



Effect of growth factors (epidermal growth factor, platelet derived growth factor, and insulin-like growth factor-1) on buffalo embryos produced *in vitro*

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ABSTRACT

The present study examined effect of epidermal growth factor (EGF) on buffalo oocytes maturation and its subsequent effect on embryo development when embryos cultured in media supplemented either with insulin-like growth factor-1 (IGF-1) or/and platelet-derived growth factor (PDGF). Slaughterhouse derived excellent to good quality oocytes were matured for 24 h in TCM-199 supplemented with epidermal growth factor (EGF, 20ng/ml) and without EGF (control), 10% follicular fluid and 10% fetal bovine serum. Matured oocytes were fertilized with frozen buffalo bull semen. Presumptive zygotes were divided and cultured in 4 groups each: Group 1 (control, containing mSOF+ β -mercaptoethanol, 100 μ M), group 2 (control + IGF-1, 100ng/ml), group 3 (control+ PDGF, 1ng/ml) and group 4 (control+ IGF-1 +PDGF). Maturation rate was higher in EGF supplemented group compared to control group (85.7% vs 67.5%). Cleavage rate was also higher in EGF supplemented groups. Overall developmental rate of *in vitro* produced embryos was significantly higher in EGF supplemented group except in PDGF supplemented groups. PDGF supplemented group showed highest cleavage when oocytes matured with EGF (42.0% and 45.5%) but was not able to convert proportionate embryos to blastocyst (15.9% and 18.5%) while IGF-1 supplemented group showed highest percentage of blastocysts (29.7%). These results indicated that addition of IGF-1 (100ng/ml) improved the cleavage and blastocyst production rate, whereas PDGF (1ng/ml) though improved cleavage significantly but showed comparatively low blastocyst production rate. It can be concluded that oocytes matured in EGF (20ng/ml) and subsequently cultured in IGF-1 (100ng/ml) supplemented media have greater embryonic developmental potential.

Key words: Buffalo, EGF, Embryonic developmental block, IGF-1, PDGF

Buffaloes have low reproductive potential. Efforts have been initiated in recent years to augment the reproductive potential of these animals using biotechnology (Madan *et al.* 1994). *In vitro* embryo production (IVEP) system was successfully used for producing large number of buffalo embryos and pregnancies (Gasparrini *et al.* 2000, Madan 2005, De Rosa *et al.* 2006, Mishra *et al.* 2010) from abattoir-derived ovaries, but the success rate is low. Little information is available on *in vitro* maturation and fertilization of buffalo oocytes. In buffaloes, despite a similar maturation rate (87 versus 94%) a significantly lower cleavage rate (65 versus 84%) was observed in comparison to cattle (Gasparrini 2002, Neglia *et al.* 2003). It is therefore, a matter of concern to further improve IVEP so that it can be widely used for buffalo. *In vitro* cultured bovine and buffalo embryos show developmental arrest during transition from a regulatory

program governed by proteins transcribed maternally synthesized by mRNA stored in the oocytes to a regulatory program controlled by proteins synthesized from embryonic genome (Barnes and Eyestone 1990). In buffaloes, zygotic genome activation (ZGA) primarily occurs in the fourth cell cycle (Rajhans *et al.* 2006). Development of most of the embryos are arrested at the ZGA stage, which is known as developmental block (Memili and First 2000) and must rely on mRNAs transcribed from its own genome to continue further development.

Relief, from this developmental block was reported with the addition of growth factors, which play a significant role in stage specific manner. Epidermal growth factor (EGF, 20ng/ml) enhances the buffalo oocyte maturation and embryo development *in vitro* (Purohit *et al.* 2005, Singhal *et al.* 2009, Mishra *et al.* 2010). EGF supplementation to the maturation media doubled the blastocyst formation (13 to 27%) (Grazul-Bilska *et al.* 2003). Platelet-derived growth factor (PDGF, 1ng/ml) promotes the development of *in vitro* fertilized bovine embryos cultured singly from 8–16 cell stage to

