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AUTHORS: Goodarzi, A; Zare Shahneh, A; Kohram, H; Sadeghi, M; Moazenizadeh, M H; Fouladi-Nashta, A; Dadashpour Davachi, N

JOURNAL: THERIOGENOLOGY

PUBLISHER: Elsevier

PUBLICATION DATE: January 2018

DOI: 10.1016/j.theriogenology.2017.10.009

Accepted Manuscript

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Abbas Goodarzi, Ahmad Zare Shahneh, Hamid Kohram, Mostafa Sadeghi, Mohamed Hussein Moazeni zadeh, Ali Fouladi-Nashta, Navid Dadashpour Davachi

PII: S0093-691X(17)30483-1

DOI: 10.1016/j.theriogenology.2017.10.009

Reference: THE 14292

To appear in: *Theriogenology*

Received Date: 14 April 2017

Revised Date: 1 October 2017

Accepted Date: 7 October 2017

Please cite this article as: Goodarzi A, Zare Shahneh A, Kohram H, Sadeghi M, Moazeni zadeh MH, Fouladi-Nashta A, Dadashpour Davachi N, Effect of melatonin supplementation in the long-term preservation of the sheep ovaries at different temperatures and subsequent in vitro embryo production, *Theriogenology* (2017), doi: 10.1016/j.theriogenology.2017.10.009.

Revised

Effect of melatonin supplementation in the long-term preservation of the sheep ovaries at different temperatures and subsequent in vitro embryo production.

Abbas Goodarzi[a], Ahmad Zare Shahneh[a*], Hamid Kohram[a], Mostafa Sadeghi[a], Mohamed Hussein Moazeni zadeh[a], Ali Fouladi-Nashta[b], Navid Dadashpour Davachi[c].

[a] Department of Animal Science, Faculty College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran

[b] Royal Veterinary College, Reproduction Research Group, Hawkshead Campus, Hatfield, UK.

[c] Department of Research, Breeding and Production of Laboratory Animals, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran

* Correspondence: azareh@ut.ac.ir

Abstract

Investigations in the past decades have shown that oocytes developmental competence following in vitro fertilization is greatly influenced by an interval between isolation of the ovaries immediately after death/slaughter and oocytes recovery from the visible follicles. In order to determine the optimal conditions for long-term preservation of ovaries, an experiment was conducted with adding different doses of melatonin (0 (C), 500 (M1), 600 (M2), 700 (M3) and 800 (M4) µM) as an antioxidant to sheep ovaries preservation medium (PBS) maintained at 4 and 20°C for 24 hours. The effects on in vitro embryo production (IVEP) parameters including maturation, fertilization, cleavage, and blastocyst rates and the total number of blastomere were evaluated after the ovaries preservation. Melatonin reduced the decline in fertilization rate as an indicator of success in vitro maturation ($P \leq 0.05$). Furthermore, ovarian storage time had significant negative effect ($P \leq 0.05$) on IVEP parameters. Supplementation with melatonin increased the total cell number of blastocysts as an indicator of embryo quality (i.e. mean blastomeric cells in 4℃ groups: 86.00±3.00, 98.50±3.5, 111.5±1.5, 125.5±2.00 and 126.50±5.5 for C, M1, M2, M3 and M4. respectively). Overall, the results showed that the use of melatonin antioxidant in the ovaries storage medium had beneficial effects on sheep oocytes development and embryos quality.

Key words: melatonin, ovary, preservation medium, sheep

1. Introduction

Sheep is an important species in animal husbandry industry, and a critical research model to development of assisted reproductive technologies (ARTs) in human and endangered species[1, 2].Cumulus-oocyte-complexes (COCs) from both small and large antral follicles can be isolated directly from ovarian tissue and matured in vitro to obtain mature gametes. However, collection of oocytes for ART from live animals is costly. The use of ovaries from slaughtered animals has provided an affordable source of oocytes for researchers and laboratories involved in animal ART [2, 3]. However, integrating ovarian transport into in vitro embryo production (IVEP) protocols has been an important challenge in large countries and/or in low resource settings where the slaughterhouse is located far from the laboratory [4]. The long distance transportation of ovaries to the laboratory has adverse effects on oocyte quality in terms of nuclear maturation and developmental competence after the in vitro maturation (IVM) and fertilization (IVF) [5]. Ovaries need to be collected and returned to the laboratory instantly after the slaughter in order to make

