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Elucidating the Impact of Secretome Derived from Mesenchymal Stem Cell and Uterine Epithelial Cells During *In Vitro* Blastocyst Production in Buffalo

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Abstract

One challenge that needs to be addressed in animal embryo production is to create the appropriate *in vitro* culture to improve the blastocyst rate and produce high-quality embryos. Buffalo Mesenchymal Stem Cells (MSCs) were derived from Wharton's jelly and expanded *in vitro*. Conditioned media (secretome) was collected from well-characterized WJ-MSCs at 3rd passage. Similarly, buffalo Uterine Epithelial Cells (UECs) were derived from nongravid uteri and expanded *in vitro*. The secretome was collected from a well-characterized first passage UECs monolayer primed with steroid hormones (progesterone 3.14ng/ml and estradiol-17β 5 31pg/ml). Culture media was replaced with non-serum media, and the media was collected after 72h. Day 4 IVF-derived embryos were cultured in three groups: in regular mSOF media (Group I), mSOF replaced with 50% CM derived from MSCs (Group II), and mSOF replaced with 50% CM from steroid-treated UECs (Group III). Blastocyst rates were evaluated on day 09 post IVF. The blastocyst rate in group II was significantly higher ($p < 0.05$) than the control group, which was further enhanced in group III. *In vitro* co-culture of embryos with the secretome derived from mesenchymal stem cells or steroid-treated UECs improved the blastocyst rate. UECs and their secretions are essential to establish uterine receptivity and to mimic the internal *in vivo* environment.

Keywords: Buffalo Embryos, Blastocysts, Mesenchymal Stem Cell, Secretome, Uterine Epithelial Cells

1. Introduction

India is the leading country in the world for its tremendous and rapidly growing markets for milk and milk products. The rise in demand for buffalo meat and milk necessitates rapid propagation of superior germplasm, which can be identified, selected, and disseminated[1]. Despite myriad successful milestones in improving embryo quality, there is considerably lower buffalo fertility due to several reproductive and non-reproductive problems. In bovine, several hormones and growth factors like progesterone, interferon-tau, and maternal immune complex play a key role in establishing pregnancy and maternal recognition for successful pregnancy. About 20-25 % of *in vitro* oocytes develop into blastocysts by an *in vitro* embryo production technique, which is considerably low, probably because *in vitro* conditions cannot mimic the *in vivo* conditions[2]. For a successful conception in ruminants, it is a prerequisite to have a "good quality embryo"; "maternal recognition of pregnancy,"; a "receptive uterus" during the stage of implantation of conceptus to the endometrium of the uterus; and a well-developed immune system[3]. Therefore, synchronous signaling between the embryo and uterine endometrium during the pre-implantation period is crucial for embryo implantation, placentation, and normal embryo development[3]. After fertilization, the bovine embryo remains in the oviduct for around four days and moves into the uterus at the 16-cell or

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early morula stage, approximately[4]. In cattle the embryo starts developing and forms a blastocyst by day 7, hatched from ZP (Zona Pellucida) between day 09 and day 10, elongation initiates, and filamentous conceptus is formed, which attaches to the uterine epithelium by day 19 in the uterus[5]. Improving *in vitro* embryo culture may further enhance blastocyst quality and quantity. In the mammalian reproductive tract, the oviduct secretes various growth factors and cytokines, which play an essential role in the development of the initial stages of pre-implantation-embryos. Supplementation of *in vitro* embryo culture medium with multiple growth factors has been shown to reduce apoptosis and degeneration and increase blastocyst formation, cell number, and hatching rate in several animal species[6]. *In vitro* oocyte maturation with oocyte secretory factors improves buffalo oocytes' developmental competence[7]. Supplementing all these growth factors together becomes very expensive and cumbersome. It has been reported that Mesenchymal Stem Cells (MSCs) secrete several cytokines or growth factors[8,9].

Thus, the present study aimed to elucidate the impact of secretome derived from steroid-treated uterine epithelial cells and mesenchymal stem cells on *in vitro* blastocyst production in buffalo.