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morula stage (Lim and Hansel 1996). PDGF was also detected from bovine oviductal secretions (Gondolfi and Moor 1987). Larson *et al.* (1992) suggested that following acceleration of fourth cell cycle, PDGF inhibits further embryonic development in bovine. Addition of IGF-1 (100ng/ml) in embryo culture media enhances bovine preimplantation embryo development (Sirisathien *et al.* 2003) and supplementation of maturation media with IGF-1 (100ng/ml) increases oocyte maturation and subsequent embryo development in buffalo (Singhal *et al.* 2009). IGF-1 was also detected in oviductal cell secretion (Stevenson and Wathes 1996). IGF-1 acts as survival factor for preimplantation mammalian embryos exposed to heat stress (Block and Hansen 2007). IGF-1 serves as anti apoptotic factor during oocyte maturation (Wasielak and Bogacki 2007) and embryo development (Makarevich and Markkula 2002). Somatic cell co-culture like bovine oviductal epithelial cell (BOEC) overcame the developmental arrest of bovine embryos *in vitro* (Eyestone and First 1989) and this supportive effect might be due to the secretion of some growth factors and free radical scavenging effect of somatic cells.

Systematic study of these growth factors in buffalo embryo development has so far not been done. Therefore, the present work aimed to improve *in vitro* maturation rate of buffalo oocytes and subsequent survival of *in vitro* produced embryos using different growth factors like EGF, PDGF and IGF-1.

MATERIALS AND METHODS

All the chemicals used for the present study were procured commercially, unless otherwise mentioned.

Experimental design: The experiment was designed as randomized complete blocks with each block consisting of a group of presumptive zygotes obtained from same conditions of maturation, fertilization and embryo culture. Each group consisted of 5 cultures each. Developmental competence of embryos was analyzed in terms of embryos (percentage) reached to the particular stage in relation to the total cleaved oocytes. Embryos that did not cleave at least once were not included in the analysis to eliminate oocytes that were not competent to mature or to be fertilized.

In vitro oocyte maturation: Buffalo ovaries were collected in 0.9% normal saline solution (NSS) fortified with antibiotics at 35–37°C temperature and carried to the laboratory within 1h. Ovaries were washed in fresh, sterile physiological saline to remove any contaminants and rinsed in 70% ethyl alcohol for a few minutes to eliminate surface organisms. All follicles measuring 3–8 mm were aspirated with the help of 18-gauge needle attached to 5 ml syringe containing pre-warmed oocyte collection media (OCM). Oocytes with at least 4 intact layers of cumulus cells and homogenous cytoplasm (grade excellent and good quality) were selected for *in vitro* maturation (IVM). Cumulus oocyte complexes (COCs) were washed 4–5 times in handling media (TCM-199 with hepes + 10% FCS + BSA (3 mg/ml) and

matured in media (TCM-199+ 10% FCS+ 10% follicular fluid + 3 mg/ml BSA) with EGF, 20ng/ml or without EGF). Ten to fifteen COCs were placed in 50 µl droplets of respective maturation media overlaid with mineral oil in a 35 mm petridish and incubated in CO₂ incubator at 38.5°C in a humidified atmosphere of 5% CO₂ in air for 24 h.

In vitro fertilization (IVF) of matured oocytes: Frozen buffalo bull semen of single fertile bull was used throughout the study. Briefly, frozen semen straw (0.25 ml) was gently shaken in air for 20 sec and thawed in water at 37°C for 1 min. Contents were transferred in 15 ml sterile tube. Semen was washed twice in Fert-TALP media (containing fatty acid free BSA 6mg/ml, heparin 10 µg/ml, pyruvate 0.2 mM). After final wash pellet was reconstituted in Fert-TALP media to get final sperm concentration around 1–2 × 10⁶ sperms/ml. *In vitro* matured oocytes were washed thrice with the Fert-TALP and a group of 10–15 oocytes were placed in 100 µl of processed semen suspension under mineral oil and co-incubated at 38.5°C in a humidified atmosphere of 5% CO₂ in air for 18 h.

