International Journal of Agriculture, Environment and Biotechnology
Citation: *IJAEB*: **15**(03): 733-738, September 2022
DOI: 10.30954/0974-1712.03.2022.10

ASSOCIATION FOR AGRICULTURE ENVIRONMENT AND BIOTECHNOLOGY

ANIMAL HUSBANDRY

Augmentation of Developmental Competence of Immature Cattle Oocytes Supplemented with Growth Factors in Culture Media

Shiva Prasad, Rohit Kumar, Muthu Karunakaran, Ashok Santra and Subrata K. Das*

Animal Biotechnology Lab, Eastern Regional Station, ICAR-National Dairy Research Institute, Kalyani, West Bengal, India

*Corresponding author: subratakdas1@gmail.com (**ORCID ID:** 0000-0002-9276-001X)

Paper No. 1048 **Received:** 16-04-2022 **Revised:** 22-07-2022 **Accepted:** 29-08-2022

ABSTRACT

The aim of the present study was to produce cattle embryos through *in vitro* maturation, fertilization and culture by supplementing culture media with insulin-like growth factor-1 individually and in combination with epidermal growth factor. Cattle ovaries were collected from the abattoir and carried to the laboratory within 3-4 h in normal saline, maintaining 30-35 °C. Cumulus oocyte complexes (COCs) were collected from visible surface follicles and matured *in-vitro* in maturation media supplemented with (i) insulin-like growth factor-1 and (ii) in combination with epidermal growth factor at 38.5 °C in CO_2 incubator with maximum humidity. After 24 h matured, oocytes were allowed for fertilization with capacitated sperms in Fert-BO media at 38.5 °C in CO_2 incubator. After 15-18 h, oocytes were cultured in 100 µl droplets of mCR2^{aa} medium supplemented with growth factor. After 48 h, cleavage was checked and further co-cultured with oviductal cells for development. In the first experiment, the cleavage rate (63.27±2.17^{a}) and morula formation rate (20.09±2.57^{a}) were significantly ($p<0.05$) higher in the treatment group @ 50 ng/ml of IGF-1 as compared to the control group. In contrast, in the second experiment the cleavage rate (70.00±2.16^{a}) and morula formation rate (25.30±1.45^{a}) were significantly ($p<0.05$) higher in the treatment group @ 50 + 10 ng/ml of IGF-1+ EGF, as compared to control group. From the present study, it could be concluded that insulin-like growth factor-1 may have induced cleavage after fertilization and helps in early embryo developmental growth.

HIGHLIGHTS

- IGF-1 @ 50 ng/ml and in combination with EGF @ 10 ng/ml enhanced the cleavage rate and helped in early embryonic developmental growth significantly.

Keywords: Cattle, oocyte, embryo, IGF-1, EGF, IVF

Assisted reproductive techniques like *in vitro* maturation and fertilization have great potential for faster multiplication of superior germplasm or cryopreserving the embryos for future use (Cognie *et al.* 2003). *In vitro* production of embryos involves oocytes recovery, maturation, and fertilization with capacitated spermatozoa and culture of the produced embryos (Malakar *et al.* 2007; Singh and Das, 2014). Production of embryos through *in vitro* maturation and fertilization has been improved using different macromolecule supplementation in the medium (Herrick *et al.* 2004, Singh *et al.* 2020). Following the early report of Staigmiller and Moor (1984), in which the addition of granulosa cells, gonadotropins, and estradiol to the culture media was found to enable the sheep COCs to mature outside follicles, supplementation of the IVM media with gonadotropins and estradiol has been found to be essential for the acquisition of developmental

How to cite this article: Prasad, S., Kumar, R., Karunakaran, M., Santra, A. and Das, S.K. (2022). Augmentation of Developmental Competence of Immature Cattle Oocytes Supplemented with Growth Factors in Culture Media. *Int. J. Ag. Env. Biotech.*, **15**(03): 733-738.

