Embryo development through *in vitro* maturation and fertilization of cattle oocytes

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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 serum, hormone and fresh follicular fluid from slaughter house ovaries. Cattle ovaries were collected from Kolkata slaughter house and brought to the laboratory within 3-4 h in normal saline maintaining 30-35 ºC. 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) and matured *in-vitro* for 24 h in maturation media (Washing media + 5 µg/ml FSH-P + 5% Follicular fluid) at 38.5 ºC 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 ºC in CO_2 incubator. At the end of 16-18 h presumptive zygotes were separated from sperm-oocytes co-incubation by washing in TCM-199 supplemented with 10% FBS and cultured for cleavage in EDM and RVCL media. After 48 h cleavage was checked and further co-cultured with oviductal cells in RM and RVCL mediafor further development. In this study, overall 67.01% oocytes were cleaved, and developed to different stages of embryos viz. 39.00% morula and 8.37% blastocyst. The cleavage rate in EDM was 65.72% and in RVCL it was 68.01%. Embryos reached to morula and blastocyst stage in RM was 38.03% and 7.97% respectively, whereas in RVCL it was 39.72% and 8.67% respectively. The results show that slaughtered house derived immature cattle oocytes could be matured and fertilized *in vitro* and embryo could be produced both in EDM/RM and RVCL medium, however, RVCL media showed comparatively better result than the EDM/RM for embryo development.
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1. Introduction

Production of large number of embryos through assisted reproductive techniques like *in vitro* maturation and *in vitro* fertilization have great potential for research, production of offspring, 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 (Malakar et. al., 2007, Cognie et. al., 2004). *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). The developmental competence of *in vitro* matured ruminant oocytes is, however, low under common IVM conditions (Trounson, 1992). 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 and Younis 1989, Fukushima and Fukui 1985). Supplementation of the IVM media with fetal calf serum (Staigmiller and Moor, 1984; Totey et al., 1993) or estrus cow serum (Brackett and Younis 1989, Madan et al., 1994) has also been found to be necessary for achieving high maturation rates for cattle and buffalo oocytes. 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 IVMFC by supplementing culture media with fresh follicular fluid, serum and hormone, culturing presumptive zygotes/embryos in EDM and RVCL media.

2. Materials and methods:

All plastic wares were used from Tarson Products Pvt. Ltd. (Kolkata, India) and chemicals/ biochemicals/ mineral 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 in vitro maturation*

Cattle ovaries were collected at an abattoir 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 + 3 mg/ml BSA + 50 µg/ml gentamycin) 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, C and D grade of cumulus oocytes complexes (COCs) with homogenous, evenly granulated cytoplasm were used for maturation. *In vitro* maturation was carried out as described earlier (Meena and Das, 2006, Malakar et al. 2007). Briefly, all the COCs were washed 3-4 times in washing medium (TCM-199 + 10% FBS + 27 µg/ml Sod. Pyruvate + 50 µg/ml gentamycin) followed by 2-3 times in maturation medium (Washing media + 5 µg/ml FSH-P + 5% Follicular fluid). Then groups of 10-20 COCs were placed in 50 µl 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.

2.2 *Sperm preparation and in vitro fertilization*

The spermatozoa used for *in vitro* fertilization throughout the study were from the same batch of same donor. The spermatozoa were prepared for insemination as described by Chauhan et al. (1997). Briefly, 2-3 straws of frozen-thawed ejaculated cattle semen were suspended for swim-up in quenched BO medium (Bracket et al, 1975) with 10 µg/ml heparin and 10 mM caffeine. Progressively motile sperm cells were washed with 5 ml of BO medium at 1800 rpm for 5 min. The pellet was dissolved in 2 ml of fertilization-BO (Fert-BO) medium and centrifuged at 1800 rpm for 5 min. Finally the pellet was dissolved in 200 µl Fert-BO medium. Motile sperm cells were placed in 50 µl droplets of Fert-BO medium containing 0.5% BSA, 10 µg/ml heparin and 10 mM caffeine in a 35 mm petri dish, covered with mineral oil and placed at 38.5 °C in a 5% CO_2 incubator with maximum humidity for 30 min before insemination to *in vitro* matured oocytes culture drop.

After 24 h, maturation medium from the maturation culture drop was replaced with Fert-BO medium gradually and carefully without disturbing oocytes. The motile spermatozoa (2-4 million spermatozoa/ml) were inseminated into droplets and placed in 5% CO_2 incubator at 38.5 °C with maximum humidity for 16-18 h for fertilization.

2.3 *Culture of oviduct epithelial cells and embryo culture*

Oviducts were dissected carefully and washed 4-5 times with washing media. The 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 washing medium. Cell chunks were washed 5-6 times in washing medium and then cultured into 100 µl droplet of maturation media. After 24 h, vibrant with good cilliary movement cells were picked up and washed in washing media and cultured in 100 µl droplet of maturation media overlaid with mineral oil.

