Developmental Competence of Buffalo (*Bubalus bubalis*) Pluripotent Embryonic Stem Cells Over Different Homologous Feeder Layers and the Comparative Evaluation with Various Extracellular Matrices

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Background and Objectives: Use of somatic cells as a feeder layer to maintain the embryonic stem cells (ESCs) in undifferentiated state limits the stem cell research design, since experimental data may result from a combined ESCs and feeder cell response to various stimuli. Therefore, present study was designed to evaluate the developmental competence of the buffalo ESCs over different homogenous feeders and compare with various extracellular matrices using different concentrations of LIF.

Methods and Results: Inner cell masses (ICMs) of *in vitro* hatched blastocysts were cultured onto homologous feeders viz. fetal fibroblast, granulosa and oviductal cell feeder layers and synthetic matrices viz. fibronectin, collagen type I and matrigel in culture medium. Developmental efficiency was found higher for ESCs cultured on fetal fibroblast and granulosa layers (83.33%) followed by fibronectin (77.78%) at 30 ng LIF. Oviductal feeder was found to be the least efficient feeder showing only 11.11% undifferentiated primary ESC colonies at 30 ng LIF. However, neither feeder layer nor synthetic matrix could support the development of primary colonies at 10 ng LIF. Expression of SSEA-4, TRA-1-60 and Oct-4 were found positive in ESC colonies from all the feeders and synthetic matrices with 20 ng and 30 ng LIF.

Conclusions: Fetal fibroblast and granulosa cell while, amongst synthetic matrices, fibronectin were found to be equally efficient to support the growth and maintenance of ESCs pluripotency with 30 ng LIF. This well-defined culture conditions may provide an animal model for culturing human embryonic stem cells in the xeno-free or feeder-free conditions for future clinical applications.

Keywords: Buffalo, Embryonic stem cell, Extracellular matrix, Feeder layer, Pluripotency

Introduction

In recent past, widespread investigations have been car-

ried out to establish feeder-free culture conditions to maintain the embryonic stem cells (ESCs) in undifferentiated state using extracellular matrices (1-4) for the therapeutic and regenerative medicine. However, still various types of cells like: fibroblasts (1, 5), granulosa (6) and oviductal (7) as feeder layer have been using for the culture and establishment of ESCs in various animal species. Moreover, use of somatic cell as feeder layers limits the stem cell research design, since experimental data may result from a combined ESC and feeder cell response to various stimuli. In spite of this progress, there is a still great

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scope for the comparative studies using different feeders vis-a-vis feeder-free conditions for the establishment of buffalo ESCs. Therefore, the choice of an optimal strategy for stem cell culture need to elaborate with more defined culture condition, capable to adapt the derivation of embryonic stem cells for the clinical application of cell therapy (8).

ES cell culture requires an extracellular support as feeder layers which may provide appropriate conditions in order to prevent spontaneous differentiation. It serves as an attachment matrix for the ESCs and secretes certain growth factors such as leukemia inhibitory factor (LIF) that may stimulate ESC growth and inhibit their differentiation as well as remove toxins from the culture medium (9, 10). Thus, advantages to use synthetic matrices are no DNA contamination while doing RT-PCR studies for marker genes besides easy manipulation of the ESC colonies. Very limited literature is available for the establishment of ESC culture in buffaloes (11, 12). Finally, use of any feeder layer increases the work load and subsequently limits derivation of ESCs in larger scale. Thus, the development of feeder-free culture will allow more reproducible culture conditions, facilitate scale-up and potentiate the clinical use of cells differentiated from cultured ESCs. With this background, the present study was undertaken with the objectives to evaluate the impact of homologous feeder layers, its comparison with feeder-free conditions having an appropriate combination of leukemia inhibitory factor (LIF) on the development of pluripotent embryonic stem cells derived from *in vitro* produced buffalo blastocyst.

Materials and Methods

Materials

All the chemicals used in this study were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise indicated. Buffalo ovaries from random stages of the estrous cycle were collected from local abattoir immediately after slaughter and transported in 0.9% normal saline at 25~30°C to the laboratory within two hours.

Experimental design

Experiment I: To find out the optimal dose of LIF, mechanically isolated ICMs of buffalo blastocyst were cultured separately in three different doses of LIF viz. 10, 20 and 30 ng/ml onto fetal fibroblast monolayer to evaluate the attachment time, colony formation and their growth.

