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Original Article

Synergistic impact of α-linolenic acid and α-tocopherol on *in vitro* maturation and culture of buffalo oocytes

Impacto sinérgico do ácido α-linolênico e α-tocoferol na maturação *in vitro* e cultura de oócitos de búfalo

A. Azam[a]*, R. Ejaz[a], S. Qadeer[b], S. Irum[c], A. Ul-Husna[d], S. Ullah[b], Q. Shahzad[e], T. Akhtar[f] and S. Akhter[g]

[a]Shaheed Benazir Bhutto Women University, Department of Zoology, Peshawar, Pakistan
[b]University of Education, Division of Science & Technology, Department of Zoology, Lahore, Pakistan
[c]University of Gujrat, Department of Zoology, Gujrat, Pakistan
[d]University of Haripur, Department of Zoology, Haripur, Pakistan
[e]Bovine IVF Laboratory, Ovatech (Shandong) Biotechnology, Dongying, China
[f]Buffalo Research Institute Pattoki, Kasur, Lahore, Pakistan
[g]Pir Mehr Ali Shah Arid Agriculture University, Department of Zoology, Rawalpindi, Pakistan

Abstract

The objective of the current study was to investigate the synergistic impact of α-Tocopherol and α-Linolenic acid (100 µM) on IVM and IVC of Nili Ravi buffalo oocytes. Oocytes were obtained from the ovaries of slaughtered buffaloes within two hours after slaughter and brought to laboratory. Buffalo cumulus oocyte complexes were placed randomly in the five experimental groups included; GROUP 1: Maturation media (MM) + 100 µM ALA (control), GROUP 2: MM + 100 µM ALA + 50µM α-Tocopherol, GROUP 3: MM + 100 µM ALA + 100µM α-Tocopherol, GROUP 4: MM + 100 µM ALA + 200 µM α-Tocopherol and GROUP 5: MM + 100 µM ALA + 300 µM α-Tocopherol under an atmosphere of 5% CO2 in air at 38.5 °C for 22-24 h. Cumulus expansion and nuclear maturation status was determined (Experiment 1). In experiment 2, oocytes were matured as in experiment 1. The matured oocytes were then fertilized in Tyrode's Albumin Lactate Pyruvate (TALP) medium for about 20 h and cultured in synthetic oviductal fluid (SOF) medium to determine effect of α-Linolenic acid (100 µM) and α-Tocopherol in IVM medium on IVC of presumptive zygotes. To study the effect of α-Linolenic acid (100 µM) in IVM media and increasing concentration of α-tocopherol in the culture media on early embryo development (Experiment 3), the presumptive zygotes were randomly distributed into the five experimental groups with increasing concentration of α-tocopherol in culture media. Higher percentage of MII stage oocytes in experiment 1(65.2±2.0), embryos at morula stage in experiment 2 (30.4±1.5) and experiment 3 (22.2±2.0) were obtained. However, overall results for cumulus cell expansion, maturation of oocyte to MII stage and subsequent embryo development among treatments remain statistically similar ($P > 0.05$). Supplementation of α-tocopherol in maturation media having α-Linolenic acid and/or in embryo culture media did not further enhance *in vitro* maturation of oocyte or embryo production.

Keywords: α-linolenic acid, α-tocopherol, buffalo, nuclear maturation, embryonic development.