The presumptive zygotes were separated from the drop at the end of 16-18 h sperm-oocyte co-incubation and were washed with TCM-199 supplemented with 10 % FBS and then cultured for 48 h in EDM (TCM-199 100 µg/ml glutamine, 30 µg/ml sodium pyruvate, 50 µg/ml genatamycin, 10 mg/ml BSA, 10 µl/ml EAA and 5 µl/ml non-EAA) and RVCL medium at 38.5 °C in a 5% CO_2 incubator with maximum humidity. The cleavage rate was recorded after 48 h of incubation and then further co-cultured with oviduct epithelial cells in 100 µl droplet of replacement medium (RM) and RVCL medium for further development.

3. Results and Discussion:

In the present study, a total of 779 cattle oocytes were isolated from apparently non-atretic surface follicles of cattle ovaries collected from slaughter house. Among these oocytes 171 were graded as A (21.95%), 194 as graded B (24.90%), 225 as graded C (28.88%) and 189 as graded D (24.26%). All A, B, C and D 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. A total of 570 (73.17%) immature oocytes found matured (Table.1). It was observed that quality of follicular fluid affect the maturation of oocytes. Follicular fluid from a single follicle was better instead of mixing from number of follicles. All 570 *in vitro* matured oocytes were used for *in vitro* fertilization. Capacitated spermatozoa were co-incubated for 16-18 h and after sperm-oocytes co-incubation; presumptive zygotes were freed from attached sperm and cumulus cells followed by incubated in EDM and RVCL medium separately for

cleavage. After 48 h post incubation cleaved embryos were co-cultured with vibrant oviduct cells. In this study, overall 67.01% oocytes were cleaved, and developed to different cell stages of embryos viz. 39.00% morula stage and 8.37% blastocyst stage. The cleavage rate in EDM was 65.72% and in RVCL it was 68.01%. The formation of morula and blastocyst stage in replacement media was 38.03% and 7.97% respectively, whereas in RVCL it was 39.72% and 8.67% respectively (Table.2). The majority of uncleaved oocytes showed uneven pigmentation, darkening of cytoplasm or vacuolization. Such oocytes were removed from the culture drop after 48 h of incubation. The results of this study demonstrated the *in vitro* development of embryos through *in vitro* maturation and *in vitro* fertilization of oocytes collected from slaughter house ovaries. Yang et. al. (1995) developed *in vitro* matured and fertilized bovine embryos in a controlled design experiments and produced 25% blastocyst. Sugiyama et. al. (2003) demonstrated the temperature effect on the early embryonic development and produce 4.5 % morulae and blastocyst on fourth day of culture compared to 10.5% for the control group. The result shows that immature cattle oocytes could be matured and fertilized *in vitro* and embryo could be produced both in EDM/RM and RVCL medium, however, RVCL media is comparatively better than the EDM/RM for embryo development.

Table.1: *In vitro* maturation of oocytes

Total No. of immature oocytes used for IVM	Grade A (%)	Grade B (%)	Grade C (%)	Grade D (%)	Total No. of oocytes matured (%)
779	171 (21.95)	194 (24.90)	225 (28.89)	189 (24.26)	570 (73.17)

Table.2: *In vitro* fertilization and *in vitro* embryo development

Culture medium for		No. of matured oocytes fertilized in Fert-BO medium	No. of oocytes cleaved (%)	No. of embryos at morula stage (%)	No. of embryos at blastocyst stage (%)
Cleavage	Development				
EDM	Replacement media (RM)	248	163 (65.72)	62 (38.03)	13 (7.97)
RVCL	RVCL	322	219 (68.01)	87 (39.72)	19 (8.67)
Total		570	382 (67.01)	149 (39.00)	32 (8.37)

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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. Cognie Y, Poulin N, Locatelli Y, Mermillod P. State-of-the-art production, conservation and transfer of in vitro produced embryos in small ruminants. Reprod Fert Dev 2004; 16: 437-445.
6. 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.
7. 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.
8. 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.
9. 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.
10. Meena CR. Das SK. Development of water buffalo (*Bubalus bubalis*) embryos from in vitro matured oocytes reconstructed with fetal skin fibroblast cells as donor nuclei. Anim Reprod Sci 2006; 93: 258-267.
11. 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.
12. Sugiyama S, McGowan M, Kafi M, Philips N, Mary Y. Effects of increased ambient temperature on the development of in-vitro-derived bovine zygotes. Theriogenology 2003; 60(6): 1039-1047.
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: 153-1171.
14. Trounson A. The production of ruminant embryos *in vitro*. Anim Reprod Sci 1992; 28:125-137.
15. Yang BK, Giles JR, Yang X, Foote RH. Development of *in vitro* matured/*in vitro* fertilized bovine oocytes in a simple defined (KSOM) medium. J Reprod Dev 1995; 41: 213-218.

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