Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

JEZS Journal of Entomology and Zoology Studies

E-ISSN: 2320-7078
P-ISSN: 2349-6800
www.entomoljournal.com
JEZS 2020; 8(3): 175-180
© 2020 JEZS
Received: 17-03-2020
Accepted: 19-04-2020

Trilok Gocher
Department of Veterinary Gynaecology and Obstetrics, College of Veterinary and Animal Science Bikaner, RAJUVAS Bikaner, Rajasthan, India

Govind Narayan Purohit
Department of Veterinary Gynaecology and Obstetrics, College of Veterinary and Animal Science Bikaner, RAJUVAS Bikaner, Rajasthan, India

Sasi G
Department of Veterinary Gynaecology and Obstetrics, College of Veterinary and Animal Science Bikaner, RAJUVAS Bikaner, Rajasthan, India

Corresponding Author:
Trilok Gocher
Department of Veterinary Gynaecology and Obstetrics, College of Veterinary and Animal Science Bikaner, RAJUVAS Bikaner, Rajasthan, India

The effect of supplementation of different antioxidants during maturation of caprine oocytes *in vitro*

Trilok Gocher, Govind Narayan Purohit and Sasi G

Abstract
This study was carried out to compare the effect of supplementing maturation medium with different antioxidants on the *in vitro* maturation of immature oocytes. In order to evaluate the effects of antioxidants culturable grade oocytes (total 528) were matured *in vitro* in TCM-199 (with 20 IU/ml eCG, 20 IU/ml hCG, 1 µg/ml estradiol, 25 mM Hepes, 0.25 mM pyruvate and antibiotics) that was randomly supplemented with either 10 µg/ml of melatonin (MLT), 50 µg/ml of L-ascorbic acid (LAA), 1mM taurine (TAU) or without any antioxidants (Control) for 28 hours. On completion of *in vitro* maturation the oocytes were evaluated for cumulus expansion and nuclear maturation. The best cumulus expansion (+++) was achieved in TCM-199 medium supplemented with MLT followed by LAA supplementation. After evaluation of cumulus expansion, the same oocytes were freed of cumulus cells, fixed, stained and assessed for nuclear maturation. Significantly higher ($P<0.05$) proportions of oocytes were matured *in vitro* in medium supplemented with MLT compared to medium supplemented with LAA, TAU and control. It was concluded that enriching the maturation medium with melatonin or L-ascorbic acid improves the cumulus cell expansion of *in vitro* matured goat oocytes and melatonin also improves the nuclear maturation.

Keywords: Goat, *in vitro* maturation, oocytes, melatonin, L-ascorbic acid, taurine

Introduction
During recent years there has been an increasing interest in large scale *in vitro* production of goat embryos through *in vitro* maturation, *in vitro* fertilization and *in vitro* culture of oocytes for faster multiplication of superior germplasm (Rahman *et al*., 2008) [45]. The techniques of *in vitro* maturation (IVM) of follicular oocytes, their fertilization with *in vitro* capacitated spermatozoa and the *in vitro* culture of the resulting embryos have been successfully established for goat (De Smedt *et al*., 1992; Keskintepe *et al*., 1994) [15, 26].
The medium for *in vitro* culture of oocytes requires the supplementation of gonadotrophins, growth factors and other substances however; the *in vitro* maturation rates have been modest because of the biochemical state of oocytes, and interactions between the oocytes and cumulus cells (Canipari, 2000; Khazaei and Aghaz, 2017) [11, 27].
Multiple factors likely contribute to the overall poor quality of *in vitro* maturation of oocytes. One of the important factors may be the oxidative stress (OS). The generation of pro-oxidants such as reactive oxygen species (ROS) is an invariable phenomenon in the culture conditions. It is possible that OS also influences oocyte development *in vitro*. On the other hand, ROS are considered signal molecules in oocyte physiology and their impact on maturation promoting factor (MPF) destabilization has recently been reported (Premkumar and Chaube, 2016; Tiwari *et al*., 2016; Khazaei and Aghaz, 2017) [43, 58, 27].
OS is caused by an imbalance between pro-oxidants and antioxidants (Al-Gubory *et al*., 2010) [4]. This ratio could change with increased levels of pro-oxidants, such as ROS, or a decrease in antioxidant defense mechanisms (Ruder *et al*., 2009; Burton and Jauniaux, 2011) [47, 10]. ROS represents a wide class of molecules that indicate the collection of free radicals (hydroxyl ion, superoxide, etc.), non-radicals (ozone, single oxygen, lipid peroxides, hydrogen peroxide) and oxygen derivatives (Agarwal and Prabakaran, 2005) [1]. They are highly reactive and unstable. Hence, ROS can react with nucleic acids, lipids, proteins, and carbohydrates to acquire an electron and become stable. These reactions induce a cascade of subsequent chain reactions that eventually result in cell damage (Attaran *et al.,* 2000; Szczepanska *et al*., 2003) [6, 52]. ROS can diffuse and pass through cell membranes and alter most types of cellular molecules

