International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2017; 5(5): 1359-1362

Received: 12-07-2017
Accepted: 14-08-2017

Sonowal J
PhD Scholar, Department of animal Biotechnology, IVRI, Izatnagar, Uttar Pradesh, India

Barua PM
Professor, Department of ARGO, CVSc, AAU, Khanapara, Assam, India

Borah P
Professor and Head, Department of Animal Biotechnology, CVSc, AAU, Khanapara, Assam, India

Dutta DJ
Professor, department of Veterinary Physiology, CVSc, AAU, Guwahati, Assam, India

Hazarika G
PhD Scholar, Department of Animal Biotechnology, CVSc, AAU, Khanapara, Assam, India

Gogoi C
MVSc Scholar, Department of Veterinary Pharmacology, CVSc, AAU, Khanapara, Assam, India

Deuri N
SRF, ICAR-NRC pig, Rani, Assam, India

Das A
PhD Scholar, department of ARGO, CVSc, AAU, Khanapara, Assam, Inida

Borgohain I
PhD Scholar, Department of Animal Biotechnology, CVSc, AAU, Khanapara, Assam, India

Pathak B
MVSc Scholar, Department of Animal Biotechnology, CVSc, AAU, Khanapara, Assam, India

Correspondence
Sonowal J
PhD Scholar, Department of animal Biotechnology, IVRI, Izatnagar, Uttar Pradesh, India

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

Sonowal J, Barua PM, Borah P, Dutta DJ, Hazarika G, Gogoi C, Deuri N, Das A, Borgohain I and Pathak B

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% CO_2 in a CO_2 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) [6]. 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 (LN_2) at -196 °C. So the ultra-structure and cytoskeleton of the oocytes are damaged (Abedelahi *et al.*, 2010) [1].
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; Abedelahi *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-mercaptoethnol, 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.*,

2012; Kitagawa *et al.*, 2014) [12, 13, 17]. 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 alcoxyl 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) [29], 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% CO_2 in a CO_2 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 Hurtt *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) [5], 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, 3].

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 H_2O_2 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].

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

Supplements	Concentrations	*In-Vitro* Maturation	
		Cumulus Cells Expansion (%) MEAN ± SE	Polar Body Formation (%) MEAN ± SE
Vitamin-E	100 µM	69.95^{b}±3.20	56.23^{bc}±1.61
Vitamin-C	100 µM	71.19^{b}±2.63	54.57^{bc}±1.69
Vitamin-E + Vitamin-C	100µM + 100µM	83.23^{a}±3.00	61.25^{ab}±3.38
Vitrified control	0 µM	51.67^{c} ±1.94	30.26^{d}±0.16
Non-vitrified Control	0 µM	85.18^{a} ±2.57	65.38^{a} ±1.83

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

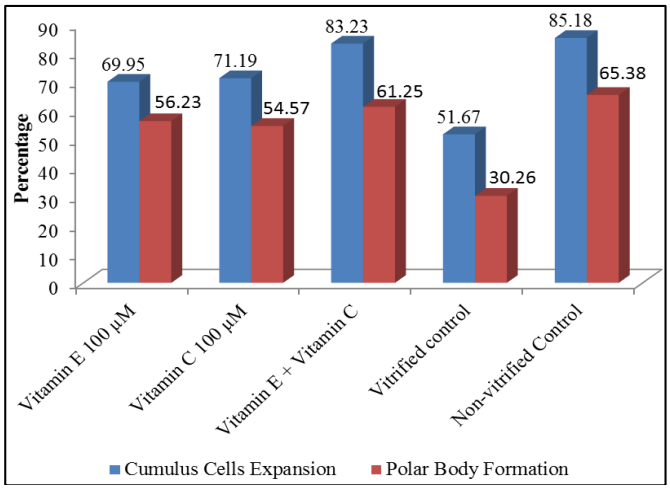

Fig 1: Percentage of oocytes showing cumulus cells expansion and polar body formation after *in-vitro* maturation in VITAMIN E (100 µM), VITAMIN C (100 µM), VITAMIN E (100 µM) + VITAMIN C (100 µM) supplemented tcm-199 media and vitrified control and non-vitrified control media

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.

