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Effect of growth factor and antioxidant on *in vitro* maturation of oocytes and cleavage rates of *in vitro* produced Indian buffalo (*Bubalus bubalis)* embryos

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ABSTRACT

The present study was carried out to evaluate the effect of growth factor in combination with antioxidant on the *in vitro* maturation (IVM) rate of oocytes and cleavage rates of *in vitro* fertilized (IVF) Indian *Bubalus bubalis* embryos. The cumulus oocyte complexes (COCs) were collected from slaughterhouse ovaries by aspirating follicles, and kept in maturation media (MM) for 24 h. The MM consisted of Group-1 (Control MM) - TCM 199 + 10% FBS + PMSG (20 IU/mL) + hCG (10 IU/mL) + Sodium pyruvate (0.80 mM) + L-glutamine (2 mM) + Gentamicin (50 µg/mL), Group-2 - Control MM + Epidermal growth factor (EGF) (10 ng/mL) + β-mercaptoethanol (β-ME) (25 µM), Group-3 - Control MM + EGF (20 ng/mL) + β-ME (100 µM), Group-4 - Control MM + EGF (100 ng/mL) + β-ME (500 µM). After maturation in diverse maturation media, the media used for IVF and the subsequent development of embryos was the same in all groups. The present results revealed that 20 ng/mL EGF + 100 µM β-ME concentrations were optimum and showed a significant effect on oocyte maturation and further development. Also high concentrations of EGF + β-ME (100 ng/mL + 500 µM), showed a decrease in the cumulus expansion rate, polar body formation rate and cleavage rates. A significant improvement in cleavage rate was observed when oocytes were matured in maturation medium with 20 ng/mL EGF + 100 µM β-ME and capacitation and fertilization was carried out in BO medium compared with TALP medium (64.8 ± 3.8 vs 44.1 ± 2.9). No significant difference in cleavage rate was observed for oocytes matured

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in the control group, control group + 10 ng/mL EGF + 25 μM β-ME and control group + 100 ng/mL EGF + 500 μM β-ME, between BO and TALP medium, indicating the beneficial effect of the addition of 20 ng/mL EGF + 100 μM β-ME to the maturation media for improved *Bubalus bubalis* embryo production under *in vitro* culture.

Key words: oocyte, growth factor, antioxidant, *in vitro* maturation, *in vitro* fertilization, cleavage rate

Introduction

Inherent reproductive problems limit the productivity of buffalo (*Bubalus bubalis*), which is an important species in India in terms of milk, meat as well as draft. *In vitro* embryo production would be an effective technique to improve the efficacy of transferable embryo production. In fact, progress in the use of embryo transfer in buffalo is slow due to poor superovulatory response. *In vitro* embryo production improvement is important for production of embryos of high quality, for use in animal biotechnology and biomedical research (FEUGANG et al., 2009). Comparison of supplements to the media for buffalo oocytes maturation, fertilization and embryo culture have yielded varying results (GASPARRINI et al., 2006).

In mammals, oocyte and embryo culture are negatively affected by increased oxidative stress. *In vitro* culture results in higher oxygen concentrations than the *in vivo* environment, leading to an increased level of reactive oxygen species (ROS), including superoxide anions, hydrogen peroxide and hydroxyl radicals that cause lipid peroxidation of the cellular membrane. Buffalo oocytes are very sensitive to oxidative stress, because of their high lipid content (BONI et al., 1992).

KIM et al. (2004) reported that glutathione (GSH) functions in many ways, such as in maintaining the redox state in cells, improving formation of male pronucleus, protein and DNA synthesis, and reduction of disulfides. The GSH content of oocytes increases during maturation of oocytes in the ovary, which is used for cell protection in later steps (BEHESHTI et al., 2011). Synthesis of GSH during oocyte maturation has been reported in the oocytes of pigs (YOSHIDA, 1993) and cattle (DE MATOS et al., 1996). It was reported that in the presence of low molecular weight, thiol containing precursors of GSH, such as cysteamine and β-mercaptoethanol in the IVM medium, increases the GSH content of oocytes after maturation and this has a beneficial effect on the development of 6-8-cell stage bovine embryo up to the blastocyst stage (GASPARRINI et al., 2003; BEHESHTI et al., 2011). It has also been shown that supplementation of thiol compounds, such as cysteamine and β- mercaptoethanol, increases GSH synthesis during bovine and ovine IVM, which affects embryo development and quality (DE MATOS et al., 2002). The number of oocytes exhibiting pronuclei formation in buffaloes increased when IVM medium was supplemented with β-mercaptoethanol (SONGSASEN and APIMETEETUMRONG, 2002).

