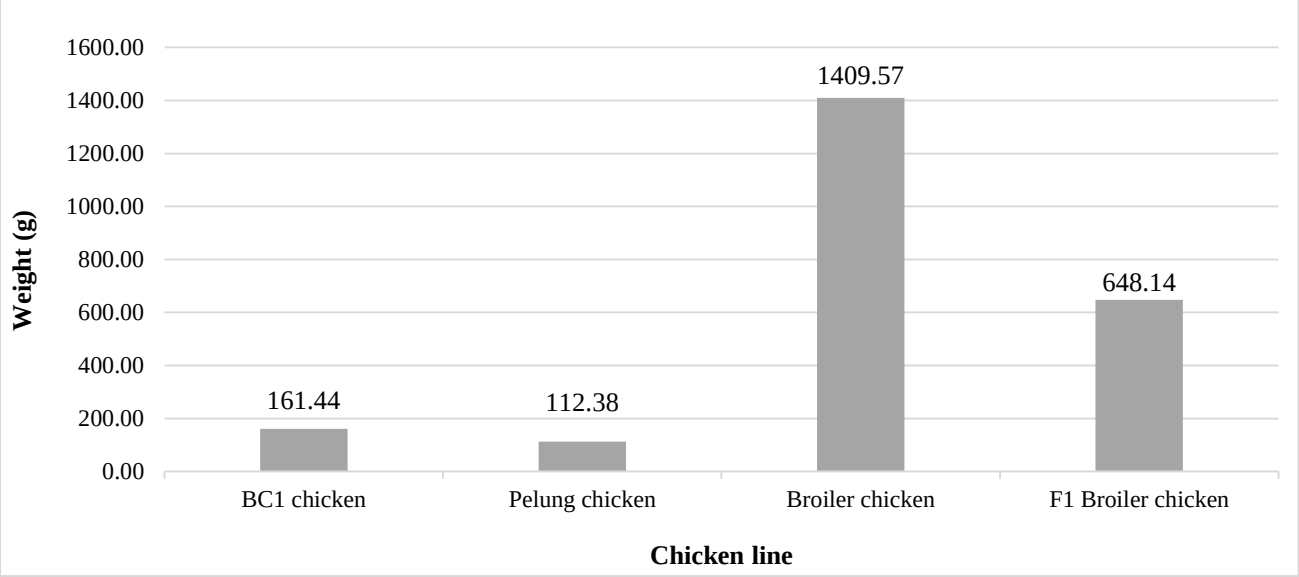

Figure 1. Comparison of mean body weights of BC_1, Pelung, Broiler and F_1 Broiler chickens for seven weeks

Table 1. The differences of chicken weights in BC_1, Pelung, Broiler and F_1 Broiler chickens during seven weeks

Chicken line	Age (week)						
	1	2	3	4	5	6	7
Hybrid BC_1	48.67± 6.18^b	65.33± 9.64^b	95.17± 24.37^b	122± 4306^b	156.4± 43.79^b	230.92± 45.49^b	419.08± 100.6^b
Broiler	194.0± 0.00^d	461.00±0.00^d	842.00±0.00^d	1309±0.00^d	1817± 0.00^d	2347± 0.00^d	2897± 0.00^d
Pelung	32.33±2.52^a	44.33±11.93^a	57.33±17.21^a	84.00±27.22^a	124.3±30.10^a	185.67±42.19^a	258.67±54.09^a
F_1 broiler	94.30±0.00^c	230.00±0.00^c	387.00±0.00^c	583.70±0.00^c	833± 0.00^c	1100.3±0.00^c	1308.70±0.00^c

