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Synthetic Oviductal Fluid Medium with Isologous Oviductal Epithelial Cells Improves the Development of *In Vitro* Produced Nili Ravi Buffalo (*Bubalus bubalis*) Embryos

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AA, SA and QS planned and executed the project. AA, SA, AUH, SQ, RE analyzed the results and wrote the manuscript. NU and TA provided technical assistance. SA supervised the whole project.

Key words
Buffalo, Oviductal cell, Co-culture, Conditioned media, IVF.

ABSTRACT

The objective of the current study was to investigate and compare the role of buffalo oviductal epithelial cell (BOEC) co-culture and conditioned culture media, on *in vitro* development of Nili Ravi buffalo embryos. Oocytes were obtained from the ovaries of slaughtered buffaloes within two hours after slaughter and brought to laboratory. After *in vitro* maturation (IVM) in Tissue Culture Medium-199 (TCM199) for 24 h and fertilization (IVF) in Tyrode's Albumin Lactate Pyruvate (TALP) medium for about 20 h, the presumptive zygotes were randomly distributed into 6 culture groups; Group I: Synthetic Oviductal Fluid (SOF) medium alone, Group II: SOF + co-culture, Group III: Conditioned SOF, Group IV: M199 alone, Group V: M199 + co-culture and Group VI: Conditioned M199 for *in vitro* culture (IVC). The percentage of embryos capable of crossing 8-16 cell block and reaching morula stage were higher ($P < 0.05$) in Group II and Group III compared to other treatments. Both treatment groups were equally effective ($P > 0.05$). Results also indicated that synthetic oviductal fluid (SOF) medium was found better than M199 when co-cultured and conditioned with buffalo oviductal epithelial cells for culturing buffalo embryos. Conditioned SOF is recommended to be used as IVC media in future studies as it eliminates contamination problem, remove batch to batch variation, long-lived and is readily available.

INTRODUCTION

Among the highest milk-producing breeds of buffalo, Nili Ravi is the principle breed in Pakistan. Although, this breed has potential to produce more than 5000 liters of milk/lactation (Bilal *et al*., 2006), the milk yield of this breed is no more than 1800-2500 liters/lactation. The reason is poor genetic selection that can be improved by technologies like artificial insemination and *in vitro* embryo production/transfer but so far AI has met little success (Asma-ul-Husna *et al*., 2017). *In vitro* embryo production (IVEP) that extends the genetically superior male and female germ-plasm simultaneously is the widely accepted assisted reproductive technique around the world but not yet has been optimized for Nili Ravi buffalo. It is relevant to mention that previous studies conducted on Nili Ravi buffalo reported an *in vitro* block to development at about 8-16 cell stage that can be attributed to sub-optimal culture conditions (Jamil *et al*., 2011; Patel *et al*., 2013). Adequate culture medium is requisite for an *in vitro* system that can efficiently provide nutrients such as glucose, amino acids and fatty acids to the developing embryo. Different *in vitro* culture media like TCM-199, mCR2 or mSOF have been adopted for the culture of buffalo embryos. But, the main shortcoming was slow growth rate and low percentage of transferable stage embryos.

Secretory epithelial cells of the oviduct play an important role particularly during early pregnancy by secreting specific glycoproteins that are required for the embryonic development in the female reproductive tract (Murray, 1997). An ongoing hypothesis suggests that oviductal epithelial cells co-culture enhance the development of pre-implantation embryos by one or more mechanisms. Firstly, these cells produce and release specific embryotrophic factors (Oliphant, 1986) such as insulin-like growth factors (IGF), transforming growth

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factor (TGF) and leukemia inhibitory factor (LIF) that are required by early stage embryos to maintain normal rate of development (Gandolfi and Moor, 1987). Another possibility is that these cells also contribute antioxidant enzymes like manganese superoxide dismutase (MnSOD), Cu-Zn-superoxide dismutase (CuZnSOD), glutathione peroxidase (GPX), glutamyle cysteine synthetase (GCS) and catalase (CAT) which inhibit production of toxic radicals and embryotoxic substances, such as ammonia in the culture medium (Nancarrow and Hill, 1994). For efficient embryotrophic activity, recovery of oviductal epithelial cells from fresh tissue is prerequisite, which is one of the limitations of co-culture (Xu *et al*., 1992). Another limitation for co-culture is that culture media may get contaminated by the oviductal cells that cause risk of infecting the embryos (Avery *et al*., 1993). To reduce the variability, one alternative in co-culture systems could be the use of cell lines that maintain primary culture attributes. An even better alternative is the conditioned media; the spent medium collected from cultured cells has been developed and successfully used. These cultured cells secrete vast variety of proteins including growth factors, cytokines, chemokines, extracellular matrix proteins and metabolites such as lactate, pyruvic acid, amino acids, and glucose into the medium in which they grow (Freshney, 1999; Lopera-Vásquez *et al*., 2016) that support the early embryonic development. Conditioned medium is readily available as it can be prepared and stored, easy alternative, unaffected by freezing and thawing and same batch can be used for several replicates (Lopera-Vásquez *et al*., 2016; Eyestone *et al*., 1991).

