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In vitro production of early stage buffalo embryos in modified synthetic oviductal fluid (mSOF) medium

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ABSTRACT

The objective of the present study was to observe the developmental rates and the stage of development since fertilization of *in vitro* produced early stage buffalo embryos. Buffalo cumulus-oocyte complexes (COC's) obtained from slaughterhouse ovaries were matured and fertilized *in vitro*. The fertilized oocytes (400) were then cultured in modified synthetic oviductal fluid (mSOF) medium containing bovine serum albumin (BSA) and fetal bovine serum (FBS) and evaluated for the developmental stages of preimplantation early stage embryos up to morula on 48 h, 72 h, 96 h and 7th day post fertilization. Highest percentage of 8 cell embryos were obtained followed by 16 cell, 2 cell, 4 cell and morula at the end of *in vitro* culture (IVC). Similarly, the mean embryo percent of 2 cell, 4 cell, 8 cell, 16 cell and morula stage at the end of IVC was 8.20±1.28, 6.50±0.97, 12.19±1.03, 11.83±0.96 and 5.27±0.87. The overall embryos developed at the end of IVC were 43.98±2.11%. The percentage of oocytes which arrested and subsequently degenerated were 56.02% till day 7 post-insemination. It can be concluded that early stage buffalo embryos till morulae can be produced by using modified synthetic oviductal fluid (mSOF) medium as embryo development medium but for further development, the supplementation of different growth factors required.

Key words: Buffalo, Cumulus oocyte complex (COC's), *In vitro* fertilization, Early stage embryos, Modified synthetic oviductal fluid

In the last decade, concern in buffalo reproduction has incredibly improved worldwide with the application of latest reproductive biotechnologies in livestock. Buffaloes are reported to have low reproductive performances due to weak/silent estrus signs, seasonal anestrous, delayed puberty, delayed first calving, late post partum conception and a long calving interval (Warriach *et al*. 2015) that needs to be augmented as soon as possible. Out of currently employed techniques for improvement of buffalo reproduction, *in vitro* embryo production (IVEP) holds great promises. This technique utilizes the retrieval of oocytes from slaughtered buffaloes and encompasses the techniques like *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC). This technology allows the predictable supply of embryos from ovaries of slaughtered females. To date, IVEP has given considerable success in both cattle and buffalo, achieving a success rate ranging from 30-50% development of preimplantation stage embryo *in vitro*. This technology does not only offer optimization of high-quality dams, but also allows the preservation and rapid multiplication of genetically superior characters by making embryos available for cloning, sexing and nuclear transfer. This paper discusses the production of quality early stage embryos *in vitro* using the modified synthetic oviductal fluid (mSOF) medium.

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MATERIALS AND METHODS

In vitro *embryo production:* Buffalo ovaries were obtained from local abattoir and transported to the laboratory in 0.9% sterile saline supplemented with 50 µg/ml gentamycin sulphate at 35–37ºC within 2–3 h. Ovaries were washed 4–5 times with physiological saline solution and oocytes were aspirated from all visible ovarian follicles of 3–8 mm diameter in working oocyte collection medium (W-OCM) [Tissue culture medium–199 (TCM-199) containing 10% Fetal bovine serum (FBS)] using a 18-gauge needle fitted to a 5 ml syringe and kept undisturbed in an incubator at 37ºC. After 15–20 min, supernatant was discarded and oocytes were recovered from sediment under a stereozoom microscope (Bausch and Lomb). The COCs were evaluated and graded (Table 1) on the basis of cellular investment and homogeneity (Chauhan *et al.* 1998).

In vitro *maturation (IVM):* Compact cumulus oocytes complexes (COCs) with an unexpanded cumulus having more than 5 layers of cumulus cells and evenly granular homogenous ooplasm (grade A) and 2–4 layers of cumulus cells and with homogenous cytoplasm (grade B) were

Table 1. Grading of cumulous oocyte complex's (COC's) before maturation

COCs Grade	COCs Characteristics
Grade A	COCs with an unexpanded cumulus cells having at least 5 layers of cumulus cells with homogenous cytoplasm
Grade B	COCs with 2–4 layers of cumulus cells and with homogenous cytoplasm
Grade C	Oocytes partially denuded of cumulus cells with irregular shrunken cytoplasm
Grade D	Oocytes completely denuded of cumulus cells with irregular cytoplasm

selected for *in vitro* maturation. Selected COCs of grade A and B were washed five times in maturation medium (TCM-199 supplemented with 10% FBS, 0.5 µg/ml follicular stimulating hormone (FSH), 10 IU/ml luteinizing hormone (LH), 1 µg/ml 17-β-estradiol, 2.0 mg/ml sodium pyruvate, 200 mM β-mercaptoethanol, 200 mM cysteamine and 50 µg/ml gentamycin sulphate). Oocytes (10–15) were kept for maturation in 100 µl microdrops of maturation medium overlaid with mineral oil in a 35 mm culture dish for 24 h at 38.5ºC and 5% CO_2 in air with 95% relative humidity.

