Ameliorating effect of vitamin E on *in vitro* development of preimplantation buffalo embryos

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Abstract

Purpose Oxidative stress has been implicated in the etiology of defective embryo development. Vitamin E is an effective lipid-soluble antioxidant, protecting cell membranes from peroxidative damage. In this context, this study was undertaken to find if supplementation of vitamin E in culture medium could ameliorate the developmental competence of preimplantation buffalo embryos.

Methods Vitamin E was supplemented in maturation/embryo culture medium at concentrations of 0, 50, 100, 200 and 400 μM . The developmental competence of buffalo embryos was assessed by observing the cleavage, morulae, blastocyst rate, total cell count and comet assay.

Results Vitamin E had no significant effect in maturation medium. Vitamin E in embryo culture medium under 5% O_2 significantly reduced blastocyst formation in the 400 μM supplemented group. Culture under 20% O_2 enhanced the frequency of blastocyst formation, total cell count and significantly reduced comet tail in the 100 μM supplemented group ($P < 0.001$) when compared with the control. Vitamin E in ECM for the first 72 h of culture period enhanced blastocyst rate and total cell number in the 100 μM group ($P < 0.001$) when compared with the control.

Conclusion Our results demonstrate that the addition of Vitamin E may enhance the developmental competence of buffalo embryos *in vitro* by protecting them from oxidative stress.

Keywords Blastocyst · *In vitro* fertilization · Matured oocytes · Oxidative stress · Vitamin E

Introduction

Buffalo is an important livestock resource and is well adapted to a hot and humid climate and play a distinct role in the economy of farmers. Reproductive efficiency is the primary factor affecting productivity and is hampered in the female buffalo by various factors which include delayed puberty, silent estrous, a long postpartum period before the return to estrous and low conception rates [1]. As the application of superovulation and embryo transfer technologies in the buffalo has been only marginally successful [2], there is an increasing interest in the production of embryos through the use of *in vitro* fertilization (IVF) technology. Although the *in vitro* embryo production efficiency has improved, embryo yield and development to term are still very low. The entire *in vitro* system has been developed and established in buffalo species by extrapolating information acquired in more studied species such as cattle. The acquisition of more insights into buffalo embryo physiology, metabolism, and culture requirements is critical to optimize the efficiency of advanced reproductive strategies in this species.

Reactive oxygen species (ROS) are produced by embryo metabolism [3] and culture environment [4, 5]. Oxidative injury may be responsible for numerous types of embryo damage, which include mitochondrial alterations, embryo

Capsule Buffalo embryos are prone to oxidative stress due to high lipid content; supplementation of vitamin E enhanced their developmental competence by protecting them from oxidative stress.

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cell block and apoptosis [6]. In efforts to curtail these potentially damaging effects, cells have evolved complex antioxidant defense mechanisms including those which enzymatically neutralize many of these ROS and those which use small molecule scavengers like vitamins A, C and E, and also sulphur compounds which include reduced glutathione (GSH), hypotaurine, taurine and cysteamine (CSH).

Embryo protection against ROS depends, in part, upon an endogenous pool of antioxidant enzymes [7]. Despite the endogenous protective mechanisms in the oocytes and embryos, the oxygen free radicals mostly affect mitochondrial DNA, proteins and lipids as well as the cytoplasm, where they disturb the ratio of glutathione to glutathione disulphide [8]. Enzymes dedicated to protecting the cell from these ROS include superoxide dismutase (SOD), which converts superoxide anions into oxygen and hydrogen peroxide; and glutathione peroxidase and catalase, both of which catalyze the conversion of hydrogen peroxide into water and oxygen. Although the presence of these enzymatic protectants is extensive intracellularly, their protective roles extracellularly are limited as their levels are low in extracellular compartments. Instead, small molecule scavengers like vitamin C and vitamin E seem to play a more important role in the extracellular milieu. Significant amounts of CSH have been detected in the follicular fluid of the cow, sow, goat and dog [9].