Source of Support: None; **Conflict of Interest:** None

capacity of oocytes in cattle (Brackett *et al.*1989). Supplementation of the IVM media with FCS (Totey *et al.* 1993; Daniel *et al.* 2015) or estrus cow serum (Madan *et al.* 1994) has also been found to be necessary for achieving high maturation rates for cattle and buffalo oocytes. Supplementing the culture media with various growth factors such as insulin-like growth factor-1(IGF-1), epidermal growth factor (EGF) and other growth factors also boost up the developmental competence of *in vitro* cultured bovine oocytes and embryo production (Neira *et al.* 2010, Thongkittidilok *et al.* 2015; Prasad *et al.* 2018; Umdor *et al.* 2021). Various combinations of growth factors positively affect blastocyst formation rates in cultured bovine embryos (Sirisathien *et al.* 2003; Ahumada *et al.* 2013; Saeed-Zidane *et al.* 2019). IGF-1 is shown to have a positive effect during *in vitro* maturation and has been reported in follicular fluid at levels, which can stimulate meiosis (Raty *et al.* 2011; Qu *et al.* 2016). The present study was conducted by supplementing culture media with insulin like growth factor-1.

MATERIALS AND METHODS

A study was made to produce cattle embryos through *in vitro* maturation, fertilization and culture by supplementing culture media with insulin-like growth factor-1. In this study, all plastic wares were used from Tarson Products Pvt. Ltd. (Kolkata, India) and chemicals/ biochemicals/ paraffin oil from Sigma-Aldrich Chemicals Co.(St. Louis, MO, USA); the 0.22 μm disposable syringe filters were used from Millipore Corp., Bedford, MA, USA. Disposable, nontoxic, non-pyrogenic plastic syringes and sterile disposable 19 gauge hypodermic needles of Dispovan make, Kolkata, India, unless otherwise mentioned.

Oocytes collection and *in vitro* maturation

Fresh cattle ovaries were collected at an abattoir immediate after slaughter and transported within 3 to 4 h to the laboratory in isotonic saline solution supplemented with penicillin (100 IU/ml), and streptomycin (50 l/ml) maintained at 30-35°C. Follicular oocytes from apparently non-atretic surface follicles (3-8 mm in diameter) were aspirated with 19 gauge hypodermic needle to a 5 ml disposable plastic syringe containing oocyte aspiration medium (TCM-199 + DPBS + 0.3% BSA + 50 μg/ml gentamicin sulfate) and categorized into A grade (>5 layers of cumulus cells), B grade (3-5 layer of cumulus cells), C grade (<3 layer of cumulus cells) and D grade (partial/without the layer of cumulus cells). All A, B, and C-grade oocytes complexes with a compact cumulus cell layer and homogenous, evenly granulated cytoplasm were used for maturation. The *in vitro* maturation was done as described earlier (Das *et al.* 1996). All the COCs were washed 4-6 times in washing medium (TCM-199 + 10% FBS + 0.81 mM sodium pyruvate + 50 μg/ml gentamicin sulfate), followed by 2-3 times in maturation medium (TCM-199 + 5% follicular fluid +10% FBS + 5 μg/ ml FSH-P + 0.33 mM sodium pyruvate +50μM β-Mercaptoethanol + 50 μg/ml gentamicin sulphate + IGF-1 or IGF-1 + EGF). Then groups of 20-25 COCs were placed in 100 droplets of maturation medium, covered with sterile mineral oil in a 35 mm petri dish, and incubated for 24 h at 38.5 °C in a 5% CO_2 incubator with maximum humidity.

Sperm preparation and in vitro fertilization

The spermatozoa used for IVF throughout the study were from the same donor that had been tested for IVF earlier. The spermatozoa were prepared for fertilization as described earlier (Das *et al.* 2013). Briefly, two straws of frozen-thawed cattle semen were suspended in 8 ml of Working Bracket Oliphant (WBO) medium (Brackett and Oliphant 1975) with 10 μg/ml heparin and 0.57 mM caffeine sodium benzoate and 1.23 mM sodium pyruvate and incubated for swim-up at 38.5°C. After 15 minutes of incubation, progressively motile sperm cells were taken by collecting 4 ml of WBO medium from the top and centrifuged at 2000 rpm for 5 min. After that, the supernatant was removed, and the pellet was dissolved in 1.5 ml of BO medium and centrifuged at 2000 rpm for 5 min. Finally, the pellet was dissolved in l ml of Fertilization Bracket Oliphant (FBO). In the treatment group, the FBO was supplement with different concentration of IGF-I (25, 50 and 75 ng/ml) or IGF-1(25, 50 and 75ng/ml) +EGF (5, 10 and 20 ng/ml). The *in vitro* matured oocytes were washed twice with the FBO medium in the same maturation drop and inseminated with capacitated motile spermatozoa (2-4 million spermatozoa/ml), and placed in 5% CO_2 incubator at 38.5°C for 15-18 h with maximum humidity.