(nucleic acids, proteins, and lipids), leading to mitochondrial alterations (Kowaltowski and Vercesi, 1999) [28], meiotic arrest in the oocytes (Nakamura *et al*., 2002) [40], embryonic block, and cell death (Hashimoto *et al*., 2000) [23].

Oxidative stress is known to have a negative effect on *in vitro* maturation and embryonic development of oocytes (Guerin *et al*., 2001; Matos De *et al*., 2002; Ozturkler *et al*., 2010) [19][36][42]. Various studies have been conducted to measure the effects of antioxidants on oocyte maturation and early embryo development (Matos De and Furnus, 2000; Cetica *et al*., 2001; Guerin *et al*., 2001; Tarin *et al*., 2002) [35, 12, 19, 55]. Antioxidants scavenge ROS, which helps maintain the cell oxidant/antioxidant balance. On the other hand, antioxidants are the compounds which either suppress the formation of ROS or oppose their actions (Khazaei and Aghaz, 2017) [27].

The effects of antioxidant supplementation to IVM media have been studied in various mammalian species (Deleuze and Goudet, 2010; Aghaz *et al*., 2015; Rodrigues-Cunha *et al*., 2016) [16, 2, 46]. However, it is not yet clear which antioxidant is the most efficient to support the development and quality of caprine embryos. The present study examined the effect of three different antioxidants on *in vitro* maturation of goat oocytes.

Materials and methods

Collection of ovaries

Ovaries were collected from a local abattoir (during August 2019 to December 2019) in sterile Normal Saline Solution (NSS 0.85%) supplemented with antibiotics (Penicillin 100 IU/ml, Streptomycin 50 µg/ml) at 30-35º C in an iso-thermic container and transported to the laboratory within 2-7 hours of slaughter.

In the laboratory, the surrounding tissues were trimmed off and the ovaries were washed with sterile NSS. The ovaries were then exposed to 70% ethyl alcohol for 30 seconds and finally washed in modified DPBS.

Oocyte collection

Oocytes from apparently non-atretic surface follicles of goat ovaries were collected by aspirating the follicle with a 18-20 gauge needle attached to a 5 c.c. disposable syringe in a sterilized petridish containing the oocyte collection medium as described previously (Nagar and Purohit, 2005)[39]. The oocytes surrounded by a compact cumulus mass with an evenly granulated cytoplasm were selected under a stereomicroscope (Olympus, Japan) and washed 5-6 times in a oocyte collection media followed by 3 washing in washing media (TCM 199, buffered with Hepes 25mM plus Pyruvate 0.25 mM and antibiotics, pH 7.2-7.4).

In vitro maturation

The COC's were randomly divided into four groups of approximately equal number of oocytes (10 replicates in each group with 10-15 oocytes in each replicate) and cultured in TCM-199 media with or without addition of different antioxidants:-

Control: The COC's were cultured in TCM-199 supplemented with 20 IU/ml eCG (Folligon®, MSD Animal Health), 20 IU/ml hCG (Chorulon®, MSD Animal Health) and 1 µg/ml estradiol (Hi-media), 25 mM Hepes, 0.25 mM Pyruvate and antibiotics in 50-100 µl maturation media for 28 hrs at 38±1º C and 5% CO_2 in humidified air in a CO_2 incubator.

MLT Group: The COC's were matured as per Control group with addition of melatonin (10 µg/ml) in the maturation media.

LAA Group: The COC's were matured as per Control group with addition of L- ascorbic acid (50 µg/ml) in the maturation media.

TAU Group: The COC's were matured as per Control group with addition of taurine (1mM) in the maturation media.

