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Effect of L-Ascorbic acid on *in-vitro* maturation of vitrified-Thawed bovine oocytes

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Abstract
The present study was conducted to assess the effect of supplementation of L-ascorbic acid (vitamin C) on *in-vitro* maturation (IVM) of vitrified-thawed bovine oocytes. A total of 264 normal vitrified-thawed bovine follicular oocytes were divided into four experimental groups consisting of 66 oocytes in each group. *In-vitro* maturation was done in the medium supplemented with different concentrations of vitamin C @ 50 µM, 100 µM and 200 µM. In the control group, *in-vitro* maturation was done in medium without addition of vitamin C. *In-vitro* maturation was conducted for 24 hrs at 38.5ºC in presence of 5% CO_2 in a CO_2 incubator. The mean percentages of vitrified oocytes in medium supplemented with 50 µM, 100 µM and 200 µM vitamin C were found to be 52.79 ± 1.39 and 33.56 ± 2.18,71.19 ± 2.63 and 54.57 ± 1.69and 51.17 ± 3.59 and 30.43 ± 2.01, respectively in respect of cumulus cell expansion and polar body formation. The percentage of both cumulus cell expansion and polar body formation in medium supplemented with 100 µM vitamin C was found to be significantly higher ($P<0.01$) than that of the 50 µM and 200 µM vitamin C supplemented groups. The mean percentages of cumulus cell expansion and polar body formation in the vitrified control group were 51.67 ± 1.94 and 30.26 ± 0.16, respectively. Hence, it could be concluded that *in-vitro* maturation in medium supplemented with 100 µM vitamin C enhances the growth of vitrified-thawed bovine oocytes.

Keywords: cryopreservation, IVM, Vitamin C, Bovine, oocytes

Introduction
Gamete cryopreservation is an important tool in many aspects of assisted reproductive biotechnology like human infertility treatment, livestock production, endangered species preservation and basic stem cell research (Dutta *et al.*, 2013; Chaves *et al.*, 2017; Fathi *et al.*, 2017) [4, 3, 5]. However, the rate of *in-vitro* oocyte maturation, fertilization and production of embryo are very less due to the effects of cryopreservation. During cryopreservation, cytoskeleton is damaged within mature oocytes due to disruption of sub-cortical actin network, microtubule depolymerization, abnormal spindle configuration and chromosome scattering (Prentice and Anzar, 2011; Brambillasca *et al.*,2013; Dutta *et al.*, 2013; Hwang and Hochi, 2014) [15, 2, 4, 8]. For enhancing the rate of maturation, additives like antioxidants with the base medium TCM-199 may be effective. In case of non-vitrified oocytes of porcine, mice, bovine, ovine and caprine, antioxidants like vitamin C (L-ascorbic acid), β-mercaptoethnol and vitamin E (α-tocopherol) at specific concentration enhance the rates of maturation and further processes of *in-vitro* culture of oocytes (Thiyagarajanand Valivittan, 2009; Nadri *et al.*, 2009; Natarajan *et al.*, 2010; Gupta *et al.*, 2010) [16, 12, 13, 7]. This type of non-enzymatic antioxidants protects the oocytes from harmful reactive oxygen species (ROS), which leads to oxidative stress and alterations of the cell functions (Gupta *et al.*, 2010; Kere *et al.*, 2012; Kitagawa *et al.*, 2014) [7, 9].

L-ascorbic acid is found in extra-cellular fluid, which has an important role to inhibit lipid peroxidation and biosynthesis of collagen (Geesin *et al.*, 1991) [6]. The functions of antioxidants are associated with lowering the DNA damage and other parameters of cell damage *in-vitro.* Hence, the present study was undertaken to assess the effects of supplementation of vitamin C in the growth medium on *in-vitro* maturation of vitrified bovine oocytes at different concentrations.

Materials and Methods

The media and chemicals used to conduct the present study were procured from Sigma-Aldrich.

Bovine ovaries were collected soon after slaughter (within 2 hours) of the animals and carried to the laboratory in a thermos flask maintaining the temperature of normal saline solution (NSS) at 37 ºC with antibiotic. The cumulus-oocyte-complexes (COCs) were recovered from follicles (3-5 mm in diameter) by aspiration and slicing techniques. Grade A+B COCs were selected for vitrification and *in-vitro* maturation processes. A total of 264 vitrified-rehydrated COCs were equally divided into four groups consisting 66 numbers in each. In three groups, the COCs were *in-vitro* cultured separately in TCM-199 medium supplemented with vitamin C @ 50, 100 and 200 µM, respectively. The fourth group of vitrified-rehydrated oocytes was *in-vitro* cultured in TCM-199 medium without vitamin C supplementation. In another group, 66 non-vitrified COCs were *in-vitro* cultured in TCM-199 medium and it was considered as the control group.