Antioxidant vitamins help to reduce oxidant damage by acting as a sink to the spare electrons [10, 11]. Tocopherols are membrane soluble lipids which react with free radicals and reactive oxygen species. Earlier studies indicate that culture of bovine embryos with vitamin E resulted in development of more embryos to early and expanded blastocysts, compared to embryos cultured in the control medium [12] and studies on porcine suggest that blastocyst quality of porcine somatic cell nuclear transfer and IVF embryos was improved when embryo culture media was supplemented with vitamin E [13]. Since the effect of vitamin E has not been evaluated in buffalo, an attempt has been made to assess the role of vitamin E in *in vitro* production of buffalo embryos through its supplementation in maturation and embryo culture medium.

Materials and methods

All chemicals used in the experiment were purchased from Sigma Chemicals, unless otherwise indicated.

Oocyte collection

Buffalo ovaries were obtained from a local abattoir (Chennai Corporation Abattoir, Chennai, Tamilnadu) and

transported to the laboratory in 0.9% saline supplemented with 50 µg/mL gentamycin, within two hours of slaughter. The ovaries were washed repeatedly in normal saline. Cumulus oocyte complex (COC) were isolated from the follicles by slicing method [14]. The isolated oocytes were washed three times in Tyrode's lactate - N- [2-Hydroxyethyl] Piperazine-N'- [2- ethanesulphonic acid] (TL-HEPES) medium and classified into three categories (good, fair, poor) according to the character of the cumulus cells. Oocytes with more than 4 layers of compact cumulus cells and uniform cytoplasm were classified as good quality oocytes; oocytes with less than 4 layers or with a partial cumulus mass were classified as fair quality oocytes; and oocytes without cumulus cells with a uniform cytoplasm were classified as poor quality oocytes.

In vitro maturation

Only the oocytes that were completely surrounded by at least 4 to 5 layers of unexpanded cumulus cells and had a homogenous ooplasm were used for maturation. The collected oocytes were washed three times in TL-HEPES medium. The last two washings were in the maturation medium composed of TCM 199 (Invitrogen Corporation, USA) supplemented with 10% fetal calf serum (FCS, Gibco laboratories, Grand Island, USA) 0.5 µg/mL FSH, 5.0 µg/mL LH and 1 µg/mL Estradiol (E2). Twenty oocytes were placed in 100 µL droplets of the maturation medium covered with sterile paraffin oil and incubated at 39°C under 5% CO₂ in air and 95% relative humidity for 24 h.

In vitro fertilization

Frozen straws (0.5 ml) of buffalo semen were procured from Tamilnadu Veterinary and Animal Sciences University, Chennai. Two 0.5 ml straws, thawed for 60 sec at 35°C were emptied into 15 mL air-tight tube and washed with Brackett and Oliphant (BO) medium containing heparin 10 µg/mL [15] without BSA by centrifugation at 700 g for 5 min. The sperm pellet was mixed with 2 mL of BSA-free BO medium containing 10 mM of caffeine (sodium caffeine benzoate) and 10 µg/mL heparin in air-tight tube and placed into the incubator at 38.5°C for swim up for 1 h, after which the top 1.8 mL of the semen suspension containing motile spermatozoa was removed through micropipettes and washed twice with BSA-free BO medium. The sperm pellets were resuspended in 1 mL of BSA-free BO medium supplemented with caffeine and heparin in the same concentrations. This was further diluted with 1 mL of BO medium containing 20 mg/mL BSA supplemented with 10 µg/mL heparin. Matured buffalo oocytes were washed in BO medium. The oocytes were then distributed to 10 per

50 μL drop of fertilization medium under paraffin oil. Two μL of swim up spermatozoa was then added to 50 μL fertilization drops to give a final sperm concentration of 2×10^6 . The oocyte and spermatozoa were incubated at 39 °C under 5% CO_2 in air for 24 h.

In vitro embryo culture

After incubation with spermatozoa, the ova were removed from the fertilization drops. The cumulus cells were stripped off by repeated pipetting through a small-bore pipette and cultured with modified synthetic oviductal fluid [16], containing 3 mg/mL of BSA, 0.6 mM sodium pyruvate, 2% (v/v) BME essential and 1% (v/v) MEM non essential amino acids, 100 $\mu\text{g/mL}$ penicillin and streptomycin and cultured for 8 days at 39°C under 5% CO_2 , 5% O_2 and 90% N_2 (or) 5% CO_2 in air, which is approximately 20% O_2 .