In vitro culture of in vitro fertilized oocytes: After 18 h co-incubation, presumptive zygotes from both of maturation groups were separated from the sperm droplets and divided into four groups each; they were washed with the respective *in vitro* culture (IVC) media. Basal media (control group) consisting of modified synthetic oviductal fluid (mSOF as Tervit *et al.* 1972) supplemented with 100µM βME and 3 mg/ml fatty acid free BSA. Culture groups consisted of: group 1 (control, mSOF+ βME, 100µM); group 2 (control+ IGF-1, 100ng/ml); group 3 (control+ PDGF, 1ng/ml); group 4 (control+ IGF-1, 100ng/ml + PDGF, 1ng/ml). Culture conditions were kept same for all the groups and the media was changed at every alternate day with respective freshly prepared overnight quenched media. Cleavage of embryos was recorded at 48 hpi of culture and embryo development was recorded at every 24 h till blastocyst development or day 9 post insemination whichever earlier.

Statistical analysis: The results obtained for *in vitro* fertilization and embryo culture were analyzed using one-way ANOVA and has been presented in the form of total embryos (percentage) in relation with total oocytes cleaved. A 5% significance level was used as level of significance.

RESULTS AND DISCUSSION

The present study was undertaken to examine the effect of epidermal growth factor (EGF) on buffalo oocyte maturation and its subsequent effect on embryo development when embryos cultured *in vitro* in different media supplemented with insulin-like growth factor-1 (IGF-1) and platelet derived growth factor (PDGF). Performance of EGF on oocyte maturation showed significantly ($P<0.05$) higher nuclear maturation rate (85.7% vs 67.5%), first polar body release (70% vs 60%) and cumulus expansion (grade A⁺ vs grade A). Cleavage as well as embryo development was

Table.1. Developmental competence of embryos when oocytes matured without EGF or with EGF (20 ng/ml) and resulting embryos cultured in different media supplemented with IGF-1 (100ng/ml) or PDGF (1ng/ml)

Treatment at maturation	Treatment at embryo culture		Oocytes cultured	Cleavage (%)	8–16 cell (%)	Morula (%)	Blastocyst (%)
No EGF (control)	βME (100μM)	Group 1	400	90 (22.5) ^a	70 (77.8) ^a	36 (40.0) ^a	16 (17.8) ^a
	βME (100 μM)+IGF (100ng/ml)	Group 2	386	96 (24.9) ^a	79 (82.3) ^b	45 (46.9) ^b	21 (21.8) ^b
	βME (100μM)+PDGF (1ng/ml)	Group 3	405	124 (30.6) ^b	98 (79.0) ^a	61 (49.2) ^b	28 (22.6) ^b
	IGF (100ng/ml)+ PDGF (1ng/ml)	Group 4	395	126 (31.9) ^b	98 (77.8) ^a	52 (41.3) ^a	27 (21.4) ^b
EGF (20ng/ml)	βME (100μM)	Group 1	401	129 (32.2) ^a	99 (76.7) ^a	60 (46.5) ^a	33 (25.6) ^a
	βME (100 μM)+IGF (100ng/ml)	Group 2	395	145 (36.7) ^a	120 (82.8) ^b	75 (51.7) ^a	43 (29.7) ^b
	βME (100μM)+PDGF (1ng/ml)	Group 3	405	170 (42.0) ^b	122 (71.8) ^c	51 (30.0) ^b	27 (15.9) ^c
	βME (100 μM)+IGF (100ng/ml)+ PDGF (1ng/ml)	Group 4	391	178 (45.5) ^b	126 (70.8) ^c	63 (35.4) ^b	33 (18.5) ^c

Values (%) in respective group of maturation having different superscript differ significantly ($P<0.05$). Percentage of 8–16 cell, morula and blastocyst embryos are presented in relation to total oocytes cleaved.

significantly ($P<0.05$) higher in EGF supplemented group except in PDGF supplemented groups (Table 1). In PDGF supplemented groups matured in EGF showed lower developmental potential even after the increased cleavage rate, viz. blastocyst percentage in group 3 (22.6% vs 15.9%) and in group 4 (21.4% vs 18.5%). The cleavage rate increased in IGF-1 treated group compared to the control group (36.7% vs. 32.2%) and it was further increased significantly ($P<0.05$) in PDGF supplemented groups (Table 1), group 3 and 4 (42.0% and 45.5%) respectively when oocytes matured in EGF supplemented media. Embryos reached to 8–16 cell stage, morula and blastocyst stage was highest in IGF-1 supplemented group i.e. 82.8%, 51.7% and 29.7% respectively (Table 1). The percentage of cleaved embryos reached to blastocyst stage increased non-significantly ($P<0.05$) in IGF-1 supplemented group 2 than the group 1 (29.7% vs. 25.6%) but it reduced significantly ($P<0.05$) in PDGF supplemented group 3 (15.9%) and addition of IGF-1 to the PDGF (group 4) increased the blastocyst production rate (18.5%) compared to PDGF alone (15.9%).

