RESEARCH ARTICLE

Effect of Alpha-Tocopherol Supplementation in TCM199 Medium on *In Vitro* Maturation and Cleavage of Buffalo Oocytes

Vijayalakshmi[1]*, Shrikant Kulkarni[1], KB Sathisha[1], HM Yathish[1], MH Girish[1], GS Naveen Kumar[2], RG Bijurkar[1], Ambadas[1], Yogesh[1], SM Kartikesh[1]

Abstract

An experiment was conducted to assess the effect of supplementing α-tocopherol at two different concentrations to the *in vitro* maturation (IVM) medium (TCM 199 culture medium) on *in vitro* maturation and cleavage of buffalo oocytes. Ovaries were collected from local slaughter house. Oocytes were collected by aspiration method and were matured in IVM medium (T0, control), IVM medium supplemented with α-tocopherol @ 10 µg/ml (T1) and IVM medium supplemented with α-tocopherol @ 20 µg/ml (T2). The mean cytoplasmic maturation rate was 82.39 ± 0.81, 88.81 ± 1.08, 81.67 ± 1.82 % and nuclear maturation rate was 62.50 ± 8.33, 87.50 ± 5.59, 66.66 ± 7.68 % in T0, T1 and T2 groups, respectively. The fertilized oocytes reaching 2-cell and 4-cell stages of cleavage for T0, T1 and T2 groups were 14.92 ± 1.52, 32.39 ± 1.01 and 16.39 ± 1.25 %, respectively. Significantly ($p < 0.05$) higher level of cytoplasmic maturation rate, nuclear maturation rate and cleavage rate was observed in IVM medium supplemented with α-tocopherol @10 µg/ml than other two groups.

Key words: α-tocopherol, Buffalo oocytes, Cleavage, *In vitro* maturation, *In vitro* fertilization.

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Introduction

Inherent reproductive problems limit the productivity of buffalo. Assisted reproductive technologies such as artificial insemination (AI), superovulation, *in vitro* fertilization (IVF), and embryo transfer (ET) have been introduced to overcome these problems, to increase the number of offspring from selected females, and to reduce the generation intervals in buffalo. Laboratory production of embryos (IVF technology) provides an excellent and inexpensive source of embryos for carrying on basic research in developmental physiology, farm animal breeding, and for commercial application of the emerging bio-techniques like cloning and transgenesis (Suresh *et al*., 2009). *In vitro* culture results in higher level of oxygen than *in vivo* environment, leading to an increased level of reactive oxygen species (ROS) that cause lipid peroxidation of cellular membranes. Oxidative stress has recently appeared as one of the most important factors contributing to low oocyte quality *in vitro*. Oxidative oocyte injury is detrimental to fertility potential (Guérin *et al.,* 2001) and early embryo development (Kitagawa *et al.,* 2004). Alpha-tocopherol (an active form of Vitamin-E) is the predominant lipid soluble antioxidant which is found to have powerful function as an antioxidant *in vitro* to protect the cells from ROS (Hamideh *et al.,* 2015). Hence the present experiment was designed with an aim to assess the effect of supplementing α-tocopherol at two different concentrations to the *in vitro* maturation medium on *maturation* and subsequently on *in vitro* fertilization (IVF) and cleavage of buffalo oocytes.

[1]Department of Veterinary Physiology and Biochemistry, Veterinary College, Nandi Nagar, KVAFSU, Bidar – 585 226, India.

[2]Veterinary College, Hassan, Karnataka, India

Corresponding Author: Vijayalakshmi, Department of Veterinary Physiology and Biochemistry, Veterinary College, Nandi Nagar, KVAFSU, Bidar – 585 226, India, e-mail: vijju4254@gmail.com

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Materials and Methods

Buffalo ovaries were collected from organised abattoir, M/s. Frigerio Conserva Allana Limited, Zaheerabad located near Bidar, Karnataka. Immediately after slaughter, the ovaries were collected and placed into thermos (27°C to 30°C) in normal saline (0.9% NaCl) containing gentamicin (50 µg/ml) and transported to the laboratory within two hours post

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slaughter. The ovaries were processed and cumulus oocyte complexes (COCs) were recovered from matured follicles into oocyte collection media (OCM) as per standard aspiration method described by Suresh and Maurya (2000).