effective use of the oocytes contained within [6]. The type of transportation medium [3, 6-8], storage time [3-16], as well as, its temperature during transportation of the ovaries [4, 5, 10-16] are among the factors affecting subsequent follicular and oocyte survival, and oocyte developmental competence [7]. Consequently, many studies have been conducted in order to improve oocyte preservation by modifying the transportation solutions. These studies have demonstrated that the transportation of ovaries is possible without a considerable harm to oocyte and follicles [4, 7, 10, 17]. However, there seems to be differences between animal species in sensitivity of the oocytes to the transportation conditions. For example in bovine, when the ovaries were stored for 7 hours at 4°C, 20°C, and 39°C, none of the oocytes from the ovaries stored at 4°C and 39°C developed to blastocyst stage compared to the other group [13].Whereas in mice, storage of ovaries at 4°C for up to 24 hours did not affect the number of mature gametes which could be collected or fertilized post-orthotropic transplantation [10].

Normal cellular metabolism produces reactive oxygen species (ROS) and reactive nitrogen species (RNS), which regulate diverse cell functions. Reactive species, nevertheless are highly reactive with lipid, protein, and nucleic acid resulting in a loss of membrane integrity, structural, or functional changes in proteins, and damage in nucleic acids referred to as oxidative stress [18]. During the ovary transportation to laboratory, the stoppage of blood flow reduces oxygen and energy supply, and put ovaries under ischemic conditions [19]. Ischemia damages follicles viability and luteal function in ovaries; oxygen free radicals in particular, are major contributors to organ damage during preservation [20]. Furthermore, the antioxidant system of ovary cells is compromised during the preservation. To prevent the damage due to the reactive species, the cells possess a number of antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) [7]. The balance between ROS and antioxidants within the follicle seems to be critical to the function of oocyte and granulosa cells [4, 7, 21]. During the ovary transportation, ROS and RNS generation in the follicular microenvironment by oocyte and somatic cells accompanied by reduced levels of antioxidant enzymes may cause oxidative stress-mediated apoptosis in follicles [22].

The main goal in ovaries preservation is to maintain the function of the oocyte enclosed in follicle for in vitro maturation and fertilization. Accordingly, many studies investigated the effects of different antioxidants to reduce the damage caused by free oxygen radicals during ovaries preservation [6-8, 15]. Several studies indicated that the imbalance between ROS production and antioxidant activities could cause oxidative stress [7, 23]. In cow, oocyte meiotic competence could be maintained when ovaries were stored in saline supplemented with epigallocatechingallate compared to glutathione [6]. Experimental data from the feline model showed that oocytes from ovaries preserved in PBS supplemented with SOD reduced the percentage of apoptosis in COCs compared to control group [7].

The protective effects of melatonin as a powerful direct scavenger of free radicals are well documented in the recent years [21, 23-29]. Melatonin, because of its amphiphilic nature, diffuses broadly in diverse subcellular compartments barriers. It is also a highly effective antioxidant and anti-apoptotic agent, which due to its direct scavenging of toxic oxygen derivatives and its ability to reduce of ROS and RNS, prevents oxidative and nitrosative damages to all macromolecules in all compartments of a cell[30].Therefore, we hypothesized that the use of this antioxidant in preservation medium may also have beneficial effects by reducing oxidative stress in ovaries during long term preservation. In the present study, we used the sheep as an experimental model to examine the effects of supplementing the ovaries transport medium with melatonin at two different temperatures on blastocyst rate and quality after IVEP.

2. Materials and methods

Except otherwise indicated, chemicals were purchased from Sigma Chemical Co. (Saint Louis, MO, USA). Adult testes and ovaries (without distinguished corpus luteum) were obtained from slaughtered Lory-Bakhtiary sheep aged 2 to 3 years without apparent abnormality in reproductive tract. Due to the low solubility of melatonin in water, it was dissolved in ethanol before adding to PBS.