Several factors affect *in vitro* maturation and *in vitro* embryo culture. These factors are either selection of proper maturation and culture medium, the quality of oocytes or the growth factors added.

The present study revealed that supplementation of maturation media with EGF (20ng/ml) increased the oocyte maturation, cleavage, 8–16 cell embryos, morula and blastocyst production rate. Similar results were reported in buffalo (Chauhan *et al.* 1999, Purohit *et al.* 2005, Singhal *et al.* 2009, Mishra *et al.* 2010) and in bovine (Keefer *et al.* 1994, Lonergan *et al.* 1996). EGF accelerates the meiotic cycle of bovine oocytes possibly by increased LH and MAP kinase activity during early stage of *in vitro* maturation (Sakaguchi *et al.* 2002). Bovine oocyte activation is associated with an increase in calcium channel mediated events (Tosi *et al.* 2002) and it is thought that growth factors alter these events by activating protein synthesis.

IGF-1 (100ng/ml) supplemented group was found to be superior in terms of overall embryonic development. When IGF-1 was supplied with PDGF it enhanced the survivability of the cleaved embryos, thus increasing the blastocyst percentage. Similar results were reported by Sirisathein *et al.* (2003) and Glabowski *et al.* (2004) in bovine. IGF-1 supplementation improved the percentage and survival of morula and blastocyst development *in vitro* in buffalo (Mishra *et al.* 2010). *In vitro* produced embryos cultured with IGF-1 improved pregnancy and calving rate following transfer to lactating cows during the summer season (Block and Hansen 2007). Different growth factors play important role during preimplantation embryo development and IGF-1 is one of the most important growth factors for cellular differentiation (Schultz and Heyner 1993). IGF-1 increases glucose uptake (Pantaleon and Kaye 1996), protein synthesis, increases embryonic metabolism and cell proliferation there by increases total cell number and quality of embryos produced (Doherty *et al.* 1994), thus affecting embryo development. IGF-1 (100ng/ml) was shown to have an anti-apoptotic activity in preimplantation bovine embryos cultured *in vitro* (Sirisathein *et al.* 2003, Wasielek and Bogacki 2007) and the same way it might have increased the survival of group 4 (PDGF+IGF-1) embryos. Expression of IGF-1 gene was not detected in buffalo preimplantation embryos, while the genes for IGF-1 receptor was reported (Vikash Chandra *et al.* 2011). Thus, supplementation of IGF-1 for embryo culture seems to be necessary at least for buffalo.

Supplementation of PDGF in the culture medium increased cleavage rate significantly ($P<0.05$) compared to all other groups, but at the same time it increased developmental block at 8–16 cell stage and decreased the proportion of cleaved embryos reaching to blastocyst stage. These findings are in contradiction with findings in bovine (Lim and Hansel 1996). Eckert and Neimann (1996) reported that addition of PDGF on day 1 of embryo culture could not support embryo development while PDGF supplementation

on day 3 of embryo culture increased the embryo development to the morula and blastocyst. PDGF acts as proliferation agent in cultured somatic cells or cultured embryos by shortening cell cycle time through involving mechanism which includes synthesis of regulatory subunits of mitosis promoting factors (Jaskulski *et al.* 1988). Larson *et al.* (1992) reported that PDGF following its acceleratory effect at fourth cell cycle inhibits further embryo development. Larson *et al.* (1992) also reported that prolonged exposure of embryos to PDGF reduces the embryo development and also reduces the receptor for other growth factors like EGF. It may therefore be hypothesized that PDGF plays stimulatory role at particular cell cycle and for a limited time. Higher developmental block at 8–16 cell in all groups may be due to the stage of zygotic genome activation as it occurs at fourth cell cycle in buffalo (Rajhans *et al.* 2006).

