Effects of Addition of Melatonin and L-Arginine on Cooled Semen Parameter of Iraqi Local Breed Rams in Vitro

A. A. Omar[1], M. N. Ahmed[2], Audai sabah Asker[3], A. F. Majeed[4], R.M. Awad[5]

[1]Assist. Prof., Coll. of Veterinary Medicine, University of Fallujah, Anbar, Iraq, [2]Assist Prof. Coll. of Applied Science, University of Fallujah, Anbar, Iraq, [3]Assist Prof. Assist Prof. Coll. of Agriculture, University of Anbar, Anbar, Iraq, [4]Prof., [5] Researcher,Coll. of Veterinary Medicine, University of Fallujah, Anbar, Iraq

Abstract

The current study was carried out on six Iraqi breed rams, aged between 2 to 4 years, during the period from Nov. 2018 to the Mar. 2019. Semen samples were collected from each ram with electro ejaculator at the morning, one ejaculate per week over a time of 16 weeks. Pooled semen for all rams were used to evaluate the characteristics of semen to avoid individual variation between the animals**.** The volume and the color offresh semen was recorded directly, then semen was evaluated for mass and individual motility, live/dead percentage and sperm abnormalities. Semen samples were diluted 1:10 with a Tris-based extender, and divided into seven parts (each part 2ml), 0.1µml, 1.0µml & 3.0µml concentration of melatonin were added to the T_1, T_2 and T_3 respectively, L-arginine were added to the T_4, T_5 and T_6 in 0.001µmol, 0.1µmol and 1.0µml concentration respectively while T_7 serve as a control without any addition. All treatments cooled at 4°c. Then semen parameters evaluated. It was concluded from this study that addition of 0.1 µmol melatonin significantly protected ram sperm cell from cold storage that induced negative effects on sperm function. However the addition of L-arginine have no beneficial effect on cooled ram semen.

Keywords: *semen characteristics, melatonin, L-arginine, anti-oxidants materials.*

Introduction

Artificial insemination (A.I) plays an important role in sheep industry. It participates in distribution of Superior genetic materials from a little numbers of rams to a large numbers of ewes [(32)]. These distribution of genetic materials leads to improvement of pure bred ewes [(5)] via increase meat, milk and wool production [(45)]. Mammalian and ram semen seems to be more susceptible and sensitive to oxidative stress resulting from reactive oxygen species (ROS) produced from metabolic activity of cellular components of semen during storage due to a high content of sperm cell membrane of unsaturated fatty acids phosphor lipid [(4, 16, 20, 34)]. Although the oxidation is important for life, but it may cause a harmful effect due to the formation of free radicals that cause a damage to sperm cells [(20)]. The oxidative stress may cause a reduction ın reproductive performance of ram through its effect on characteristics of seminal fluid, due to production of ROS, which have a great role in lipid peroxidation in the sperm membranes with production of fatty acids peroxides that leads to a decrease in sperm motility and reduced their ability of fertilization [(11)]. In order to reduce the effect of these free radicals or prevent their action, antioxidant either natural or synthetic were used [(39, 44)] Arginine is an amino acids of alkaline groups having a positive charge which includes; Lysine and Histadine, acts as antioxidant through its stimulation of Glutathione peroxidase which acts on Hydrogen peroxide (H_2O_2) and organic peroxide which is a site compounds resulted from metabolic activity of the body to water and oxygen ions. This peroxide when left to react with iron or copper ions it produced free radical of Hydroxyl that have a strong reaction [(49).] Arginine metabolism in the body resulted from the effect Arginase enzyme that leads to production of nitric oxide (No), ornithine and urea [(47)]. 1t has been reported by many authors that treatment with arginine effect the male and female reproductive system