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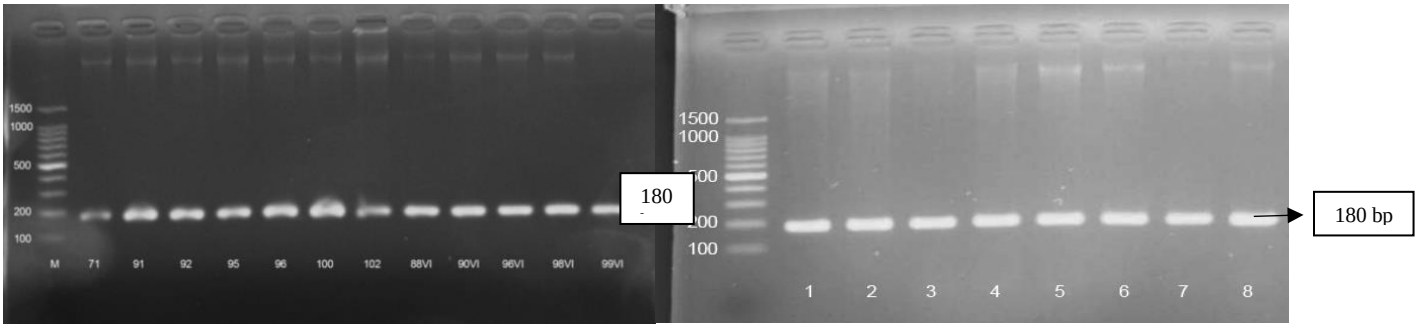
Pituitary positive transcription factor-1 gene polymorphism

The molecular selection was performed as a way to improve genetic quality. This study was aimed to detect the presence of PIT-1 gene polymorphism on the weight growth of hybrid chickens. The Pituitary Positive Transcription Factor-1 (PIT-1, POU1F1, or GHF1) gene in chickens was located on chromosome 1 with a length of 14 kb as a genetic marker that had been used to aid in the early selections based on the relationship between markers and the expected quantitative traits (Yamada et al., 1993). The PIT-1 gene was one of the genes that were closely related to the growth and productivity of chickens because the PIT-1 gene controlled the expressions of the coding genes for growth hormone and prolactin hormone (Miyai et al., 2005). Therefore, it could be express that the PIT-1 gene was a gene candidate that had the prospect of being used as a genetic marker in the local chicken selection program. Electrophoresis was carried out to determine the results of DNA fragments amplification by PCR. The results of electrophoresis can be seen in figure 2 and figure 3.

Based on exon 6 PIT-1 gene visualization, the 12 BC_1 samples had a nucleotide length of 180 bp (Figure 2a). In the PCR result of the exon 6 PIT-1 gene (Figure 2b), the samples 1-4 are broiler chickens that have nucleotide length 180 bp and the samples 5-8 are Pelung chickens also have nucleotide length 180 bp. DNA amplification results showed good fragments that were shown by the appearance of thick and clear DNA bands, then from the amplification results by PCR, a sequencing process was carried out to determine the nucleotide sequences of the genes. The alignment of the exon 6 PIT-1 gene is shown in table 2.



(a) (b)

Figure 2. The results of exon 6 Pituitary Positive Transcription Factor-1 gene (180 bp) amplification by PCR (a) hybrid BC_1 chickens (b) 1, 2, 3, 4: Broiler chickens; 5, 6, 7,8: Pelung chickens

Table 2. The single nucleotide polymorphism of exon 6 pituitary positive transcription factor-1 gene

Sample No.	PIT-1 gene polymorphism		Haplotype	Chicken weight on day 49 (g)
	Exon 6			
	Substitution	Substitution		
AJ236855	A	T	Reference	-
Hybrid BC_1 1	A	T	Reference	680.00
Hybrid BC_1 2	A	T	Reference	490.00
Hybrid BC_1 3	A	T	Reference	493.00
Hybrid BC_1 4	A	T	Reference	471.00
Hybrid BC_1 5	A	T	Reference	369.00
Hybrid BC_1 6	A	T	Reference	414.00
Hybrid BC_1 7	A	T	Reference	352.00
Hybrid BC_1 8	A	T	Reference	348.00
Hybrid BC_1 9	A	T	Reference	368.00
Hybrid $BC_1$10	A	T	Reference	338.00
Hybrid BC_1 11	A	T	Reference	349.00
Hybrid BC_1 12	A	T	Reference	357.00
Broiler 1	G	A	1	-
Broiler 2	G	A	1	-
Broiler 3	G	A	1	-
Broiler 4	G	A	1	-
Pelung 1	G	A	1	321.00
Pelung 2	G	A	1	224.00
Pelung 3	G	A	1	231.00