Resumo

O objetivo do presente estudo foi investigar o impacto sinérgico do α-tocoferol e do ácido α-linolênico (100 µM) na MIV e CIV de oócitos de búfala Nili Ravi. Os oócitos foram obtidos dos ovários de búfalos abatidos duas horas após o abate e levados ao laboratório. Complexos de oócitos cumulus de búfalo foram colocados aleatoriamente nos cinco grupos experimentais incluídos; GRUPO 1: Meio de maturação (MM) + 100 µM ALA (controle), GRUPO 2: MM + 100 µM ALA + 50 µM α-tocoferol, GRUPO 3: MM + 100 µM ALA + 100 µM α-tocoferol, GRUPO 4: MM + 100 µM ALA + 200 µM α-tocoferol e GRUPO 5: MM + 100 µM ALA + 300 µM α-tocoferol sob uma atmosfera de 5% de CO2 em ar a 38,5 °C por 22-24 h. A expansão cumulus e o estado de maturação nuclear foram determinados (Experimento 1). No experimento 2, os oócitos foram maturados como no experimento 1. Os oócitos maturados foram então fertilizados em meio de Tyrode's Albumina Lactato Piruvato (TALP) por cerca de 20 h e cultivados em meio de fluido oviductal sintético (SOF) para determinar o efeito do ácido α-linolênico (100 µM) e α-tocoferol em meio IVM em IVC de presumíveis zigotos. Para estudar o efeito do ácido α-linolênico (100 µM) em meio IVM e aumentar a concentração de α-tocoferol no meio de cultura no desenvolvimento inicial do embrião (Experimento 3), os presumíveis zigotos foram distribuídos aleatoriamente nos cinco grupos experimentais com concentração crescente de α-tocoferol em meios de cultura. Maior porcentagem de oócitos em estágio MII no experimento 1

*e-mail: asimaazam786@gmail.com
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(65,2 ± 2,0), embriões em estágio de mórula no experimento 2 (30,4 ± 1,5) e experimento 3 (22,2 ± 2,0) foram obtidos. No entanto, os resultados gerais para a expansão das células do cumulus, maturação do oócito para o estágio MII e desenvolvimento embrionário subsequente entre os tratamentos permanecem estatisticamente semelhantes (P> 0,05). A suplementação de α-tocoferol em meios de maturação com ácido α-linolênico e / ou em meios de cultura de embriões não aumentou ainda mais a maturação in vitro de oócitos ou a produção de embriões.

Palavras-chave: ácido α-linolênico, α-tocoferol, búfalo, maturação nuclear, desenvolvimento embrionário.

1. Introduction

Dietary lipid supplementation is believed to enhance fertilizing potential in animals by increasing ovulatory follicle size, progesterone concentration in plasma and lifespan of corpus luteum (Sharma et al., 2020). Supplementing ruminants' diet with fats improves quality of the blastocysts obtained. Likewise, the *in vitro* bovine, ovine and porcine oocyte maturation and high quality embryos are obtained when fatty acids supplemented media was used (Marei et al., 2009; Ghaffarilaleh et al., 2014; Jeon et al ., 2020). We have previously shown that α-linolenic acid (100 µM) enriched *in vitro* growth media enhanced the rates of oocyte maturation and pre-implantation embryo development in Nili Ravi buffalo (Azam et al., 2017). Apart from the useful role of α-linolenic acid, it is also susceptible to lipid peroxidation. As rising the fatty acids in media also increase the lipid droplets in oocyte which enhance lipid peroxidation in the cells (Halliwell and Chirico, 1993). Lipid peroxidation implicates production of reactive oxygen species that may obstruct oocyte quality for further development (Khalil et al., 2013).

The reactive oxygen species (ROS) like hydrogen peroxide, hydroxyl radicals and superoxide anions are produced naturally by developing embryos as they interact with their adjacent environment (Nasr-Esfahani and Johnson, 1991; Khurana and Niemann, 2000; Guerin et al., 2001). But complex nature of oviductal fluid i.e. hormones, defense agents, growth factors, glyco-proteins and antioxidants (Gandolfi et al., 1993; Bavister, 1995; Ozdaş et al., 2006; Aviles et al., 2010) ensures protection of embryos against oxidative stress and ROS (Guerin et al., 2001). Whereas, ROS are produced in an excess amount during *in vitro* culture of oocytes (Goto et al., 1993) and due to absence of counteract mechanism against oxidative stress the developing embryos are more liable to be harmed (Johnson and Nasr-Esfahani, 1994; Guerin et al., 2001) and are of low quality (Enright et al., 2000; Sadeesh et al., 2014).