Corresponding author:
A. A. Omar
Email:dr.aliabd@uofallujah.edu.iq

through the increase blood supply of genital organ and increase sexual desire [5, 31]. Kaya et al. [25] observed that injection of arginine intramuscular increase sperm motility, sexual desire and accelerate ejaculation in the ram. The mechanism of arginine action act to increase sperm motility might be due to increase synthesis of ATP that supplied sperm with energy [33]. Melatonin (N-acetyl-5-methoxytryptamine) or it may called dark hormone, secreted mainly from pineal gland, but it has been recognized to be synthesized in many other sites [36]. It consists mainly from the amino acid tryptophane, and its receptors present in a numbers of tissues and organs of the body. It control or regulates daily and seasonal harmony of the biological activities in the body and increase sensitivity of leydig cells to interstitial-cell stimulating hormone (ICSH) [9]. Also it controls many serious physiological events, including metabolism, sleeping, circadian rhythms and body temperature stability [12, 28, 43]. Furthermore, it acts as anti-oxidant and anti-apoptotic substance [48] through its ability to remove different types of free radicals such as ROS, H_2O_2, OH^- and activate the production and catalyze of enzymatic antioxidant like; superoxide dismutase (SOD), Glutathione peroxidase (GOX) and Catalase (CAT) [27, 37, 39]. Melatonin have the ability of enhancing body immunity [19]. And has been used to boost the maturation of ova and to improve embryo development in many species, sheep, cattle and buffalo [2, 30, 46]. Deng et al. [13] reported that treatment of Rams with melatonin increase the testosterone levels in the interstitial cells of the testes through increase Insulin like growth factor from sertoli cells which regulates synthesis and secretion of testosterone due to presence of melatonin receptors on sertoli cells membranes [15]. It has been observed that addition of antioxidant such as melatonin or L-arginine protect sperm cells form harmful effect of ROS and improve sperm activity during its storage in unfrozen state [1, 6, 29]. The objective of the Current study was to investigate the effect of addition melatonin and L-arginine Tris extender on cooled ram Semen in vitro.

Materials and Methods

The current study was carried out on six Iraqi local breed rams aged between 2-4 years, Presented in the farm of college of veterinary medicine, University of Fallujah Al-Anbar province during the period from November 2018 to the March 2019. The animals were fed alfalfa and hay the water was given in a free choice. All animals were healthy and treated for internal and external parasite and vaccinated with Co-Baghdad and Pox. Semen samples were collected from each ram with electro ejaculator (Electro jac5/a neogen company, U.S.A.) at the morning one ejaculate per week over a time of 16 weeks (sixteen samples program). Samples were taken and put it in a water bath at 38°C. Pooled semen for all rams were used to evaluate the characteristics of semen to avoid individual variation between the animals. 1ml of semen sample was taken to determine its parameters. Volume of the semen was directly measured by reading of graduated marks of collecting tubes. Color of semen has been visually evaluated according to Salisbury et al.[40]. Mass motility has been done by putting a drop of fresh undiluted semen on a warm slide at 36°C and examined under light microscope supplied with heat stage at 100× magnification. Estimate the swirl grade according to Chenoveth et al.[10], the grades include Rapid swirl (very good), slower swirl (good), general oscillation (fair), sporadic oscillation (poor). One drop of fresh semen plus one drop of sodium citrate were taken to estimate the individual motility. Two smears of semen stained with eosin-nigrosin, were prepared [8] and used to determine the percent of dead/ alive and morphological abnormal spermatozoa (primary and secondary) according to Bielański et al. [7]. Sperm concentration was calculated with hemocytometer chamber [40]. Semen samples were diluted 1:10 with a Tris-based extender according to concentration of spermatozoa (Tris: 24.2gm, citric acid: 13.4gm, fructose: 10gm, glycerin: 64^{ml}, egg yolk: 192^{ml}, distal water up to one liter) described by Eidan et al. [17]. Diluted semen was taken and divided into seven parts (each part 2ml), 0.1µml, 1.0µml & 3.0µml concentration of melatonin were added to the T1, T2, T3 respectively, L-arginine were added to the T4, T5 and T6 in 0.001µmol, 0.1µmol and 1.0µml concentration respectively while T7 serve as a control without any addition. All treatments cooled gradually via addition a piece of ice until it reaches 4°c within 2h. Semen parameters evaluated after dilution and cooling. Statistical analysis was applied using Tuckey's - W procedure and chi-square test according to Steel and Torrie [42].