Evaluation of oocyte maturation:

On completion of *in vitro* maturation all the oocytes were assessed for a subjective cumulus expansion and then they were freed of cumulus mass, fixed, stained and evaluated for nuclear maturation.

The cumulus cell expansion was assessed as described previously (Lorenzo *et al*., 1994; Kumar and Purohit, 2004) [32, 29]. Briefly, a subjective scoring system in which: 0 indicated no detectable response; + indicating minimum observable response and +++ indicated the maximum degree of expansion, where all the layers of cumulus cells expand, even those closest to the oocyte. The number and proportion of oocytes with different degree of cumulus expansion were recorded after 28 hours of *in vitro* maturation.

The nuclear maturation of oocytes was evaluated as per Kumar and Purohit (2004) [29]. Additionally, after 28 hrs of maturation, all oocytes from different groups were collected for staining. The surrounding cumulus cells were removed by vortexing for 1 minute or pipeting with a fine capillary tube or keeping them in TCM-199 with hyaluronidase (0.3%). The oocytes were placed in the center of an area delineated by two paraffin wax bars on a clean grease free glass slide. The denuded oocytes were compressed gently with a cover slip to hold and were fixed for 24 hrs in acetic acid and methanol [1:3(v:v)] and stained with 1% aceto-orcein or 2% Giemsa's stain for evaluation of nuclear status. The different stages of meiotic maturation were classified as follows:

Germinal Vesicle (G.V.): Oocytes with distinct nuclear envelops and chromatin around the nucleus were present. Individual filaments or bivalent were not visible.

Metaphase-I (M-I): The chromosomes were maximally condensed and present as cluster (polar view of thin line: equatorial view).

Ana Telophase-I (AT-I): The chromosomes were under division or segregation and spindle were not yet detached. This includes all the stages between beginning of chromosomal separation (Anaphase) and cytokinesis (Formation of 1st polar body).

Metaphase-II (M-II): A reduced number of chromatin and 1st polar body (if available) were present.

Statistical analysis

The percentage values were subjected to arcsine transformation before applying statistical analysis. The data on cumulus expansion and nuclear maturation were analysed by a one way ANOVA to verify differences, between the treatment groups and control, within treatment groups, followed by the Duncan multiple range test between group mean. The significance of difference between the mean values was determined at $P<0.05$. Results are expressed as mean ± SEM.

Results

Cumulus expansion

Cumulus expansion evaluation of oocytes revealed that the mean number and proportion of oocytes that showed maximum expansion (+++) was highest in MLT group followed by that in LAA, TAU and control groups (Table 1 and 2). Significantly higher ($P<0.05$) (+++) cumulus expansion was shown in MLT treated and LAA groups compared to control. Addition of taurine did not show significant difference in +++ degree cumulus expansion compared to control. The respective mean number and proportion of oocytes that showed + and no expansion was significantly lower in the MLT group only whereas the oocytes showing ++ expansion was not different between any of the groups.

Table 1: Cumulus expansion stages of *in vitro* matured goat oocytes

Treatment	No. of replicates	Total number of oocytes matured	Cumulus Expansion			
			0	+	++	+++
Control	10	125	24	32	37	32
MLT	10	136	9	11	32	84
LAA	10	135	18	21	38	58
TAU	10	132	19	29	36	48
Total		528	70	93	143	222

Table 2: Mean and proportion of stages of cumulus expansion of goat oocytes matured *in vitro*

Treatment	0	+	++	+++
Control	$2.4^{b} \pm 0.34$ (19.20%)	$3.2^{b} \pm 0.249$ (25.60%)	3.7 ± 0.26 (29.60%)	$3.2^{a} \pm 0.416$ (25.60%)
MLT	$0.9^{a} \pm 0.233$ (6.62%)	$1.1^{a} \pm 0.277$ (8.09%)	3.2 ± 0.389 (23.53%)	$8.4^{c} \pm 0.686$ (61.76%)
LAA	$1.8^{ab} \pm 0.249$ (13.33%)	$2.1^{ab} \pm 0.277$ (15.56%)	3.8 ± 0.389 (28.15%)	$5.8^{b} \pm 0.646$ (42.96%)
TAU	$1.9^{ab} \pm 0.277$ (14.39%)	$2.9^{b} \pm 0.407$ (21.97%)	3.6 ± 0.306 (27.27%)	$4.8^{ab} \pm 0.533$ (36.36%)