Cell number analysis

Embryos were evaluated for the appearance and morphological quality by determining the cell numbers. Expanded day 8 blastocysts from each treatment groups were fixed and stained as per the method described herewith [17]. These expanded blastocysts were then transferred individually onto glass microscopic slides and after drying at room temperature, fixed with 70% ethanol for 24 h. The fixed blastocysts were stained with 10 $\mu\text{g/mL}$ bisbenzamide (Hoechst 33342) and 2.3 % sodium citrate. The slides were observed under an epifluorescence microscope, fitted with excitation filter (330 to 380 nm) and barrier filter (420 nm). The total numbers of nuclei were counted in each blastocyst.

Comet assay for detecting DNA damage in individual embryo

DNA damage in individual embryos that developed under high oxygen concentration was assessed by comet assay [18]. After 3 days of culture, embryos cultured under 20% O_2 with/without vitamin E supplement were removed and used for analysis of DNA damage by comet assay. Ten to twenty embryos were washed twice in PBS plus polyvinylpyrrolidone (4 mg/mL). Embryos in each experimental group were then transferred to a 200 μL drop of 1% low-melting temperature agarose (Bangalore, Genei) in PBS at 39°C; the agarose drop was placed on a 35-mm plastic Petri dish. Using a stereo dissecting microscope to visualize the embryos, the embryos were gently mixed with the 1% low-melting temperature agarose and then captured in a total volume of about 10 μL with a mouth-operated glass pipette. The embryos were then quickly placed on a microscope slide glass, which was initially coated with 1%

high-melting temperature agarose (Bangalore, Genei). The slides were then placed on ice for 5 min to solidify the agarose. The embryos were then lysed by incubating the slides for 3 h at ambient temperature in lysing buffer, which was composed of 10 mM Tris, pH 10, containing 1% sodium sarcosinate, 2.5 mM NaCl, 100 mM $\text{Na}_2\text{-EDTA}$, 1% Triton X-100, and 10 $\mu\text{g/mL}$ proteinase K. The slides were then removed from the lysing solution and placed on a horizontal gel electrophoresis unit. The unit was filled with fresh electrophoresis buffer (1 mM $\text{Na}_2\text{-EDTA}$, 300 mM NaOH) to a level of 0.25 cm above the slides, and the slides were equilibrated in the electrophoresis buffer for 20 min. Electrophoresis was then conducted for 20 min at 25 V. After the electrophoresis, the slides were neutralized by immersing them in 0.4 M Tris-HCl (pH 7.5) for 5 min at ambient temperature. DNA was stained by adding a 20 μL drop of acridine orange (5 $\mu\text{g/mL}$) to the slide for 2 min followed by 1 min of washing in distilled water. Observation of DNA was carried out under a fluorescence microscope. DNA damage was quantified by measuring the length of the streak of DNA comet tail between the edge of the zona pellucida and the end of the visible comet tail. The length was calculated by referring to a photograph of a micrometer at the same magnification as the embryos.

Experimental design

Vitamin E (α -tocopherol) was first dissolved in 95% ethanol as a 2000-strength stock solution, stored in the dark at 4°C, and then (18–20 h prior to culture) diluted in culture medium to get the required concentration of vitamin E [12]. The concentration of ethanol during maturation or culture was less than 0.05%. In control groups, 0.05% ethanol was added in maturation/ culture medium according to the experimental design.

In experiment I, varying concentrations of vitamin E (0, 50, 100, 200 and 400 μM) were supplemented to the maturation medium alone and embryos were allowed to develop under 5% O_2 . In experiment II, varying concentrations of vitamin E (0, 50, 100, 200 and 400 μM) were added to embryo culture medium for the entire culture period of 8 days and embryos were allowed to develop under 5% O_2 . In experiment III, various concentration of vitamin E was supplemented in ECM for the entire culture period and the embryos were allowed to develop under 20% O_2 . In experiment IV, vitamin E was added to the embryo culture medium for the first 72 h of embryo development of 8-day culture and embryos were developed under 5% O_2 . The embryo culture medium was replaced with fresh medium after 72 hrs, to avoid toxic accumulation of ammonia. Vitamin E was also added to the fresh embryo culture medium.