Experiment II: To find out the best homologous feeder

layer and extracellular matrices, mechanically isolated ICMs of blastocyst were cultured separately onto three different homologous feeder layer (fetal fibroblast, granulosa and oviductal cell) and matrices (collagen type-I, fibronectin and matrigel) along with three different doses of LIF (10, 20 and 30 ng/ml) in terms of attachment time, colony formation and their growth.

Experiment III: Based on the results of experiment I and II, developmental competence was compared between two types of culture system viz. homologous feeder vs. extracellular matrices. Three independent experiments were conducted for each culture system.

In vitro embryo production

Buffalo embryos were produced *in vitro* as per our laboratory established protocol (1). In brief, cumulus oocytes complexes (COCs) were collected by aspiration of antral follicles. Collected COCs were matured in tissue culture medium-199 (TCM-199) supplemented with 10% fetal bovine serum (FBS), 0.25 mM sodium pyruvate, 0.68 mM L-glutamine, 0.5 µg/ml FSH, 5 µg/ml LH, 1 µg/ml estradiol and 20 ng/ml epidermal growth factor for 24 h under sterile embryo culture tested mineral oil at 38.5°C in a moist atmosphere of 5% CO₂ in air. Frozen-thawed buffalo bull semen was used for *in vitro* fertilization of *in vitro* matured oocytes. Sperms were washed twice in fertilization (FERT-TALP) medium containing heparin (10 µg/ml), fatty acids free BSA (6 mg/ml) and sodium pyruvate (0.25 mM) by centrifugation at 850 g for 10 min each. Sperm motility and concentration ($1\sim2\times10^6$) were assessed with a hemocytometer. *In vitro* matured oocytes were washed in TALP medium and incubated with 70 µl droplets of sperm suspension (15~20 oocytes/droplet). After 18 h co-incubation of sperms and eggs, presumptive zygotes were removed from fertilization droplets and washed in embryo development medium (EDM) containing BSA (3 mg/ml), 10% FBS, sodium pyruvate (0.25 mM), L-glutamine (0.68 mM) with essential and non-essential amino acids. Presumptive zygotes were washed and cultured (10~15 zygotes/50 µl droplets) in embryo development medium at 38°C, 5% CO₂ in air with maximum humidity for their development until blastocyst stage.

Feeder layer preparation

Preparation of fetal fibroblast monolayer: Pregnant uteri were collected from local abattoir to obtain fetus (approx. 40 days old) for the preparation of feeder layer. Fetus was properly washed several times with DPBS. Head, bones and abdominal viscera were removed and rest of the fetus was minced into small pieces. The minced tissue

pieces were then transferred into 75 mm² conical flasks and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine and 50 µg/ml gentamycin in 5% CO₂ in air with maximum humidity at 38.5°C. The explants were removed after 24 hrs and the fetal fibroblasts were allowed to grow till confluency.

Preparation of granulosa cell monolayer

Granulosa cells were collected from follicular fluid obtained by aspirating the larger follicles with a diameter of 2~8 mm. Clumps of granulosa cells were separated into single cells using repeated pipetting and washed 8~10 times properly with DPBS. Cells were then cultured in DMEM supplemented with 10% FBS and 2 mM L-Glutamine and 50 µg/ml gentamycin sulphate in 5% CO₂ at 38.5°C in humidified air.

Preparation of oviductal cell monolayer

Buffalo oviducts of early luteal phase (trimmed free of surrounding tissue and ligated at the infundibulum and at the isthmus-uterus junction) were used to collect oviductal cells by squeezing the oviduct with sterilized glass slide along the ampulla. Cell sheets were separated mechanically by repeated pipetting and washed thoroughly in DPBS. Cells were then cultured in DMEM supplemented with 10% FBS and 2 mM L-Glutamine at 38.5°C in a humidified atmosphere of 5% CO₂ in air.

Medium were changed every 24 hrs for all the feeder layers. Fibroblasts were used as feeder layer for ESC culture after third passage only. Feeder layers were inactivated with mitomycin-C (10 µg/ml) for 3 hrs prior to its use for ESC culture.