Co-culture system/conditioned medium can be developed using oviductal cells of different species and the effect of oviductal cells on embryonic development is non-species-specific (Pavasuthipaisit *et al*., 1994). However, it has also been claimed that co-culture systems using heterologous oviductal cells monolayer may increase the risk of genetic contaminations (Nematollahi-mahani *et al*., 2009). Therefore, more efficient culture environment either co-cultured or conditioned with oviductal cells from same species (isologous) needs to be evaluated to alleviate the danger of genetic inter-species contamination.

In the present study, the competence of culture environment (either co-cultured or conditioned with buffalo oviductal epithelial cells) for developing pre-implantation buffalo embryos in two different culture media (Synthetic Oviductal Fluid media and Medium 199) was investigated.

MATERIALS AND METHODS

Collection of oocytes from buffalo ovaries

Buffalo ovaries (900) were collected during breeding season from slaughtered animals and transferred to the IVF laboratory, Buffalo Research Institute (BRI), Pattoki within two hours in a thermos containing sterilized phosphate buffered saline (PBS) kept at 33-35°C. Fresh PBS was used to wash the ovaries immediately after arrival. Sterile disposable plastic syringe (10 ml) fitted with 18 gauge needle was used to aspire immature cumulus-oocyte complexes (COCs) from 2-8 mm visible follicles. Searching for COCs were done under stereomicroscope and collected in PBS. COCs were classified as grade A, B, C and D, on the basis of their cumulus investment and ooplasm homogeneity (Azam *et al*., 2017). Only grade A and B oocytes (1000) were processed for IVM.

In vitro *maturation (IVM) of oocytes*

Selected COCs were washed twice in PBS (37ºC) and twice in pre-equilibrated maturation medium: TCM-199 supplemented with 0.02 IU/mL FSH, 1 µg/mL estradiol-17β (E2), 10.0 ng/mL epidermal growth factor (EGF), 50 µg/mL gentamicin and 10% Fetal calf serum (FCS).

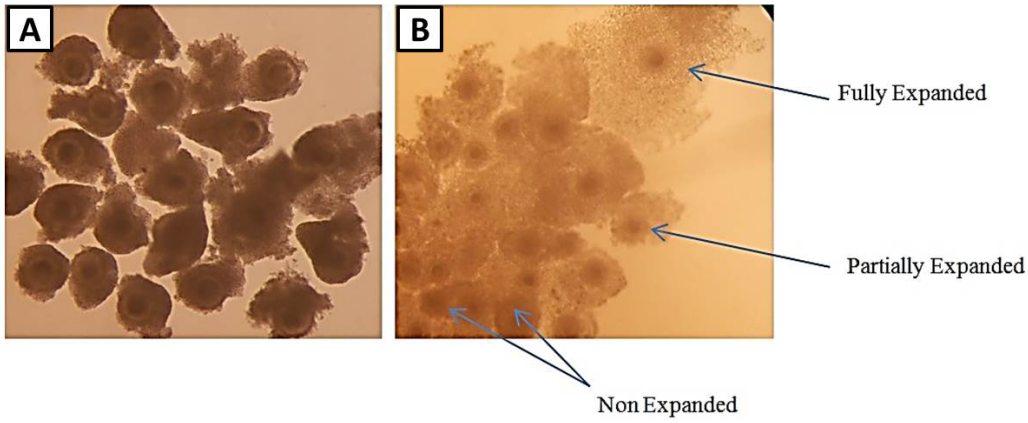

Fig. 1. Cumulus oocyte complexes (COCs) before (A) and after (B) 24 h of maturation.