To enhance quality of *in vitro* produced embryos, the supplementation of media with antioxidants is reported (Gordon, 1994; Lott et al., 2011). Alpha-tocopherol (Vitamin E) is an antioxidant that is reported to have efficient role not only in in vivo conditions but also positive effect during *in vitro* culture conditions (Miller et al., 1993; Olson and Seidel Junior, 2000) to protect the early embryo from free radicals (Wang et al., 2002). Vitamin E limits the damages to cell membranes (Niki et al., 1989; Van Metre and Callan, 2001), defends against lipid peroxidation (Wagner et al., 1996; Dalvit et al., 2005) and avoids apoptosis in mammalian cells (Carlson et al., 1993; Olson and Seidel Junior, 2000; Arias-Álvarez et al., 2018).

In our previous study (Azam et al., 2019) synthetic oviductal fluid medium co-cultured with monolayer of isologous oviductal epithelial cells further improved early embryo development. The putative mechanisms include detoxification and procurement of required metabolites and particular development stimulators into the surrounding medium (Malayer et al., 1988). Considering the role of α-tocopherol (vitamin E) as cellular control of lipid peroxidation and as an antioxidant, it was assumed that addition of α-tocopherol in the primary media with α-linolenic acid (100 µM) and culture media will further enhance *in vitro* growth and/or early embryonic maturation in buffalo. The intent was to examine the synergistic influence of increasing α-tocopherol (0 µM (control), 50 µM, 100 µM, 200 µM and 300 µM) in IVC and IVM medium on oocyte maturation and/or embryos development.

2. Materials and Methods

2.1. Collection of ovaries

A total of 2250 buffalo ovaries were collected at a local abattoir immediately after slaughter and transported back to the laboratory in a thermos flask containing sterilized pre warmed phosphate buffered saline (PBS) maintained at 33-35^{0}C within two hours after slaughter. After arrival in the laboratory, ovaries were rinsed with fresh PBS immediately.

2.2. Collection and classification of cumulus oocyte complexes (COCs)

Cumulus oocyte complexes (COCs) were collected by aspiration of 2-8 mm visible follicles present on the surface of ovaries as described earlier (Azam et al., 2017). The COCs were classified as grade A, B, C and D, based on cumulus mass investment and ooplasm homogeneity under stereomicroscope as described earlier (Azam et al., 2017). Only grade A and B oocytes were processed for IVM. A total of 1992 cumulus oocyte complexes (COCs) of grade A and B were collected.

2.3. In vitro maturation (IVM)

Selected oocytes were washed twice in PBS (37 °C) and twice in pre-equilibrated maturation medium. The washed oocytes were allocated into 100 µl of respective culture droplets of each treatment group covered with sterile mineral oil (Sigma M8410) and matured *in vitro* in a water-jacketed incubator at 38.5 °C under an atmosphere of 5% CO_2 in air with 95% humidity for 24 h. All the media and culture dishes were equilibrated at 37 °C for at least 1-2 hours before experiment. Cumulus expansion was assessed by visual assessment using stereomicroscope as 1) not expanded, 2) partially expanded or 3) fully expanded. For determination of nuclear maturation stage, COCs were

completely denuded, stained with 1% aceto-orcein and examined for metaphase II (MII) stage (Azam et al., 2017).

2.4. In vitro fertilization (IVF)

Three 0.5 mL straws of cryopreserved buffalo semen were thawed in water at 37 °C for 30 seconds. Thawed semen was placed in a 15 mL conical tube. Spermatozoa with maximum motility were collected by swim up technique (Parrish et al., 1986). About 250 µL of thawed semen was deposited at the bottom of four 15 mL tubes containing 3 mL of pre warmed sperm wash medium (TALP: modified calcium-free Tyrode's Albumin Lactate Pyruvate with 6 mg/ mL BSA fraction-V). Tubes were incubated at 45° angle for 30 minutes. Supernatant from each tube was removed and transferred into another 15 mL conical tube and centrifuged at 1600 rpm for 10 minutes. The pellet obtained after centrifugation was resuspended in pre-warmed fertilization TALP supplemented with 0.1mM hypotaurine, 0.2mM penicillamine, 0.01mM epinephrine, 10 µg/mL heparin, to get a final concentration of 2 x 10^6 live sperm mL^{-1}. After 24 hours of IVM, buffalo oocytes were washed in fertilization media and were placed in fertilization droplet (5 COCs/50µL droplet) of pre warmed fertilization medium under mineral oil with final sperm concentration of 2×10^6 mL^{-1}. The oocyte and spermatozoa were incubated at 38.5 °C under 5% CO2 with maximum humidity for 20 h (Azam et al., 2019).