Notes: *A: Adenine; G: Guanine; T: Thymine

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Based on table 2, the exon 6 PIT-1 gene in BC_1 hybrid chickens has the same nucleotide structure as the reference (AJ236855), so that these nucleotide sequences do not make new haplotypes. But Pelung chicken and broiler chicken have 2 SNP located in the coding region. The SNPs consist of 2 substitution points including Adenine to Guanine and Thymine to Adenine. Pelung chicken and broiler chicken form the same nucleotide sequence so that from the 2 SNP it will form 1 same haplotype. Table 3 is the results of the Pearson correlation test used to determine the correlation between the weight of chickens and the points of polymorphism. The results of the study showed that the two points of polymorphism were A928G substitution and T929 substitution. The substitution A928G consisted of GG genotype (mutant phenotype) in Pelung chicken with a 49th day and average weight of 258.7 g, and the AA genotype (wild type phenotype) in BC_1 hybrid chicken with weight average 419.08 g. For the second polymorphism point, T929A consisted of genotype TT (mutant phenotype) in Pelung chicken with an average weight of 49 days to 258.67 g and AA genotype (wild type phenotype) in hybrid chicken BC_1 has weight chicken average 419.08 g. Genotype frequency at A928G substitution point and T929A substitution point have the same value which is 0.5. The correlation coefficient at both points was –0.588. The A928G substitution point and T929A substitution point had a significance value smaller than 0.05, which was 0.021. Based on table 3 it can be described the mutant phenotype at both points affecting the decrease in chicken weight. So that it can be concluded that the relationship between the point of polymorphism and chicken weight was a significantly negative medium correlation. Whereas in a previous study conducted by Jiang et al. (2004) that on MR5 or exon 6 PIT-1 gene there were SNP associated significantly with the phenotypic characters of chickens' growth. A deletion occurred in C nucleotide which caused a change in the amino acid arrangement after the point of mutation, which was caused by a frameshift mutation. As a result of the frameshift mutation, the protein structure changed which was resulted in an error of protein function, or a decrease in protein formation.

Table 3. The correlation test results of Pituitary Positive Transcription Factor-1 gene polymorphism on the chicken mean weight on the 49th day

Polymorphism	**A928G Substitution**		**T929A Substitution**	
Genotype	GG (mutant)	AA (wild type)	AA (mutant)	TT (wild type)
Genotype frequency	0.5	0.5	0.5	0.5
Mean chicken weight on day 49 (g)	258.67	419.08	258.67	419.08
Correlation coefficient (r)	-0.588		-0.588	
Significant level	0.021 (P < 0.05)		0.021 (P < 0.05)	
Conclusion	Significant with moderate negative correlation		Significant with moderate negative correlation	

CONCLUSION

The conclusion showed that first backcross hybrid chickens resulting from a cross between female F_1 broiler chicken and male Pelung chicken body weight was lower with the average weight at the 7th week was 419,08 g compared to F_1 broiler chickens, but higher than the Pelung chickens. There was not exon 6 Pituitary Positive Transcription Factor-1 gene polymorphism found in first backcross hybrid chickens resulting from a cross between female F_1 broiler chickens and male Pelung chickens.

DECLARATIONS

Author`s contributions

D. Retnosari designed the plan of study, collected data and samples, contributed to analyses, and wrote the manuscript. R.Kilatsih and I.S. Maulidi checked the final form of the manuscript. Trijoko revised the research article and facilitating the experimental work. B.S. Daryono helped in designing the plan of study, facilitating the experimental work, providing the experimental tools, revising the research article.

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Competing interests

The authors have not declared any conflict of interest.

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REFERENCES

Arnedo M, Taffé P, Sahli R, Furrer H, Hirschel B, Elzi L, Weber R, Vernazza P, Bernasconi E Darioli R et al. (2007). Contribution of 20 single nucleotide polymorphisms of 13 genes to dyslipidemia associated with antiretroviral therapy. Pharmacogenet Genom,17:755-764. DOI: https://www.doi.org/10.1097/FPC.0b013e32814db8b7.

Butler JM (2009). DNA extraction from forensic samples using Chelex. Cold Spring Harbor Protocols, 4: 6. DOI: https://www.doi.org/10.1101/pdb.prot5229.