Culture of oviductal epithelial cells and presumptive embryo

Fresh oviducts were dissected carefully with blunt-end scissors and washed 3-4 times with washing media. Oviductal mucosal layer was carefully expelled by squeezing the oviduct with a sterile glass slide, and the cells were retrieved and transferred into Petri dish containing a washing medium. Cell chunks were washed in a washing medium for 5-6 times. Cell chunks were then put into 100 µl droplets of maturation media and incubated in 5% CO_2 incubator at 38.5 °C for 24 h with maximum humidity. After every 24 h half of the medium is replaced with fresh medium. At the end of 15-18 h of sperm-oocyte co-incubation, the presumptive zygotes were separated from the drop, and cumulus cells were removed by gentle, repeated pipetting in a washing medium. The zygotes were then washed 1-2 times with modified Charles Rosenkrans 2 amino acid ($mCR2^{aa}$) medium and cultured in 100 µl of $mCR2^{aa}$ medium. The treatment group, the $mCR2^{aa}$ was supplemented with IGF-I or IGF-1 + EGF. After 48 h cleaved, oocytes/embryos were shifted to 100 µl droplets of $mCR2^{aa}$ blastocyst medium and co-incubated with oviduct cells in 5% CO_2 incubator with maximum humidity at 38.5 °C for 8 days.

Experimental Design and Statistical analysis

In this study, the effect of insulin-like growth factor-I, individually and in combination with epidermal growth factor was examined on *in vitro* embryo production. The culture media were supplemented with three different concentrations of (i) insulin like growth factor-I *i.e.* 25 ng/ml, (ii) 50 ng/ml, (iii) 75 ng/ml, and (iv) insulin-like growth factor-I combined with epidermal growth factor *i.e.* 25 + 5 ng/ml, 50 + 10 ng/ml, & 75 + 20 ng/ml. The control groups were not supplemented with growth factors. IBM®Statistical Package analysed the experimental data for the Social Sciences®(SPSS version 20). Descriptive statistics were performed to calculate mean and standard errors. Post hoc analysis or significant differences between means were determined by LSD/TUKEY HSD test.

RESULTS AND DISCUSSION

Effects of insulin-like growth factor-I on different embryo developmental stages

In the present experiment, 932 immature oocytes were aspirated from fresh cattle ovaries and exhibited good cumulus expansion after IVM was subsequently used for IVF. All three different media like IVM, IVF, and IVC have been supplemented with insulin-like growth factor-1with three different concentrations. In the control group, there is no IGF-1 supplementation. The result in table 1 showed significant differences ($p<0.05$) in cleavage rate, 4-cell stage, 8-cell stage, 16-cell stage, and morula formation rate in treatment group compared to the control group. The mean percentage of cleavage and morula was significantly higher with IGF-1 (50 ng/ml) i.e. 63.27 ± 2.17 and 20.09 ± 2.57, than the control group. Apart from this, the cleavage and morula formation rate also differed significantly between all the treatment groups, but the cleavage and morula formation rate of treatment IGF-1(75 ng/ml) shows only a very slight increase *i.e.* 50.00 ± 3.17 and 8.91 ± 2.57 as compared to control and significant decrease when compared to the other treatments of IGF-I, showing the increase in the concentration of IGF-I beyond optimum level could stimulate negative effect on both cleavage and morula formation rate, whereas the mildest concentrations of IGF-I have a positive effect on the embryogenesis and further advancement, with IGF(50 ng/ml) has a higher positive effect on the cleavage and morula formation rate. As per Ahumada *et al.* (2013), supplementation

Table 1: Effects of insulin like growth factor-I on different embryo developmental stages