Statistical analysis

In each experimental group, oocytes were randomly distributed. The percentages of oocytes fertilized and embryos developed to morulae and blastocyst stage were subjected to arcsine transformation before analysis. The data for total cell count was directly subjected to analysis. All data was subjected to one-way ANOVA followed by Tukey test to determine differences in experimental groups using Statistical Package for Social Sciences (SPSS 11.0, Chicago, USA). Differences $P < 0.05$ were considered statistically significant.

Results

Experiment I

The effects of vitamin E in maturation medium are shown in Table 1. The rate of cleavage, the cleaved embryos that developed to compact morulae, blastocyst percentage and total cell count showed no significant change in the vitamin E supplemented group when compared with the control.

Experiment II

The effects of vitamin E in ECM under low oxygen tension are shown in Table 2. The rate of cleavage and the embryos that developed to compact morulae showed no significant change when compared with the control. The study revealed that the presence of 400 μM vitamin E resulted in significant reduction in the blastocyst rate ($P < 0.001$) when compared with 50 μM groups and ($P < 0.01$) when compared with the control, 100 and 200 μM groups. No significant change was observed in total cell count.

Experiment III

The effects of vitamin E in ECM, under high oxygen tension are shown in Table 3. No significant change was

observed in the rate of cleavage, while significant increase was observed in proportions of embryos that developed to morulae in 100 μM vitamin E supplemented group ($P < 0.01$) when compared with the control and ($P < 0.05$) when compared with the 400 μM supplemented group. Addition of 100 μM vitamin E enhanced blastocyst formation ($P < 0.001$) when compared with the control and ($P < 0.01$) when compared with the 50 and 400 μM supplemented groups. The proportion of embryos that developed to blastocyst stage increased significantly ($P < 0.001$) in 50, 200 & 400 μM supplemented groups when compared with the control. Total cell count increased significantly in 100 μM supplemented group ($P < 0.001$) when compared with the control and ($P < 0.05$) when compared with the 50 μM group. Supplementation of 200 μM vitamin E increased cell count significantly ($P < 0.05$) when compared with control.

The extent of DNA damage in individual embryos on day 3 of culture under 20% O_2 with or without vitamin E supplement is illustrated in Fig. 1. DNA in individual embryos that migrated was visualized as a comet tail like streak under fluorescence microscope. Embryos cultured without vitamin E in ECM exhibited comet tail, which is significantly greater ($p < 0.001$) when compared with embryos cultured with 100 μM vitamin E supplement.

Experiment IV

The effect of vitamin E in ECM for the first 72 h of embryo development is illustrated in Table 4. Cleavage rate did not differ significantly among treated groups when compared with the control. The embryos that developed to compact morulae showed a significant increase in the 100 μM group ($P < 0.05$) when compared with the 400 μM supplemented group. The proportions of embryos that developed to blastocyst increased significantly in the 100 μM supplemented group ($P < 0.001$) when compared with the control, 50 and 400 μM groups. Supplementation of 200 μM vitamin E in ECM tended to improve blastocyst rate ($P < 0.01$) when compared with the control and ($P < 0.05$) when compared with the 50 μM supplemented group. The total cell count

Table 1 Effect of Vitamin E in maturation medium on *in vitro* development of preimplantation buffalo embryos under low oxygen tension

Vitamin E Con, (μM)	No. of Oocytes inseminated(n)	Cleavage %	Morulae %	Blastocyst %	No. of blastocyst evaluated	Total cell number
0	236	60.97 $\pm$ 2.58 ^a	30.23 $\pm$ 1.05 ^a	11.19 $\pm$ 0.64 ^a	17	91.33 $\pm$ 3.98 ^a
50	242	58.96 $\pm$ 2.02 ^a	29.33 $\pm$ 1.23 ^a	11.72 $\pm$ 0.67 ^a	17	94.33 $\pm$ 4.18 ^a
100	245	63.77 $\pm$ 2.33 ^a	31.50 $\pm$ 1.26 ^a	13.25 $\pm$ 1.09 ^a	21	95.67 $\pm$ 4.02 ^a
200	238	63.67 $\pm$ 2.52 ^a	32.67 $\pm$ 1.25 ^a	12.26 $\pm$ 0.98 ^a	19	93.17 $\pm$ 4.16 ^a
400	235	62.61 $\pm$ 3.09 ^a	30.38 $\pm$ 1.29 ^a	11.50 $\pm$ 0.97 ^a	17	90.50 $\pm$ 4.27 ^a

Means in the same column with different superscripts were significantly different ($P < 0.05$)

Values are listed as Mean $\pm$ S.E.M.