Cheng HW (2010). Breeding of tomorrow's chickens to improve wellbeing. Poultry Science, 89: 805-813. DOI: https://www.doi.org/10.3382/ps.2009-00361.

Daryono BS, Roosdianto I and Saragih HTS (2010). Phenotypical characters in hybrids chicken of crossbreeds between pelung and cemani. Jurnal Veteriner, 11 (4): 257-263. Available at: https://ojs.unud.ac.id/index.php/jvet/article/view/3460/2494.

Hidayat C and Asmarasari SA (2015). Native chicken production in Indonesia: a review. Indonesian Journal of Animal Science, 17 (1): 1-11. DOI: https://www.doi.org/10.25077/jpi.17.1.1-11.2015.

Jiang R, Li J, Qu L, Li H and Yang N (2004). A new single nucleotide polymorphism in the chicken pituitary-specific transcription factor (POU1F1) gene associated with growth rate. Animal Genetics, 35: 344-346. DOI: https://www.doi.org/10.1111/j.1365-2052.2004.01164.x.

Miyai S, Yoshimura S, Iwasaki Y, Takekoshi S, Lloyd RV and Osamura R (2005). Induction of GH, PRL, and TSH beta mRNA by transfection of PIT-1 in a human pituitary adenoma-derived cell line. Cell Tissue,322:269-277. DOI: https://www.doi.org/10.1007/s00441-005-0033-z.

Nie Q, Meixia F, Liang X, Min Z, Zhangmin L, Zipin L, Guohuang W, Wensen B, Canjian L, Wei Z et al. (2008). The PIT-1 gene polymorphisms were associated with chicken growth traits. Genetics, 9: 20. DOI: https://www.doi.org/ 10.1186/1471-2156-9-20.

Oktafiantari R (2016). Ghrelin gene polymorphisms encoding growth in chicken [(*Gallus gallus gallus* (Linnaeus, 1758)] second generation of backcross derived crosses between ♀ Pelung with ♂ the first generation of Backcross. Thesis. Faculty of Biology Universitas Gadjah Mada, Indonesia, p. 30.

Roosdianto I (2010). Inheritance of Phenotypic Characters of chickens (F_1) results of crosses of chicken (*Gallus gallus domesticus* Linnaeus, 1758) Pelung with Broiler. Thesis. Faculty of Biology Universitas Gadjah Mada, Indonesia, pp. 24-48.

Sanger F, Nicklen S and Coulson AR (1977). DNA sequencing with chainterminating inhibitors. Proceedings of the National Academy of Sciences of the United States of America, 74 (12): 5463-5467. DOI: https://www.doi.org/10.1073/pnas.74.12.5463.

Sulandari S, Zein MSA, Payanti S, Sartika T, Astuti M, Widyastuti T, Sujana E, Darana S, Setiawan I and Garnida D (2007). Indonesian Local Chicken Biodiversity Resources: Benefits and Potential. Biology Research Center. Bogor, LIPI, Indonesia.

Suryaman (2010). The Comparison of morphometry of native chicken, pelung chicken and first breed chicken (F_1) result from crossbreeding between Pelung and native chickens age 5-12 Weeks. Thesis. Faculty of Animal Science, IPB University, Bogor, Indonesia. Available at: http://repository.ipb.ac.id/handle/123456789/12721.

Van As P, Buys N, Onagbesan OM and Decuypere E (2000). Complementary DNA cloning and ontogenic expression of pituitary-specific transcription factor of chickens (*Gallus domesticus*) from the pituitary gland. General and Comparative Endocrinology, 120: 127–136. DOI: https://www.doi.org/10.1006/gcen.2000.7529.

Yamada S, Hata J and Yamashita S (1993). Molecular cloning of fish Pit-1 cDNA and its functional binding to promoter of gene expressed in the pituitary. Journal of Biological Chemistry, 268(32): 24361-24366. Available at: https://www.jbc.org/content/268/32/24361.long.

Zein MSA and Sulandari S (2009). Pedigree investigation of Indonesian chicken by sequences hypervariable-1 d-loop mitochondrial dna. Jurnal Veteriner, 10(1): 41-49. Available at: http://ojs.unud.ac.id/index.php/jvet/article/view/3345.

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