The epidermal growth factor receptor (EGFR) signalling pathway plays a pivotal role in cell-cell communication in both vertebrates and invertebrates. It also participates

in specification of cell fate and coordinates cell proliferation. Binding of EGFR with its ligand, EGF (on the cell surface), triggers receptor dimerization which leads to transphosphorylation of multiple tyrosine residues, and recruitment of a plethora of enzymes and adaptor proteins essential for cell survival and cell proliferation. Amongst the various factors studied for the complex events that eventually prepare oocytes for fertilization, epidermal growth factor (EGF) is known to induce resumption of meiosis in oocytes of various mammalian species, such as rats (FENG et al., 1989); mice (DAS et al., 1992); pig (SIROTKIN et al., 2000); cattle (NANDI et al., 2002); sheep (GULER et al., 2000), buffalo (KUMAR and PUROHIT, 2004); dogs (BOLAMBA et al., 2006); horses (LINDBLOOM et al., 2008). The possible mechanisms of EGF action on oocyte maturation are either disruption of oocyte communication with cumulus cells (DECKEL and SHERIZLY, 1985) or creation of a positive maturational signal (DOWNS, 1989) and mediation of the effect via a tyrosine kinase dependent intracellular mechanism (LORENZO et al., 2001). However, the detailed downstream events in EGF-induced meiotic resumption are not well characterized (GALL et al., 2005). Conflicting results have also been reported on the effects of EGF on *in vitro* maturation of oocytes in various mammals, including cattle (OYAMADA et al., 2004), humans (GOUD et al., 1998), dogs (BOLAMBA et al., 2006), cats (MERLO et al., 2001) and pigs (ABEYDEERA et al., 2000). The contradictory results on the effect of EGF on IVM of oocytes may be due to heterogeneous IVM conditions.

The present study was undertaken to evaluate the effect of epidermal growth factor, in combination with an antioxidant on the *in vitro* maturation rate of oocytes and cleavage rates in *in vitro* fertilized Indian *Bubalus bubalis* embryos.

Materials and methods

Reagents and media. All the chemicals and media were purchased from Sigma Chemical Co. (St Louis, MO) and disposable plastic goods were from Nunc (Roskilde, Denmark), unless otherwise mentioned.

Collection of oocytes. Buffalo ovaries (n = 1556) were obtained from the slaughterhouse (Fig.1A), they were placed in normal saline (0.9% NaCl), containing 400 IU/mL penicillin-G and 500 µg/mL streptomycin and transported to the laboratory within 6 h. The stromal tissue surrounding the ovaries was removed and oocytes from follicles (2 to 8 mm) were aspirated with a 16 gauge needle attached to a 10 mL syringe, filled with aspiration media (TCM-199 + 0.3% Bovine serum albumin (BSA) + 0.1mg/mL L-Glutamine + 50 µg/mL Gentamicin). The follicular fluid containing oocytes was pooled in a sterile 50 mL centrifuge tube (Corning, NY) and allowed to settle for 30 min at 37 °C. After settling, about 5 mL of the sediment was aspirated and placed in 10 cm diameter polystyrene sterile Petri dish (Nunclon, Demark). Oocytes with compact multi-layered cumulus investment and evenly granulated cytoplasm (Grade 1 as described by

CHAUHAN et al., 1998) were selected for in vitro maturation (Fig. 1B). The oocytes were picked up with a sterile glass pipette under a stereomicroscope and transferred to another dish containing washing medium TCM-199 supplemented with 10% fetal bovine serum (FBS), 0.09 mg/mL sodium pyruvate, 0.1 mg/mL L-glutamine and 50 µg/mL gentamicin.