2.5. In vitro embryo culture (IVC)

After IVF, presumptive zygotes were denuded by vortexing in PBS. After one washing with PBS and two with culture media; synthetic oviductal fluid medium (SOF), presumptive zygotes were transferred to 50 µL of culture drop with isologous oviductal cell monolayer and cultured in an incubator at 38.5 °C, 5% CO_2 and 95% humidity. On day 2 of development (Day 0 = day of insemination) the cleavage rate (number of oocytes cleaved/total ×100 COCs incubated) was observed, After three days of culture 40 µL of culture medium was replaced without disrupting the monolayer. Further developmental stages were evaluated and recorded every other day (Azam et al., 2019).

2.6. Experimental design

2.6.1. Experiment 1: effect of α-linolenic acid and α-tocopherol in maturation media on IVM of buffalo oocytes

A total of 662 cumulus oocyte complexes (COCs) of grade A and B, were collected by aspiration of 2-8 mm visible follicles present on the surface of 750 ovaries and processed for IVM. The α-Linolenic acid level that yielded best results in terms of oocyte maturation and early embryo development in our previous study (Azam et al., 2019) was used in this experiment. COCs were placed randomly in the five experimental groups included; GROUP 1: TCM-199 + 100 µM ALA (control), GROUP 2: MM + 100 µM ALA + 50µM α-Tocopherol, GROUP 3: MM + 100 µM ALA + 100µM α-Tocopherol, GROUP 4: MM + 100 µM ALA + 200 µM α-Tocopherol and GROUP 5: MM + 100 µM ALA + 300 µM α-Tocopherol. Results of expansion of cumulus cells and nuclear maturation status of COCs after 24 hours of maturation, from six replicates were pooled in this experiment.

2.6.2. Experiment 2: effect of α-linolenic acid and α-tocopherol in maturation media on early embryo development in buffalo oocytes

A total of 560 cumulus oocyte complexes (COCs) of grade A and B, were collected by aspiration of 2-8 mm visible follicles present on the surface of 700 ovaries and processed for IVM. All the treatment groups of maturation media were same as experiment 1. After 24 h of maturation oocytes were inseminated with prepared sperm for *in vitro* fertilization and were incubated for 20 h. After fertilization presumptive zygotes were processed for *in vitro* culture. On day 2 of development (Day 0 = day of insemination) the cleavage rate (number of oocytes cleaved/total ×100 COCs incubated) was observed. Further developmental stages were evaluated and recorded every other day. Results of early embryo development from five replicates were pooled in this experiment.

2.6.3. Experiment 3: effect of α-linolenic acid (ALA) in IVM media and α-tocopherol in culture media on subsequent embryo development

A total of 770 cumulus oocyte complexes (COCs) of grade A and B, were collected by aspiration of 2-8 mm visible follicles present on the surface of 850 ovaries and processed for IVM. Oocytes were allocated to IVM medium supplemented with α-Linolenic acid (100 µM). After fertilization presumptive zygotes were placed randomly in the five experimental groups with increasing concentration of α-tocopherol in culture media (0 µM (control), 50 µM, 100µM, 200µM and 300µM α-Tocopherol). Results of early embryo development from five replicates were pooled in this experiment.

2.7. Statistical analysis

Data of cumulus expansion, nuclear maturation stages of *in vitro* matured oocytes, and subsequent developmental stages were recorded and analyzed by one-way analysis of variance (ANOVA) at 5% level of significance.