In Vitro Maturation and Cleavage Study

The COCs were evaluated and graded according to cumulus morphology under a stereomicroscope (Motic, Germany). Only excellent (>5 layers of cumulus cells and evenly granulated cytoplasm) and good (>3 layers of cumulus cells and evenly granulated cytoplasm) COCs were isolated and used for the experiment. The separated COCs were washed three times in the *in vitro* maturation (IVM) medium [TCM199 culture medium containing 10 % FCS (Fetal calf serum), 5% sterile BFF (Buffalo follicular fluid), 0.3% BSA, 50 IU/ml Gonadotrophin, 10 IU/ml of hCG and 50 µg/ml gentamicin]. After washing, 20-25 COCs were cultured in 50 µl droplets of IVM medium (control group, T0) in 35 mm sterile petridish. The droplet was covered with warm non-toxic mineral oil and cultured at 38°C, 5% CO_2, 90-95% RH for 24 hrs in CO_2 incubator (Nuaire, USA). For treatments, the IVM medium was supplemented with α-tocopherol (*Sigma-Aldrich, USA*) @ 10 µg/ml (T1) and 20 µg/ml (T2). The experiment was repeated in 6 replicates for all the groups.

After 24 hr of maturation period, the cytoplasmic maturation was assessed (Kobayashi *et al*., 1994) under inverted microscope as per the degree of cumulus expansion and graded as degree 0 (no cumulus cell expansion), degree 1 (cumulus cells were non-homogenously spread and clustered cells were still observed) and degree 2 (cumulus cells were homogenously spread and clustered cells were no longer present). The cytoplasmic maturation rate (CMR) was calculated as:

$$\text{Cytoplasmic maturation rate (\%)} = \frac{\text{Total number of degree 1 and 2 matured oocytes}}{\text{Number of oocytes utilised for maturation}} \times 100$$

The nuclear maturation was assessed by nuclear staining under phase contrast microscope as per method of Wael *et al.*(2013) and they are graded as germinal vesicle (GV) stage, germinal vesicle breakdown (GVBD) stage, M1 and M2 stage. Nuclear maturation rate was calculated as:

$$\text{Nuclear maturation rate (\%)} = \frac{\text{Number of oocytes in GVBD, M-I and M-II stage}}{\text{Total number of matured oocytes stained}} \text{X } 100$$

To prepare the sperms for the IVF, semen straw was thawed in warm water (35-37° C) for 1 min, emptied in to 5 ml of Tyrode's Albumin Lactate Pyruvate (TALP) medium supplemented with 3 mg/ml BSA, 10 µg/ml heparin, 0.25 mM pyruvate (Sperm TALP medium) in a centrifuge tube and centrifuged at 800-1000 rpm for 5 min. The supernatant was removed and process was repeated again by adding 5 ml sperm TALP. Sperm pellet was dissolved in 1 ml of TALP medium supplemented with 6 mg/ml fatty acid free BSA, 10 µg/ml heparin, 0.25 mM pyruvate (Fertilization TALP medium), kept in CO_2 incubator for 30 min before inseminating the matured oocytes. The sperm concentration was adjusted to 4-5×10^6 cell/ml.

Following IVM, only degree 1 and 2 cumulus expanded oocytes were considered as matured and utilized for *in vitro* fertilization. They were washed several times with fertilization TALP medium to remove expanded cumulus cells. Denuded oocytes were transferred to 60 µl droplet of fertilization TALP medium in 35 mm sterile petridish and were inseminated with 40 µl of processed spermatozoa. The droplet was covered with sterile mineral oil and kept in CO_2 incubator at 38°C, 5% CO_2, 90-95% RH for 18 hr. After 18 hr of sperm oocyte co-incubation, oocytes were washed several times with modified synthetic oviductal fluid (mSOF) to remove the attached and dead spermatozoa from the oocytes. Washed oocytes were cultured in mSOF supplemented with 0.8% BSA, essential and nonessential amino acids. Cleavage rate was observed after 48 hr of culture, and it was calculated as:

$$\text{Cleavage rate (\%)} = \frac{\text{Number of cleaved oocytes (2-4 cell stage embryos) at 48 hr}}{\text{Number of oocytes utilised for maturation } \textit{in vitro} \text{ fertilization}} \text{X } 100$$

The data was subjected to statistical analysis using General Linear Model (GLM) procedures of SAS 9.3 to assess the significant difference at 5% level between the groups for cytoplasmic maturation rate, nuclear maturation rate and cleavage rate.

Results and Discussion

The mean cytoplasmic and nuclear maturation rate of buffalo oocytes cultured in IVM medium supplemented with two different concentrations of α-tocopherol are presented in Table 1 and 2, respectively. In the present study, the oocytes cultured in IVM medium supplemented with α-tocopherol @ 10 µg/ml showed significantly ($p < 0.05$) higher level of cytoplasmic and nuclear maturation rate assessed (Figs. 1 and 2) than the control group (T0) and α-tocopherol 20 µg/ml (T2) group. The beneficial effect of addition of tocopherol to the maturation media in improving cytoplasmic and nuclear maturation of oocytes has also been documented earlier by Cavalcante *et al.* (2009) in dogs, and Miclea *et al.* (2009) and Ileana *et al.* (2010) in pigs. Dalvit *et al.* (2005) reported that during *in vitro* maturation, α-tocopherol content naturally present in the membranes of COCs diminished by 50% in bovine, indicating the partial loss of antioxidant activity during the period of *in vitro* culture. Therefore, the favourable effect of additional supplementation of α-tocopherol in the