The washed oocytes were randomly allocated into 100 µl of respective culture droplets of each treatment group covered with sterile mineral oil (Sigma M8410) and matured *in vitro* in a water-jacketed incubator at 38.5ºC under an atmosphere of 5% CO_2 in air with 95% humidity for 24 h. After 24 h of maturation, cumulus cell expansion was assessed by visual assessment using stereomicroscope. Oocytes with fully and partially expanded cumulus cells were taken as mature oocytes and oocytes with no cumulus expansion were not processed further. Criterion is shown in Figure 1. Data were collected in ten independent repeats. All media and culture dishes were equilibrated at 38.5°C in CO2 incubator for at least 1-2 h before experiment.

In vitro *fertilization (IVF) of oocytes*

Three 0.5 ml straws of cryopreserved buffalo semen were thawed in water at 37°C for 30 seconds. Thawed semen was placed in a 15 ml conical tube. Spermatozoa with maximum motility were collected by swim up technique (Parrish *et al*., 1986). About 250 µL of thawed semen was deposited at the bottom of four 15 mL tubes containing 3 mL of pre warmed sperm wash medium (TALP: modified calcium-free Tyrode's Albumin Lactate Pyruvate with 6 mg/ml BSA fraction-V). Tubes were incubated at 45° angle for 30 min. Supernatant from each tube was removed and transferred into another 15 mL conical tube and centrifuged at 1600 rpm for 10 min. The pellet obtained after centrifugation of supernatant was assessed for sperm motility, and concentration was determined using an improved Neubauer Cell Counting Chamber. Subsequently, to get a final concentration of 2 x 10^6 live sperm mL^{-1}, the sperm pellet was re-suspended in pre-warmed fertilization TALP supplemented with 0.1mM hypotaurine, 0.2mM penicillamine, 0.01mM epinephrine and 10 µg/mL heparin.

After 24 h of maturation, buffalo oocytes were washed in fertilization media and were placed in fertilization droplet (5 COCs/50 µL droplet) of pre warmed fertilization media under mineral oil with final sperm concentration of 2×10^6 mL^{-1}. The oocyte and spermatozoa were co-incubated at 38.5°C under 5 % CO2 with maximum humidity for 20 h (Gasparrini *et al*., 2008).

Preparation of oviductal cell monolayer

Processing of buffalo oviductal epithelial cells for preparation of monolayer was performed as described by (Lopera-Vásquez *et al*., 2016; Nandi *et al*., 2006). Oviducts ipsilateral to the corpus luteum at the mid-luteal phase of the estrous cycle were collected from buffaloes at local slaughterhouse, sealed in a plastic bag and transported to the laboratory on ice. The oviducts were washed with Dulbecco's phosphate-buffered saline (DPBS) and extra tissue was removed. The oviducts were compressed with a glass slide and oviductal epithelial cells were extracted in a petri dish. Cells were flushed through 26-gauge needle attached to a 10 ml syringe to get single cell suspension. The recovered cells were washed twice in wash medium (DPBS + 0.3% BSA) by centrifugation at 2000 x g for 10 min and then for 5 min at 1000 x g with respective culture medium. The supernatant was discarded and the cells were diluted with respective culture medium (M199 + 5% FCS or SOF + 5% FCS) to a concentration of 1 x 10^6 cells/ mL. 50 µL of this cell suspension was then transferred to a 4-well dish (Nunc, Roskilde, Denmark) under mineral oil and incubated at 38.5°C in an atmosphere of 5% CO_2 in air with 95% humidity. After 72 h monolayer of oviductal epithelial cells was observed at the base of culture droplet.

Preparation of conditioned media

For preparation of conditioned medium of oviductal epithelial cells, oviductal cell suspension (5 mL) with a total of 2.5 x 10^6 cells from respective culture media (M199 + 5% FCS or SOF + 5% FCS) were transferred to corning culture flasks and cultured at 38.5°C in a humidified incubator with 5% CO2 in air. The culture medium was renewed with respective culture media (M199 + 5% FCS or SOF + 5% FCS), once after 48 h of culture to remove unattached cells. Culture was continued for 3 to 5 days, till they were 80-100% confluent (Lopera-Vásquez *et al*., 2016; Maeda *et al*., 1996). The cultures were centrifuged at 300 x g for 5 min, and the supernatant was collected. Supernatant, called the conditioned medium was filtered through a 0.22 micron filter and used for embryo culture as conditioned M199 and conditioned SOF.

