Effect of α-tocopherol and l-ascorbic acid on in-vitro maturation of vitrified bovine oocytes

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Abstract
The aim of the study was to evaluate the effects of vitamin E (α-Tocopherol) and Vitamin C (L-Ascorbic Acid) on the rates of in-vitro maturation in respect to cumulus cell expansion and polar body formation of vitrified bovine oocytes. A total of 264 vitrified oocytes were equally divided in to four experimental groups and subjected to in vitro maturation as vitrified control (without antioxidant), and groups supplemented with Vitamin E @100µM, Vitamin C @ 100µM and combination of Vitamin E @100µM + Vitamin C @ 100µM. In another group, 66 numbers of non-vitrified Cumulus Oocyte Complexes (COCs) were in-vitro cultured in TCM-199 medium and was considered as the control group. TCM-199 supplemented with Oestradiol 17β + p-FSH + Follicular fluid + Gentamicin + Sodium pyruvate + Cysteamine + FBS were taken as base medium. In-vitro maturation was conducted for 24 hrs at 38.5°C in presence of 5% CO₂ in a CO₂ incubator. Among the treatment groups, the group with vitamin E @ 100 µM + vitamin C @ 100 µM supplemented in TCM-199 showed the highest rate of in-vitro maturation of vitrified bovine follicular oocytes. Both vitamin E (100 µM) and vitamin C (100 µM) supplemented in TCM-199 improved the rate of in-vitro maturation of vitrified bovine follicular oocytes compared to the vitrified control group. It was concluded that supplementation of α-Tocopherol and L-Ascorbic acid with TCM-199 enhances the rate of in-vitro maturation of vitrified-thawed bovine oocytes.

Keywords: bovine, oocytes, antioxidant, vitrification, IVM.

Introduction
Vitrification is the method used for preserving the gametes of human, livestock (Bovine, Ovine, Caprine and Porcine), lab animals (mice, rabbit) and endangered species (Chaves et al., 2017) [8]. However, the success rate of maturation of vitrified oocyte is very less than non-vitrified oocytes (Purohit et al., 2012; Dutta et al., 2013) [26, 11]. Vitrification of oocytes depends on several factors like type and concentration of cryoprotectants, suitable cryopreservation techniques, number of equilibrations, warming steps and cryopreservation devices, etc. During cryopreservation, a large number of oocytes lose their physical and chemical properties in the process of cytoplasmic fluid extraction with cryoprotectants and solidification in Liquid nitrogen (LN₂) at -196 °C. So the ultra-structure and cytoskeleton of the oocytes are damaged (Abdelalai et al., 2010) [11]. Cryopreservation increases the reactive oxygen species (ROS) production due to lipid peroxidation. High concentration of ROS induces oxidative stress which damages the cell membrane and cause fragmentation of DNA followed by cell apoptosis just after warming (Hurtt et al., 2000; Abdelalai et al., 2010; Hadi et al., 2011; Rho et al., 2012) [15, 1, 14, 28]. Cumulus oocyte complex has the capacity to control the ROS production at the time of in-vitro maturation. Therefore, grading of oocytes is important to enhance the rate of oocyte maturation. Basically, A+B graded oocytes are selected for in-vitro maturation, where more than 3-4 layers of cumulus cells are surrounded the oocytes (John et al., 2015) [16]. Cumulus cells provide the antioxidant system to the oocytes using enzymatic activities of Glutathione peroxidase (GPx) and catalase etc. (Cetica et al., 2001; Khalil et al., 2013) [4, 18]. Antioxidants like vitamin C (L-ascorbic acid), β-mercaptoethanol, Cu, Mg, Zn, Se and vitamin E (α-tocopherol) at specific concentration enhance the rate of maturation and further the process of in-vitro culture of oocytes (Thiyagarajan and Valivittan, 2009; Nadri et al., 2009; Natarajan et al., 2010; Gupta et al., 2010) [29, 23, 13]. These are the primary free radical scavengers in mammalian cell membranes (Geesin et al., 1991; Gupta et al., 2010; Kere et al.,
Vitamin E, a lipid soluble agent catalyze the GPx activity and protects the cell from ROS. Ascorbic acid is an extracellular antioxidant which is not only biologically active in promotion of collagen synthesis, production of hormone, reducing agent of oxygen and cytocromes c and a, but can also protect membranes against lipid peroxidation and free radical formation (Luck et al., 1995; Miclea et al., 2012) [20, 22]. Against lipid peroxidation, α-tocopherol act by removing peroxyl and alcoxy radicals, generating the poorly reactive tocopheryl radical (Olson and Seidel, 2000) [24]. In case of vitrified oocytes, the level of α-tocopherol is very less which might be fulfilled by supplementation of extracellular antioxidants like ascorbic acid. α-tocopherol can be regenerated by ascorbic acid from tocopheroxyl (Chow, 1991; Dalvit et al., 2005) [7, 9, 10]. Combination of vitamin E and vitamin C shows positive effects on non-vitrified oocytes of bovine (Thiyagarajan and Valivittan, 2009) [20], ovine (John et al., 2015) [16] and porcine (Ulloa et al., 2008) [31]. Hence, the present study was conducted with a view to assess the effects of supplementation of vitamin E and vitamin C at specific concentration in TCM-199, either separately or in combination, on in-vitro maturation of vitrified bovine oocytes.