Resultand Discussion

The Characteristics of fresh semen of local Iraqi

rams in non-breeding season are shown in table:1. There were no statistical difference (P≤0.05) in semen parameters between rams in different ejaculates of the same ram. These results indicates that the semen is of good quality even when it collected in non-breed

Table: 2 show semen parameters after diluted with Tris based extender with addition of melatonin and L-arginine in different concentration (0.1, 1.0, 3.0) µmol and (0.001, 0.1, 1.0) µmol respectively. There were a statistical differences (P≤0.05) in individual motility %, dead/ alive% and sperm abnormalities between 0.1µmol melatonin concentrations as compared with L-arginine or control treated semen. Similar observation has been reported by many investigators [6. 23, 26]. The addition of antioxidant such as melatonin to ram semen [6] has been shown to protect sperm against harmful effects of reactive oxygen species (ROS) and improve sperm motility during sperm liquid storage or in unfrozen state.

Table: 3 showed the effect of addition of melatonin and L-arginine after cooling on the percentage of dead/ alive sperm, which showed a statistical difference (P≤0.05) between different treated semen. In the current study it observed that melatonin addition to the diluent in vitro enhanced the cooled preservation capacity of ram sperm in a dose of 0.1µmol and efficiently maintained sperm motility and other parameters over 48h of cooled storage. Melatonin is an indole derivative secreted rhythmically from pineal gland, plays an important role in the reproductive functions in mammals [37]. Melatonin presents in seminal plasma, having multiple actions on different physiological process, as its metabolites are indirect anti-oxidants and powerful direct scavargers that protected sperm cells from free radicals raised by their metabolism [3, 24]. Melatonin also modulating the glutathione activity to improve mitochondrial health state and functions [18]. L-arginine is found to effect the reproductive process. O'Flaherty et al. [35] determined that L-arginine has a protective effect on spermatozoa against the sperm plasma membrane lipid peroxidation and enhances the cell metabolism. It has been reported that low concentration of L-arginine increase sperm motility, whereas high L-arginine concentration decreases sperm motility [21, 22]. In addition, it has been found that nitric oxide synthesized from L-arginine might help or assist to induce acrosome reaction, sperm chemotaxis, and sperm egg interaction [22, 38]. In current study the ineffectiveness of L-arginine on ram semen in vitro might be due to the high dose usage as explained by [21, 22, 29].

Table: 2 show the percentage of individual motility of ram semen after dilution and addition of L-Arginine and Melatonin

Treatment		After dilution	After cooling		
			24 h	48 h	72 h
		Individual motility%			
T1	0.1µmol	71.66 ± 2.80a	60.83 ± 3.51a	51.66 ± 3.07a	45.83 ± 3.27a
T2	1µmol	65.83 ± 2.47a	56.66 ± 2.78a	45 ± 2.23b	36.6 ± 3.07b
T3	3µmol	58.33 ± 2.54b	46.66 ± 2.10b	41.66 ± 2.10b	33.3 ± 3.07b
T4	0.001µmol	56.66 ± 1.92b	41.66 ± 1.66b	39.16 ± 0.83b	30 ± 2.23b
T5	0.1µmol	52.5 ± 1.55b	42.5 ± 1.11b	31.66 ± 1.05c	19.16 ± 2.38c
T6	1.0µmol	40 ± 0c	32.5 ± 1.11c	25.83 ± 0.83c	13.33 ± 2.47c
T7	Control	63.33 ± 3.04b	50 ± 2.23b	45 ± 2.23b	36.66 ± 2.1b
Values: Mean ± SE. Different letter show statistical difference at (P≤0.05).					