In vitro *culture (IVC) of oocytes*

After IVF, the presumptive zygotes were denuded by repeated pipetting in PBS and washed twice in culture media. Denuded presumptive zygotes (792) were transferred to the 50 µl droplet of embryo culture according to the experimental group and were cultured in an incubator at 38.5ºC, 5% CO_2 and 95% humidity. Experimental groups used in this study were: Group I: SOF alone, Group II: SOF + co-culture, Group III: conditioned SOF, Group IV: M199 alone, Group V: M199 + co-culture and Group VI: conditioned M199. Both culture media (SOF and M199) in all treatment groups were supplemented with 5% FCS. On day 2 of development (Day 0 = day of insemination) the cleavage rate (number of oocytes cleaved/total ×100 COCs incubated) was observed. Further developmental stages (4-8 cell stage, > 8 cell stage and Morula) were evaluated and recorded every other day. Criterion is shown in Figure 2.

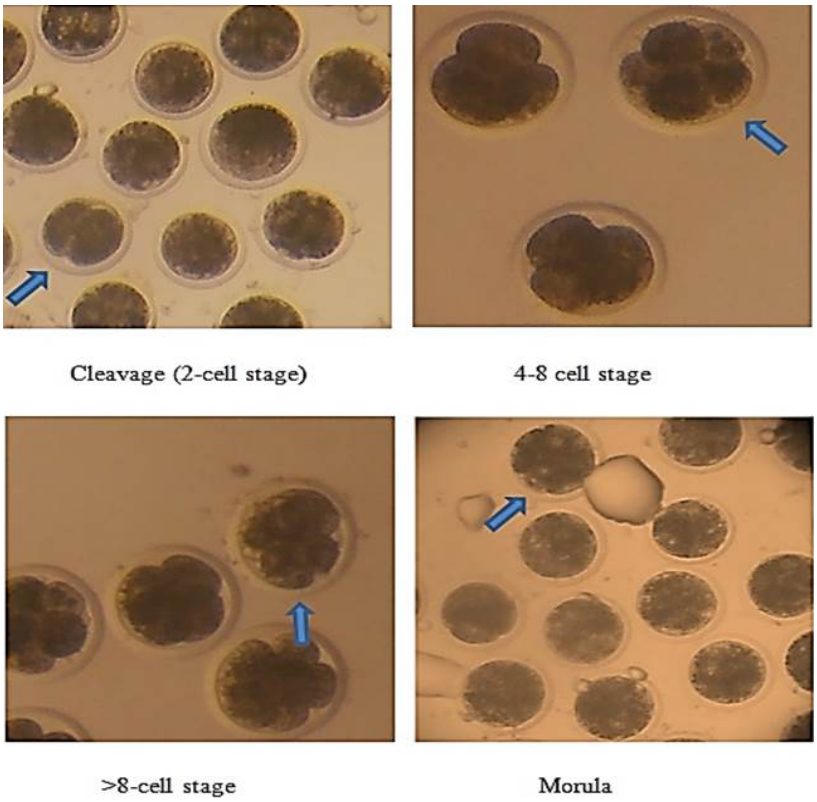

Fig. 2. Assessment criteria for developmental stages.

Statistical analysis

Data on all developmental stages were recorded and analyzed by one-way analysis of variance (ANOVA) at 5% level of significance. Duncan's multiple range test (DMRT) was used to compare treatment means.

RESULTS

A total of 1440 oocytes were recovered from 900 ovaries (1.6/ovary), out of which, a total of 1000 oocytes (A and B grade) were processed for *in vitro* maturation (IVM). Matured oocytes were inseminated and 792 presumptive zygotes were randomly placed in culture treatments (SOF alone, SOF + co-culture, Conditioned SOF, M199 alone, M199 co-culture and Conditioned M199). The ability of presumptive zygotes to develop in culture media (SOF and M199) alone, co-cultured with buffalo oviductal cells or conditioned media was studied. The data on the effect of different embryo culture systems on cleavage rate and further development of buffalo embryos (4-8 cell stage, >8 cell stage and Morula) are shown in Table I. Development of presumptive zygotes to the 2-cell stage in all treatment groups was similar ($P > 0.05$), ranging from 50 to 57% of COCs existing at the beginning of experiment. Development up to the 4- to 8-cell stage was recorded higher ($P < 0.05$) with SOF + co-culture compared to M199 alone and conditioned M199 (47% vs. 35% and 39%, respectively), while development in SOF co-culture, Conditioned SOF, SOF alone and M199 + co-culture groups was similar ($P > 0.05$), ranging from 40 to 47%.