The Follicular fluids were collected from visible follicles (3-6 mm) of bovine ovaries and centrifuged at 37 ºC, at 10,000 rpm for 30 minutes. The collected supernatant was then heat inactivated at 56 ºC for 30 minutes followed by filtering and stored at -20 ºC for future use.

The TCM-199 medium supplemented with Follicular Fluid + Oestradiol 17β + p-FSH + Gentamicin + Sodium pyruvate + Cysteamine + FBS was used for *in-vitro* maturation of bovine oocytes. *In-vitro* maturation was carried out in an environment of 5% CO_2 in a humidified environment at 38.5 ºC for 24 hrs in a CO_2 incubator. The data obtained from the study were analyzed statistically using SAS enterprise Guide 4.3.

Results and Discussions

Effects of vitamin C on *in-vitro* maturation of vitrified-thawed bovine oocytes are shown in Table 1. The mean percentages of cumulus cell expansion and polar body formation in non-vitrified control and vitrified control groups were found to be 85.18 ± 2.57 and 65.38 ± 1.83 and 51.67 ± 1.94 and 30.26 ± 0.16, respectively. Duncan's Multiple Range Test (DMRT) indicated that the mean percentages of both cumulus cells expansion and polar body formation in the non-vitrified (control) group were significantly higher (P<0.01) than that of the vitrified control (without antioxidant) group of bovine follicular oocytes. This indicated that vitrification might arrest *in-vitro* maturation (Moawad *et al.*, 2012; Dutta *et al.*, 2013) [4]. It might be due to the toxic effects of the cryoprotectants like glycerol and ethylene glycol to the ultra-structure of the oocytes. Vitrification and exposure of oocytes to the cryoprotectants enhance disorganization of actin filaments and thus cause alteration of the secondary structure of the proteins of zona pellucida as well as the carbohydrate residues as cumulus cells establish physical contact with the zona pellucida of oocytes through actin filaments within trans-zonal process (Bogliolo *et al.*, 2012) [1]. Thus, the rates of IVM of vitrified oocytes were significantly lower compared to the non-vitrified oocytes. Pepis *et al.* (2000) [14] and Dutta *et al.* (2013) [4] also reported that low dose and mixture of cryoprotectants reduce the individual toxicity to the cell.

Groups		Vitamin C	Per cent (Mean ± SE) oocyte Maturation (N= 66)	
			Cumulus Cells Expansion	Polar Body Formation
Treatment		50 µM	$52.79^{b} \pm 1.39$	$33.56^{b} \pm 2.18$
		100 µM	$71.19^{a} \pm 2.63$	$54.57^{a} \pm 1.69$
		200 µM	$51.17^{b} \pm 3.59$	$30.43^{b} \pm 2.01$
Control	Vitrified	0 µM	$51.67^{b} \pm 1.94$	$30.26^{b} \pm 0.16$
	Non-vitrified		$85.18^{a} \pm 2.57$	$65.38^{a} \pm 1.83$

*Means in a column with different superscripts differ significantly (P<0.05)

Table 1: Per cent cumulus cells expansion and polar body formation of vitrified and non-vitrified Oocytes in vitamin C supplemented (50, 100 and 200 µM) treatment and control media

The rate of cumulus cells expansion and polar body formation in medium supplemented with vitamin C @ 50 µM, 100 µM and 200 µM were found to be 52.79 ± 1.39 and 33.56 ± 2.18, 71.19 ± 2.63 and 54.57 ± 1.69and 51.17 ± 3.59 and 30.43 ± 2.01 per cent, respectively. DMRT indicated that the mean percentages of both cumulus cells expansion and polar body formation in the group supplemented with vitamin C @ 100 µM was significantly higher (P<0.01) than that of 50 µM and 200 µM vitamin C supplemented as well as vitrified control groups. The results suggested that L-ascorbic acid increases meiotic resumption of vitrified-thawed bovine oocytes at certain concentrations. The antioxidant properties of vitamin C (L-ascorbic acid) enable it to protect tissues from reactive oxygen species such as O^{2-}, OH^{-}, H_2O_2, $'O_2$, OCl^{-}, NO and metal-oxygen complexes (Nadri *et al*., 2009) [12], which leads to improved developmental ability. Vitamin C supports the glutathion peroxidase (GPx) activity and catalase activity in the COCs, which also regenerates other intra-cellular antioxidants like alpha-tocopherol, selenium etc. and balances the intracellular antioxidant concentrations. *In-vitro* maturation medium supplemented with vitamin C at the lower concentration enhances cytoplasmic maturation and subsequent development, whereas higher concentration of vitamin C induces apoptosis (Tilly and Tilly, 1995; Kere *et al.*, 2012) [17, 9].

From the present study, it could be concluded that supplementation of Vitamin C @100 µM in TCM-199 medium could significantly improve the IVM rates of vitrified bovine oocytes.

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