In vitro *fertilization (IVF):* Evaluation of maturation was done on the basis of visual assessment and degree of cumulus expansion (Table 2) under inverted microscope as per Kobayashi *et al*. (1994). The mature oocytes with non-homogenous spread of cumulus cells and visibility of clustered cells (degree 1) and homogenous spread of cumulus cells and no visibility of clustered cells (degree 2) were subjected to *in vitro* fertilization in Tyrode-albumin-lactate-pyruvate (TALP) supplemented with 0.2 mM/ml sodium pyruvate, 6 mg/ml fatty-acid-free bovine serum albumin (BSA), 10 µg/ml heparin and 50 µg/ml gentamycin sulphate. A single frozen buffalo semen straw (0.25 ml) was thawed at 37ºC for 1 min. Semen from a straw was added to 1 ml of sperm-TALP in 15 ml centrifuge tube and kept in CO_2 incubator for 1 h (swim up method). After one hour, 850 µl of supernatant in 15 ml of centrifuge tube was taken and the final volume made up to 5 ml by adding sperm-TALP and then centrifuged at 1,000 rpm for 5 min. The supernatant was discarded and again the procedure was repeated with fresh sperm-TALP medium. The supernatant was again discarded and the pellet was dissolved in 350 µl of FERT- TALP medium with fatty acid-free bovine serum albumin (BSA, 6 mg/ml) and heparin (10 µg/ml) and adjusted the final sperm concentration to 1×10^6/ml and kept in CO_2 incubator for about 35 min before inseminating matured oocytes for capacitation. COCs (10–15) were introduced into 100 µl droplets of processed buffalo spermatozoa and kept in an incubator with 5% CO_2 at 38.5ºC with maximum humidity for 18 h.

Table 2. Grading of matured COC's

Degree	Cumulus expansion
Degree-0	No cumulus expansion
Degree-1 (Moderate expansion)	Non-homogenous spread of cumulus cells and visibility of clustered cells
Degree-2 (Fully expanded)	Homogenous spread of cumulus cells and no visibility of clustered cells

In vitro *culture (IVC) of embryos:* After 10 h of sperm oocytes co-incubation, the oocytes (presumptive zygotes) were taken out from fertilization drop and serially washed 8–10 times in FERT- TALP medium. After washing, 10–15 oocytes were further transferred into 100 µl of FERT-TALP medium. The droplets were covered with warm mineral oil and finally placed in CO_2 incubator at 38.5°C with 5% CO_2 and humidified air for further 6 h. After 18 h of fertilization, the oocytes (presumptive zygotes) were taken out from fertilization drop and serially washed 8–10 times in modified synthetic oviductal fluid (mSOF) medium comprising of 3 mg/ml BSA, 0.25 mM sodium pyruvate with 1% essential and nonessential amino acids, 0.68 mM L-glutamine and 50 µg/ml gentamycin sulphate. After washing, 10–15 oocytes were further transferred into 100 µl of mSOF. The droplets were covered with warm mineral oil and finally placed in CO_2 incubator at 38.5°C with 5% CO_2 and humidified air for 48 to 72 h post insemination for *in vitro* culture (IVC). The fertilization rate was recorded after 48–72 h of post insemination and further observations were made to monitor the development of embryos up to morula stage till 7th day of culture.

Statistical analysis: All the experimental data were analyzed by Sigma Software (SPSS-16.0) using one way ANOVA. The collection day wise (n=56) descriptive statistics was applied to calculate Mean±SEM among different parameter as specified above.