2. Materials and Methods

2.1 Isolation of Uterine Luminal Epithelial Cells and Collection of Conditioned Media

Buffalo uterine epithelial cells were isolated by enzymatic method using 0.25% trypsin–EDTA as per the protocol described by Pandey *et al.*[2] Fresh bubaline uteri samples at the early- to mid-luteal phase of the estrous cycle were collected from a local abattoir. Uterus was washed 3-4 times with Normal Saline Solution (NSS) fortified with 50µg/mL gentamycin sulphate solution (G1272, Sigma, USA. The uterine horns were separated from the uterus and flushed with 10-15mLwarm phosphate buffer saline (70011-044, Gibco Life Technologies) (1X PBS) containing 0.1% Bovine Serum Albumin (BSA) (05482; Sigma) and 100µg/ml gentamycin using 10mL syringe two times to ensure proper removal of debris from the lumen. After flushing, 10-15 mL of 0.25% trypsin-EDTA (T4799, Sigma; E6758, Sigma) was infused into the uterine lumen, and the horn was sealed from the other side with cotton thread. The uterine horns were incubated at 38.5°C for 60min in a shaker incubator (Matrix Eco Solution, India) with 70-100 moves/min. Uterine contents in suspension were aspirated from the uterine lumen with a 10 mL syringe fitted with an 18-gauge needle. The cell suspension was centrifuged at 250g for 10 min. The pellet was resuspended in high-glucose DMEM (D5796, Sigma) containing 10% FBS (Lot# 2106467RP, gibco) and antibiotic-antimycotic solution (Lot# 2257208, Gibco), centrifuged twice at 250g for 6 min to ensure proper washing of pellets. Finally, the pellet was resuspended in 10% DMEM and strained with a 70 µm mesh size cell strainer (352350, BD Falcon™, USA). Cell viability and concentration were determined using the 0.4% trypan blue dye (T8154, Sigma) and a Neubauer's hemocytometer chamber under a phase contrast microscope. The cells were seeded in 24 well plate (142475, Thermo Scientific) @ $5x10^4$ cells/cm^2 and incubated at 38.5 °C in a 5% CO_2 incubator with maximum relative humidity. After 24 h of culture, the uterine Luminal Epithelial cells (LE) were collected along with the media and re-plated into a new plate. The culture medium was changed every 48 h till 70-80 % cell confluence. After attaining 70-80 % confluence, cells were passaged using Accutase® (A6964; Sigma) and again reseeded at 5 x 10^4 cells/cm^2 concentration.

The first Passage (P1) Uterine Epithelial Cells (UECs) monolayer with 60-70 % confluence was exposed to steroid hormones to mimic the internal milieu of the first few days of the estrous cycle. Estradiol-17β was supplemented at 10pg/mL for 24h. After that, the culture medium was changed with one containing progesterone 3.14ng/mL and estradiol-17β 5pg/mL on alternate days for another 5 days[2], and the culture media was replaced with non-serum culture medium on the last day of treatment and incubated for 72h. After 72h, conditioned media were collected by centrifuging at high speed and stored at -20°C till further use.

2.2 Isolation and Culture of Wharton's Jelly-derived Buffalo MSCs (bWJ-MSCs) and Collection of Conditioned Media

The umbilical cord obtained from full-term delivery of buffalo was processed to isolate WJ-MSCs[10]. The umbilical cord was appropriately rinsed in normal saline;

the umbilical cord vessels were removed manually from cord segments. The exposed mesenchymal connective tissues (Wharton's jelly) were cut into very small pieces or explants of approximately 1–2 mm and washed 5 to 6 times in Dulbecco's PBS. These explants were cultured in 24 well plate using DMEM medium fortified with 15% FBS and 1µg/mL gentamycin and maintained at 37°C in a humidified atmosphere of 5% CO_2. The explants were observed for attachment within 72hr of plating. Wharton's jelly fragments explants were removed from culture dishes a week after plating, and the expanded cells were cultured for at least 7 more days. The medium was replenished every four days. After two weeks, when attached cells were 80% confluent, they were passaged. For passaging, cells were harvested by enzymatic digestion, where cells were detached by treatment with accutase (Sigma) for 10 min.