In vitro maturation. The maturation medium (MM): Group-1-Control - TCM-199 + 10% FBS + PMSG (20 IU/mL) + hCG (10 IU/mL) + Sodium pyruvate (0.80 mM) + L-Glutamine (2 mM) + Gentamicin (50 µg/mL), Group-II- Control MM + EGF (10 ng/mL) + β-ME (25 µM), Group-III- Control MM + EGF (20 ng/mL) + β-ME (100 µM) and Group-IV- Control MM + EGF (100 ng/mL) + β-ME (500 µM). The pH of the medium was adjusted to 7.4 and it was filtered through 0.2 µm filter (Pal life Sciences, Ann Arbor, USA) just before use. The cumulus oocyte complexes were washed several times with the IVM medium and groups of 10-15 COCs were placed in 100 µL droplets of the IVM medium, covered with sterilized mineral oil in a 35- mm Petri dish and cultured for 24 h under 5% CO_2 at 38.5 °C (Fig. 1C).

Cumulus expansion assessment. Cumulus expansion was graded according to a 0-4 scale as described by FAGBOHUN and DOWNS (1990). Using this scale, score 0 indicates no expansion, characterized by the detachment of cumulus cells from the oocyte, to assume a flattened monolayer of fibroblastic appearance leaving a partially or fully denuded oocyte. A score of 1 indicates no expansion but spherical cumulus cells , remaining compact around the oocyte. For a score 2 complex, only the outer most layer of cumulus cells have expanded, a score 3 complex has all layers prominently expanded except the corona radiata and a score of 4 indicates the maximum degree of expansion including the corona radiata (cells most proximal to the oocyte).

A representative number of expanded COCs from each group (n = 40) were subjected to nuclear maturation. The nuclear examination of the COCs was assessed at 24 h of *in vitro* culture after removing the surrounding cumulus cells and zona pellucida, as described by SHAH et al. (2008). Briefly, cumulus oocyte complexes with expanded cumulus were transferred into 1.5 mL micro centrifuge tube containing 500 µL hyaluronidase in T2 (Where T denotes M-99 supplemented with 2 mM L-glutamine, 0.2 mM sodium pyruvate, 50 µg/mL gentamicin and following numbers denotes 2% FBS) and incubated for 1 min at 38.5 °C, followed by vortexing (2 min). Completely denuded oocytes with eventually granular cytoplasm (Fig. 1D) were selected and incubated in pronase (2 mg/mL in T10) for 10 min at 38.5 °C; oocytes with completely digested zona pelluida (Fig. 1E) were transferred to T20 and incubated for 10 min at 38.5 °C. Zona free oocytes were observed and evaluated morphologically at 20× magnification under a phase contract microscope for nuclear status, and oocytes with a polar body (PB) were classified in Metaphase phase II (MII) stage of maturation (Fig. 1F). Successful nuclear maturation was also confirmed when denuded oocytes were transferred to 10 µL of glycerol bisbenzimide drops (Hoechst

33342 - Invitrogen, USA) and mounted on slides for fluorescence microscopic analysis of the nuclear maturation stage (Landim-Alvarenga, 2001) (Fig. 1G).

Sperm capacitation. Spermatozoa were capacitated using both Bracket and Oliphant (BO) media (BRACKET and OLIPHANT, 1975) and Tyrodes Albumin Lactate (TALP) media (BAVISTER and YANAGIMACHI, 1977). Frozen semen from buffalo bulls in 0.25 mL straws was thawed in a water bath at 37 °C for 1 min. Semen was obtained from the semen lab of the institute. In all experiments, frozen semen from the same bull was used.

For sperm capacitated in BO medium, spermatozoa were washed twice in a semen washing solution of BO medium, containing 20 µg/mL heparin plus 3.383 mg/mL caffeine (NIWA and OHGODA, 1998) by centrifugation at 2500 rpm for 5 min. After centrifugation the supernatant was discarded and pellets with spermatozoa were maintained in an incubator at 37 °C, 5% CO_2 for 1 h for capacitation (IRRITANI and NIWA, 1997). The spermatozoa were re-suspended in 1 mL of semen diluent solution of BO medium and the sperm number was counted using a hemocytometer and adjusted to 2×10^6/mL.