Table 1: Effect of α-tocopherol supplementation in IVM medium on cytoplasmic maturation rate (CMR %) of buffalo oocytes

Groups	No. of replicate	Culturable oocytes	Degree of COC expansion (%)			No. of oocytes matured (degree 1 and 2)	CMR (%)
			0	1	2		
Control (T0)	6	142	17.43 ± 0.81 (25)	30.07 ± 1.30 (43)	52.45 ± 1.58 (74)	117	82.39 ± 0.81[a]
T1	6	143	10.95 ± 1.07 (16)	32.14 ± 2.7 (46)	56.91 ± 3.64 (81)	127	88.81 ± 1.08[b]
T2	6	131	18.00 ± 1.81 (24)	29.44 ± 2.12 (38)	52.57 ± 2.54 (69)	107	81.67 ± 1.82[a]

Mean values with different superscripts differ significantly ($p \leq 0.05$).
The values in parenthesis are number of oocytes in respective degree of cumulus expansion.

Table 2: Effect of α-tocopherol supplementation in IVM medium on nuclear maturation rate (NMR, %) of buffalo oocytes

Groups	No. of replicates	Number of oocytes assessed	Stage of nuclear maturation (%)				NMR (%)
			GV	GVBD	M-I	M-II with PB	
Control	6	24	37.50 ± 10.70 (9)	12.50 ± 5.59 (3)	20.83 ± 4.16 (5)	29.17 ± 7.68 (7)	62.50 ± 8.33[a] (15)
T1	6	24	12.50 ± 5.59 (3)	20.83 ± 4.16 (5)	25.00 ± 0.00 (6)	41.67 ± 5.27 (10)	87.50 ± 5.59[b] (21)
T2	6	24	33.33 ± 10.5 (8)	8.33 ± 5.27 (2)	20.83 ± 7.68 (5)	37.50 ± 5.59 (9)	66.66 ± 7.68[a] (16)

Mean values with different superscripts differ significantly ($p \leq 0.05$).
The values in parenthesis are number of oocytes showing different stages of nuclear maturation.

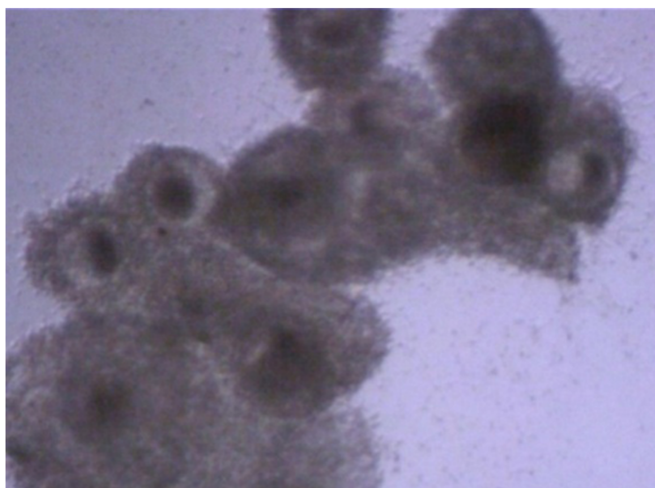
Fig. 1: Matured oocytes with expanded cumulus complex

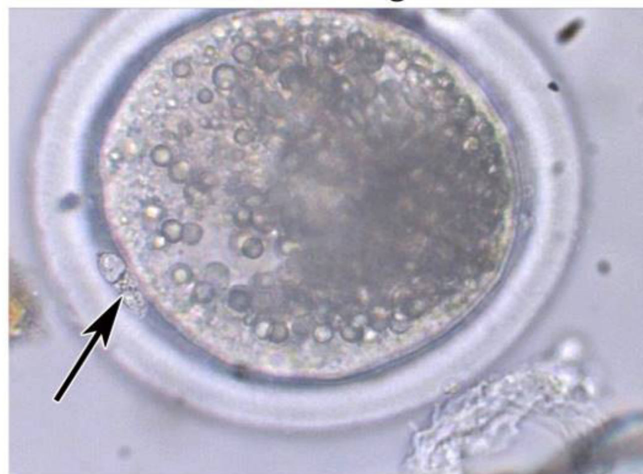
Fig. 2: Matured oocyte showing polar body

IVM medium on oocyte maturation rate can be attributed to the fact that α-tocopherol protects the polyunsaturated fatty acids in membranes against free radicals. It is probable that the lipid soluble α-tocopherol could be better distributed throughout the lipid rich environment of the oocyte and thus contributes to maintaining cell viability. Other possible functions of α-tocopherol is, it prevents the DNA fragmentation of cumulus cells and therefore, maintaining the GSH synthesis in COCs (Tao *et al.,* 2004). In contrast to our findings, Natarajan *et al.* (2010) and Hamideh *et al.* (2015) have concluded that supplementation of α-tocopherol in maturation medium had no beneficial effect on ovine oocyte maturation.