Table 2 Effect of Vitamin E in Embryo culture medium on *in vitro* development of preimplantation buffalo embryos under low oxygen tension

Vitamin E Con.(μ M)	No. of Oocytes inseminated(n)	Cleavage %	Morulae %	Blastocyst %	No. of Blastocyst evaluated	Total cell number
0	230	64.36 $\pm$ 3.09 ^a	31.61 $\pm$ 0.82 ^a	11.57 $\pm$ 0.69 ^a	19	90.17 $\pm$ 3.80 ^a
50	241	60.13 $\pm$ 2.58 ^a	30.05 $\pm$ 1.50 ^a	12.98 $\pm$ 0.80 ^a	19	93.67 $\pm$ 3.33 ^a
100	248	62.92 $\pm$ 2.53 ^a	31.21 $\pm$ 1.23 ^a	12.05 $\pm$ 0.78 ^a	19	93.00 $\pm$ 4.12 ^a
200	237	60.73 $\pm$ 2.68 ^a	32.37 $\pm$ 1.25 ^a	11.64 $\pm$ 0.76 ^a	17	87.66 $\pm$ 3.56 ^a
400	240	58.10 $\pm$ 2.17 ^a	29.39 $\pm$ 1.60 ^a	7.84 $\pm$ 0.58 ^b	11	81.83 $\pm$ 3.66 ^a

Means in the same column with different superscripts were significantly different ($P<0.05$)

Values are listed as Mean $\pm$ S.E.M.

increased significantly ($P<0.01$) in the 100 μ M group when compared with the control and the 50 μ M supplemented group.

Discussion

The development of a suitable culture system is essential for successful production of embryos *in vitro*. Since oxidative stress during culture has been emphasized as one of the main factors responsible for low production and poor quality of *in vitro* produced embryos, it has been suggested that environmental factors such as exposure to light, greater oxygen tension and culture medium composition induce changes in embryo metabolism leading to an imbalance in the production and degradation of ROS [19, 20].

Our results show that supplementation of vitamin E in maturation medium and subsequent culture of embryos under 5% O₂ had no beneficial effect on developmental competence of buffalo oocytes. Addition of vitamin E to the maturation medium failed to improve the blastocyst percentage and total cell numbers, suggesting that the antioxidant exert no effect on nuclear and/or cytoplasmic maturation thereby increasing the developmental com-

petence of buffalo embryos. This was in agreement with the previous studies [21] in bovine, which demonstrated the active form of vitamin E had no effect on developmental competence in maturation medium.

Earlier studies suggest that the most important factors for the IVM of oocytes are the composition of the culture medium and the quality of the immature oocytes, particularly the presence of cumulus cells which play a critical role in protecting oocytes against apoptosis induced by oxidative stress [22]. In converse, earlier report in bovine suggests that the production of ROS in denuded oocytes from immature COC's was unaltered by maturation, indicating that culture conditions employed were not responsible for oxidative stress in the female gamete [23].

The presence of vitamin E in ECM, throughout the culture period under 5% O₂ had significantly reduced the blastocyst development in 400 μ M supplemented group. Vitamin E is a potent lipid-soluble antioxidant. Despite its low concentration in cell membranes it is considered the main lipid-soluble antioxidant in the body, although it has been also shown to have pro-oxidant activity [24]. Earlier reports have demonstrated that embryos cultured *in vitro* under 5% O₂ have higher developmental rates than those cultured under 20% O₂ [25, 26]. ROS have a number of effects in cells, under physiological concentration they

Table 3 Effect of Vitamin E in Embryo culture medium on *in vitro* development of preimplantation buffalo embryos under high oxygen tension