Table: 3 show the percentage of dead sperm after dilution and addition of L-Arginine and Melatonin					
Treatments		After dilution	After cooling		
			24 h	48 h	72 h
		Dead %			
T1	0.1µmol	18.66 ± 0.91a	19.5 ± 0.92a	23.33 ± 9.52a	27.33 ± 1.72a
T2	1µmol	21.66 ± 1.22a	23.5 ± 0.92a	26 ± 10.61a	32 ± 1.67b
T3	3µmol	26 ± 0b	22.16 ± 2.16a	26.33 ± 10.75a	36 ± 1.89b
T4	0.001µmol	27 ± 0.44b	31 ± 0b	36 ± 14.69b	42 ± 0c
T5	0.1µmol	31.83 ± 0.9b	34.83 ± 0.74b	38.83 ± 15.85b	54.33 ± 0.21d
T6	1.0µmol	37.5 ± 2.72c	40.33 ± 3.33c	46.83 ± 19.11c	65 ± 0.63d
T7	Control	19 ± 0a	22 ± 0a	29 ±11.83b	35 ± 1.26b
Values: Mean ± SE. Different letter show statistical difference at (P≤0.05).					

The results of table-4 showed that there was a statistical difference (P≤0.05) between different treatments and doses as compared with the control one. The lower percentage of sperm abnormalities were observed in melatonin treatment with different doses especially at 0.1Mmol. Similar observations have been made by [13, 14, 15]. It has been reported that melatonin promotes development of haploid germ cells from early developing spermatogenic cells of sheep under in vitro environment [13] It has been found that melatonin acts as anti-oxidant through their ability to remove various types of free radicals such as ROS, H_2O_2, OH^- and superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) [37]. The result of L-arginine treatments were disagreed with several authors [25, 33, 49]. The ineffective results of L-arginıne might be due the concentration applied or used that decrease sperm motility and increase abnormalities [21, 22].

Conclusion

It was concluded from this study that addition of 0.1µmol melatonin significantly protected ram sperm cell from cold storage that induced negative effects on sperm function. However the addition of L-arginine have no beneficial effect on ram semen.

Table: 4 show the percentage of abnormalities of cooled ram semen after dilution and addition of L-Arginine and Melatonin

Treatments		After dilution	After cooling		
			24 h	48 h	72 h
		Abnormality %			
T1	0.1µmol	6.58 ± 0.42a	7.25 ± 0.25a	8.33 ± 017a	9.67 ± 0.33a
T2	1µmol	8.08 ± 0.42a	9.75 ± 0.25a	10.67 ± 033b	12.58 ± 0.42b
T3	3µmol	9.58 ± 0.42b	10.25 ± 0.25b	12.75 ± 0.25b	15.83 ± 0.67b
T4	0.001µmol	12.66 ± 0.67c	14 ± 1b	19.83 ± 083c	22.33 ± 1.33c
T5	0.1µmol	16 ± 0c	19 ± 0c	21 ± 0c	24 ± 1c
T6	1.0µmol	19.33 ± 0.33d	23.33 ± 033c	32.17 ± 0.17d	34.5 ± 0.5d
T7	Control	14.66 ± 0.33c	18.5 ± 0.5b	21.83 ± 0.17c	30.83 ± 1.78c

Values: Mean ± SE.

Different letter show statistical difference at (P≤0.05).

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq

Conflict of Interest: The authors declare that they have no conflict of interest.

Funding: Self-funding

References

1. Abdulkareem TA, Alzaidi OH. Effect of adding aqueous extract of Melissa officinalis leaves and some other antioxidants to milk–based extender on post-cooling and post-cryopreservative sperm's individual motility and live sperm percentage of Holstein bulls. Al-Anbar Journal of Veterinary Sciences. 2018;11(1):37-53.
2. Abecia JA, Forcada F, Zuniga O. The effect of melatonin on the secretion of progesterone in sheep and on the development of ovine embryos in vitro. Veterinary research communications. 2002 Mar 1;26(2):151-8.
3. Adriaens I, Jacquet P, Cortvrindt R, Janssen K, Smitz J. Melatonin has dose-dependent effects on folliculogenesis, oocyte maturation capacity and steroidogenesis. Toxicology. 2006 Dec 7;228(2-3):333-43.
4. Agarwal A, Makker K, Sharma R. Clinical relevance of oxidative stress in male factor infertility: an update. American journal of reproductive immunology. 2008 Jan;59(1):2-11.
5. Alawiy IK, Mohammed TR, Majeed AF. Effect of