2.1 Experimental design

There were two experimental groups in the present study design. Two different temperatures (4 and 20 °C) and 4 concentrations of melatonin (M1: 500, M2: 600, M3: 700 and M4: 800 µM) were considered as the treatments. In experiment 1 we analyzed the effect of maintaining ovaries in PBS supplemented with four concentrations of melatonin (M1- M4) at 4°C for a period of 24 h prior to oocyte retrieval. As the control group(C) ovaries were maintained in PBS without melatonin supplementation for a period of 24 hours before oocyte collection. Fertilization was carried out using the freshly collected sperm from ram testicles of the same breed at the time of ovaries collection.

In the experiment 2 we assessed the effect of maintaining ovaries at 20℃ for a period of 24h in PBS supplemented with (M1-M4) or without (C) melatonin.

To evaluate the accuracy of the laboratory procedure during this study we also had considered IVF cycles with standard condition (SC) concurrent with experiment 1, 2.

2.2 Ovaries collection

Ovine ovaries were collected immediately after slaughter and transferred into PBS containing 100 µg/mL penicillin–streptomycin (Gibco; Grand Island, NY, USA) as a primary repository. Afterwards, the collected ovaries were washed three times in sterile PBS and then randomly divided between the treatment conditions in thermos flask containing PBS (C) and PBS ± melatonin (M1-M4). The insulation efficiency of the thermos flask in maintaining a constant temperature was tested prior to conducting the experiments. After arrival in laboratory the SC group was instantly conformed for embryo production and the other groups were preserved for 24 h in the experimental conditions.

2.3 In vitro maturation of oocyte

Oocyte collection and in vitro maturation method were carried out as previously described [31], with some modifications. In brief, ovarian antral follicles 2-6 mm in diameter were aspirated (45 ovaries per treatment group) using an aspiration pump (MEDAP Sekretsauger P7040; Tilburg, The Netherlands) fitted with a disposable vacuum line (length-35 cm, the internal diameter of 3 mm). The flow rate was set at 10 mL H_2O/min using an attached disposable 20-gauge needle. Next, oocytes with more than three layers of cumulus cells and uniform ooplasm were selected for IVM [31-33]. The TCM199 medium used for IVM was supplemented with 10% fetal bovine serum (FBS) (Cat. Number: A6003), 0.2 mM sodium pyruvate, 5 µg/mL of gentamicin, 10 µg/mL of ovine follicle-stimulating hormone (oFSH), and 1 µg/mL of estradiol. COCs were cultured for 24 hours in a 50 µl droplet of maturation medium (approximately 10 oocytes per drop) under mineral oil at 38.5 ℃ in an atmosphere of 5% CO2 with maximum humidity. After 24 hours oocyte with expanded cumulus considered as mature and selected for next stage.

2.4 Sperm preparation, in vitro fertilization (IVF) and in vitro culture (IVC), and staining methods

In vitro fertilization was carried out as previously described [34] with some modifications. Briefly, fresh spermatozoa were obtained from the slaughtered Lory-Bakhtiary rams (2-3 years old) in fertilization day. After transport of the testicles to the laboratory in a cool box (5℃), all the blood and connective tissues were removed aseptically in a cold room with the temperature of 5℃. For sperm recovery, 1 mL tuberculin syringe attached with 22-gauge needle was inserted into the vas deference. The content of the vas deference was aspirated gently and the recovered spermatozoa was diluted 1:100 in sperm-TALP and storage in 4℃ less dan one houre. Samples with more than 60% progressive motility, which had normal appearance, were selected for IVF. Motile spermatozoa were separated by percoll gradient (45% over 90%). The fertilization medium included 12 mM KCL, 25 mM $NaHCO_3$, 90 mM NaCl, 0.5 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 10 mM sodium lactate, 3 mg/mL BSA (fatty acid free), 50 µg/mL gentamicin. At least 15 min prior to insemination the sperm suspension was transferred into a droplet of fertilization medium immediately prior to co-culture adjusted to a final concentration of 1×10^6 cells/ml. Co-incubation of gametes (day 0; IVF) was carried out under 5% CO2/5% O2 in air for 18 hours at 38.5 ℃. After co-incubation, cumulus cells and attached spermatozoa to zona pellucida were removed by repeatedly pipetting.The presumptive fertilized eggs were cultured at 38.5℃ under 5% CO, 5% O_2, 90% N_2 for the next 7 days in synthetic oviduct fluid (SOF), used as in vitro culture (IVC) medium[31]. Two days after IVC the cleavage rate of the embryo were recorded. At day 8 after insemination, the rate of embryo development to the blastocyst stage was recorded.