Vitamin E Con.(μ M)	No. of Oocytes inseminated(n)	Cleavage %	Morulae %	Blastocyst %	No. of Blastocyst evaluated	Total cell number
Control	235	60.05 $\pm$ 2.43 ^a	28.21 $\pm$ 1.12 ^a	9.23 $\pm$ 0.56 ^a	13	87.16 $\pm$ 3.45 ^a
50	242	58.79 $\pm$ 2.25 ^a	32.31 $\pm$ 0.98 ^{ab}	14.73 $\pm$ 0.54 ^b	21	95.67 $\pm$ 3.99 ^{ab}
100	236	64.86 $\pm$ 2.67 ^a	35.09 $\pm$ 1.25 ^b	18.79 $\pm$ 0.86 ^c	31	116.50 $\pm$ 4.30 ^c
200	248	63.42 $\pm$ 2.64 ^a	32.31 $\pm$ 1.49 ^{ab}	17.02 $\pm$ 1.03 ^{bc}	27	108.50 $\pm$ 4.99 ^{bc}
400	245	62.04 $\pm$ 2.19 ^a	30.08 $\pm$ 1.04 ^a	14.35 $\pm$ 0.77 ^b	22	102.83 $\pm$ 4.30 ^{abc}

Means in the same column with different superscripts were significantly different ($P<0.05$)

Values are listed as Mean $\pm$ S.E.M.

Fig. 1 Effect of vitamin E on DNA damage by comet assay. **a** DNA damage in buffalo embryos cultured in 20% oxygen concentration. **b** Embryos cultured under 20% oxygen supplemented with 100 μ M Vitamin E. Scale bars 50 μ m

modify and fine-tune intracellular signaling and their potentially adverse effects are prevented by different cellular antioxidant systems. Thus, the supplementation of antioxidant under low oxygen tension may scavenge the ROS,

which might help in key physiological process. Hence the reduced rate of blastocyst development obtained during supplementation of vitamin E in ECM throughout the culture period may be due to its high concentration.

In vitro culture conditions deviate from *in vivo* in many respects, but one of the critical factors appears to be oxygen tension under which embryos are cultured. The presence of vitamin E in ECM under 20% O_2 significantly increased the blastocyst rate in the vitamin E supplemented group when compared with the control. The decrease of blastocyst percentage in the control group is due to the fact that oxygen concentration in air (20%) is considerably higher than intraluminal oxygen tension in the reproductive tract of most mammals [27]. Oxygen tension at the atmospheric level is known to exert harmful effects on the development of mammalian embryos, probably due to the formation of free oxygen radicals [28]. Earlier study suggested that culture under 5% oxygen contributed to faster development of human embryos *in vitro*, resulting in a larger number of good quality and clinically applicable embryos [29]. Moreover, it is critical to minimize exposure of zygotes to the atmospheric O_2 concentration for optimal development to continue past the 8- to 16-cell stage, at least in the mouse [4]. There are inconsistent reports regarding effects of low O_2 tension on *in vitro* development of preimplantation embryos. While some studies have reported beneficial effects [30, 31] others have reported either no effect [32, 33], or even an adverse effect of low oxygen concentrations on embryonic development [34].

The effect of ROS on embryo development is paradoxical. Most studies have shown that prolonged, experimentally induced ROS production severely inhibits embryo development [35]. However, ROS may also act as second messengers in mammalian cells [36, 37]. Several genes are activated in response to alterations in ROS concentration including those for protein kinases [38], tyrosine kinases and growth factors [39]. However, it is most likely that the balance between ROS production and elimination (that is, the altered reduction–oxidation [REDOX] states), rather than

Table 4 Effect of Vitamin E in Embryo culture medium for first 72 hrs on *in vitro* development of preimplantation buffalo embryos under low oxygen tension