Arginine and Selenium with Vitamin E on WBC and the level of hormones in Iraqi ewes discharged. Al-Anbar Journal of Veterinary Sciences. 2019;12(1):6-12.

6. Ashrafi I, Kohram H, Naijian H, Bahreini M, Poorhamdollah M. Protective effect of melatonin on sperm motility parameters on liquid storage of ram semen at 5C. African Journal of Biotechnology. 2011 Jul 11;10(34):6670-4.
7. Bielański W, Dudek E, Bittmar A, Kosiniak K. Some characteristics of common abnormal forms of spermatozoa in highly fertile stallions. Journal of reproduction and fertility. Supplement. 1982;32:21.
8. Blom E. A one-minute live-dead sperm stain by means of eosin-nigrosin. Fertility and sterility. 1950;1:176-7.
9. Chen YC, Sheen JM, Tiao MM, Tain YL, Huang LT. Roles of melatonin in fetal programming in compromised pregnancies. International journal of molecular sciences. 2013 Mar;14(3):5380-401.
10. Chenoveth PJ. Semen quality assessment. InProceedings of the Applied Reproductive Strategies in Beef Cattle Workshop, Manhattan, KS, USA 2002 Sep 5 (pp. 247-254).
11. Crespilho AM, Nichi M, Guasti PN, Freitas-Dell'Aqua CP, Sá Filho MF, Maziero RR, Dell'Aqua Jr JA, Papa FO. Sperm fertility and viability following 48 h of refrigeration: Evaluation of different extenders for the preservation of bull semen in liquid state. Animal reproduction science. 2014 May 1;146(3-4):126-33.
12. Cruz MH, Leal CL, da Cruz JF, Tan DX, Reiter RJ. Role of melatonin on production and preservation of gametes and embryos: a brief review. Animal reproduction science. 2014 Mar 1;145(3-4):150-60.
13. Deng SL, Chen SR, Wang ZP, Zhang Y, Tang JX, Li J, Wang XX, Cheng JM, Jin C, Li XY, Zhang BL. Melatonin promotes development of haploid germ cells from early developing spermatogenic cells of Suffolk sheep under in vitro condition. Journal of Pineal Research. 2016 May;60(4):435-47.
14. Deng SL, Sun TC, Yu K, Wang ZP, Zhang BL, Zhang Y, Wang XX, Lian ZX, Liu YX. Melatonin reduces oxidative damage and upregulates heat shock protein 90 expression in cryopreserved human semen. Free Radical Biology and Medicine. 2017 Dec 1;113:347-54.
15. Deng SL, Wang ZP, Jin C, Kang XL, Batool A, Zhang Y, Li XY, Wang XX, Chen SR, Chang CS, Cheng CY. Melatonin promotes sheep Leydig cell testosterone secretion in a co-culture with Sertoli cells. Theriogenology. 2018 Jan 15;106:170-7.
16. Du Plessis SS, Makker K, Desai NR, Agarwal A. Impact of oxidative stress on IVF. Expert review of obstetrics & gynecology. 2008 Jul 1;3(4):539-54.
17. Eidan SM. Effect on post-cryopreserved semen characteristics of Holstein bulls of adding combinations of vitamin C and either catalase or reduced glutathione to Tris extender. Animal reproduction science. 2016 Apr 1;167:1-7.
18. El-Raey M, Badr MR, Rawash ZM, Darwish GM. Evidences for the role of melatonin as a protective additive during buffalo semen freezing. American journal of animal and veterinary sciences. 2014;9(4):252-62.
19. Haldar C. Correlation between peripheral melatonin and general immune status of domestic goat, Capra hircus: A seasonal and sex dependent variation. Small ruminant research. 2012 Oct 1;107(2-3):147-56.
20. Hamid AA, Aiyelaagbe OO, Usman LA, Ameen OM, Lawal A. Antioxidants: Its medicinal and pharmacological applications. African Journal of pure and applied chemistry. 2010 Aug 31;4(8):142-51.
21. Hassanpour H, Mirshokrai P, Shirazi A, Aminian A. Effect of nitric oxide on ram sperm motility in vitro. Pakistan journal of biological sciences: PJBS. 2007 Jul 15;10(14):2374.
22. Hassanpour H, Teshfam M, Goodarzi AK, Tajik P, Mirshokraei P. In vitro effects of l-arginine on motion parameters in ram epididymal sperm. Comparative clinical pathology. 2010 Jul 1;19(4):351-5.
23. Jang HY, Kim YH, Kim BW, Park IC, Cheong HT, Kim JT, Park CK, Kong HS, Lee HK, Yang BK. Ameliorative effects of melatonin against hydrogen peroxide□induced oxidative stress on boar sperm characteristics and subsequent in vitro embryo development. Reproduction in domestic animals. 2010 Dec;45(6):943-50.
24. Kang JT, Koo OJ, Kwon DK, Park HJ, Jang G, Kang SK, Lee BC. Effects of melatonin on in vitro maturation of porcine oocyte and expression of melatonin receptor RNA in cumulus and granulosa