Group	No. of oocytes	Cleavage (MEAN±S.E.M.)	4-cell stage (MEAN±S.E.M.)	8-cell stage (MEAN±S.E.M.)	16-cell stage (MEAN±S.E.M.)	Morulastage (MEAN±S.E.M.)
Control	216	46.50 ± 2.2^{c}	29.59 ± 3.21^{b}	17.66 ± 3.16^{c}	9.91 ± 2.72^{c}	5.18 ± 1.35^{c}
T_1 (25 ng/ml)	222	56.00 ± 2.16^{b}	38.55 ± 2.01^{b}	28.45 ± 2.76^{b}	20.91 ± 3.40^{b}	10.27 ± 1.47^{b}
T_2 (50 ng/ml)	263	63.27 ± 2.17^{a}	50.00 ± 2.90^{a}	41.18 ± 2.59^{a}	32.27 ± 3.23^{a}	20.09 ± 2.57^{a}
T_3 (75 ng/ml)	231	50.00 ± 3.17^{bc}	34.18 ± 4.42^{b}	26.18 ± 4.08^{bc}	17.91 ± 3.78^{b}	8.91 ± 2.57^{bc}

Values (Mean ± SEM) in the same column with different superscript differ significantly ($p<0.05$).

of culture media with EGF and IGF could partially avoid the harmful effect of *in vitro* culture of small groups of bovine embryos. Qu *et al.* (2016) reported that supplementation of exogenous IGF-1 to the culture medium has an apparent positive effect on the developmental competence of SCNT embryos.

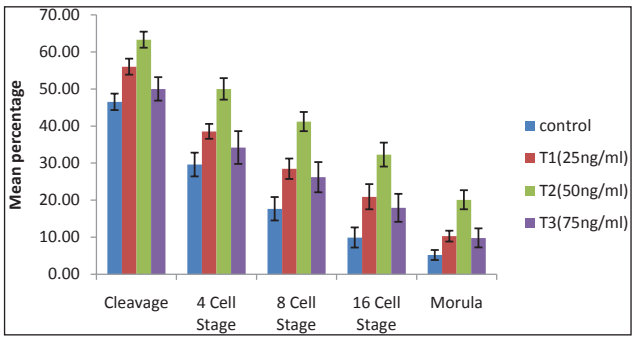

Fig. 1: Effect of different concentrations of insulin like growth factor-1 on early cattle embryo development

Effects of insulin like growth factor-I and epidermal growth factor combinedly on early embryonic developmental stages

In this experiment, 863 immature cattle oocytes were used for IVM and subsequently used for IVF. Two different groups have been examined *viz*. (i) control group: in which there is no supplementation of insulin like growth factor-1 + Epidermal growth factor, and (ii) treatment group: in which IGF-1+EGF is supplemented in all three different (IVM, IVF, and IVC) culture media with three different concentrations viz. 25 + 5 ng/ml, 50 + 10 ng/ml, and 75 + 20 ng/ml. The results indicated that significant differences ($p<0.05$) were exhibited between the control group and treatment group in the cleavage rate, 4-cell, 8-cell, 16-cell, and morula stage of early embryo development. Table 2 depicted that the cleavage rate, 4-cell, 8-cell, 16-cell and morula rates were significantly higher in the treatment group with respect to the control group. The cleavage rate and morula formation rate in the treatment group IGF-1+EGF (50+10 ng/ml) *i.e.*, 70.00 ± 2.16 and 25.30 ± 1.45 exhibited significant differences compared to the control. The cleavage rate and morula formation rate also reveal significant differences among treatment groups, but the cleavage rate of treatment group IGF-1 + EGF (75 + 20 ng/ml) showed a slight variation in cleavage rate *i.e.*, a slightly negative effect as compared to control. Still, in the morula formation they did not differ significantly, displaying that the increase in the concentration of combined IGF-1 + EGF beyond an optimum level may hurt the embryogenesis and its developmental competence. EGF and IGF-1 in combination, have been shown to accelerate the progression of meiosis and the meiotic cell cycle in bovine oocytes, possibly by increasing H1 and MAP kinase activities during the early stages of maturation (Sakaguchi *et al.* 2002). The differences between the treatment and control group may be due to the presence of the receptors (IGF-R and EGF-R) on the immature cumulus oocytes complexes and due to the maturation-promoting effect of both IGF-1 and EGF, the mitogenic role of the IGF-1.