For sperm capacitated in TALP, frozen-thawed buffalo semen was washed in a discontinuous Percoll gradient, prepared by depositing 2 mL of 90% Percoll under 2 mL of 45% Percoll in a 15 mL centrifuge tube. Semen samples were deposited on the top of the Percoll gradient and centrifuged for 30 min at 400×g. The pellet was removed and resuspended in 5 mL Sperm-TALP solution (SP-TALP) and again centrifuged for 10 min. After removal of the supernatant, spermatozoa were re-suspended in 1 mL of Fert-TALP (F-TALP base stock, supplemented with 6 mg/mL BSA plus 0.25 mM sodium pyruvate and 10 µg/mL Heparin stock) and the sperm number was counted using the hemocytometer. The final sperm concentration was adjusted to 2×10^6/mL by adding Fert-TALP solution.

In vitro fertilization. For *in vitro* fertilization (IVF) of matured buffalo oocytes, oocytes matured in maturation medium were partially denuded from the surrounding cumulus cells, to allow easy penetration of the sperm cells. They were washed twice in pre warmed IVF medium to maintain the defined component of the IVF media and IVM oocytes were fertilized *in vitro* in either BO or TALP media.

Matured oocytes in the BO media group were washed twice in BO washing media and transferred to 50 µL droplets (10-20 oocytes/ droplet) of the capacitation and fertilization BO medium (BO washing medium +10 mg/mL of fatty acid free BSA). The spermatozoa, in 50 µL of the capacitation and fertilization BO medium, were then added to the droplet containing oocytes, covered with sterile mineral oil and placed in a CO_2 incubator at 38.5°C for 18 h (CHAUHAN et al., 1998).

For the TALP group, matured oocytes were washed with HEPES - TALP and partially denuded. About 15-20 matured oocytes were placed in each well of a culture

dish containing 50 µL of fertilization media, to which 50 µL of sperm suspension was added, they were covered with sterile mineral oil and placed in a CO_2 incubator at 38.5 °C for 20-22 h (PARRISH et al., 1986).

In vitro culture. At the end of sperm- oocyte incubation, prior to transfer to the *in vitro* culture droplets, presumed zygotes were washed four times in embryo culture medium (mCR2aa containing 0.8% BSA) and cultured in this medium in a humidified CO_2 incubator at 38.5 °C; the embryo production rate was examined under an inverted microscope, to record the number of cleaved embryos at 8-16 cells after 94-96 h post-insemination (Fig. 1H).

Statistical analysis. The data were analyzed using SPSS (SPSS Inc. IL, USA). All the values are presented as mean ± SEM unless indicated otherwise. Differences among means were analyzed by one way ANOVA after arcsine transformation of the percentage data. The differences were considered significant at $P<0.05$.

Results

Recovery and in vitro maturation of buffalo oocytes. The aim of these experiments was to determine whether supplementation of epidermal growth factor, in combination with antioxidant affects the *in vitro* maturation rate of oocytes. During the entire study, 1556 ovaries were collected and 1432 (92.03%) used for aspiration of oocytes; the remaining 124 (7.97%) ovaries that were either devoid of follicles or cystic were discarded. From 1432 ovaries, a total of 2148 COCs were collected and 1829 COCs of usable quality were used for *in vitro* maturation under different experimental culture conditions. COC morphology, on the basis of compactness, provides information about the oocyte maturation quality. The cumulus expansion rate after 24 h of maturation in Group-1, Group-2, Group-3 and Group-4 was 65.7 ± 2.7, 81.5 ± 1.7, 94.4 ± 0.8 and 79.6 ± 2.4 respectively (Fig. 2).The proportion of oocytes with extruded first PB was highest in Group-3 (82.5 ± 2.9) as compared to Group-1 (57.5 ± 3.2), Group-2 (67.5 ± 3.4) and Group- 4 (62.5 ± 2.9) (Fig. 3). A significant difference was observed among the groups, at $P<0.05$, in the cumulus cell expansion and polar body extrusion rate.