Mean values within the same column with different superscript letters differ significantly ($P<0.05$)

Nuclear maturation

Significantly higher ($P<0.05$) mean number and proportion of oocytes matured *in vitro* (reached metaphase-II) in the MLT treated medium compared to untreated control (Table 3 and 4). A similar trend was observed for oocytes reaching metaphase 1. The treatment with LAA and TAU resulted in non-significantly higher mean number and proportion of oocytes maturing after 28 h of *in vitro* culture. The mean number and proportion of oocytes that were arrested at GV or Ana-telophase was not different for the treatment groups and control (Table 3 and 4).

Table 3: Nuclear maturation stages of *in vitro* matured goat oocytes

Treatment	No of replicates	Total number of oocytes matured	Nuclear stages			
			GV	M-I	AT-I	M-II
Control	10	125	29	32	17	47
MLT	10	136	25	13	14	84
LAA	10	135	25	22	21	67
TAU	10	132	28	26	20	58
Total		528	107	93	72	256

Table 4: Mean and proportion of stages nuclear maturation of goat oocytes matured *in vitro*

Treatment	GV	M-I	AT-I	M-II
Control	2.9 ± 0.433 (23.20%)	$3.2^{b}\pm0.359$ (25.60%)	1.7 ± 0.3 (13.60%)	$4.7^{a}\pm0.26$ (37.60%)
MLT	2.5 ± 0.269 (18.38%)	$1.3^{a}\pm0.3$ (9.56%)	1.4 ± 0.306 (10.29%)	$8.4^{b}\pm0.67$ (61.76%)
LAA	2.5 ± 0.342 (18.52%)	$2.2^{ab}\pm0.533$ (16.30%)	2.1 ± 0.277 (15.55%)	$6.7^{ab}\pm0.616$ (49.63%)
TAU	2.8 ± 0.291 (21.21%)	$2.6^{ab}\pm0.427$ (19.70%)	2.0 ± 0.258 (15.15%)	$5.8^{a}\pm0.416$ (43.94%)

Mean values within the same column with different superscript letters differ significantly ($P<0.05$)

Discussion

Development of improved procedures for *in vitro* maturation of goat oocytes has applications for *in vitro* embryo production and accompanying strategies for genetic improvement (Lv *et al*., 2010) [33]. Mammalian cumulus cells play a very important role during oocyte growth and maturation. They are known to supply nutrients (Eppig, 1982; Haghighat and Van Winkle, 1990; Laurincık *et al*., 1992) [18, 22, 30] and/or messenger molecules for oocyte development (Lawrence *et al*., 1978; Thibault *et al*., 1987; Buccione *et al*., 1990) [31, 56, 9], and to mediate the effects of hormones on oocytes (Zuelke and Brackett, 1990) [61]. Moreover, cumulus cell expansion is considered an important marker for oocyte maturation (Chen *et al*., 1990; Qian *et al*., 2003) [13, 44] and is essential for fertilization, subsequent cleavage, and blastocyst development (Gutnisky *et al*., 2007) [21].

Our results confirmed that melatonin and L-ascorbic acid supplementation to IVM medium had potentially significant effects on the degree of cumulus cell expansion proven to be optimal for nuclear maturation. The same promoting effects of melatonin on cumulus cell expansion were reported in porcine oocytes (Kang *et al*., 2009b) [25]. Nevertheless, it is not clear whether this enhancing effect was exerted via its receptors or its direct and indirect antioxidant activities. As an antioxidant, melatonin might protect cumulus cells against apoptosis (Sugino *et al*., 2000; Na *et al*., 2005; Taniguchi *et al*., 2009; Kang *et al*., 2009b) [51, 38, 54, 25] and enhance their expansion (El-Raey *et al*., 2011) [17]. The same beneficial

effects of ascorbic acid on cumulus cell expansion were reported by Miclea *et al*. (2011) [37] on porcine oocytes.