In summary, it can be concluded that supplementation of maturation media with EGF improved maturation and subsequent embryo development. Supplementation of IGF-1 in culture media was best in terms of cleavage as well as blastocyst production rate when oocytes matured in EGF supplemented media. Though, the maximum cleavage rate was recorded in PDGF group but it did not translate them into the proportionate number of blastocysts. PDGF might be beneficial at particular stage of embryo development. This needs some more studies.

REFERENCES

- Barnes F L and Eyestone W H. 1990. Early cleavage and the maternal zygotic transition in bovine embryos. *Theriogenology* **33**: 141–52.
- Block J and Hansen P J. 2007. Interaction between season and culture with insulin-like growth factor-1 on survival of *in vitro* produced embryos following transfer to lactating dairy cows. *Theriogenology* **67** (9): 1518–29.
- Chauhan M S, Singla S K, Palta P, Manik R S and Madan M L. 1999. Effect of EGF on cumulus expansion, mitotic maturation and development of buffalo oocytes *in vitro*. *Veterinary Record* **144**: 266–70.
- De Rosa A, Di Palo R, Attanasio L, Moaco E, Campanile G and Gasparrini B. 2006. Open pulled straw vitrification for *in vitro* produced buffalo (*Bubalus bubalis*) embryos. *Reproduction Fertility and Development* **18** (2): 153–53.
- Doherty A S, Temeles G L and Schultz R M. 1994. Temporal pattern of IGF-1 expression during mouse preimplantation embryogenesis. *Molecular Reproduction and Development* **37** (1): 21–26.
- Eckert J and Niemann H. 1996. Effects of platelet-derived growth factor (PDGF) on the *in vitro* production of bovine embryos in protein-free media. *Theriogenology* **46** (2): 307–20.
- Eyestone W H and First N L. 1989. Co-culture of early cattle embryos to the blastocyst stage with oviductal tissue or in conditioned medium. *Journal of Reproduction and Fertility* **85**: 715–20.
- Gasparrini B, Neglia G, Di Palo R, Campanile G and Zicarelli L. 2000. Effect of cysteamine during *in vitro* maturation on buffalo embryo development. *Theriogenology* **54**: 1537–42.
- Gasparrini B. 2002. *In vitro* embryo production in buffalo species: state of art. *Theriogenology* **57** (1): 237–57.
- Glabowski W, Kurzawa R, Wiszniewski B, Baczkowski T, Marchlewicz M and Brelic P. 2004. Growth factors effects on preimplantation development of mouse embryos exposed to tumor necrosis factor alpha. *Journal of Reproductive Biology* **5** (1): 83–99.
- Gondolfi F and Moor R M. 1987. Stimulation of early embryonic development in the sheep by co-culture with oviduct epithelial cells. *Journal of Reproduction and Fertility* **81**: 23–28.
- Grazul-Bilska A T, Choi J T, Bilska J J, Weigl R M, Kirsch J D, Kraft K C, Reynolds L P and Redmer D A. 2003. Effect of epidermal growth factor on early embryonic development after *in vitro* fertilization of oocytes collected from ewes treated with follicle stimulating hormone. *Theriogenology* **59**: 1449–57.
- Jaskulski D, Gatti C, Travali S, Calabretta B and Baserga R. 1988. Regulation of the proliferating cell nuclear antigen cyclin and thymidine kinase mRNA levels by growth factors. *Journal of Biological Chemistry* **263**: 10175–79.
- Keefer C L, Stice S L, Paprcki A M and Golueke P. 1994. *In vitro* culture of bovine IVM-IVF embryos. Cooperative interaction among embryo and the role of growth factors. *Theriogenology* **41**: 1323–31.
- Larson R C, Ignatz G G and Currie W B. 1992. Platelet-derived growth factor (PDGF) stimulates development of bovine embryos during the fourth cell cycle. *Development* **115**: 821–26.
- Lim J M and Hansel W. 1996. Role of growth factors in the development of bovine embryos fertilized *in vitro* and cultured singly in a defined medium. *Reproduction Fertility and Development* **8**: 1199–1205.
- Loneragan P, Carolan C, Langendonck A V, Donway I, Khatri H and Mermillod P. 1996. Role of epidermal growth factor in bovine oocyte maturation and preimplantation development *in vitro*. *Biology of Reproduction* **54**: 1420–29.
- Madan M L. 2005. Animal biotechnology: applications and economic implications in developing countries. *Rev. Sci. Tech. Off International Epizootics* **24** (1): 127–39.
- Madan M L, Chauhan M S, Singla S K and Manik R S. 1994. Pregnancies established from water buffalo (*Bubalus bubalis*) blastocysts derived from *in vitro* matured *in vitro* fertilized oocytes and co-cultured with cumulus and oviductal cells. *Theriogenology* **42**: 591–600.
- Makarevich A V and Markkula M. 2002. Apoptosis and cell proliferation potential of bovine embryos stimulated with insulin-like growth factor-1 during *in vitro* maturation. *Biology of Reproduction* **66**: 386–92.
- Memili E and First N L. 2000. Zygotic and embryonic gene expression in cow: a review of timing and mechanisms of early gene expression as compared with other species. *Zygote* **8**: 87–96.
- Mishra A, Vikash C and Taru Sharma G. 2010. Effect of epidermal growth factor on *in vitro* maturation of buffalo oocytes and subsequent development with insulin-like growth factor-1 and β -mercaptoethanol. *Indian Journal of Animal Sciences* **80** (8): 721–24.
- Neglia G, Gasparrini B, Caracciolo di Brienza V, Di Palo R, Campanile G, Presicce G A and Zicarelli L. 2003. Bovine and buffalo *in vitro* embryo production using oocytes derived from