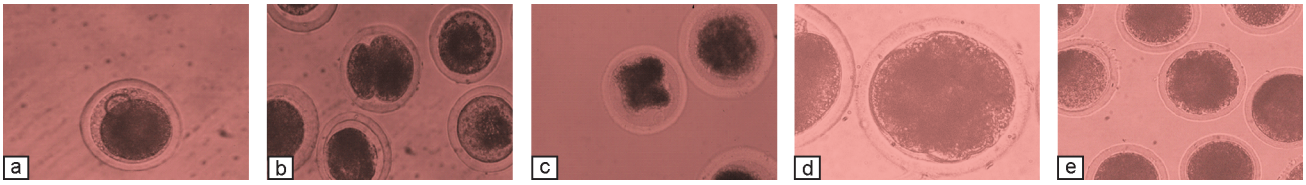
RESULTS AND DISCUSSION

The *in vitro* culture of fertilized oocytes in the present study was assessed 48 h after IVF. Presumptive zygotes were placed in mSOF culture media for further development and observed (Table 3) after 48, 72, 96 h and 7th day post fertilization. The early stage embryos (Fig. 1) developed at the end of IVC were 43.98±2.11%. In the present study at the end of 7th day of culture, mean cleavage percent was

Table 3. Mean embryo percent of different embryonic cell stage observed at 7th day of *in vitro* culture (IVC)

Embryonic cell stage	Number of embryos (X)	Overall embryo percent	Mean embryo percent (n= 56)
2 cell	77	8.40	8.20±1.28
4 cell	61	6.65	6.50±0.97
8 cell	114	12.43	12.19±1.03
16 cell	108	11.78	11.83±0.96
Morula	40	4.36	5.27±0.87
Overall developed	400	43.62	43.98±2.11
Overall undeveloped	517	56.38	56.02±2.11

N, Number of culturable oocytes utilized for IVF (917); X, number of embryos in particular cell stage; n, number of collection days.



Polar body 2 cell embryo 4 cell embryo 8 cell embryo 16 cell embryo

Fig. 1. Different cell stages of presumptive embryos.

43.98±2.11% which was more or less similar to the findings of earlier workers, viz. Raza *et al.* 2001 (42.66%), Mishra *et al.* 2010 (42.63%) and Sadeesh *et al.* 2014 (44.10±2.9%). However, the supplementation of culture media was different from the present study. Raza *et al.* (2001) cultured the presumptive zygotes in *IVF*-Tl media and reported the cleaved embryos only up to 2 cell stage while Mishra *et al.* (2010) supplemented the maturation media with 20 ng/ml epidermal growth factor (EGF) and embryo culture media with 100 ng/ml insulin like growth factor (IGF-1) and 100 µM βME. Sadeesh *et al.* (2014) incorporated the mCR2aa embryo culture media supplemented with 0.8% bovine serum albumin and the maturation media supplemented with 20 ng/ml EGF and 100 µM βME. The excellent quality early stage embryos obtained through the mSOF media in the present study showed the potency of the media utilized without even adding the growth factors like epidermal or insulin like growth factors.

On the contrary, the mean fertilization percent reported by earlier workers were higher than that obtained in present study, viz. Lojkic *et al.* 2012 (82.15±3.7%), Serrano *et al.* 2012 (80.49%) and Uger 2015 (73.80±4.08%). Higher cleavage percent obtained by earlier workers may be due to incorporation of various types of culture media as well as the supplements like βME, activin-A, EGF, IGF-1. Serrano *et al.* (2012), Lojkic *et al.* (2012) and Uger (2015) carried out the *in vitro* embryo culture in synthetic oviductal fluid medium as in present study but, Serrano *et al.* (2012) added 30µl/ml βME to reduce the oxidative stress in presumptive zygotes during *in vitro* embryo culture. Lojkic *et al.* (2012) supplemented 100 µM/ml cysteamine in the basic embryo culture media and obtained higher cleavage percent suggesting that early stage embryos are probably more sensitive to oxidative stress because of exhausted GSH pool synthesized during IVM. Uger (2015) added 10 µl/ml βME in the embryo culture media to reduce the oxidative stress.

Morula stage embryos obtained in the present study at the end of 7th day of culture were 5.27±0.87% which were higher than that obtained by Bag *et al.* (2010). They reported 4.6% of morulae (up to 16 cell stages). However, morula % as reported by Mishra *et al.* (2010), Cevik *et al.* (2014) and Uger (2015) was 35.26, 34.20±4.23 and 44.60±2.84, respectively. This may be due to the supplementation of various components like antioxidants, growth factors. Mishra *et al.* (2010) added EGF, IGF-1 and βME and achieved greater morula percent while without adding these supplements they obtained only 10.25% morula.

