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Biomedicine and Nursing

MARSLAND PRESS
Multidisciplinary Academic Journal Publisher

Cattle embryos development through i*n vitro* techniques using thyroxine hormone as a media supplement

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Abstract: The present study was conducted to produce cattle embryos through *in vitro* maturation, fertilization and culture by supplementing culture media with thyroxine hormone (T4). Cattle ovaries were collected from slaughter house and brought to the laboratory within 3-4 h in normal saline maintaining 30-35 ºC. Immature oocytes were collected from visible surface follicles (3-8 mm) in the aspiration media (TCM-199 + DPBS + 3 mg/ml BSA + 50 µg/ml gentamycin) by 19 g hypodermic needle. The COCs were washed thoroughly 5-6 times in washing media (TCM-199 + 10%FBS + 27 µg/ml sod. pyruvate + 50 µg/ml gentamycin sulfate) and matured *in-vitro* for 24 h in maturation media (TCM-199 + 10% FBS + 5 µg/ ml FSH-P + 0.33 mM sodium pyruvate + 50 µM β-mercaptoethanol + 50µg/ml gentamicin sulfate) supplemented with thyroxine hormone (T4) with three different concentrations (*viz*.20, 50 and 100 ng/ml) at 38.5 ºC in CO_2 incubator with maximum humidity. After 24 h oocytes were allowed for fertilization with in vitro capacitated sperms in Fert-BO media at 38.5 ºC in CO_2 incubator. After 15-18 h of sperm-oocyte co-incubation, the cumulus cells were washed off from the oocytes by gentle pipetting in washing medium. The oocytes were then washed 1-2 times with RVCL media and cultured in 100 µl RVCL medium supplemented with T4, and cultured for cleavage. After 48 h of culture cleavage was checked and further co-cultured with oviductal cells for development. In the present study the cleavage rate and morula rate were significantly higher in the treatment group as compared to control group. The mean percentage of cleavage rate was 23.80 ± 2.04 in control group. The highest mean percentage of cleavage rate was 52.59 ± 5.93 in 100 ng/ml treatment group. From the present study it could be concluded that thyroxine hormone may have some role to increase the cleavage rate after fertilization.

[Aman Kumar Singh, Prakash C, Rohit K, A.Santra and Subrata K. Das. **Cattle embryos development through i*n vitro* techniques using thyroxine hormone as a media supplement.** *Biomedicine and Nursing* 2022;8(2):15-18]. ISSN 2379-8211(print);ISSN 2379-8203(online). http://www.nbmedicine.org 3. doi:10.7537/marsbnj080222.03.

Key words: Cattle, oocyte, embryo, thyroxine, IVF

1. Introduction

Production of large number of embryos through assisted reproductive techniques like *in vitro* maturation and *in vitro* fertilization have great potential for production of offspring and faster multiplication of superior germplasm or cryopreserve the embryos for future use (Cognie et. al., 2003). Slaughterhouse derived ovaries provide a cheap and abundant source of large number of oocytes either for production of offspring or research purpose (Das et al., 2013; Malakar et. al., 2007). *In vitro* production of embryos involves oocytes recovery, maturation and fertilization with capacitated spermatozoa and culture of the produced embryos. Production of embryos from *in vitro* maturation and fertilization has been improved using different macromolecule supplementation in medium (Herrick et. al., 2004). Improvement of developmental competence of mammalian oocytes by supplementation of IVM media with hormones and serum supplements has been the subject of many investigations. Following the early report of Staigmiller and Moor, 1984, in which addition of granulose 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 acquisition of developmental capacity of oocytes in cattle (Brackett et al., 1989, Fukushima and Fukui, 1985). Supplementation of the IVM media with FCS (Staigmiller and Moor, 1984; Totey et al., 1993) or estrus cow serum (Brackett et al., 1989; Madan et al., 1994) has also been found to be necessary for achieving high maturation rates for cattle and buffalo oocytes. Supplementation of IVM media with follicular fluid has been found to increase the maturation rates for buffalo oocytes (Das et al., 1996). All these studies however, employed an IVM culture medium which was supplemented with gonadotropins, estradiol and a serum source. The present study was conducted to produce cattle embryos through *in vitro* maturation, fertilization and culture by additional supplementing of culture media with thyroxine hormone followed by culturing presumptive zygotes in RVCL media.

2. Materials and methods

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 and non-pyrogenic plastic syringes and sterile disposable 19 gauge hypodermic needles of Dispovan make, Kolkata, India unless otherwise mentioned.