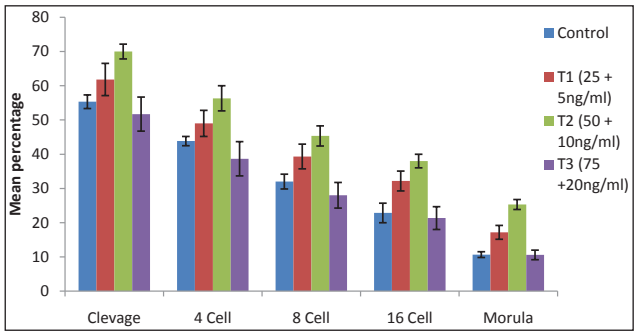

Fig. 2: Effect of different concentration of insulin like growth factor-1and epidermal growth factor in combination on the early embryo development in cattle

Table 2: Effect of insulin like growth factor-I and epidermal growth factor on different embryo developmental stages in cattle

Group	No. of oocytes	Cleavage (MEAN±S.E.M.)	4-cell stage (MEAN±S.E.M.)	8-cell stage (MEAN±S.E.M.)	16-cell stage (MEAN±S.E.M.)	Morulastage (MEAN±S.E.M.)
Control	137	55.33±1.99^{b}	43.83±1.35^{b}	32.00±2.16^{b}	22.83±2.85^{b}	10.67±0.84^{c}
T_1 (25+5 ng/ml)	218	61.83±4.70^{ab}	49.00±3.81^{ab}	39.33±3.61^{ab}	32.17±2.90^{a}	17.17±2.02^{b}
T_2 (50+10 ng/ml)	257	70.00±2.16^{a}	56.33±3.67^{a}	45.33±2.94^{a}	38.00±1.98^{a}	25.30±1.45^{a}
T_3 (75+20 ng/ml)	251	51.72±4.9^{b}	38.67±1.9^{b}	28.00±3.74^{b}	21.33±3.33^{b}	10.57±1.39^{c}

Values (Mean±SEM) in the same column with different superscript differ significantly ($p<0.05$).

CONCLUSION

Insulin-like growth factor-1, individually and in combination with epidermal growth factor, has been used as a culture media supplementation in three different concentrations for early embryonic development by IVF. The growth factor supplementation in combination was satisfactory for *in vitro* embryo production. Nevertheless, the system was not efficient when embryos were produced from <3 mm follicles and cultured *in vitro*. The results indicated that insulin-like growth factor-1 @ 50 ng/ml and in combination with EGF @ 50+10ng/ml was superior in early embryo production.

ACKNOWLEDGMENTS

The authors acknowledge sincere thanks to the Director, National Dairy Research Institute, Karnal, Joint Director (Research), National Dairy Research Institute, Karnal and Head, Eastern Regional Station, National Dairy Research Institute, Kalyani for providing funds and necessary facilities to carry out the work.

REFERENCES

Ahumada, C.J., Salvador, I., Cebrian-Serrano, A., Lopera, R. and Silvestre, M.A. 2013. Effect of supplementation of different growth factors in embryo culture medium with a small number of bovine embryos on in vitro embryo development and quality. *Animal,* **7**(3): 455- 462.

Brackett, B.G. and Oliphant, G. 1975. Capacitation of rabbit spermatozoa *in vitro*. *Biol. Reproduc.,* **12**: 260-274.

Brackett, B.G., Younis, A.I. and Fayrer-Hosken, R.A. 1989. Enhanced viability after *in vitro* fertilization of bovine oocytes matured *in vitro* with high concentrations of luteinizing hormone. *Fertil. Steril.*, **52**(2): 319-324.

Cognie, Y., Baril, G., Poulin, N. and Mermillod, P. 2003. Current status of embryo technologies in sheep and goat. *Theriogenol.*, **59**: 171-188.

Daniel, R.A., Carolina, A.P.C., Laura, L.G.L., Roberta, C.G., Guilherme, F.R., Aderson, M.I., Joao, C.T.P., Gisele, M. and Flavia, L.L. 2015. Supplementation of fetal bovine serum alters histone modification H3R26me2 during preimplantation development of *in vitro* produced bovine embryos. *Pesq. Vet. Bras.*, **35**(7).

Das, S.K., Chauhan, M.S., Palta, P., Katiyar, P.K. and Madan, M.L. 1996. Replacement of fetal bovine serum and FSH with buffalo follicular fluid in *in-vitro* maturation of buffalo oocytes. *Theriogenol.*, **45**(1): 245.

Das, S.K., Sharma, A.K., Mahapatra, S.K., Chatterjee, A., Bhatia, V. and Mohanty, A.K., 2013. Purification of cattle oviduct specific proteins and their effect on *in-vitro* embryo development. *Livestock Sci.,* **152**(1): 88-93.