Examination of the IVF results carried out according to the previously described procedures [31]. Briefly, 24 hours after insemination one fourth of zygote were removed randomly from the culture medium and washed twice in PBS-PVP (Polyvinylpyrrolidone, 1mg/mL). Then the zygote were fixed on a 100 µL drop of paraformaldehyde solution [4% (w/v) in PBS, pH 7.4], at room temperature. In the final step, the zygote were removed from the fixation drop and washed three times in PBS-PVP. Afterwards, zygotes were transferred to 100µl drop of PBS-PVP containing 1mg/mL Hoechst 33342 for assessment of fertilization rate. The slides were examined under a fluorescent microscope with a UV filter showing the sperm head and pronuclei having a blue appearance. The same method was used for counting the total cells in blastocysts day 8 after insemination.

2.5 Statistical analysis

The GLM procedure of the SAS (SAS, Inc., Cary, NC, USA) was used for the analysis of variance (ANOVA).The Duncan test was taken into account for comparisons of mean values with a significant main effect. P values <0.05 were considered statistically significant. Data are presented as mean ± SEM, total number of blastomeric are presented as number.

3. Results

As expected, the standard condition group which was used as a control to assure the accuracy of IVEP procedure, resulted in higher maturation, fertilization, blastocyst rates and mean blastomeric cells (93.12±0.47, 84.62±0.75, 28.27±1.73 and 171.5±4.50, respectively). The

oocytes in this group were retrieved from ovaries which were transported to the laboratory in less than three hours in PBS at 37.5□,

3.1 Effects of storage condition at 4℃

The effect of melatonin supplementation in ovaries preservation for 24 h at 4℃ is presented in Table 1. The results show that oocyte maturation rate was affected by treatment (*P<0.05*). Only M3 (700) and M4 (800) increased maturation rates compared to the control, and the rates were similar to M1 and M2 groups. Higher concentration of melatonin (M3 and M4) improved fertilization and cleavage rates compared to M1 and control group (p <0.05). Moreover, the highest melatonin supplementation (M4: 800) increased morula rates compared to all groups. Interestingly, some of the cleaved embryos in control group developed to blastocyst stage. The percentages of zygotes undergoing blastocyst in oocytes obtained from ovaries of theM3 and M4 groups were similar (16.95±0.59 vs. 18.19±0.33, respectively) but in all melatonin-treated groups were significantly higher (P<0.05) than C group. The total cell number was significantly greater for M3-M4 compared with M1 and C blastocysts (Figure 1. a-f).

3.2 Effects of storage condition at 20℃

As shown in Table 2, supplementation of different concentration of melatonin to ovaries preservation medium led to significant increase in oocyte maturation rate evaluated based on cumulus expansion ($p < 0.05$). There were no significant differences between 500, 700, and 800 µM treatment groups. No differences were observed in the maturation rate of oocytes from PBS alone (control) with 600 µM melatonin (M2) group. Also, fertilization rate were not significant between M3 and M4 groups ($p < 0.05$). Higher concentration of melatonin (M4; 800 µM) resulted in the best fertilization rate. Oocytes from control group and low melatonin groups had inferior fertilization rate ($p < 0.05$). Similarly, the percent of cleaved zygote in melatonin-treated groups was higher than the control group and the highest concentration of melatonin resulted in higher cleavage rate (p<0.05), except compared to M3 group. Moreover, the highest melatonin concentration showed the highest morula rates compared to all groups (P<0.05). No blastocyst was formed from the control group. Melatonin supplementation resulted in significant increase in blastocyst formation with the higher doses (700 and 800 µM) resulting in higher blastocyst rate than the lower doses (500 and 600 µM melatonin). Similar results were obtained for blastocyst quality as compared by the mean number of blastomeric cells being significantly higher in M3 and M4 groups than the low melatonin groups ($p < 0.05$) (Figure 1. B-F).