cells. Journal of pineal research. 2009 Jan;46(1):22-8.

25. Kaya SO, Gur S, Kaya E. The effect of l-arginine on erection time, sperm quality and the seminal plasma arginase activity in rams. Revue De Medecine Veterinaire. 2019 Jan 1;170(4–6):73-9.

26. Khalifa MA. International Journal of Animal Research (ISSN: 2575-7822) Effect of supplementing ram semen extender with melatonin on oxidative stress indices and physical properties of chilled spermatozoa. Journal of Animal Research. 2017;1:14.

27. Li Y, Zhang Z, He C, Zhu K, Xu Z, Ma T, Tao J, Liu G. Melatonin protects porcine oocyte in vitro maturation from heat stress. Journal of pineal research. 2015 Oct;59(3):365-75.

28. Lincoln GA, Clarke IJ, Hut RA, Hazlerigg DG. Characterizing a mammalian circannual pacemaker. Science. 2006 Dec 22;314(5807):1941-4.

29. Maidin MS, Adanan NF, Aminudin MT, Tawang A. In vitro supplements improves motility and progressive score of spermatozoa in jermasia goats. APCBEE procedia. 2014 Jan 1;8:329-33.

30. Marthol H, Hilz MJ. Weibliche sexuelle Funktionsstörungen: Klassifikation, Diagnostik und Therapie. Fortschritte der Neurologie· Psychiatrie. 2004 Mar;72(03):121-35.

31. Maxwell WM, Watson PF. Recent progress in the preservation of ram semen. Animal Reproduction Science. 1996 Apr 1;42(1-4):55-65.

32. Manjunatha BM, Devaraj M, Gupta PS, Ravindra JP, Nandi S. Effect of taurine and melatonin in the culture medium on buffalo in vitro embryo development. Reproduction in Domestic Animals. 2009 Feb;44(1):12-6.

33. Medeiros CM, Forell F, Oliveira AT, Rodrigues JL. Current status of sperm cryopreservation: why isn't it better?. Theriogenology. 2002 Jan 1;57(1):327-44.

34. Mohammed OA, Abdulkareem TA, Ibrahim FF, Al-Zaidi OH, Latif WE, Alwan SH. EFFECT OF ADDING PENTOXIFYLLINE AND NITRIC OXIDE TO TRIS EXTENDER ON SOME POST-CRYOPRESERVED SEMEN ATTRIBUTES OF HOLSTEIN BULLS. The Iraqi Journal of Agricultural Science. 2020;51(2):619-28.