In vitro fertilization of buffalo oocytes. A total of 986 *in vitro* matured oocytes were subjected to IVF from four groups. The present results reveal that the cleavage rates of buffalo oocytes matured in MM with 20 ng/mL EGF + 100 µM β-ME (Group 3) was higher when capacitation and fertilization was carried out in BO medium than in TALP medium (64.8 ± 3.83 vs 44.1 ± 2.9). For oocytes matured in MM Group-1, Group- 2 and Group- 4 there was no significant difference ($P>0.05$) between the BO and TALP medium for the cleavage rates of buffalo oocytes (34.1 ± 0.3 vs 26.2 ± 3.1; 35.8 ± 0.5 vs 29.4 ± 4.4; 40.2 ± 1.1 vs 34.8 ± 1.8) (Fig. 4).

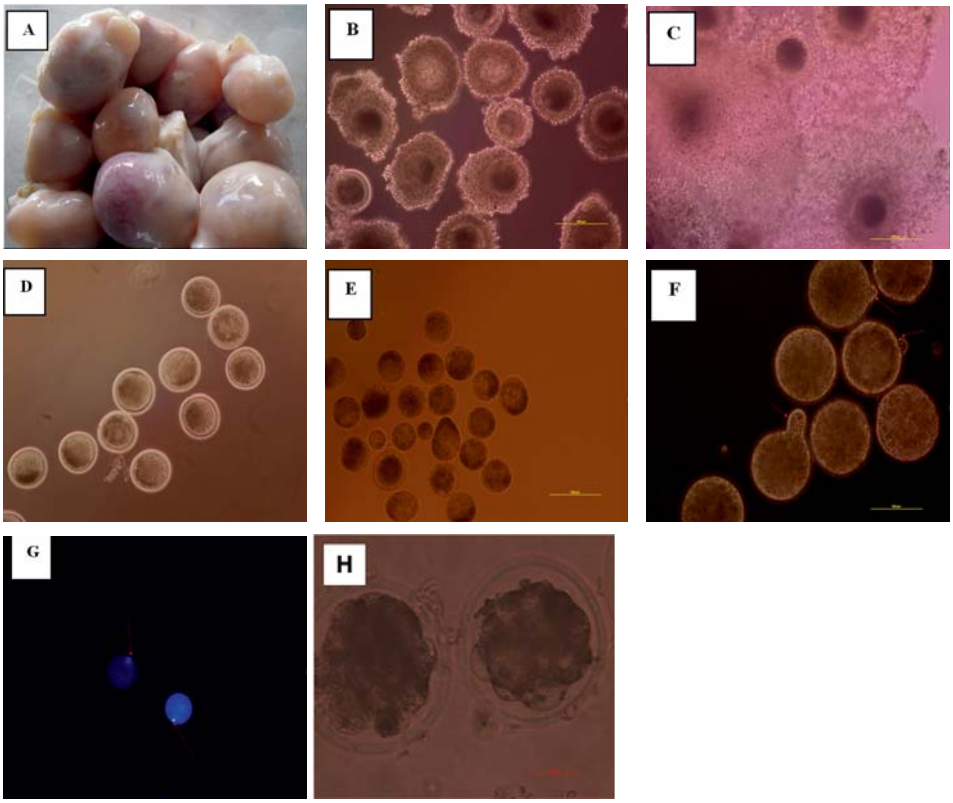

Fig. 1. Buffalo ovaries obtained from slaughterhouse (A); Immature buffalo oocytes of usable quality (B); *In vitro* matured oocytes with expanded cumulus mass (C); *In vitro* matured oocytes after hyaluronidase treatment with zona pellucida (D); *In vitro* matured oocytes after pronase treatment without zona pellucida (E); *In vitro* matured oocytes with polar body in bright light (F) and under UV light (G); A representative photograph of IVF embryos at eight cell stage (H).