During the present study the proportion of oocytes that matured *in vitro* (reached M-II stage) was significantly ($P<0.05$) higher for melatonin supplemented (10 µg/ml) maturation medium compared to taurine supplemented and control group and non significantly higher compared to L-ascorbic acid supplemented medium. The same promoting effects of melatonin on maturation of oocytes were reported in juvenile goats (Soto-Heras *et al*., 2018) [49]. Also, melatonin was reported to enhance meiotic maturation of porcine (Kang *et al*., 2009a) [24], buffalo (Manjunatha *et al*., 2009) [34], bovine (El-Raey *et al*., 2011; Tian *et al*., 2014) [17, 57], ovine (Barros *et al*., 2020) [8] and mouse oocytes *in vitro* (Ahn and Bae, 2004; Na *et al*., 2005) [3, 38]. It is well known that melatonin has a positive antioxidant effect by directly reducing ROS generated during IVM (reviewed by Tamura *et al*., 2013) [53] and activating antioxidant enzymes (catalase, Cu/Zn superoxide dismutase and glutathione peroxidase) by epigenetic regulation of the genome (reviewed by Tomas-Zapico and Coto-Montes, 2005) [59]. Moreover, it avoids the harmful consequences of ROS in the oocytes (Tripathi *et al*., 2011; Banerjee *et al*., 2012; Song *et al*., 2016) [60, 7, 48].

This study demonstrated that supplementation of maturation medium with L-ascorbic acid improved the nuclear maturation rate but non significantly. Similar to our results, Dalvit *et al*. (2005) [14] and Sovernigo *et al*. (2017) [50] working with bovine and Ozturkler *et al*. (2010) [42] with ovine oocytes found that addition to the maturation medium of L-ascorbic acid failed to vary the percentage of meiotic maturation significantly, suggesting that this natural antioxidant exerts less observable effect on the nuclear maturation of oocytes during the course of IVM.

In this study, nuclear maturation rate did not improve significantly by supplementing the maturation medium with 1 mM taurine. Similar to our results, Manjunatha *et al*. (2009) [34] and Lv *et al*. (2010) [33] determined no beneficial effect on maturation rate of oocyte *in vitro* when added taurine and hypotaurine respectively. *In vivo*, hypotaurine and taurine are synthesized and secreted by oviductal epithelial cells (Guerin *et al*., 1995) [20]. Hypotaurine can neutralize hydroxyl radicals and prevent lipid peroxidation (Alvarez and Storey, 1983) [5]. The by-product of hypotaurine, after free radical scavenging, is taurine. Taurine has indirect antioxidant effects: it contributes to limiting the deleterious effects of ROS by neutralizing cytotoxic aldehydes, the end products of the peroxidation cascade reaction (Ogasawara *et al*., 1993) [41]. In this study, supplementing the maturation medium with taurine did not find a positive effect on oocyte maturation. This might be due to the *in vitro* culture system used in the study.

It was concluded that enriching the maturation medium with melatonin or L-ascorbic acid improves the cumulus cell expansion of *in vitro* matured goat oocytes and melatonin also improves the nuclear maturation.