Wharton's jelly MSCs (3rd passage) were cultured up to 80% confluence in 24 well plates. These cells were washed twice with DMEM without serum and incubated with the same media (0.5ml per well was added) at 37°C and 5% CO_2. The conditioned media was collected at 72 hours of culture after incubation and centrifuged at 6000rpm for 10 minutes. The supernatant was collected as CM and stored at -20°C till further use.

2.3 *In Vitro* Embryo Production

2.3.1 Oocyte Retrieval and Grading

Buffalo ovaries were collected from a local slaughterhouse and transported to the laboratory in pre-warmed Normal Saline Solution (NSS) at 37°C within 3-4 h of slaughter. Ovaries were thoroughly washed 5-6 times with pre-warmed (37°C) NSS. All the visible ovarian follicles were aspirated using an 18 gauge needle fitted with a 5mL syringe using oocyte collection media. The supernatant follicular fluid was removed, the Cumulus-Oocyte Complexes (COCs) were obtained, and culture-grade COCs were utilized for further culture[11,12].

2.3.2 In Vitro Oocyte Maturation (IVM)

Selected COCs were washed 4-5 times in maturation media consisting of TCM 199 (HEPES modified), 5µg/mLLH (Cat# L5269, Sigma, Sigma), 0.5µg/mLFSH (Cat#F2293, Sigma, Sigma), 1µg/mLestradiol-17β (Cat#E4389, Sigma), 20ng/mLEGF (Cat# E4127, Sigma), 0.25mM sodium pyruvate, 0.68mM L-glutamine, 10µg/mL gentamicin, 3mg/mLBSA, and 10% FBS to minimize OCM concentration into the final drop of oocyte maturation medium. Thereafter, 10-15 COCs were kept for *in vitro* maturation in 50µL droplets of maturation medium in a 35mm culture dish overlaid with mineral oil (Cat#M8410, Sigma) for 24h at 38.5°C and 5% CO_2 in the air with maximum relative humidity.

2.3.3 In Vitro Fertilization (IVF)

Frozen thawed buffalo bull semen from a single bull was used for the *in vitro* fertilization of matured oocytes throughout the study. A semen straw (0.25mL) was thawed at 37°C for 30 sec, and contents were transferred to a 15mL centrifuge tube suspended in 4mL FerTALP (Tyrode-albumin-lactate-pyruvate) media supplemented with 0.2mM Sodium Pyruvate, 6mg/mL BSA and 20µg/mL heparin. The semen was washed twice in FerTALP medium by centrifugation at 600rpm for 10 min each. Approximately 90–100 µL remaining sperm pellet was resuspended and kept in a CO_2 incubator for 15 min for swim-up, and a 5µL aliquot was taken to determine the sperm concentration. The final sperm concentration was adjusted to 2×10^6 spermatozoa/mL using FerTALP medium, and the sperm samples were kept in the incubator with 5% CO_2 at 38.5°C with maximum humidity to allow the motile sperm to reach the supernatant of the media by swim-up technique. Progressively motile sperm from the sperm suspension was placed as 50µL semen droplets overlaid with the mineral oil, and 10–15 *in vitro* matured COCs were washed in FerTALP medium and co-incubated with semen droplets for 18h with 5% CO_2 in air at 38.5°C with maximum relative humidity. The fertilization rate was accessed based on second polar body released.

2.3.4 In Vitro Culture (IVC)

After 18h co-incubation, the presumptive zygotes were washed 5-6 times in Embryo Developmental Media (EDM) comprising of 3mg/mL BSA (fatty acid-free), 0.25mM sodium pyruvate with 1% essential and non-essential amino acids, 0.68mM L-glutamine, 100ng/mL IGF-1 and 50µg/mL gentamycin sulphate. About 10-15 presumptive zygotes were transferred in 50µL drop of EDM without FBS for the initial development of 48h.