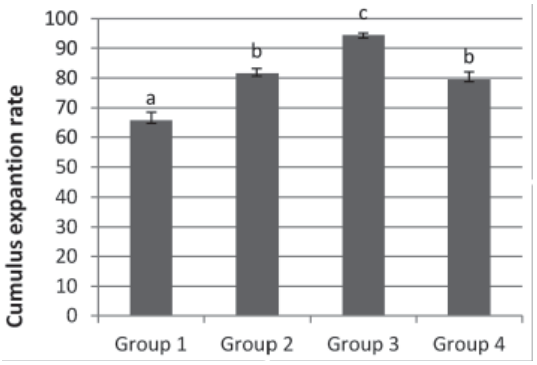

Fig. 2. Cumulus expansion rate in different concentration of EGF + β -ME. Bars with different superscript differ significantly (P<0.05). The standard error is indicated by error bars. The vertical axis indicates cumulus expansion rate, and the horizontal axis indicates the culture medium groups.

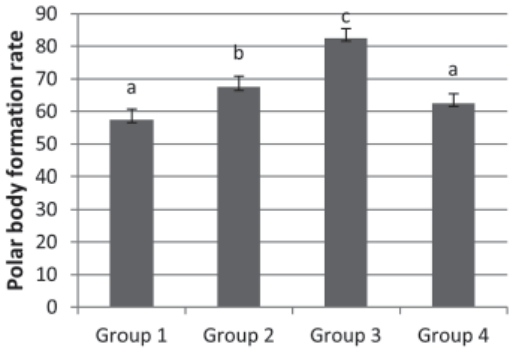

Fig. 3. Polar body formation rate in different concentrations of EGF + β -ME. Bars with different superscript differ significantly (P<0.05). The standard error is indicated by error bars. The vertical axis indicates polar body formation rate, and the horizontal axis indicates the culture medium groups.

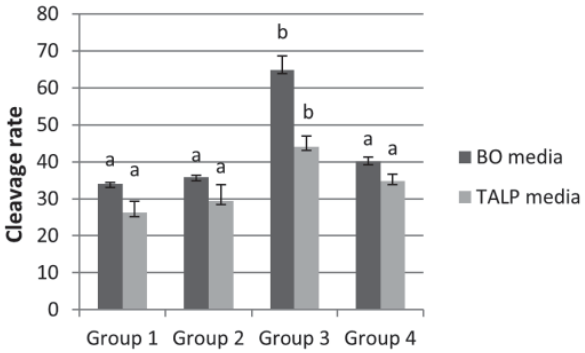

Fig. 4. *In vitro* embryo production rate of buffalo oocytes obtained from different maturation media with sperm capacitated in either BO or TALP. Bars with different superscript differ significantly ($P<0.05$). The standard error is indicated by error bars. The vertical axis indicates cleavage rate either in BO or TALP medium, and the horizontal axis indicates the culture medium groups.

Discussion

In order to enhance *Bubalus bubalis* oocyte maturation rates, it is necessary to modify a suitable oocyte maturation medium with various supplements. The process of meiotic maturation and acquisition of developmental competence determines the ability of the oocyte to undergo successful fertilization, cleavage, and embryonic development. These important steps are dependent on a variety of factors that lead to proper nuclear and cytoplasmic maturation (TROUNSON et al., 2001). Oocyte meiotic maturation is a complex process that involves germinal vesicle breakdown, chromosome condensation and segregation formation of the metaphase plate, completion of meiosis I, extrusion of the first PB and arrest at Metaphase-II (MODINA et al., 2001). It is well known that oocyte developmental potential is a reflection of proper cytoplasmic maturation. Even though most bovine oocytes resume meiosis and progress to Metaphase-II following IVM (DESOUSA et al., 1998), cytoplasmic *in vitro* maturation is generally compromised, leading to low rates of development. In the *in vitro* culture system of human COCs, the number of cumulus cells in COCs before culture correlated positively ($P\leq0.05$) with the developmental competence of oocytes after *in vitro* maturation (RACEDO et al., 2007), suggesting that a sufficient number of cumulus cells is required to support oocyte maturation. To mimic the changes observed in *in vivo* oocytes, immature COCs from early stage follicles were cultured with a specific temporal sequence of PMSG, hCG, along with different concentrations of EGF and β-ME in four groups. The addition of EGF + β-ME during the maturation of bovine COCs increased cumulus expansion, polar body extrusion and the proportion of oocytes that reached the Metaphase-II stage. Our

findings also support the fact that EGF and β-ME act in cumulus-enclosed oocytes to stimulate the cumulus expansion rate up to 94.4% and the polar body formation rate up to 82.5%.