Preparation of extracellular matrices coated plates

The matrices of collagen type-I (extracted from human placenta), fibronectin (from bovine plasma) and matrigel (murine sarcoma basement membrane) were procured from Sigma Chemical Company (St Louis, MO, USA) and used in this study for culture of ES cells. The working solution of different types of matrices was prepared as per the manufacture's instruction. In brief, matrix of 1% collagen gel solution (1.0 mg/ml in 0.1 M acetic acid), fibronectin (1.0 µg/ml) and matrigel (20 µg/ml) were prepared by mixing (v/v) with 2X DMEM (20% FBS, 200 U/mL penicillin, and 200 µg/ml streptomycin). The pH of the mixtures was neutralized immediately by titration with 0.1 mol/L NaOH. Each of them (200 µl) were pipetted separately into 24 well culture plate (Nunc) and incubated for 20 to 30 minutes at 37°C and 5% CO₂ to allow gelling of the mixture.

Embryonic stem cell culture

Pluripotent embryonic stem cells were mechanically isolated using two fine needles from inner cell masses (ICMs) of blastocyst under stereozoom microscope (Olympus, Japan). To find out the optimal dose of LIF, isolated ICMs were cultured with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% FBS, 2 mM L-Glutamine, 0.1 mM β-Mercaptoethanol, 2% NEAA, 1% ITS and gentamycin sulphate (50 µg/ml) in presence of three different concentrations of LIF (10, 20 and 30 ng/ml) along with bFGF (10 ng/ml) onto mitomycin-C treated fetal fibroblast feeder layer at 38.5°C and 5% CO₂ in humidified air for the derivation of primary ES cell colonies.

Embryonic stem cell culture on homogenous feeder and feeder free culture system

To evaluate the developmental competence of primary ES cell colonies over feeder and feeder free culture system, third passaged ES cell colonies were seeded onto different homogenous feeder layers (fetal fibroblast, granulosa and oviductal cell) and synthetic matrices (collagen type-I, fibronectin and matrigel) in 2.0 ml undifferentiating basic ES cell culture medium (DMEM, supplemented with 20% FBS, 1% non-essential amino acids, 0.1 mM β-mercaptoethanol, 10 ng/ml bFGF) in presence of three different concentrations of LIF (10, 20 and 30 ng/ml). Developmental potential was evaluated based upon the time of attachment, formation of undifferentiated primary colonies and their characterization. Culture medium was replenished after every 48 hrs and culture was routinely observed under phase contrast microscope (CKX-41, Olympus, Shinjuku, Tokyo, Japan). Formation of dome shaped structure was examined 8, 9 days after the seeding of ICMs over mitomycin C-treated feeder layers and synthetic matrices. To further propagate, the primary embryonic stem cell colonies were mechanically dispersed into two to three small clumps by using a micropipette and then transferred to a fresh feeder layer. These cells were passaged mechanically up to the tenth passages with the similar culture conditions. ESCs were routinely passaged every 4, 5 days and the medium was changed every 48 h.

Alkaline phosphatase staining

Primary ESC colonies cultured for 7~9 days over homologous feeders and synthetic matrices were subjected to alkaline phosphatase (AP) staining for characterization. Medium was removed from the culture and the cells were fixed in 4% paraformaldehyde prepared in DPBS for 10 min. After the fixation, ESCs were washed properly with DPBS and incubated with 25 mM Tris-HCl and 150 mM

NaCl containing 8 mM $MgCl_2$, Naphthol AS-MX Phosphate (0.4 mg/ml) and FastRed TR salt (1 mg/ml) for 30 min at 37°C.

Immunolocalization of Oct-4, SSEA-4 and TRA-1-60

For characterization, ESCs were fixed in 4% formalin solution prepared in PBS for 10 min at 37°C and then cells were permeabilized with 0.1% Triton-X in PBS for 30 min at 37°C (for Oct-4 only). Non-specific binding was blocked by incubating with donkey serum (1 : 10 dilution in PBS with 2.5% BSA) for 30 min at 37°C. Primary antibodies included SSEA-1 (1 : 50, Santa Cruz Biotechnology, Santa Cruz, CA, USA), SSEA-4 (1 : 50, Santa Cruz), TRA-1-60 (1 : 50, Santa Cruz), TRA-1-81 (1 : 50, Santa Cruz) and Oct-4 (1 : 100, Santa Cruz). Localization of antigens was done with anti-goat IgG secondary antibodies conjugated with FITC or Texas Red (1 : 500, Santa Cruz). Immunolocalization of SSEA-1, SSEA-4, TRA-1-60, TRA-1-81 and Oct-4 proteins in ESCs were viewed under fluorescent microscope (IX 71, Olympus, Shinjuku, Tokyo, Japan). Negative controls were processed in the same manner except the omission of primary antibody.