References

1. Agarwal A, Prabakaran SA. Mechanism, measurement and prevention of oxidative stress in male reproductive physiology. Indian J Exp Biol. 2005; 43(11):963-974.
2. Aghaz F, Hajarian H, Shabankareh HK, Abdolmohammadi A. Effect of sericin supplementation in maturation medium on cumulus cell expansion, oocyte nuclear maturation and subsequent embryo development in Sanjabi ewes during the breeding season. Theriogenology. 2015; 84(9):1631-1635.
3. Ahn HJ, Bae IH. Effects of melatonin on the meiotic maturation of mouse oocytes *in vitro*. Korean J Fertil Steril. 2004; 3:155-168.
4. Al-Gubory KH, Fowler PA, Garrel C. The roles of cellular reactive oxygen species, oxidative stress and antioxidants in pregnancy outcomes. Int J Biochem Cell Biol. 2010; 42(10):1634-1650.
5. Alvarez JG, Storey BT. Taurine, hypotaurine, epinephrine and albumin inhibit lipid peroxidation in rabbit spermatozoa and protect against loss of motility. Biol Reprod. 1983; 29:548-555.
6. Attaran M, Pasqualotto E, Falcone T, Goldberg JM, Miller KF, Agarwal A *et al*. The effect of follicular fluid reactive oxygen species on the outcome of *in vitro* fertilization. Int J Fertil Womens Med. 2000; 45(5):314-320.
7. Banerjee J, Maitra D, Diamond MP, Abu-Soud HM. Melatonin prevents hypochlorous acid-induced alterations in microtubule and chromosomal structure in metaphase-II mouse oocytes. J Pineal Res. 2012; 53:122-128.
8. Barros VRP, Monte APO, Santos JMS, Lins TLBG, Cavalcante AYP, Gouveia BB *et al*. Effects of melatonin on the *in vitro* growth of early natral follicles and maturation of ovine oocytes. Domest Anim Endocrinol. 2020; 71:106386.
9. Buccione R, Schroeder AC, Eppig JJ. Interactions between somatic cells and germ cells throughout mammalian oogenesis. Biol Reprod. 1990; 43:543-547.
10. Burton GJ, Jauniaux E. Oxidative stress. Best Pract Res Clin Obstet Gynaecol. 2011; 25(3):287-299.
11. Canipari R. Oocyte-granulosa cell interactions. Hum Reprod Update. 2000; 6(3):279-289.
12. Cetica PD, Pintos LN, Dalvit GC, Beconi MT. Antioxidant enzyme activity and oxidative stress in bovine oocyte *in vitro* maturation. IUBMB Life. 2001; 51:57-64.
13. Chen L, Wert SE, Hendrix EM, Russell PT, Cannon M, Larsen WJ. Hyaluronic acid synthesis and gap junction endocytosis are necessary for normal expansion of the cumulus mass. Mol Reprod Dev. 1990; 26:236-247.
14. Dalvit G, Llanes SP, Descalzo A, Insani M, Beconi M, Cetica P. Effect of alpha-tocopherol and ascorbic acid on bovine oocytes *in vitro* maturation. Reprod Domest Anim. 2005; 40:93-97.
15. De Smedt V, Crozet N, Ahmed-Ali M, Martino A, Cognie Y. *In vitro* maturation and fertilization of goat oocytes. Theriogenology. 1992; 37(5):1049-1060.
16. Deleuze S, Goudet G. Cysteamine supplementation of *in vitro* maturation media: a review. Reprod Domest Anim. 2010; 45(6):e476-482.
17. El-Raey M, Geshi M, Somfai T, Kaneda M, Hirako M, Abdel-Ghaffar AE *et al*. Evidence of melatonin synthesis in the cumulus oocyte complexes and its role in enhancing oocyte maturation *in vitro* in cattle. Mol Reprod Dev. 2011; 78:250-262.
18. Eppig JJ. The relationship between cumulus cell-oocyte coupling, oocyte meiotic maturation, and cumulus expansion. Dev Biol. 1982; 89:268-272.
19. Guerin P, El-Mouatassim S, Menezo Y. Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. Hum Reprod Update. 2001; 7:175-189.