The 20 ng/mL EGF + 100 μM β-ME concentrations were optimum and had a significant effect on oocyte maturation and further development. At high concentrations of EGF + β-ME (100 ng/mL + 500 μM), a decrease in cumulus expansion rate and polar body formation rate was observed. This demonstrates that higher dosage of EGF + β-ME partially inhibits the maturation process, whereas, a low dosage of EGF + β-ME (10 ng/mL + 25 μM) causes inhibition of oocyte maturation and further development, due to binding of the culture media component to EGF + β-ME.

In this study, supplementation of β-ME improved buffalo oocyte *in vitro* maturation. The highest rate of oocyte IVM was in the 100 μM β-ME treatment. There is evidence that oxidative stress had negative effects on *in vitro* mammalian embryo development. Antioxidant systems, such as superoxide dismutase, catalase, and thiol compounds, scavenge ROS in mammalian cells (BEHESHTI et al., 2011). Buffalo oocytes are capable of synthesizing GSH *in vitro* (GASPARRINI et al., 2003). It is shown that adequate synthesis of GSH in the maturation period is very important for later embryo development (DE MATOS et al., 2002). GSH is a non-protein sulphahydrl compound and plays an important role in protecting mammalian cells from oxidative stress, and its intracellular synthesis is very important in oocyte cytoplasmic maturation (GASPARRINI et al., 2008). The addition of β-ME to the bovine and ovine IVM medium increased GSH synthesis, and improved embryo development and quality (DE MATOS et al., 2002). Previous reports indicated that addition of 100 μM β-ME improved blastocyst formation and their cell number in bovine oocytes (KOBAYASHI et al., 2006). DEA et al. (2011) reported that the addition of low molecular weight thiol compounds e.g. cysteamine and β-ME, led to low oxidative stress in many species.

Growth factors such as growth hormone (GH) and EGF may be viewed as local regulators involved in the subtle coordination of cellular proliferation and differentiation. In that regard, EGF stimulates distinct cellular functions, which suggests a possible effect on early development of mammalian embryos (TERUEL et al., 2000). The present study demonstrated that epidermal growth factor enhanced cumulus expansion in buffalo cumulus oocyte complexes. In our study 20 ng/mL EGF was more beneficial on IVP embryo development than other concentrations examined. Many workers have reported that EGF contributes to the promotion of oocyte maturation (DOWNS, 1989; SANBUISSHO et al., 1991), germinal vesicle breakdown (GVBD), polar body formation (DAS et al., 1992) and cleavage of the oocytes (COSKUN et al., 1991). MTANGO et al. (2003) reported that the addition of EGF to the culture medium resulted in higher developmental capacities than other growth factors used, after the blastocysts had been vitrified, thawed and cultured. EGF is naturally present in the follicular fluid of several species (LONERGAN et al., 1996),

and is capable of stimulating meiotic resumption in a variety of species. DAS et al. (1992) showed that the positive effect of follicular fluid on IVM of mouse oocytes was largely due to the presence of EGF. Thus, the beneficial effects of EGF on IVM of buffalo oocytes were likely enhanced in the absence of buffalo follicular fluid in the current study.

An ideal medium for IVF is one that provides an environment conducive to penetration of the oocytes by the sperm. MADAN et al. (1994) reported that the fertilization media used in IVF not only affects the proportion of oocytes capable of undergoing fertilization, but also their subsequent development. The current study revealed higher fertilization with BO medium than TALP; these results were similar to those reported by BRACKET and OLIPHANT (1975) for rabbit and RAVINDRANATHA et al. (2003) for buffalo. Our study indicated that the basic sperm processing and fertilization medium Brackett-Oliphant (BO) resulted in higher cleavage rates, which was in contrast to some earlier reports (PAVASUTHIPAISIT et al., 1992; TOTEY et al., 1996). This discrepancy may be because of differences in sperm processing procedures or diverse additives in the media, due to lab to lab variation, cultural conditions, handling procedures and temperature variations at different places, also BRACKET and OLIPHANT (1975) indicated that the fertilizing ability of the ova depends on individual variations.