Materials and Methods

The media and chemicals used to conduct the present study were procured from Sigma-Aldrich, USA. Bovine oocytes were recovered from abattoir ovaries soon after slaughter of the animals and carried to the laboratory in a thermos flask containing warm (37 °C) normal saline solution (NSS) with antibiotics (Gentamicin + Penicillin). The cumulus-oocyte-complexes (COCs) were recovered from the follicles (3-5 mm diameter) by aspiration and slicing techniques. A total of 264 vitrified-rehydrated COCs were equally divided into four groups consisting 66 in each group. In three groups, the COCs were separately in-vitro cultured in TCM-199 medium supplemented with vitamin E @ 100 µM, vitamin C @ 100 µM and combination of vitamin E + vitamin C @ 100 µM each. The fourth group of vitrified-rehydrated oocytes was in-vitro cultured in TCM-199 medium without supplementation of vitamin E or vitamin C. In another group, 66 numbers of non-vitrified COCs were in-vitro cultured in TCM-199 medium and it was considered as the control group. The TCM-199 + Follicular Fluid + Oestradiol 17β + p-FSH + Gentamicin + Sodium pyruvate + Cysteamine + FBS was used for in-vitro maturation of bovine oocytes. In-vitro maturation was done at a temperature of 38.5 °C for 24 hr in an environment of 5% CO₂ in a CO₂ incubator. The data obtained from the study were analyzed statistically using SAS Enterprise Guide 4.3.

Result and Discussion

The mean percentage of in-vitro matured bovine follicular oocytes of non-vitrified (Control) and vitrified (without antioxidant) groups was found to be 85.18 ± 2.57 and 51.67 ± 1.94, respectively in terms of cumulus cell expansion and 65.38 ± 1.83 and 30.26 ± 0.16, respectively in terms of polar body formation. DMRT (Duncan’s Multiple Range Test) indicated that the mean percentage of both cumulus cells expansion and polar body formation in the non-vitrified (control) group was significantly higher (P<0.01) than the vitrified control (without antioxidant) group of bovine follicular oocytes. The findings of the present study were comparable to that of Hurt et al. (2000) [15] in case of vitrified control (without antioxidant) group of bovine follicular oocytes, while the values were not in conformity to that of Martins et al. (2005) [21], Cetin et al. (2006) [9], Hadi et al. (2011) [14], and Rho et al. (2012) [28]. The discrepancy of findings in various studies might be due to the variation in the technique of vitrification, thawing and diluting out of cryoprotectants, species, status of ovary and the animals. Lower percentage of n-vitro matured oocytes might be caused by ultra-structural damage and interruption of the oocytes, deleterious effects on chromosome, viscosity, osmotic injury and possible toxic effect of glycerol and ethylene glycol. A good vitrifying capacity can be achieved in a mixture of cryoprotectants with no major alteration of ultra-structure in bovine oocytes. Mixture of cryoprotectants could decrease individual toxicity (Papis et al., 2000; Cocchia et al., 2010; Purohit et al., 2012; Dutta et al., 2013; Bhat et al., 2013) [25, 8, 26, 11, 31].