20. Guerin P, Tappaz M, Guillard J, Menezo Y. Mise en evidence de la cysteine sultanate decarboxylase (EC 4.1.1.129) dans les cellules epitheliales tubaires de vache et de chevre en culture. C R Acad Sci. 1995; 318:523-528.
21. Gutnisky C, Dalvit GC, Pintos LN, Thompson JG, Beconi MT, Cetica PD. Influence of hyaluronic acid synthesis and cumulus mucification on bovine oocyte *in vitro* maturation, fertilization and embryo development. Reprod Fertil Dev. 2007; 19:488-497.
22. Haghighat N, Van Winkle LJ. Developmental change in follicular cell enhanced amino acid uptake into mouse oocytes that depends on intact gap junctions and transport system. Gly J Exp Zool. 1990; 253:71-82.
23. Hashimoto S, Minami N, Yamada M, Imai H. Excessive concentration of glucose during *in vitro* maturation impairs the developmental competence of bovine oocytes after *in vitro* fertilization: relevance to intracellular reactive oxygen species and glutathione contents. Mol Reprod Dev. 2000; 56(4):520-526.
24. Kang JT, Koo OJ, Kwon DK, Park HJ, Jang G, Kang SK *et al*. Effects of melatonin on *in vitro* maturation of porcine oocyte and expression of melatonin receptor RNA in cumulus and granulose cells. J Pineal Res. 2009a; 46:22-28.
25. Kang JT, Koo OJ, Kwon DK, Park SJ, Atikuzzaman M, Gomez N *et al*. Effects of melatonin on preimplantation development of porcine parthenogenetic embryos. Reprod Fertil Dev. 2009b; 22:327-328.
26. Keskintepe L, Darwish GM, Kenimer AT, Brackett BG. Term development of caprine embryos derived from immature oocytes *in vitro*. Theriogenology. 1994; 42(3):527-535.
27. Khazaei M, Aghaz F. Reactive oxygen species generation and use of antioxidants during *in vitro* maturation of oocytes. Int J Fertil Steril. 2017; 11(2):63-70.
28. Kowaltowski AJ, Vercesi AE. Mitochondrial damage induced by conditions of oxidative stress. Free Radic Biol Med. 1999; 26(3-4):463-471.
29. Kumar D, Purohit GN. Effect of epidermal and insulin-like growth factor-1 on cumulus expansion, nuclear maturation and fertilization of buffalo cumulus oocyte complexes in simple serum free media DMEM and Ham's F-10. Vet Arhiv. 2004; 74:13-25.
30. Laurincık J, Kroslak P, Hyttel P, Pivko J, Sirotkin AV. Bovine cumulus expansion and corona-oocyte disconnection during culture *in vitro*. Reprod Nutr Dev. 1992; 32:151-161.
31. Lawrence TH, Beers WH, Guila NB. Transmission of hormonal stimulation by cell-to-cell communication. Nature. 1978; 272:501-506.
32. Lorenzo PL, Lorenzo MJ, Illera JC, Illera M. Enhancement of cumulus expansion and nuclear maturation during bovine oocyte maturation *in vitro* by the addition of epidermal growth factors and insulin-like growth factors. J Reprod Fert. 1994; 101:697-701.
33. Lv L, Yue W, Liu W, Ren Y, Li F, Lee KB *et al*. Effect of oocyte selection, estradiol and antioxidant treatment on *in vitro* maturation of oocytes collected from prepubertal Boer goats. Italian J Anim Sci. 2010; 9(1):e11.
34. Manjunatha BM, Devaraj M, Gupta PSP, Ravindra JP, Nandi S. Effect of taurine and melatonin in the culture medium on buffalo *in vitro* embryo development. Reprod Domest Anim. 2009; 44:12-16.
35. Matos De DG, Furnus CC. The importance of having high glutathione level after bovine *in vitro* maturation on embryo development effect of beta-mercaptoethanol, cysteine and cystine. Theriogenology. 2000; 53:761-771.
36. Matos De DG, Gasparrini B, Pasqualini SR, Thompson JG. Effect of glutathione synthesis stimulation during *in vitro* maturation of ovine oocytes on embryo development and intracellular peroxide content. Theriogenology. 2002; 57:1443-1451.
37. Miclea I, Pacala N, Zahan M, Hettig A, Roman I, Miclea V. Influence of alpha-tocopherol and ascorbic acid on swine oocyte viability and maturation. Bull UASVM Anim Sci Biotech. 2011; 68:338-345.
38. Na K, Kim J, Lee J, Yoon T, Cha K, Lee D. Effect of melatonin on the maturation of mouse GV oocytes and apoptosis of cumulus cells *in vitro*. Fertil Steril. 2005; 84:103Abst.
39. Nagar D, Purohit GN. Effect of epidermal growth factor on maturation and fertilization *in vitro* of goat follicular oocytes in a serum free or serum supplemented medium. Vet Arhiv. 2005; 75(6):459-467.
40. Nakamura Y, Yamagata Y, Sugino N, Takayama H, Kato H. Nitric oxide inhibits oocyte meiotic maturation. Biol Reprod. 2002; 67(5):1588-1592.
41. Ogasawara M, Nakamura T, Koyama I, Nemoto M, Yoshida T. Reactivity of taurine with aldehydes and its physiological role. Chem Pharm Bull. 1993; 41:2172-2175.
42. Ozturkler Y, Yildiz S, Gungor O, Pancarci SM, Kacar C, Ari UC. The effect of L-Ergothioneine and L-Ascorbic Acid on the *in vitro* maturation (IVM) and embryonic development (IVC) of sheep oocytes. Kafkas Univ Vet Fak Derg. 2010; 16(5):757-763.
43. Premkumar KV, Chaube SK. Increased level of reactive oxygen species persuades postovulatory aging-mediated spontaneous egg activation in rat eggs cultured *in vitro*. *In vitro* Cell Dev Biol Anim. 2016; 52(5):576-588.
44. Qian Y, Shi WQ, Ding JT, Sha JH, Fan BQ. Predictive value of the area of expanded cumulus mass on development of porcine oocytes matured and fertilized *in vitro*. J Reprod Dev. 2003; 49:167-174.
45. Rahman ANMA, Abdullah RB, Wan-Khadijah WE. *In vitro* maturation of oocytes with special reference to goat: A review. Biotechnol. 2008; 7:599-611.
46. Rodrigues-Cunha MC, Mesquita LG, Bressan F, Collado MD, Balieiro JC, Schwarz KR. Effect of melatonin during IVM in defined medium on oocyte meiosis, oxidative stress and subsequent embryo development. Theriogenology. 2016; 86(7):1685-1694.
47. Ruder EH, Hartman TJ, Goldman MB. Impact of oxidative stress on female fertility. Curr Opin Obstet Gynaecol. 2009; 21(3):219-222.
48. Song C, Peng W, Yin S, Zhao J, Fu B, Zhang J *et al*. Melatonin improves age-induced fertility decline and attenuates ovarian mitochondrial oxidative stress in mice. Sci Rep. 2016; 6:35165.
49. Soto-Heras S, Roura M, Catala MG, Menendez-Blanco I, Izquierdo D, Fouladi-Nashta AA *et al*. Beneficial effects of melatonin on *in vitro* embryo production from juvenile goat oocytes. Reprod Fertil Dev. 2018; 30:253-261.
50. Sovernigo TC, Adona PR, Monzani PS, Guemra S, Barros FDA, Lopes FG *et al*. Effects of supplementation of medium with different antioxidants during *in vitro*

