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 Hapes, 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) [43]. 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; Keskinetpe et al., 1994) [15, 20]. 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) [11]. 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) [39], 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) [13, 12, 19, 59]. 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 (Khazaee 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 Heps 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 Heps, 0.25 mM Pyruvate and antibiotics in 50-100 µl maturation media for 28 hrs at 38±1º C and 5% CO2 in humidified air in a CO2 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 pipetting 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 envelopes 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 chromat in 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

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

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0</th>
<th>+</th>
<th>++</th>
<th>+++</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.4 ± 0.34 (19.20%)</td>
<td>3.2 ± 0.249 (25.60%)</td>
<td>3.7 ± 0.26 (29.60%)</td>
<td>3.2 ± 0.416 (25.60%)</td>
</tr>
<tr>
<td>MLT</td>
<td>0.9 ± 0.233 (6.62%)</td>
<td>1.1 ± 0.277 (8.09%)</td>
<td>3.2 ± 0.389 (23.53%)</td>
<td>8.4 ± 0.686 (61.76%)</td>
</tr>
<tr>
<td>LAA</td>
<td>1.8 ± 0.249 (13.33%)</td>
<td>2.1 ± 0.277 (15.56%)</td>
<td>3.8 ± 0.389 (28.15%)</td>
<td>5.8 ± 0.646 (42.96%)</td>
</tr>
<tr>
<td>TAU</td>
<td>1.9 ± 0.277 (14.39%)</td>
<td>2.9 ± 0.407 (21.97%)</td>
<td>3.6 ± 0.306 (27.27%)</td>
<td>4.8 ± 0.533 (36.36%)</td>
</tr>
</tbody>
</table>

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 Anatalephase was not different for the treatment groups and control (Table 3 and 4).

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No of replicates</th>
<th>Total number of oocytes matured</th>
<th>Nuclear stages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>125</td>
<td>29 32 17 47</td>
</tr>
<tr>
<td>MLT</td>
<td>10</td>
<td>136</td>
<td>25 13 14 84</td>
</tr>
<tr>
<td>LAA</td>
<td>10</td>
<td>135</td>
<td>25 22 21 67</td>
</tr>
<tr>
<td>TAU</td>
<td>10</td>
<td>132</td>
<td>28 26 20 58</td>
</tr>
<tr>
<td>Total</td>
<td>528</td>
<td></td>
<td>107 93 72 256</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GV</th>
<th>M-I</th>
<th>AT-I</th>
<th>M-II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.9±0.433 (23.20%)</td>
<td>3.2±0.359 (25.60%)</td>
<td>1.7±0.3 (13.60%)</td>
<td>4.7±0.26 (37.60%)</td>
</tr>
<tr>
<td>MLT</td>
<td>2.5±0.269 (18.38%)</td>
<td>1.3±0.3 (9.56%)</td>
<td>1.4±0.306 (10.29%)</td>
<td>8.4±0.67 (61.76%)</td>
</tr>
<tr>
<td>LAA</td>
<td>2.5±0.342 (18.52%)</td>
<td>2.2±0.533 (16.30%)</td>
<td>2.1±0.277 (15.55%)</td>
<td>6.7±0.616 (49.63%)</td>
</tr>
<tr>
<td>TAU</td>
<td>2.8±0.291 (21.21%)</td>
<td>2.6±0.427 (19.70%)</td>
<td>2.0±0.258 (15.15%)</td>
<td>5.8±0.416 (43.94%)</td>
</tr>
</tbody>
</table>

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; Buccone 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, 41] 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) 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., 2009) [34], bovine (Manjunatha et al., 2009) [34], ovine (El-Raey et al., 2011; Tian et al., 2014) [17, 57], and mouse oocytes (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) [39]. 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 Soverno 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) [13] 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 is a product of hypotaurine, after free radical scavenging, 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.

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