Conclusion

The present study showed that maturation media used differ in buffalo oocytes maturation. Supplementation with 20 ng/mL EGF + 100 μM β-ME in maturation medium resulted in a greater percentage of *in vitro* maturation in comparison with 0 ng/mL EGF + 0 μM β-ME, 10 ng/mL EGF + 25 μM β-ME and 100 ng/mL EGF + 500 μM β-ME. The *in vitro* matured oocytes derived from maturation media supplemented with 20 ng/mL EGF + 100 μM β-ME when fertilized with sperm processed in BO medium had higher ability to cleave the oocytes compared with TALP.

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SADEESH, E. M., F. SHAH, A. K. BALHARA, S. M. K. THIRUMARAN, S. YADAV, P. S. YADAV: Učinak faktora rasta i antioksidanta na *in vitro* dozrijevanje i diobu oocita *in vitro* proizvedenih embrija indijskog bivola (*Bubalus bubalis*). Vet. arhiv 84, 459-474, 2014

SAŽETAK

Istraživanje je poduzeto s ciljem da se procijeni učinak kombinacije čimbenika rasta i antioksidanta na *in vitro* dozrijevanje oocita i diobu *in vitro* proizvedenih zametaka indijskog bivola (*Bubalus bubalis*). Kompleksi kumulus oocita bili su prikupljeni s jajnika na klaonici aspiracijom folikula i držani u mediju za dozrijevanje tijekom 24 sata. Medij za dozrijevanje 1. skupine (kontrolne) sadržavao je TCM 199, 10% FBS, PMSG (20 IJ/mL), hCG (10 IJ/mL), natrijev piruvat (0,80 mM), L-glutamin (2 mM) i gentamicin (50 μg/mL). Mediji za dozrijevanje pokusnih skupina bili su sljedećeg sastava: 2. skupina, isto kao kontrolna uz dodatak epidermalnog faktora rasta EGF (10 ng/mL) i β-merkaptoetanola (β-ME) (25 μM); 3. skupina, kao i medij u kontrolnoj skupini s dodatkom EGF (20 ng/mL) i β-ME (100 μM) i 4. skupina, kao i kontrolna uz dodatak EGF (100 ng/mL) i β-ME (500 μM). Nakon dozrijevanja u različitim medijima, za *in vitro* oplodnju i razvoj embrija u svim skupinama korišten je isti medij. Rezultati pokazuju da su koncntracije od 20 ng/mL EGF i 100 μM β-ME bile optimalne i pokazivale značajan učinak na dozrijevanje oocita i budući razvoj. Također, visoke koncentracije EGF i β-ME (100 ng/mL + 500 μM) pokazale su smanjenje stupnja ekspanzije kumulusa, stupnja formiranja

polarnog tijela i stupnja diobe. Signifikantno poboljšanje stupnja diobe opaženo je kod dozrijevanja oocita u mediju kojem je dodano 20 ng/mL EGF i 100 μM β-ME, a kapacitacija i oplodnja provedena je u BO mediju u usporedbi s TALP medijem (64,8 ± 3,8 prema 44,1 ± 2,9). Signifikantna razlika nije utvrđena za stupanj diobe kod oocita koje su dozrijevale u kontrolnoj skupini, u skupini s dodatkom 10 ng/mL EGF i 25 μM β-ME, skupini s dodatkom 100 ng/mL EGF i 500 μM β-ME, kao i između BO and TALP medija. Navedeno ukazuje na povoljan učinak dodavanja 20 ng/mL EGF i 100 μM β-ME u medij za dozrijevanje embrija indijskog bivola (*Bubalus bubalis*) koji su proizvedeni uz pomoć *in vitro* kulture.

Ključne riječi: oocite, čimbenik rasta, antioksidant, dozrijevanje *in vitro*, oplodnja *in vitro*, stupanj diobe