35. O'Flaherty C, Rodriguez P, Srivastava S. L-arginine promotes capacitation and acrosome reaction in cryopreserved bovine spermatozoa. Biochimica et Biophysica Acta (BBA)-General Subjects. 2004 Sep 24;1674(2):215-21.

36. Omar SM, Mohammed BM. ANTIGENOTOXIC EFFECTS OF MELATONIN AGAINST CHROMOSOME DAMAGE INDUCED BY 7, 12-DYMETHYLBENZ (a) ANTHRACENE. The Iraqi Journal of Agricultural Science. 2020;51(3):916-23.

37. Reiter RJ, Tan DX, Osuna C, Gitto E. Actions of melatonin in the reduction of oxidative stress. Journal of biomedical science. 2000;7(6):444-58.

38. Revelli A, Costamagna C, Moffa F, Aldieri E, Ochetti S, Bosia A, Massobrio M, Lindblom B, Ghigo D. Signaling pathway of nitric oxide-induced acrosome reaction in human spermatozoa. Biology of reproduction. 2001 Jun 1;64(6):1708-12.

39. Rodriguez C, Mayo JC, Sainz RM, Antolín I, Herrera F, Martín V, Reiter RJ. Regulation of antioxidant enzymes: a significant role for melatonin. Journal of pineal research. 2004 Jan;36(1):1-9.

40. Salisbury GW, VanDemark NL, Lodge JR. Physiology of reproduction and artificial insemination of cattle. WH Freeman and Company.; 1978.

41. Stanner SA, Hughes J, Kelly CN, Buttriss J. A review of the epidemiological evidence for the 'antioxidant hypothesis'. Public health nutrition. 2004 May;7(3):407-22.

42. d Steel RG, Torrie JH. Principles and procedures of statistics: a biometrical approach. McGraw-Hill; 1986.

43. Tian X, Wang F, He C, Zhang L, Tan D, Reiter RJ, Xu J, Ji P, Liu G. Beneficial effects of melatonin on bovine oocytes maturation: a mechanistic approach. Journal of pineal research. 2014 Oct;57(3):239-47.

44. Tomaino A, Cimino F, Zimbalatti V, Venuti V, Sulfaro V, De Pasquale A, Saija A. Influence of heating on antioxidant activity and the chemical composition of some spice essential oils. Food chemistry. 2005 Mar 1;89(4):549-54.

45. Unal NE, Akcapinar HA, Atasoy FA, Aytac ME. Some reproductive and growth traits of crossbred genotypes produced by crossing local sheep breeds of Kivircik x White Karaman and Chios x White Karaman in steppe conditions. Archives Animal Breeding. 2006 Oct 10;49(1):55-63.

46. Wu Z, Hou Y, Hu S, Bazer FW, Meininger CJ,

McNeal CJ, Wu G. Catabolism and safety of supplemental L-arginine in animals. Amino acids. 2016 Jul 1;48(7):1541-52.

47. Zhao XM, Hao HS, Du WH, Zhao SJ, Wang HY, Wang N, Wang D, Liu Y, Qin T, Zhu HB. Melatonin inhibits apoptosis and improves the developmental potential of vitrified bovine oocytes. Journal of pineal research. 2016 Mar;60(2):132-41.

48. Wang F, Tian X, Zhou Y, Tan D, Zhu S, Dai Y, Liu G. Melatonin improves the quality of in vitro produced (IVP) bovine embryos: implications for blastocyst development, cryotolerance, and modifications of relevant gene expression. PloS one. 2014 Apr 2;9(4):e93641.

49. Zheng P, Song Y, Tian Y, Zhang H, Yu B, He J, Mao X, Yu J, Luo Y, Luo J, Huang Z. Dietary arginine supplementation affects intestinal function by enhancing antioxidant capacity of a nitric oxide–independent pathway in low-birth-weight piglets. The Journal of nutrition. 2018 Nov 1;148(11):1751-9.