3. Results

3.1. Experiment 1: effect of α-linolenic acid and α-tocopherol in maturation media on IVM of buffalo oocytes

The data on the degree of cumulus expansion and nuclear maturation of buffalo COCs matured in the *in vitro* maturation media supplemented with 100 µM ALA and different concentrations of α-tocopherol are shown in Figure 1 and Table 1 respectively. Supplementation of different concentrations of α-tocopherol (0 (control), 50, 100, 200 and 300 µM) in the maturation media having 100 µM ALA did not differ ($P > 0.05$) in degree of cumulus expansion and nuclear maturation rate of buffalo oocytes as compared to control.

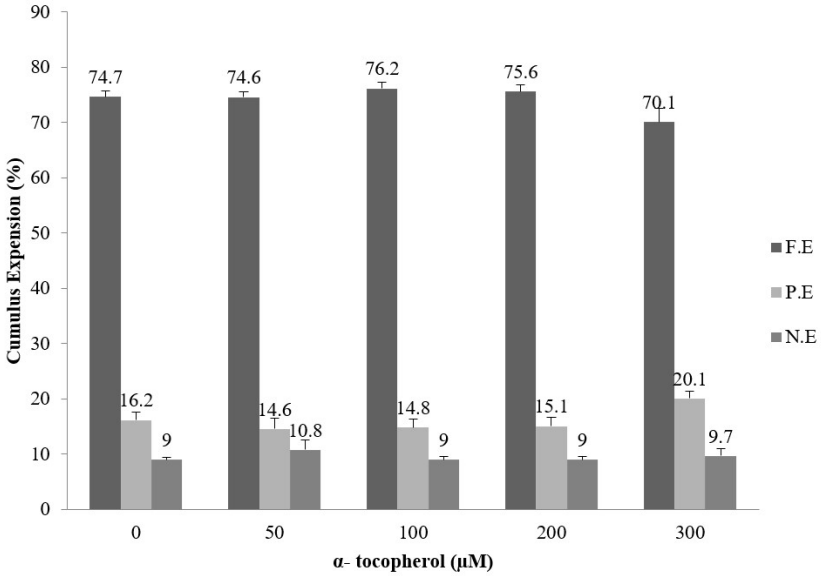

Figure 1. Cumulus expansion rate of buffalo oocytes after 24 hours of maturation in IVM medium supplemented with ALA (100µM) and different concentrations of α-tocopherol. Data were collected in six independent repeats. All the values were statistically non-significant among treatments (P > 0.05). F.E: Fully Expanded; P.E: Partially Expanded; N.E: Not Expanded

Table 1. Nuclear maturation status of buffalo oocytes after 24 hours of maturation in IVM medium supplemented with ALA (100µM) and different concentrations of α-tocopherol.

Treatments ALA+α-tocopherol	No. of COCs	Nuclear Maturation Status (Mean percentage±SEM)			
		GV N (%±SEM)	GVBD N (%±SEM)	MI N (%±SEM)	MII N (%±SEM)
100µM+0 µM	154	2(1.3±1.4)	34 (22.1±1.0)	24 (15.6±1.8)	94 (61.0±1.7)
100µM+50 µM	130	0 (0.0±0.0)	28 (21.5±1.0)	22 (16.9±1.2)	80 (61.5±0.6)
100µM+100 µM	122	2 (1.6±1.2)	26 (21.3±0.9)	16 (13.1±1.6)	78 (63.9±2.3)
100µM+200 µM	132	2 (1.5±1.1)	28 (21.2±1.8)	16 (12.1±1.4)	86 (65.2±2.0)
100µM+300 µM	124	2 (1.6±1.12	28 (22.6±1.9)	20 (16.1±2.3)	74 (59.7±0.9)

Data were collected in six independent repeats. All the values were statistically non-significant (P > 0.05) among treatments. GV: Germinal Vesicle; GVBD: Germinal Vesicle Breakdown; MI: Metaphase I; MII: Metaphase II; N: Total number of COCs.

3.2. Experiment 2: effect of α-linolenic acid and α-tocopherol in maturation media on early embryo development in buffalo oocytes

The data on effect of supplementation of ALA (100 µM) combined with different concentrations of α-tocopherol in the *in vitro* maturation medium on cleavage rate and embryonic development stage are shown in Table 2. The cleavage rate, development of embryo up to 4-8 cell stage, >8 cell stage and morula did not vary (P > 0.05) when oocytes were matured in the *in vitro* maturation media supplemented with 100 µM ALA combined with α-tocopherol at 0 (control), 50, 100, 200 and 300 µM.