2.1 Oocytes collection and maturation in vitro

Fresh cattle ovaries were collected at slaughter house immediate after slaughter and transported to the laboratory within 3 to 4 h in isotonic saline solution supplemented with penicillin (100 IU/ml) and streptomycin (50 µl/ml) maintained at 30-35°C. Follicular oocytes from apparently non-atretic surface follicles (3 to 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 layer of cumulus cells), B grade (3-5 layer of cumulus cells), C grade (<3 layer of cumulus cells) and D grade (partial/without 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. All the COCs were washed 5-6 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 + 10% FBS + 5 µg/ ml FSH-P + 0.33 mM sodium pyruvate + 50 µM β-mercaptoethanol + 50 µg/ml gentamicin sulphate). Then groups of 20-25 COCs were placed in 100 µl droplets of maturation medium supplemented with three different concentration (20, 50 1nd 100 ng/ml) of thyroxine hormone, 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.

2.2 Sperm preparation and in vitro fertilization

The spermatozoa used for IVF throughout the study were from the same donor. The spermatozoa were prepared for fertilization as described earlier (Chauhan et al., 1997). Briefly, two straws of frozen-thawed cattle semen were suspended in 8 ml of Working Bracket Oliphant (WBO) medium (Bracket and Oliphant., 1975) with 10 µg/ml heparin and 0.57 mM caffeine sodium benzoate and 1.23mM 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 6 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 6 min. Finally, the pellet was dissolved in l ml of Fertilization Bracket Oliphant (FBO). In the treatment group, the FBO was supplemented with different concentration of T_4 (20, 50 and 100 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.

2.3 Culture of oviductal cells and embryo culture

Fresh oviducts were dissected carefully with blunt end scissors and washed 4-5 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 petridish containing washing medium. Cell chunks were washed in washing medium for 5-6 times and 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 24 h of incubation good vibrant cells were picked up and cultured in 100 µl droplet of maturation media covered with mineral oil. After every 48 h half of the medium used to replace 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 washing medium. The zygotes were then washed 1-2 times with RVCL media and cultured in 100 µl droplet of RVCL medium. In the treatment group, the droplet was supplemented with different concentrations (20, 50 and 100 ng/ml) of T4. After 48 h of incubation cleavage was observed under microscope and embryos were shifted to 100 µl droplets of RVCL medium for further development and

co-incubated with oviduct cells in 5% CO_2 incubator with maximum humidity at 38.5 °C for 8 days.

2.4 Experimental Design and Statistical analysis

In the experiment, the effect of thyroxine hormone on *in vitro* fertilized embryo production was examined. The culture media were supplemented with three different concentrations of thyroxine hormone *i.e.* 1) 20 ng/ml, 2) 50 ng/ml, 3) 100 ng/ml. The control group was not supplemented with the hormone. The data analysis was done by IBM® Statistical Package 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.

3. Results & Discussion:

In the present study, a total of 707 immature cattle oocytes were isolated from apparently non-atretic surface follicles of ovaries collected from slaughter house. Among these oocytes all A, B, C grade COCs were *in vitro* matured for 24 h in maturation medium. After 24 h of incubation, a marked cumulus cells expansion was observed under microscope for maturation. Capacitated spermatozoa were co-incubated for 15-18 h and after sperm-oocytes co-incubation presumptive zygotes were freed from the attached sperm and incubated in RVCL medium supplemented with thyroxine hormone (T4) separately for cleavage. After 48 h post incubation cleaved embryos were co-cultured with vibrant oviduct cells. In the present study, the cleavage rate and morula rate were significantly higher in the treatment group as compared to control group. The mean percentage of the cleavage rate were significantly higher in T4 (100 ng/ml) *i.e.* 52.59±5.93 and also higher in morula stage (3.57±0.43) than the control group (Table). The cleavage rate in T4 (100 ng/ml) were not significantly differ than the T4 (20 ng/ml) and T4 (50 ng/ml). The morula formation rate in T4 (100 ng/ml) category was significantly higher than the T4 (20 ng/ml) and T4 (50 ng/ml). This treatment shows that the higher concentration of T4 (100 ng/ml) helped to improve the cleavage rate and further also improve the morula development as compare to control group. The Costa et al (2013) supplemented the T4 only in IVM media with the concentration of 25 nM, 50 nM & 100 nM, and found that there was no significant differences occurred in the cleavage stage (75.10%, 74.10% & 78.70%) with the control group (79.00%). The differences between control and treatment group may be due to the presence and expression of thyroid hormone receptor (TRα and TRβ) in the immature oocytes and cumulus cells which may have a role to increase cleavage rate and embryo growth.