References

1. Abedelahi A, Salehnia M, Allameh AA, Davoodi D. Sodium selenite improves the *in vitro* follicular development by reducing the reactive oxygen species level and increasing the total antioxidant capacity and glutathione peroxide activity. Human Reprod. 2010; 25(4):977-985.
2. Asard H, Smirnoff J, May N. Vitamin C: its functions and biochemistry in animals and plants. BIOS Scie. Publ., New York, 2004, 191-208.
3. Bhat MH, Yaqoob SH, Khan FA, Waheed SM, Sharma V, Vajta G *et al*. Open pulled straw vitrification of *in-vitro* maturated sheep oocytes using different cryoprotectants. Small Rumi. Res. 2013, 1-5
4. 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.
5. Cetin Y, Bastan A. Cryopreservation of immature bovine oocytes by vitrification in straws. Anim. Reprod. Sci. 2006; 92:29-36.
6. Chaves DF, Corbin E, Almiñana C, Locatelli Y, Souza-Fabjan JMG, Bhat MH *et al*. Vitrification of immature and *in vitro* matured bovine cumulus-oocyte complexes: effects on oocyte structure and embryo development. Livestock Science, http://dx.doi.org/10.1016/j.livsci.2017.02.022, 2017.
7. Chow CK. Vitamin E and oxidative stress. Free Radic Biol Med. 1991; 11:215-232.
8. Cocchia N, Ciani F, Russo M, El Rass R, Rosapane I, Avallone L *et al*. Immature cat oocyte vitrification in open pulled straws (OPSs) using a cryoprotectant mixture. Cryobiol. 2010; 60:229-234.
9. Dalvit G, Lanes SP, Descalzo A, Insani M, Beconi M, Cetica P. Effect of α-Tocopherol and ascorbic acid on bovine oocyte *in-vitro* maturation. Reprod. Dome. Anim. 2005; 40(2):93-97.
10. Dalvit GC, Cetica PD, Pintos LN, Beconi MT. Reactive oxygen species in bovine embryo *in vitro* production. Biocell. 2005; 29(2):209-212.
11. Dutta DJ, Dev H, Raj H. *In-vitro* blastocyst development of post-thaw vitrified bovine oocytes. Vet. World. 2013; 6(10):730-733.
12. Geesin JC, Hendricks LJ, Falkenstein PA, Gordon JS, Berg RA. Regulation of collagen synthesis by ascorbic acid: Characterization of the role of ascorbate-stimulated lipid peroxidation, Arch. Biochem. Biophys. 1991; 290:127-132.
13. Gupta MK, Lee H. Cryopreservation of oocytes and embryos by vitrification. Korean. J. Reprod. Med. 2010; 37(4):267-291.
14. Hadi H, Wahid H, Rosnina Y, Daliri M, Dashtizad M, Holmes R *et al*. Nuclear maturation of immature bovine oocytes after vitrification using open pulled straw and cryotop methods. African J. Biotechnology. 2011; 10(2):2334-2339
15. Hurtt AE, Landin-Alvarenga F, Seidel GE Jr, Squires EL. Vitrification of immature and mature equine and bovine oocytes in an ethylene glycol, ficoll and sucrose solution using open-pulled straws. Theriogenology. 2000; 54:119-128.
16. John A, Joseph M, Vijayakumaran V, Manoj CJ. Effect of Oocyte Retrieval Techniques on Yield and Quality of Caprine Oocytes. IOSR Journal of Agri. and Vet. Sci. 2015; 8(4):50-52.
17. Kere M, Siriboon C, Lo NW, Ju JC. Ascorbic acid improves the developmental competence of porcine oocytes after parthenogenetic activation and somatic cell nuclear transplantation. Euro. J. Appl. Sci. 2012; 4(4):182-190.

18. Khalil AW, Mare FAW, Khalid M. Protective effects of antioxidants on linoleic acid treated bovine oocytes during maturation and subsequent embryo development. Theriogenology. 2013; 80(2):161-168.
19. Kitagawa Y, Suzuki K, Yoneda A, Watanabe T. Effect of oxygen concentration and antioxidants on the *in vitro* developmental ability, production of reactive oxygen species (ROS), and DNA fragmentation in porcine embryos. Theriogenology. 2004; 62:1186-1197.
20. Luck MR, Jeyaseelan I, Scholes RA. Ascorbic Acid and Fertility. Biology of Reprod. 1995; 52:262-266.
21. Martins RD, Costa EP, Chagas JSC, Ignacio FS, Torres CAA, McManus C. Effect of vitrification of immature bovine oocytes on *in vitro* maturation. Anim. Reprod. 2005; 2(2):128-134.
22. Miclea I, Pacala N, Hettig A, Zahan M, Miclea V. α-Tocopherol and ascorbic acid combinations Influence the Maturation of sheep oocytes. Anim. Sci. Biotech. 2012; 45(1):310-313.
23. Nadri B, Zeinoaldini S, Kohram H. Ascorbic acid effects on *in-vitro* maturation of mouse oocyte with or without cumulus cell. Afri. J. Biotec. 2009; 8(20):5627-5631.
24. Olson SE, Seidel GE Jr. Culture of *in vitro* produced bovine embryos with vitamin E improves development *in vitro* and after transfer to recipients. Biol Reprod. doi:10.1095/biolreprod62.2.248. 2000; 62:248-52.
25. Papis K, Shimizu M, Izaike Y. Factors affecting the survivability of bovine oocytes vitrified in droplets. Theriogenology. 2000; 54:651-658.
26. Purohit GN, Meena V, Solanki K. Morphological survival and subsequent *in- vitro* maturation of denuded and cumulus compact bubaline oocytes cryopreserved by ultra-rapid cooling. J. Buffalo Sci. 2012; 1(1):78-83.
27. Rehman A, Collins CS, Yang M, Kelly M, Diplock AT, Halliwell B *et al*. The effects of iron and vitamin C co-supplementation of oxidative damage to DNA in health volunteers. Biochem. Biophys. Res. Commun. 1998; 246:293-298.
28. Rho GJ, Kim S, Yoo JG, Balasubramanian S, Lee HJ, Choe SY. Microtubulin configuration and mitochondrial distribution after ultra-rapid cooling of bovine oocytes. Molec. Reprod. Deve. 2002; 63:464-470.
29. Thiyagarajan B, Valivittan K. Ameliorating effect of vitamin E on *in vitro* development of pre-implantation buffalo embryos. J. Assist. Reprod. Genet. 2009; 26:217-225.
30. Tilly JL, Tilly KI. Inhibitors of oxidative stress mimic the ability of follicle-stimulating hormone to suppress apoptosis in cultured rat ovarian follicles. Endocrinology. 1995; 136:242-252.
31. Ulloa U, CM, Yoshizawa M, Komoriya E, Mitsui A, Nagai T *et al*. The blastocyst production rate and incidence of chromosomal abnormalities by developmental stage in *in-vitro* produced porcine embryos. J Reprod. and Devel. 2008; 54:22-29.