Cleavage rate was evaluated, and embryos were further cultured in EDM supplemented with 10% FBS.

2.4 Culture of Embryos with CM Derived from Uterine Epithelial Cells and Mesenchymal Stem Cells

Day 4 cleavage embryos were co-cultured with steroid-treated uterine epithelial cells-CM (Group III) and mesenchymal stem cells-derived CM (Group II). In group II and group III, embryo culture medium (mSOF) was supplemented with 50% respective CM, while group I embryos were cultured in pure mSOF only. The embryos continued to be cultured till day 8 in the respective media while changing it on every alternate day. Blastocyst rate was calculated as the total number of blastocysts produced of total embryos cultured.

2.5 Statistical Analysis

The cleavage rate, blastocyst rate, and hatching rate were analysed using SPSS 17.0, and one-way ANOVA with Duncan post hoc test using graph-pad Prism V 5.0 software.

3. Results and Discussion

Oocytes with higher developmental competence are essential to yield higher blastocyst[13]. However, it is evident that the quality of embryos is affected by the culture environment to which they are exposed[14,15]. The composition of the culture medium mimicking the *in vivo* environment, including interleukins and growth factors, may be crucial for the success of the IVF procedures[16].

In this experiment, we used conditioned media derived from buffalo uterine epithelial cells and WJ-MSCs to demonstrate comparative *in vitro* embryo development in these two media in relation to conventional embryo culture media (Figure 1). We found that the blastocyst rate was significantly enhanced in the MSCs-CM supplemented group, which was further enhanced when embryos were cultured in UECs-CM (Table 1). Supplementation of *in vitro* culture medium with various growth factors was shown to reduce apoptosis and degeneration and increase blastocyst formation, cell number, and hatching rate in a variety of animal embryos[6]. The use of a combination of IGF-I, IGF-II, bFGF, TGF-β1, LIF, and GM-CSF in mSOF medium produced higher percentages of blastocysts on day 8 post-IVF than the use of mSOF alone[17]. *In vitro* studies demonstrated that MSCs secrete a variety of angiogenic, anti-apoptotic, and supportive factors such as VEGF, IGF-1, TGF-β, bFGF, LIF, SCF, GM-CSF, and HGF[8,9]. These growth factors/cytokines in the conditioned medium may have a stimulatory/supportive action on embryonic development *in vitro,* evidenced by our findings.

In the present study, the blastocyst rate was highest in group III (CM derived from UECs), where it is supposed that in addition to providing growth factors, UECs also secrete several other signaling molecules required for embryo development. Ovarian steroids (i.e., progesterone and estradiol from preovulatory follicles and corpus luteum) exert classical endocrine control of morphological and functional changes in the endometrium that affect embryo development and pregnancy success[18,19]. Progesterone affects blastocyst development by improving endometrial transcriptome expression[20]. The beneficial effects on blastocyst development are thought to be due to embryotrophic factors provided by the epithelial cells. Co-cultured cells during their proliferation can potentially provide bioactive factors and "cross-talk," which is absent in IVC media alone[20].

In conclusion, *in vitro* co-culture of embryos with steroid-treated uterine epithelial cells and its Conditioned Medium (CM) improved the blastocyst rate; however,

Table 1. Effect of conditioned media derived from MSCs and UECs on *in vitro* blastocysts rate

Treatment group	Day 4 embryo cultured (n)	Blastocyst rate n (%)
Group I (mSOF only)	170	29(17.06)[a]
Group II (mSOF + 50% MSCs-CM)	175	43(24.57)[b]
Group III (mSOF + 50% UECs-CM)	212	62 (29.25)[c]

Significance ($p<0.05$)