maturation of bovine oocytes on subsequent embryo production. Reprod Domest Anim. 2017; 00:1-9.
51. Sugino N, Takiguchi S, Kashida S, Karube A, Nakamura Y, Kato H. Superoxide dismutase expression in the human corpus luteum during the menstrual cycle and in early pregnancy. Mol Hum Reprod. 2000; 6:19-25.
52. Szczepanska M, Kozlik J, Skrzypczak J, Mikolajczyk M. Oxidative stress may be a piece in the endometriosis puzzle. Fertil Steril. 2003; 79(6):1288-1293.
53. Tamura H, Takasaki A, Taketani T, Tanabe M, Kizuka F, Lee L *et al*. Melatonin as a free radical scavenger in the ovarian follicle. Endocr J. 2013; 60:1-13.
54. Taniguchi K, Taketani T, Lee LM, Kizuka F, Tamura I, Sugino N. Melatonin protects granulosa cells for progesterone production as an antioxidant in human ovarian follicles. Biol Reprod. 2009; 81:378.
55. Tarin JJ, Perez-Albala S, Garcia-Perez MA, Cano A. Effect of dietary supplementation with a mixture of Vitamins C and E on fertilization of tertiary butyl hydroperoxide-treated oocytes and parthenogenetic activation in the mouse. Theriogenology. 2002; 57:869-881.
56. Thibault C, Szollosi D, Gerard M. Mammalian oocyte maturation. Reprod Nutr Dev. 1987; 27:865-896.
57. Tian X, Wang F, He C, Zhang L, Tan D, Reiter RJ *et al*. Beneficial effects of melatonin on bovine oocytes maturation: a mechanistic approach. J Pineal Res. 2014; 57:239-247.
58. Tiwari M, Prasad S, Tripathi A, Pandey AN, Singh AK, Shrivastav TG *et al*. Involvement of reactive oxygen species in meiotic cell cycle regulation and apoptosis in mammalian oocytes. Reactive Oxygen Species. 2016; 1(2):110-116.
59. Toma´s-Zapico C, Coto-Montes A. A proposed mechanism to explain the stimulatory effect of melatonin on antioxidative enzymes. J Pineal Res. 2005; 39:99-104.
60. Tripathi A, Premkumar KV, Pandey AN, Khatun S, Mishra SK, Shrivastav TG *et al*. Melatonin protects against clomiphene citrate-induced generation of hydrogen peroxide and morphological apoptotic changes in rat eggs. Eur J Pharmacol. 2011; 667:419-424.
61. Zuelke KA, Brackett BG. Luteinizing hormone enhanced *in vitro* maturation of bovine oocytes with and without protein supplementation. Biol Reprod. 1990; 43:784-787.