Herrick, J.R., Behboodi, E., Memili, E., Blash, S., Echelard, Y. and Krisher, R.L. 2004. Effect of macromolecule supplementation during *in vitro* maturation of goat oocytes on developmental potential. *Mol. Reprod. Dev.,* **69**: 338-346.

Madan, M.L., Chuhan, 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* fertilizated oocytes and co-cultured with cumulus and oviductul cells. *Theriogenol.,* **42**: 591-600.

Malakar, D., Das, S.K., Mukesh, M., Sodhi, M. and Goswami, S.L. 2007. Production of kids from *in vitro* fertilized goat embryos and their parentage assessment using microsatellite markers. *Asian-Australasian J. Animal Sci.*, **20**(6): 842-849.

Neira, J.A., Tainturier, D., Peña, M.A. and Martal, J. 2010. Effect of the association of IGF-I, IGFII, bFGF, TGF-β1, GM-CSF, and LIF on the development of bovine embryos produced *in vitro*. *Theriogenol.,* **73**(5): 595-604.

Prasad, S., Prakash, C., Rohit, K., Karunakaran, M., Santra, A. and Das, S.K. 2018. Development of cattle embryo through *in vitro* technique using epidermal growth factor as a media supplement. *Int. J. Bio-resour. Stress Manag.*, **9**(6): 691-694.

Qu, P., Li, Y., Deng, T., Jia, D., Qing, S., Su, J., Zhang, Y. and Wang, Y. 2016. Effects of Insulin-like Growth Factor-1 on Development of Somatic Cell Cloned Bovine Embryos. *Cell Reprogram.*, **18**(3): 162-170.

Räty, M., Ketoja, E., Pitkänen, T., Ahola, V., Kananen, K. and Peippo, J. 2011. *In vitro* maturation supplements affect developmental competence of bovine cumulus-oocyte complexes and embryo quality after vitrification. *Cryobiol.*, **63**(3): 245-255.

Saeed-Zidane, M., Tesfaye, D., Mohammed, S.Y., Tholen, E., Neuhoff, C., Rings, F., Held, E., Hoelker, M., Schellander, K. and Salilew-Wondim, D. 2019. Hyaluronic acid and epidermal growth factor improved the bovine embryo quality by regulating the DNA methylation and expression patterns of the focal adhesion pathway. *PLoS One*, **14**(10).

Sakaguchi, H., Misirlioglu, M., Kaya, A., First, N.L., Parrish, J.J. and Memili, E. 2002. Developmental potential of bovine oocytes cultured in different maturation and culture conditions. *Animal Reprod. Sci.,* **101**: 225-240.

Singh, A.K., Prakash, C., Rohit, K., Santra, A. and Das, S.K. 2020. Cattle embryos development through *in vitro* techniques using thyroxine hormone as a culture media supplement. *Indian Res. J. Genetics Biotechnol.,* **12**(1): 24-29.

Singh, S.B. and Das, S. K., 2014. Embryo development through *in vitro* maturation and fertilization of cattle oocytes. *Nat. Sci.,* **12**(8): 67-70.

Sirisathien, S., Hernandez-Fonseca, H. and Brackett, B. 2003. Influences of epidermal growth factor and insulin-like growth factor-I on bovine blastocyst development *in vitro*. *Animal Reprod. Sci.,* **77**: 21-32.

Staigmiller, R.B. and Moor, R.M. 1984. Effect of follicle cells on the maturation and developmental competence of ovine oocytes matured outside the follicle. *Gamete. Res.*, **9**: 221-229.

Thongkittidilok, C., Tharasanit, T., Songsasen, N., Sananmuang, T., Buarpung, S. and Techakumphu, M. 2015. Epidermal growth factor improves developmental competence and embryonic quality of singly cultured domestic cat embryos. *J. Reprod. Dev.*, **61**(4): 269-276.

Totey, S.M., Pawshe, C.H. and Singh, G.P. 1993. *In vitro* maturation and fertilization of buffalo oocytes (*Bubalus bubalis*): effects of media, hormones and sera. *Theriogenology*, **39**: 1153-1171.

Umdor, S.B., Karunakaran, M., Mandal, D.K., Santra, A. and Das, S.K. 2021. Enhancement of developmental competence of immature oocytes supplemented with growth factors in culture media. *Indian J. Animal Res.*, **55**(5): 493-497.