The better efficiency of SOF co-culture and conditioned SOF was clearly evident at the > 8 cell stage, with 34 and 36% of embryos crossing 8 cell stage compared to SOF alone, M199 +co-culture, Conditioned M199 and M199 alone supporting the development of 22%, 25%, 24% and 15% of embryos, respectively ($P < 0.05$). Furthermore highest ($P < 0.05$) percentage of embryos reaching to morula stage was maintained with SOF + co-culture and conditioned SOF (27% and 25%, respectively) compared to production of morula in SOF alone, M199 + co-culture and conditioned M199 and M199 alone (12%, 16%, 15% and 9%, respectively). The percentage of morula per cleaved embryos in different culture treatments is clearly depicted in Figure 3, which shows improved development of morula in SOF (co-culture and/or conditioned) than with M199 (co-culture and/or conditioned) after cleavage.

Table I.- Developmental rate of *in vitro* matured/fertilized buffalo oocyte in different IVC environments.

Treatments	Presumptive zygotes	Stages of embryo development (mean percentage ± SEM)			
		2- Cell n (%)	4-8 Cell n (%)	>8 cell n (%)	Morula n (%)
SOF alone	132	68 (51.5 ± 2.6)	54 (40.9 ± 3.5)^{abc}	30 (22.7 ± 2.2)^{b}	16 (12.1 ± 2.3)^{b}
SOF + co-culture	132	76 (57.6 ± 2.6)	62 (47.0 ± 1.4)^{a}	48 (36.6 ± 2.2)^{a}	36 (27.3 ± 1.8)^{a}
Conditioned SOF	132	74 (56.2 ± 2.0)	60 (45.3 ± 2.3)^{ab}	46 (34.8 ± 1.3)^{a}	34 (25.8 ± 2.6)^{a}
M199 alone	132	66 (50.2 ± 2.1)	46 (35.0 ± 3.6)^{c}	20 (15.2 ± 2.3)^{c}	12 (09.1 ± 0.5)^{c}
M199 + co-culture	132	74 (56.1 ± 1.0)	54 (40.9 ± 1.0)^{abc}	34 (25.8 ± 1.7)^{b}	22 (16.7 ± 1.6)^{b}
Conditioned M199	132	72 (54.6 ± 1.5)	52 (39.4 ± 1.3)^{bc}	32 (24.2 ± 1.2)^{b}	20 (15.2 ± 1.6)^{b}

^{a,b,c}, the values with different superscripts in the same coloumn differ significantly ($P < 0.05$). n, number of embryos.

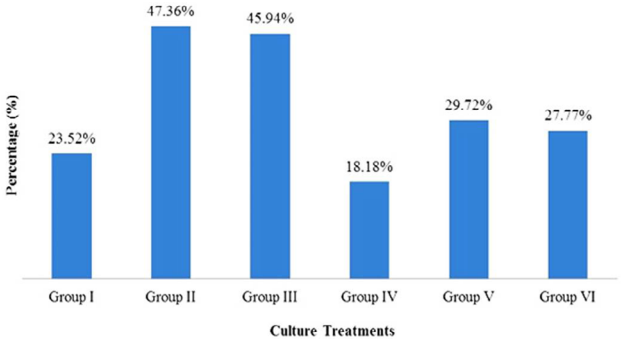

Fig. 3. Percentage of morula per cleaved zygotes in different IVC treatments (Group I, SOF alone; Group II, SOF+Co-culture; Group III, Conditioned SOF; Group IV, M199 alone; Group V, M199+Co-culture; Group VI, Conditioned M199).

DISCUSSION

The intrinsic quality of oocytes determines the embryo yield (Rizos *et al*., 2002); while post fertilization embryo culture is substantial in regulating the embryo quality (Lonergan *et al*., 2003). *In vivo*, the oviductal and uterine fluid provide appropriate amino acids, lipids, carbohydrates, proteins, nutrients, hormones, growth factors and ions for pre- and post-compaction embryonic development, respectively (Gandolfi and Moor, 1987; Ozdaş *et al*., 2006). *In vitro*, the culture medium has been developed to greatly mimic the *in vivo* conditions. In this regard, various culture media like TCM-199 medium, Charles Rosenkrans (CR) medium, ChatotZiomek-Bavister (CZB) medium and Synthetic oviductal fluid (SOF) medium were utilized for buffalo embryo culture and were found proficient for the development of buffalo embryos. However, despite of continuous refinement in the culture media, *in vitro* cultured embryos are still subjected to suboptimal conditions in the laboratory that is evident from low blastocyst yields around 10 (Nandi *et al*., 2002) to 20% (Gasparrini *et al*., 2003) which is much lower than the blastocyst yields (~30 to 40%) observed in cattle (Yang *et al*., 1998).