4. Discussion

Preservation of ovarian tissue is challenging because there are several cell types within the tissue that need to be maintained in a normal state. During the ovaries transportation to the laboratory, the stoppage of blood flow reduces energy supply, and put ovaries under ischemic conditions. As well as, storage of ovary at atmospheric level of oxygen (21%) could generate higher amounts of free radicals in follicle microenvironment, which is toxic to oocyte and other cells surrounding it. Most likely due to the formation of superoxide anion ($O_2^{\bullet-}$), this leads to the ulterior formation of $HO^{\bullet}$, H_2O_2, $NO^{\bullet}$, and $ONOO^-$, which results in oxidative stress [24, 35, 36]. The oocyte oxidative stress caused by ROS must be limited in order for a good embryo to be produced [36]. Free radicals spreads within follicle microenvironment, and react with proteins, lipids and DNA of oocyte and cumulus cells leading to cell membrane-lipid peroxidation, DNA damage and apoptosis [27, 37]. The results reported here show that the

supplementation of ovaries transport medium (PBS) with melatonin significantly maintained the developmental potential of the oocytes than PBS for 24 hours at 4 and 20℃. In particular, the oocyte competence for development the blastocyst stage was significantly improved as compared to control. Furthermore, high-dose melatonin (700 and 800 µM) during 24 h storage in both 4, 20℃ resulted in significant improvement in blastocyst quality as evidenced by greater numbers of blastomeric cells in these groups(Tables 1 and 2).

The melatonin directly suppresses free radicals derived from superoxide anion. In addition, metabolites that are formed during interaction with free radicals (i.e., cyclic 3-hydroxmelatonin, N1-acetyl-N2-formyl-5-methoxykynuramine, and N1-acetyl-5-methoxykynuramine) are high potent scavengers of toxic reactants [24]. Furthermore, it is well known that melatonin has an important indirect function in up regulation of antioxidant and down regulation of provident enzymes [30]. Brzezinski et al [38] reported that melatonin concentration in human follicular fluid is significantly higher than serum samples. Melatonin in follicular fluid besides their physiological role in steroidogenic mechanism, acts as free radical scavenger [21]. The antioxidant activities of melatonin in oxidative stress has been reported in different species. In the mouse oocyte, melatonin promote the development rate of mouse two-cell embryos to blastocysts stage [26]. Moreover, melatonin treatment increases intrafollicular concentration of melatonin and improved fertilization rate embryo transfer [39]. A recent study presented that supplementation of IVM medium with melatonin-loaded lipid-core Nano capsules (Mel-LNC) during IVEP improved cleavage and blastocyst rates of bovine embryo. Also, significantly decreased ROS levels, and down-regulated the genes involved apoptosis caspase 3 (CASPA3) B and BAX [40]. Rodriguez-Osorio et al. [37]reported that, in IVC medium melatonin supplementation improved cleavage rates in stressed conditions (H_2O_2 or 40℃) and increased blastocyst cell numbers in usual terms. Our results showed a significant effect of melatonin on developmental potential of oocytes derived from ovaries preserved in supplemented medium. In addition, our analysis demonstrated that the resultant blastocysts in the M3 (700µM) and M4 (800µM) groups were better considering the healthy blastomeric cells (Figure 1. E, e, F and f). These findings demonstrated that melatonin significantly reduced the toxic effect of ROSs that generated in ovaries during storage period.