3.3. Experiment 3: effect of α-linolenic acid (ALA) in IVM media and α-tocopherol in culture media on subsequent embryo development

The data on the effect of supplementing ALA (100 µM) in the *in vitro* maturation medium and different concentrations of α-tocopherol (0 (control), 50, 100, 200 and 300 µM) in the *in vitro* culture media on cleavage rate and developmental stages of buffalo embryos are shown in Table 3. Results indicate that values for cleavage rate and embryonic development up to 4-8 cell stage, >8 cell stage and morula did not vary when the *in vitro* culture media was supplemented with α-Tocopherol at 0 (control), 50, 100, 200 and 300 µM.

4. Discussion

Dietary source of fatty acids have been reported to affect composition of fatty acids in the follicular fluid that in turn influences molecular mechanisms in the oocyte and mitochondrial distribution and activity (Marei et al., 2010). Provision of fatty acids in the *in vitro* maturation media provides energy source to the maturing oocytes (Sturmey et al., 2009), control MAPK signaling pathway and are a direct source of prostaglandins (PGs). PGE_2 serves as a mediator of oocyte maturation and results in increased

Table 2. Effect of supplementation of IVM medium with ALA (100µM) and different concentrations of α-tocopherol on the cleavage rate and subsequent developmental of buffalo oocytes *in vitro*.

Treatments ALA+α-tocopherol in IVM Medium	No. of COCs	Developmental stages (Mean percentage±SEM)			
		Cleavage N (%±SEM)	4-8 cell embryos N (%±SEM)	>8 cell embryos N (%±SEM)	Morulla N (%±SEM)
100µM+0 µM	112	66 (58.9±2.7)	52 (46.4±3.5)	40 (35.7±2.2)	26 (23.2±2.3)
100µM+50 µM	112	66 (58.9±2.7)	50 (44.6±2.2)	40(35.7±2.2)	26 (23.2±1.2)
100µM+100 µM	112	68 (60.7±2.3)	52 (46.4±3.8)	40 (35.7±4.4)	28 (25.0±3.1)
100µM+200 µM	112	72 (64.3±5.6)	54 (48.2±3.1)	42 (37.5±1.8)	34 (30.4±1.5)
100µM+300 µM	112	68 (60.7±5.0)	52 (46.4±3.8)	42 (37.5±1.8)	28 (25.0±3.1)

Data were collected in five independent repeats. All the values were statistically non-significant (P > 0.05) among treatments.

Table 3. Effect of supplementation of IVM medium with ALA (100µM) and IVC medium with different concentrations of α-tocopherol on cleavage rate and subsequent embryonic development of buffalo oocytes *in vitro*.

Treatments ALA in IVM +α-tocopherol in IVC	No. of COCs	Developmental stages (Mean percentage±SEM)			
		Cleavage N (%±SEM)	4-8 cell embryos N (%±SEM)	>8 cell embryos N (%±SEM)	Morula N (%±SEM)
100µM+0 µM	154	86 (55.9 ±1.6)	60 (39.1±2.2)	48 (31.3±1.8)	26 (17.0±1.7)
100µM+50 µM	154	88 (57.3±2.7)	64 (41.4±2.5)	46 (29.8±2.2)	26 (17.0±1.7)
100µM+100 µM	154	94 (61.0±1.9)	66 (42.9±1.5)	50 (32.6±2.2)	28 (18.5±3.0)
100µM+200 µM	154	96 (62.3±3.8)	72 (46.8±1.4)	54 (35.1±1.2)	34 (22.2±2.0)
100µM+300 µM	154	92 (59.7±4.0)	66 (42.9±1.5)	46 (29.9±1.4)	24 (15.6±1.6)

Data were collected in five independent repeats. All the values were statistically non-significant among treatments (P > 0.05).