The rate of in-vitro maturation of experimental groups of vitrified thawed bovine oocytes in respect of Cumulus cell expansion and Polar body formation supplemented with Vitamin E @ 100 µM, vitamin C @ 100 µM and combination of vitamin E + vitamin C @ 100 µM each were found as 69.95 ± 3.20 and 56.23 ± 1.61, 71.19 ± 2.63 and 54.57 ± 1.69 and 83.23 ± 3.00 and 61.25 ± 3.38, respectively. Addition of α-Tocopherol and L-Ascorbic Acid enhanced the percentage of mitotic maturation. DMRT (Duncan’s Multiple range test) indicated that the mean percentage of cumulus cells expansion after in-vitro maturation of vitrified oocytes in media supplemented with combination of vitamin E @ 100 µM + vitamin C @ 100 µM was significantly higher (P<0.01) than the vitrified control (without antioxidant) group and the groups supplemented with vitamin E @ 100 µM or vitamin C @ 100 µM. Ulloa et al. (2008) [31] also found similar results in non-vitrified porcine oocytes, where incorporation of Vitamin E and Vitamin C in culture media enhanced in-vitro maturation of oocytes. In the antioxidant supplementary groups, there was no significant difference in polar body formation of the bovine oocytes. But, the rate of polar body formation in groups supplemented with antioxidant were significantly (P<0.01) higher than that of control (without antioxidant) group. Kitagawa et al. (2004) [19], and Olson and Seidel (2000) [24] found that the vitamin E @ 100 µM reduced H₂O₂ content, thus leading to limited DNA fragmentation and improved the developmental ability. On the other hand, Vitamin C (ascorbic acid), which functions as a general free radical scavenger, also suppressed apoptosis (Tilly and Tilly, 1995) [30]. The lower concentrations of ascorbic acid promote cell growth, whereas the higher concentrations of ascorbic acid appear to have a pro-oxidant effect which is deleterious to the cells (Rehman et al., 1998; Asard et al., 2004) [27, 2]. During in-vitro maturation, ascorbic acid maintains and regenerates the antioxidant capacity of the α-Tocopherol incorporated to COCs membranes (Dalvit et al., 2005) [9-10].
Immature cat oocyte vitrification in; Vitrification of immature oocytes.

References

Table 1: Percentages of oocytes showing cumulus cells expansion and polar body formation after in-vitro maturation in vitamin E (100 µM), VITAMIN C (100 µM), VITAMIN E (100 µM) + VITAMIN C (100 µM) supplemented tcm-199 media and vitrified control and non-vitrified control media.

<table>
<thead>
<tr>
<th>Supplements</th>
<th>Concentrations</th>
<th>In-Vitro Maturation</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Cumulus Cells Expansion (%) MEAN ± SE</td>
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<tr>
<td>Vitamin-E</td>
<td>100 µM</td>
<td>69.95±3.20</td>
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<tr>
<td>Vitamin-C</td>
<td>100 µM</td>
<td>71.19±2.63</td>
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<tr>
<td>Vitamin-E + Vitamin-C</td>
<td>100µM + 100µM</td>
<td>83.23±3.00</td>
</tr>
<tr>
<td>Vitrified control</td>
<td>0 µM</td>
<td>51.67±1.94</td>
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<tr>
<td>Non-vitrified Control</td>
<td>0 µM</td>
<td>85.18±2.57</td>
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</tbody>
</table>

*Means with different superscripts in column differ significantly (P<0.01)

Fig 1: Percentage of oocytes showing cumulus cells expansion and polar body formation after in-vitro maturation in VITAMIN E (100 µM), VITAMIN C (100 µM), VITAMIN E (100 µM) + VITAMIN C (100 µM) supplemented tcm-199 media and vitrified control and non-vitrified control media.

Conclusion
On the basis of the study it could be concluded that both vitamin E (100 µM) and vitamin C (100 µM) supplemented in TCM-199 either alone or in combination could improve the rate of in-vitro maturation of vitrified bovine follicular oocytes.