Although, improvement was noted when ovaries stored in melatonin supplemented medium, this positive effects in low temperature (4℃) were more evident than that of high temperature (20℃). The oocytes from the ovaries stored at 20℃ without melatonin (control) lost their competence to develop to blastocyst stages. Whereas, when ovaries stored at 4℃, obtained COCs in untreated group developed to blastocyst stage, although, had significantly lower rate than in melatonin treated groups (Tables 1, 2). It is well known that preservation of organs in hypothermia suppresses metabolic requirements. Consequently, it results in reduced tissue damage caused by loss of ATP, increase in pH, lipid peroxidation and proteolysis associated with ischemic condition [12, 20, 40]. Several studies investigated effects of temperature on pool of follicles in farm animal ovaries. In the Iberian red deer, preservation of ovaries in hypothermia condition significantly elevated cleavage rate [4]. Nakao et al. [13]reported that oocyte obtained from ovaries stored at 20℃ had higher competence than that of stored at 39℃ or 4℃. Results showed that temperature of preservation media affected the quality of oocytes, in which cytoplasmic membrane, microtubule, cytoskeleton and zona pellucida might be sensitive to low temperatures [5, 12]. Canine ovaries were transported for up to 4 hours at 4 and 35℃ , obtained oocyte from the ovaries stored at 4℃ had higher MII maturation rate compared with the other group [16]. In the present study, the storage of

sheep ovaries in low temperature media exhibited better results in terms of the developmental competence of oocytes and the cleavage and production of the morula and blastocysts in contrast to high temperature.

In conclusion, the present study showed that (i) supplementation of phosphate buffer saline as ovine ovaries storage media with melatonin (500, 600, 700 and 800 µM), reduced the oocytes competence decline to develop to blastocyst stage. (ii) for long preservation of ovaries (24 hours), low storage temperature (4℃) can maintain oocyte competence but higher temperature (20℃) has a detrimental effect on oocyte viability, maturation, fertilization and subsequent development after IVF; and (iii) storage of ovaries in high concentration (700, 800 µM) for 24 hours significantly maintain the oocyte quality and obtained blastocysts were better than other group, but this protective effect of melatonin were improved in cool condition. Beneficial effect of melatonin in ovine ovaries storage medium may be employed to reduce injuries mediated by ROS during ovaries preservation.

Acknowledgments

The authors would like to thank Helia Shamsi for her assistance during the laboratory procedure. We would also like to express our appreciation to the University of Tehran for financial support of for this project under grant number: 7108011/6/44. Similarly, the authors would like to acknowledge the financial support of Iran National Science Foundation under grant number: 94810027.

Table 1: Effects of ovaries preservation for 24 h at 4°C in melatonin supplemented PBS on oocyte and embryo development.

antioxidant dosage (µM)	Cumulus cells expansion%(n)	Fertilization%(n*)	Cleaved zygote on day 2 %(n)	Morula rate%(n)	Blast rate%(n)	Mean blastomere (n)
0	84.19±1.52^{c} (122/145)	72.12±2.88^{c} (18/25)	61.98±3.98^{e} (60/97)	34.09±2.09^{c} (33/97)	7.19±0.81^{c} (7/97)	86.00±3.00^{c}
500	86.83±2.21^{bc} (120/138)	71.67±1.66^{c} (18/25)	68.34±1.66^{de} (65/95)	44.11±1.89^{b} (42/95)	10.56±0.55^{b} (10/95)	98.50±3.5^{bc}
600	90.18±2.68^{abc} (128/142)	76.79±1.78^{bc} (20/26)	72.28±0.27^{cd} (73/101)	44.57±1.43^{b} (45/101)	10.90±1.10^{b} (11/101)	111.5±1.5^{b}
700	91.20±1.28^{ab} (142/156)	80.00±0.75^{ab} (24/30)	75.9±0.46^{bc} (85/112)	49.99±0.89^{b} (56/112)	16.95±0.59^{a} (19/112)	125.00±2.00^{a}
800	91.22±0.50^{ab} (135/148)	80.13±3.20^{ab} (20/25)	80.89±1.26^{ab} (89/110)	56.35±0.79^{a} (62/110)	18.19±0.33^{a} (20/110)	126.50±5.5^{a}

Each value represents the means ± SEM.

* randomly selected from zygotes for assessment IVF parameters

Table 2: . Effects of ovaries preservation for 24 h at 20°C in melatonin supplemented PBS on oocyte and embryo development. No melatonin was added to the control group.