In the present study, modified synthetic oviductal fluid medium (mSOF) containing bovine serum albumin (BSA) and fetal bovine serum (FBS) was utilized for the culture of preimplantation embryos up to morula stage. The mSOF culture medium containing BSA and FBS is widely used for producing faster developmental speed and higher developmental rate in bovine embryos (Cevik *et al.* 2014). IVC is perhaps the most important step, not only because of its longer duration of around 10 days for formation of embryos up to morula and blastocyst stage compared to that of 24 h for IVM and 6–24 h for IVF but also since the culture conditions and environment during IVC have a profound influence on the outcome of embryo development. Various simple media like Charles Rosenkrans (CR) medium (Rosenkrans *et al.* 1993), mSOF (Tervit *et al.* 1972), Chatot- Ziomek-Bavister medium (Chatot *et al.* 1989), potassium simplex optimized medium (Lawitts and Biggers 1991) and G1.2/G2.2 media (Gardner 1994) have been utilized for buffalo embryo culture and found capable of supporting the embryo development even in the absence of co-culture with somatic cells (Nandi *et al.* 2003 and Anand *et al.* 2008). IVC of buffalo zygote in mSOF gave a higher yield of embryos then in TCM-199 medium as reported by Abdoon *et al.* (2001).

In the present study, most of embryos arrested at 8 to 16 cell staged followed by 2 to 4 cell and finally morula stage. It is difficult to rescue the embryo once the block has initiated. The mechanisms of the blocks to *in vitro* development are unclear. Control of preimplantation development first depends on maternal components accumulated by the oocyte during its growth and maturation. As development proceeds, maternally inherited molecules decay and embryogenesis becomes dependent on the expression of genetic information derived from the embryonic genome. In bovine embryo, although some transcription can be detected before the first cleavage of the zygote (Memili and First 1999) or at the two-cell stage (Hyttel *et al.* 1996), ribosomal transcription only occurs at the four-cell stage (Viuff *et al.* 1998). A high transcriptional activity in the embryo is detected from the 8-cell stage (Pavlok *et al.* 1993) or even later at the 16- cell stage (Bilodeau and Panich 2002) concomitantly with marked changes in protein pattern synthesis. The functional organization of the nucleolus is not completely gained before the 8-cell or even the 16-cell stage (Laurincik *et al.* 2000). In fact, embryonic genome activation (EGA) in mammals occurs in a stepwise manner, with progressive changes in nuclear and chromatin structure regulating the

process. Products inherited from the maternal genome can be sufficient to support development until this stage. The transition from maternal to zygotic control of development (MZT) in bovine occurs between 8 and 16-cell stages, during the fourth embryonic cell cycle (Telford *et al.* 1990). In most species, MZT is a critical step characterized by a developmental block or a slowing down in cleavages under *in vitro* culture conditions. Thus, bovine embryos produced *in vitro* under suboptimal conditions showed a developmental arrest between the 8- to 16-cell stages (Camous *et al.* 1984). Under permissive *in vitro* culture conditions, the second and third cell cycles are quite short, 8 to 12 h, but the fourth cell cycle is very long, around 40 to 50 h (Barnes and Eyestone 1990) which is due to appearance of gap phases concomitantly with the major onset of zygotic transcription, and has been called the lag phase. In bovine embryos, the fourth cell cycle corresponds to the switch from maternal to zygotic control of development of MZT. The fourth cell cycle, especially sensitive to oxidative stress and oxygen tension. Here, in the present study, 12.43 and 11.78% of bovine embryos were able to develop in to 8 and 16 cell stage, respectively. Bovine oocytes collected from different follicular size can have very different developmental potential and may be only 12.43 (8 cell) and 11.78 (16 cell) percent of them have stockpiled enough messengers and proteins necessary to achieve the first four cleavages without transcription. The transition between 8 and 16 cell stages corresponds with the major outburst of embryonic transcription; however, the first four cleavages of bovine embryos can occur without zygotic transcription.

In the present study, the lower cleavage percent of *in vitro* matured and fertilized oocytes may be due to several reasons. One of the reasons may be the handling of sensitive developing embryos during transferring fresh media. The *in vitro* derived buffalo embryos are very vulnerable during development to external environment. Another reasons may be the lack of growth factors (EGF, IGF-1 activin-A) in the embryo culture media and oxidative stress that might have led to blockade of developmental stage.

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