- abattoir ovaries or collected by transvaginal follicle aspiration. *Theriogenology* **59**: 1123–30.
- Pantaleon M and Kaye P L. 1996. IGF-1 and insulin regulate glucose transport in mouse blastocysts via IGF-1 receptor. *Molecular Reproduction and Development* **44** (1): 71–76.
- Purohit G N, Brady M S and Sharma S S. 2005. Influence of EGF and IGF-1 on maturation and fertilization of buffalo cumulus oocyte complex in serum free media and their subsequent development *in vitro*. *Animal Reproduction Science* **87** (3–4): 229–39.
- Rajhans R, Sai Kumar G and Sharma G T. 2006. Expression profile of stress and metabolic marker genes during *in vitro* production of buffalo (*Bubalus bubalis*) embryos. *Journal of Reproduction Fertility and Developemnt* **18** (2): 253–54.
- Sakaguchi M, Dominko T, Yamauchi N, Leibfried Rutledge M L, Negai T and First N L. 2002. Possible mechanism for acceleration of meiotic progression of bovine follicular oocytes by growth factors *in vitro*. *Reproduction* **123**: 135–42.
- Schultz G A and Heyner S. 1993. Growth factors in preimplantation mammalian embryos. Review: *Oxford Reviews of Reproductive Biology* **15**: 43–81.
- Singhal S, Prasad S, Prasad J K and Gupta H P. 2009. Effect of including growth factors and antioxidants in maturation medium used for *in vitro* culture of buffalo oocytes recovered *in vivo*. *Animal Reproduction Science* **113** (1): 44–50.
- Sirisathien S, Hernandez-Fonseca H J and Brackett B G. 2003. Influence of epidermal growth factor and insulin-like growth factor-1 on bovine blastocyst development *in vitro*. *Animal Reproduction Science* **77** (1–2): 21–32.
- Stevenson K R and Wathes D C. 1996. Insulin-like growth factors and their binding proteins in the ovine oviduct during the oestrous cycle. *Journal of Reproduction and Fertility* **108**: 31–40.
- Tervit H R, Whittingham D G and Rowson L E A. 1972. Successful culture *in vitro* of sheep and cattle ova. *Journal of Reproduction and Fertility* **30**: 493–97.
- Tosi E, Boni R and Cuomo A. 2002. Fertilization and activation currents in bovine oocytes. *Reproduction* **124**: 835–46.
- Vikash Chandra, Sai Kumar G and Taru Sharma G. 2011. Temporal expresson pattern of insulin-like growth factors (IGF-1 and IGF-2) ligands and their receptors (IGF-1R and IGF-2R) in buffalo (*Bubalus bubalis*) embryos produced *in vitro*. *Livestock Science* **135**: 225–30.
- Wasielak M and Bogacki M. 2007. Apoptosis inhibition by insulin-like growth factor-1 (IGF-1) during *in vitro* maturation of bovine oocytes. *Journal of Reproduction and Development* **53** (2): 419–26.