The incompetency of *in vitro* culture systems to sustain development of embryos from cleavage through to blastocyst is the result of insufficiency of the culture systems used. To provide a culture environment that closely imitate *in vivo* environment of development still needs further investigations. In this respect, the system that more closely mimic the *in vivo* conditions such as co culture with feeder cells is of relevance (Rizos *et al*., 2002). Among different feeder cells studied, the oviductal cells were superior to other somatic cells and the embryotrophic properties of oviductal epithelial cells has been well defined in a range of species (Nematollahi-mahani *et al*., 2009; Eyestone and First, 1989; Rexroad and Powell, 1993; Sakkas *et al*., 1989; Smith *et al*., 1992; Kitiyanant *et al*., 1995), suggesting tissue specific but not species-specific effect (Pavasuthipaisit *et al*., 1994).

In present study, culture of buffalo oocytes using buffalo oviductal epithelial cells or conditioned medium promoted development as compared to culture in medium alone. The definitive role of conditioned medium was sufficiently clear not only for crossing the developmental block, but also getting percentage of transferable stage embryos similar to buffalo oviductal epithelial cell co-cultures. Results are in line with previous report in which it has been reported that medium conditioned by bovine oviductal cells was as effective as cell co-culture in supporting embryo development to the compact morula and blastocyst stages in bovine (Rexroad and Powell, 1993). The ability of conditioned media to perform as efficiently as direct cell co-culture could be the result of enough availability of embryotrophic factors in conditioned

media or during the conditioning period epithelial cells adequately removed inhibitory factors from the media.

The definite mechanism by which enhanced early embryo development is obtained stays uncertain; however, the putative mechanisms include detoxification and procurement of required metabolites and particular development stimulators into the surrounding medium (Malayer *et al*., 1988). Moreover, the co-cultured cells during the course of their own proliferation can potentially provide bioactive factors which are absent in IVC media alone. Consequently, the conditioned media will have the components secreted/excreted by cells, like numerous enzymes, growth factors, cytokines, chemokines, lactate, glutamate and hormones or other soluble mediators. These factors are fundamental in the processes of cell growth, differentiation, invasion and angiogenesis by regulating cell-to-cell and cell-to-extracellular matrix interactions (Dowling and Clynes, 2011).

It has been documented that embryo metabolism is different in the pre- and post-compaction stage. Within female reproductive tract, the nutrients availability changes according to preferences of nutrient by the developing embryo. In the oviductal fluid relatively higher concentrations of pyruvate and lactate, and low concentration of glucose are found. In contrast, relatively low levels of pyruvate and lactate, and a higher concentration of glucose were found in uterine fluid. Therefore, appropriate media composition is also pre-requisite for efficient embryo development at different stages of development *in vitro* (Lane and Gardner, 2007).

While comparing two basic media used in present study, it has been observed that the cleavage rate of buffalo embryos was unaffected by any of the treatments tested either using M199 or SOF as culture media. At 4- to 8-cell stage, significant difference was found between M199 alone and SOF (co-cultured or conditioned) and it became more prominent while reaching > 8-cell stage embryos. One major component that is present in the formulations of M199 is glucose that is absent in SOF (Parrish *et al*., 1989). During early development there is low level respiratory rates and limited ability to metabolize glucose as a source of energy. Glycolysis of glucose substrate may increase oxidative stress which could not be compensated by antioxidant enzymes contributed by co-cultured cells which may interfere with reactive oxygen species (ROS) production and consumption balance. In this way quality of embryos is affected which is prominent during stages of embryo genome activation, which hampered the subsequent embryo development. Further in this study, when SOF medium was conditioned/co-cultured, pronounced effect was observed for crossing 8-16 cell block and further development as compared with conditioned/co-cultured M199.

We conclude from this study that the IVC media, SOF favoured the buffalo embryo development; either co-cultured or conditioned with buffalo oviductal epithelial cells. Conditioned media proved competent like co-culture system and can be used as an alternative for developing pre-implantation buffalo embryos. The system of isologous oviductal cells or medium conditioned by these cells has further advantages of being free of the risks of genetic contamination. However, further work is desired to expose the function and nature of secretary factors released by secretary epithelial cells of buffalo oviduct.

Statement of conflict of interest

Authors have declared no conflict of interest.

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