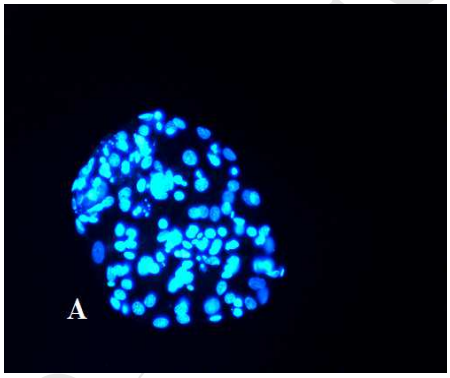
Antioxidant dosage (µM)	Cumulus cells expansion %(n)	Fertilization%(n*)	Cleaved zygote on day 2 %(n)	Morula rate%(n)	Blast rate%(n)	Mean blastomere (n)
0	75.50±1.64^{b} (119/158)	53.57±3.57^{c} (14/26)	32.42±5.59^{c} (30/93)	21.53±5.74^{c} (20/93)	0.00^{c} (0/93)	-

500	83.13±1.88 [a] (133/160)	62.08±1.19[b] (18/29)	52.53±3.47[b] (54/104)	33.38±1.24[b] (35/104)	5.83±0.17[b] (6/104)	83.50±4.50 [c]
600	79.75±0.93 [b] (138/173)	65.48±109[b] (19/29)	60.67±2.98b[b] (65/109)	33.81±0.48[b] (37/109)	7.48±0.21[b] (8/109)	89.00±4.00 [bc]
700	80.37±0.88 [a] (127/158)	75.13±1.80[ab] (21/28)	70.82±1.18[a] (75/99)	41.24±2.35b[b] (40/99)	10.36±0.36[a] (11/99)	105.50±5.5 0[ab]
800	80.29±1.04 [a] (130/158)	77.38±5.95[a] (20/26)	68.66±1.34[a] (70/104)	45.72±2.86[a] (47/104)	12.72±0.73[a] (13/104)	119.00±6.0 0[a]

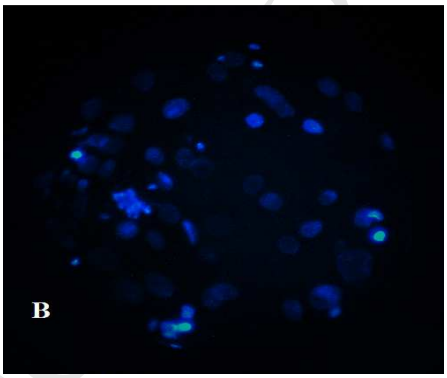
Each value represents the means ± SEM.

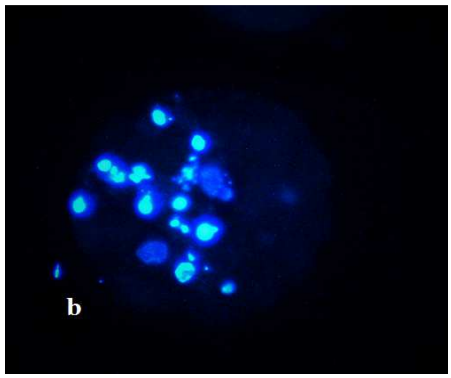
* Randomly selected from zygotes for assessment IVF parameters