MII stage oocytes and better quality embryos (Marei et al., 2009). However, in addition to valuable role of fatty acids for *in vitro* maturation of oocytes and embryo production, lipid peroxidation of fatty acids is unavoidable that may induce oxidative stress. In order to overcome the oxidative stress, different antioxidants have been studied such as vitamins (A, C, E), hypotaurine, taurine and cysteamine (Guerin et al., 2001; Thiyagarajan and Valivittan, 2009), with varying effects on *in vitro* maturation and embryo development.

Use of α-tocopherol with ALA in IVM medium has not been reported previously. In present study, the addition of α-tocopherol in maturation medium along with ALA (100 µM) did not improve the efficiency of the medium for expansion of cumulus mass and rate of nuclear maturation in buffalo oocytes *in vitro*. Perhaps the concentration of ALA used in this study has not produced oxidative stress to a level that could have been compensated by the antioxidant. As, mentioned earlier (Azam et al., 2017), ALA at higher concentration (300 µM) reduced the number of COCs with fully expanded oocytes and the rate of oocytes reaching up to MII stage. This reduced oocyte maturation rate at higher concentration of ALA, might have been due to the production of ROS in the IVM medium. Another plausible reason might be that the selected cumulus oocyte complexes were good enough that they have protected oocytes from oxidative stress. Earlier studies suggested that composition of culture medium and quality of oocyte especially presence of cumulus cells around oocytes play important role for protecting oocytes against oxidative stress (Thiyagarajan and Valivittan, 2009). These cumulus cells around the oocytes protect oocytes from changes in the intracellular environment caused by changes in the extracellular medium (Ali et al., 2003). Moreover, previous studies on cattle (Dalvit et al., 2005) and buffalo (Thiyagarajan and Valivittan, 2009), demonstrated that α-tocopherol alone in IVM medium failed to affect oocyte maturation rates, suggesting that the antioxidant have no direct effect on nuclear and/or cytoplasmic maturation.

During *in vitro* culture conditions for early embryos, increased generation of reactive oxygen species (ROS) leads to lipid peroxidation of cellular membranes (Johnson and Nasr-Esfahani, 1994) that may lead to impairment in the permeability and function of membranes, ultimately causing permanent cell damage (Olson and Seidel Junior, 2000). Pre-compaction embryos have less complex system development as compared to post-compaction embryos; therefore they are more liable to oxidative damage (Lane and Gardner, 2007). Reducing oxygen concentration in the *in vitro* culture environment enhanced the embryo development in mouse (Pabon Junior et al., 1989). Similarly, supplementing antioxidants in culture media also improved the efficiency of culture media for bovines (Marei et al., 2010; Khalil et al., 2013).

Alpha-tocopherol protects mammalian cells against lipid peroxidation (Chow, 1991) and when supplemented

in culture media is reported to increase the survival rates of embryos (Steele et al., 1974). However, results in present study demonstrated that α-tocopherol addition in the culture medium was not efficient to improve the development rate of buffalo embryos at any stage of *in vitro* culture. In earlier studies positive effects of α-tocopherol on embryo development have been reported in buffalo (Thiyagarajan and Valivittan, 2009) and cattle (Olson and Seidel Junior, 2000) during IVC. Although mammalian gametes and embryos seem capable to accept α-tocopherol, the culture environment in which it is used (i.e., co-culture with oviductal cells) is possibly responsible for its efficacy. In the present study, the use of co-culture (oviduct epithelial cell) might have helped reduced O_2 concentration in culture media and detoxified diffusible ROS by antioxidant systems present in these cells (Ouhibi et al., 1989; El-Mouatassim et al., 2000) to an extent that α-tocopherol have not shown its effect. Another possible reason might be the high number of oviductal cells used in culture media that may have consumed α-tocopherol, thereby reducing the effect of α-tocopherol on the embryos.

5. Conclusion

It is concluded from this study that supplementation of α-tocopherol in the basic media with α-linolenic acid (100 µM) and culture media with isologous oviductal epithelial cells did not improve the *in vitro* maturation and/or early embryonic development of buffalo oocytes.

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