The number of fertilized oocytes that reached 2 cells stage (Fig. 3) and 4 cells stage (Fig. 4) of cleavage and the

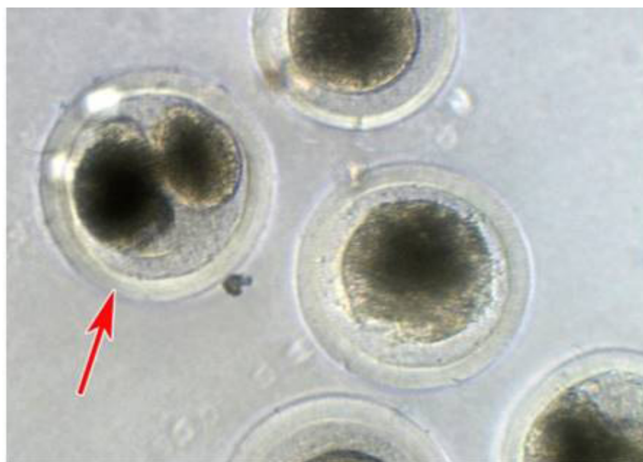
Fig. 3: Two-cell stage embryos

Table 3: Effect of α-tocopherol supplementation in IVM medium on cleavage rate (%) of *in vitro* fertilized buffalo oocytes

Groups	*No. of replicates*	*Number of matured oocytes fertilized*	*Number of fertilized oocytes*		*Cleavage rate (%)*
			2 cell stage	*4 cell stage*	
Control	6	67	10	0	14.92 ± 1.52[a] (10)
T1	6	71	19	4	32.39 ± 1.01[b] (23)
T2	6	61	9	1	16.39 ± 1.25[a] (10)

Mean values with different superscripts differ significantly ($p \leq 0.05$).
The values in parenthesis are number of fertilized oocytes showing cleavage.

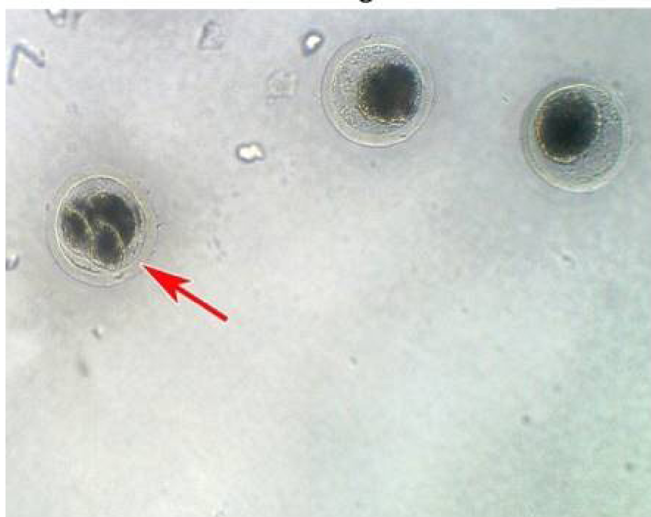

Fig. 4: Four- cell stage embryos

cleavage rates are presented in Table 3. The cleavage rate was significantly ($p < 0.05$) higher in the oocytes matured in IVM medium supplemented with α-tocopherol @ 10 µg/ml as compared to T0 and T2 groups. These findings are in agreement with the reports of Olson and Seidel (2000) in bovine, Natarajan *et al.* (2010) in ovine, and Kitagawa *et al.* (2004) and Jeong *et al.* (2006) in porcine, who have documented positive effect of α-tocopherol supplementation on early development of embryos. Feugang *et al*. (2004) reported that vitamin E could protect bovine embryos exposed to oxidative stress generated through an increase in ROS production or a decrease in antioxidant protection. Kitagawa *et al*. (2004) stated that vitamin E added to culture medium was shown to decrease the H_2O_2 content and increase the developmental ability to the blastocyst stage and the cell number in porcine IVF embryos.

Conclusion

The success of *in vitro* fertilization and *in vitro* embryo production depends on the proper cytoplasmic and nuclear maturation of oocytes. Improved cytoplasmic and nuclear maturation leads to an increase in the number of oocytes that are able to undergo fertilization and hence more embryos will be available for embryo transfer. It can be concluded from the experiment that the supplementation of α-tocopherol @ 10 µg per ml to the IVM medium is suitable approach to manage oxidative stress to improve *in vitro* maturation of buffalo oocytes and to achieve higher cleavage rate upon *in vitro* fertilization.

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