Table. Effects of thyroxine hormone on cattle early embryo developmental stages

Treatment	No. of oocytes	Cleavage stage (Mean±SEM)	Four cell (Mean±SEM)	Eight cell (Mean±SEM)	Morula (Mean±SEM)
CONTROL	113	23.80 ± 2.04^{b}	16.63 ± 2.31^{b}	5.29 ± 1.20^{c}	0.54 ± 0.54^{b}
T4 (20 ng/ml)	194	41.23 ± 3.39^{a}	30.23 ± 3.54^{a}	8.83 ± 0.47^{b}	1.04 ± 0.56^{b}
T4 (50 ng/ml)	197	45.43 ± 2.86^{a}	31.64 ± 1.24^{a}	11.06 ± 0.41^{bc}	0.89 ± 0.44^{b}
T4(100 ng/ml)	203	52.59 ± 5.93^{a}	39.58 ± 5.37^{a}	15.73 ± 2.36^{a}	3.57 ± 0.43^{a}

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

4. Conclusion:

In conclusion, the results of the present study suggested that the highest mean percentage of cleavage rate (52.59±5.93) was achieved with the supplementation of thyroxine hormone @ 100 ng/ml in treatment group.

Acknowledgment:

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 the necessary facilities to carry out the work.

Short Title (Running Head):
Effect of thyroxine hormone on IVMFC

References:

[1]. Brackett BG, Oliphant G. Capacitation of rabbit spermatozoa *in vitro*. Biol Reprod. 1975; 12:260-274.

[2]. Brackett BG, Younis AI, Fayrer-Hosken, R.A. Enhanced viability after in vitro fertilization of bovine oocytes matured in vitro with high concentrations of luteinizing hormone. Fertil Steril. 1989; 52(2):319-324.

[3]. Chauhan MS, Palta P, Das SK, Katiyar PK. Madan ML. Replacement of serum and hormone additives with follicular fluid in the IVM medium: Effects on maturation, fertilization and subsequent development of buffalo oocytes. Theriogenology. 1997; 48(3): 461-469.

[4]. Cognie Y, Baril G, Poulin N, Mermillod P. Current status of embryo technologies in sheep and goat. Theriogenology. 2003; 59: 171-188.

[5]. Costa N N, Cordeiro M S, Silva T V G, Sastre D, Santana PPB, Sa ALA, Sampaio R V, Santos SSD, Adona PR, Miranda MS, Ohashi OM. Effect of triiodothyronine on developmental competence of bovine oocytes. Theriogenology 2013; 80:295-301.

[6]. Das SK., Chauhan MS, Palta P, Katiyar PK., Madan ML. Replacement of fetal bovine serum and FSH with buffalo follicular fluid in *in-vitro* maturation of buffalo oocytes. Theriogenology, 1996; 45(1): 245.

[7]. Das SK, Sharma AK, Mahapatra SK., Chatterjee A, Bhatia V, Mohanty A K. Purification of Cattle Oviduct Specific Proteins and their Effect on *In-Vitro* Embryo Development. Livestock Science, 2013; 152(1):88-93.

[8]. Fukushima M, Fukui Y. Effects of gonadotropins and steroids on the subsequent fertilizability of extra follicular bovine oocytes cultured in vitro. Anim Reprod Sci, 1985; 9:323-332.

[9]. Herrick JR, Behboodi E, Memili E, Blash S, Echelard Y, Krisher RL. Effect of macromolecule supplementation during in vitro maturation of goat oocytes on developmental potential. Mol Reprod Dev. 2004; 69: 338-346.

[10]. Madan ML, Chuhan MS, Singla SK, Manik RS. 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. Theriogenology. 1994; 42:591-600.

[11]. Malakar D, Das SK, Mukesh M, Sodhi M, Goswami SL. Production of kids from in vitro fertilized goat embryos and their parentage assessment using microsatellite markers. Asian-Aust. J Anim Sci. 2007; 20(6): 842-846.

[12]. Staigmiller RB, Moor RM. Effect of follicle cells on the maturation and developmental competence of ovine oocytes matured outside the follicle. Gamete Res. 1984; 9:221-229.

[13]. Totey SM, Pawshi CH, Singh GP. In vitro maturation and fertilization of buffalo oocytes (*Bubalis bubalis*): effects of media, hormones and sera. Theriogenology. 1993; 9:1153-1171.

2/15/2022