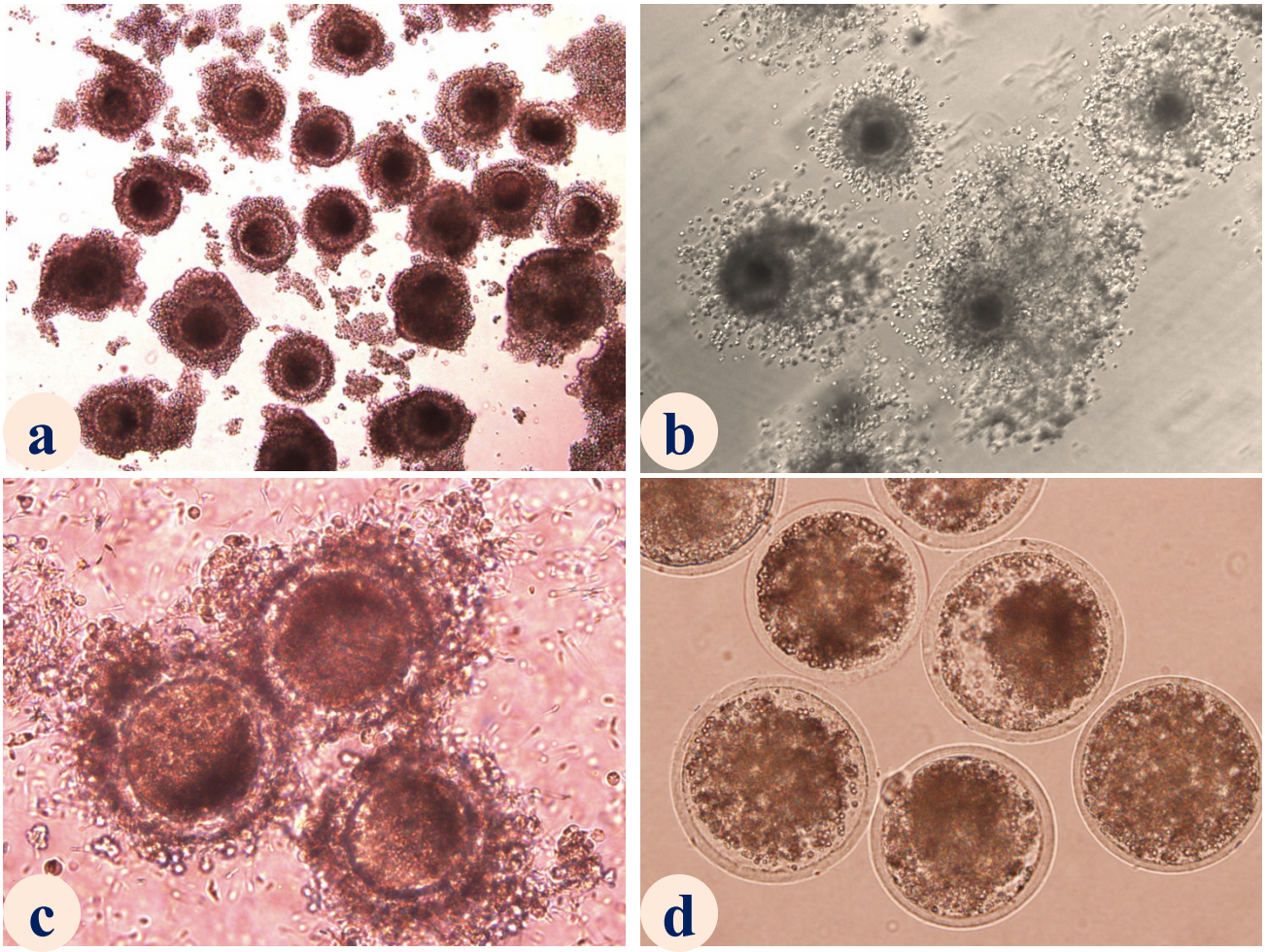

Figure 1. *In vitro* buffalo embryo production: **a.** culture grade immature oocytes (10x); **b.** in vitro matured oocytes (10x), **c.** sperm-oocyte co-incubation (IVF) (40x), **d.** *in vitro* produced blastocysts (40x)

conditioned media from mesenchymal stem cells also has the potential to enhance quality blastocysts production *in vitro* in buffalo. Thus, conditioned medium derived from steroid-primed uterine epithelial cells may be used for *in vitro* quality blastocyst production in buffalo.

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