Vitamin E Con. (μ M)	No. of Oocytes inseminated(n)	Cleavage %	Morulae %	Blastocyst %	No. of Blastocyst evaluated	Total cell number
0	232	57.65 $\pm$ 2.18 ^a	30.29 $\pm$ 1.50 ^{ab}	12.54 $\pm$ 0.82 ^a	17	90.33 $\pm$ 3.89 ^a
50	240	61.48 $\pm$ 2.69 ^a	31.97 $\pm$ 1.06 ^{ab}	13.89 $\pm$ 1.15 ^a	21	93.50 $\pm$ 3.91 ^a
100	246	67.18 $\pm$ 2.63 ^a	35.53 $\pm$ 1.67 ^b	20.56 $\pm$ 0.72 ^c	34	115.83 $\pm$ 5.09 ^b
200	239	63.89 $\pm$ 2.69 ^a	34.78 $\pm$ 0.93 ^{ab}	17.67 $\pm$ 0.96 ^{bc}	27	105.00 $\pm$ 3.75 ^{ab}
400	244	61.01 $\pm$ 2.26 ^a	30.01 $\pm$ 1.23 ^a	14.64 $\pm$ 0.69 ^{ab}	22	101.60 $\pm$ 3.96 ^{ab}

Means in the same column with different superscripts were significantly different ($P < 0.05$)

Values are listed as Mean $\pm$ S.E.M.

ROS themselves, determines these responses. Therefore, REDOX state regulation has important functions for optimal growth responses [38]. Hence the observed variation in developmental competence in a dose-dependent concentration of antioxidant supplement may be due to shift in REDOX status.

In the present study the increase in developmental competence of buffalo embryos by vitamin E may be due to its antioxidant effect. Buffalo oocytes showed an abundance of cytoplasmic granules, which give cytoplasm of oocytes and embryos a dark appearance at light microscopy, which are characterized by significant lipid content [40]. Therefore buffalo oocytes and embryos are likely to be more sensitive to oxidative damage. Moreover, earlier studies demonstrated that the culture of mouse zygotes in 20% O₂ compromises the developmental potential of resultant blastocysts, which appear to be normal on morphological assessment [41]. The supplementation of vitamin E prevents lipid peroxidation, as the free radicals are scavenged more quickly than they are able to react with fatty acid side chains or membrane proteins and cause breaking of the lipid peroxidation chain. In the present study the comet assay reveals embryos cultured under 20% oxygen tension without vitamin E are subjected to more DNA damage when compared to embryos supplemented with vitamin E, suggesting that the increase in developmental competence of buffalo embryos may be due to the antioxidant effect of vitamin E.

The ROS production by early embryos and their susceptibility to ROS vary with the stage of development. The supplementation of vitamin E in ECM for the first 72 h of embryo culture shows significant increase in blastocyst percentage and total cell count in the 100 µM group compared to the control. ROS has been implicated in the impaired development of mammalian embryos *in vitro* [28]. Earlier studies suggest that increase in H₂O₂ production *in vitro* coincides with that of developmental block which occur at the 2-cell stage, suggesting a possible relationship between the developmental block and potential rise in damaging free radicals generated from the H₂O₂ [42].

Earlier studies suggest that the gradual increase in ROS levels from the 2-cell embryo up to the late morulae stage could depend on the metabolic change undergone by the embryo during its development. The adenosine triphosphate (ATP) production increases at the onset of compaction and blastocyst development to support increased protein synthesis and activity of ion transport systems, notably the Na⁺/K⁺-dependent ATPase [43, 44]. An increase in ATP production is inferred from the increases in uptake of oxygen and energy substrates, such as pyruvate and glucose [45]. This enhancement in oxidative metabolism of the embryo could be linked to the detected increase in ROS level. Thus antioxidant supplementation is essential for the

first 72 h to enhance the developmental competence. Since at the later stage of blastocyst the decrease of ROS was detected, this may be related to onset of cellular differentiation. It has been demonstrated that, oxygen uptake diminishes in expanded blastocysts due to their lower physiological ATP demand [46], because glycolysis begins to contribute to ATP production. Moreover, shifts in intracellular REDOX state may also contribute to spatial differences in cell activity, especially after compaction, and perhaps even major embryonic events such as fertilization, genome activation and cellular differentiation [47]. Thus supplementation of vitamin E for the first 72 h is essential and may help to overcome developmental block and oxidative stress.

The *in vitro* culture conditions and high lipid content of buffalo oocytes make them more prone to oxidative stress. Thus supplementation of vitamin E in ECM under appropriate conditions ameliorates the developmental competence of preimplantation buffalo embryos *in vitro* by protecting against oxidative stress. Further specifically designed experiments are required to find the exact mechanism by which vitamin E contributes to the developmental competence of buffalo embryos and to confirm the present hypothesis.

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