SC



C





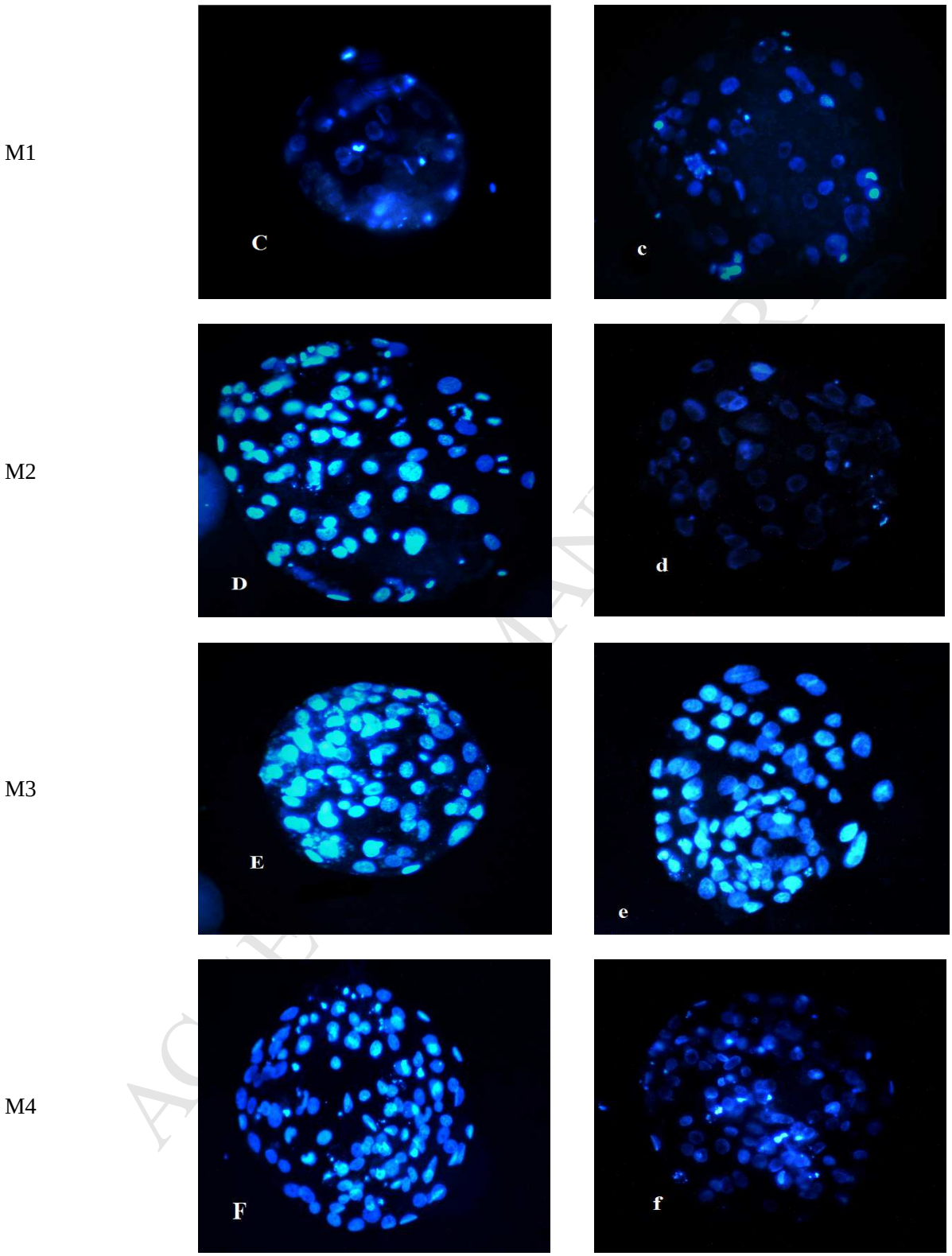


Figure 1. Example of blastocysts 7 days. Stained with Hoechst fluorescent dye. SC: Blastocyst obtained in conventional method (up to 3h storage in 37.5℃). C: from control group (ovaries stored without melatonin

supplementation) at 4℃ [B] or20℃ [b]. M1-M4: Blastocysts from ovaries stored with 500, 600, 700 or 800 µM melatonin at 4 [C, D, E and F] or 20oC [c, d, e and f]. A high-resolution version of panels A, B, b, C, c, D, d, E, e, F and f of this image are available as Virtual Microscope eSlides: VM04430, VM04431, VM04432, VM04433, VM04434, VM04435, VM04436, VM04437, VM04438, VM04439 and VM04440, respectively.

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Highlight

- Effect of melatonin supplementation in ovaries storage medium for up 24 hours in 4 and 20℃ was studied on oocyte quality and embryo production in sheep.
- Oocyte competence, fertilization rate, morula and blastocyst improved by melatonin (500, 600, 700 and 800 µM) supplementation in ovaries storage medium.
- Storage of ovaries in high concentration (700, 800 µM) for 24 hours significantly maintain the oocyte quality and obtained blastocysts were better than other groups.