Reverse transcriptase-polymerase chain reaction analysis (RT-PCR)

Expression of Oct-4 gene was assessed in primary ES cell colonies cultured for 7~9 days on different feeder layers and under feeder-free conditions on synthetic matrices. cDNA was reverse transcribed from RNA isolated from three primary ESCs colonies developed on each feeder and synthetic matrix and then subjected to polymerase chain reaction for gene amplification. Reverse transcription was carried out using FastLane cell to cDNA kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. Briefly, excess of media was removed and cells were washed with wash buffer before they were lysed with lysis buffer. Cell lysate was then mixed with genomic DNA wipeout buffer to eliminate genomic DNA contamination by incubating for 5 min at 42°C. Reaction was reverse transcribed using Quantiscript Reverse Transcriptase by incubating at 42°C for 30 min followed by incubation at 95°C for 3 min to obtain cDNA. For amplification of Oct-4 gene, PCR reaction was carried out using 3.0 μ l of cDNA, 3.0 μ l $MgCl_2$ (25 mM), 1.0 μ l dNTP mix (10 mM), and 1.0 μ l of Oct-4 specific sequence forward 5'-GAAAGACGTCCGAGTGTGGT-3' and reverse 5'-TCC CCCTGTGAAAGGAGAC-3' primers (20 pmol) in 5.0 μ l PCR reaction buffer. Finally, 0.5 μ l of Taq DNA polymerase (5 U/ml, Fermentas, Vilnius, Lithuania) was added, and the final volume of 25 μ l was made with nuclease

free water. PCR program had an initial heating at 95°C for 3 min, samples were amplified for 35 cycles (95°C for 45 s, 50°C for 45 s, and 72°C for 1 min), final extension at 72°C incubation continued for 10 min. PCR products were run on 1.5% agarose gel containing ethidium bromide (0.5 μ g/ml) and visualized using a gel documentation system (Alpha Imager-2200, Alpha Innotech Corporation, Santa Clara, CA, USA). The electrophoresed PCR products were run simultaneously with 100 bp ladder for determining their tentative molecular sizes. Reference gene β -actin was included as an endogenous control to evaluate the quality of cDNA.

Statistical analysis

Embryos, ES cell colony formation and PCR data of different feeder layers and feeder-free conditions were presented as mean $\pm$ SEM and analyzed by one-way ANOVA using Graph Pad Statistical Software (Peter Russell, Royal Veterinary College) at 0.05% and 0.01% level of significance. Differences with $p < 0.05$ and $p < 0.001$ were considered significant statistically.

Results

In vitro embryo production

Total of 2,255 ovaries were used for present study from which 2,294 good quality of oocytes were subjected for *in vitro* embryo production (Fig. 1). Oocyte recovery rate was found to be 1.02 oocyte per ovary and developmental competence of *in vitro* matured oocytes was 70.93%, 70.00%, 42.91% and 20.98% for 2~4 cells (48~72 hpi), 8~16 cells (72~96 hpi), morula (120~144 hpi) and blastocyst (168~192 hpi) stages, respectively (Table 1). Out of the 1,651 cleaved oocytes, a total of 345 embryos reached to blastocyst stage (20.98%) during *in vitro* culture with cleavage rate of 70.93% in the present study (Table 1).

Preparation of homologous feeders

Three homologous feeders were prepared from fetal fibroblasts, granulosa and oviductal cells for the *in vitro* culture of ESCs (Fig. 2). Amongst the developed feeders, fetal fibroblasts and granulosa cells showed attachment after 24 hrs of seeding, while oviductal cells took 72 hrs for attachment. Cultured fibroblast reached to confluency after 6~7 days, it was further passaged to get sub-cultures. Mitomycin-C treated homogenous feeders were used for ESC culture after third passage. Fetal fibroblast and granulosa cell feeders were found to be most stable after mitomycin-C treatment even for more than a week, while the oviductal cells showed detachment after 4~5 days of primary culture.

Fig. 1. Different developmental stages of buffalo embryos produced *in vitro* (A) group of immature oocytes, (B) group of matured oocytes showing cumulus cell expansion, (C) 2~4 cell stage of embryos, (D) 8~16 cell stage embryos, (E) compact morula, (F) expanded blastocyst, (G) hatching blastocyst and (H) hatched blastocyst showing clear inner cell masses (ICMs) and trophoctodermal cells (TE). Scale bar: 50 μ m.

Table 1. Developmental competence of cultured oocytes matured and fertilized *in vitro*

No. of ovaries	No. of culturable oocytes	Oocyte recovery rate (oocyte/ovary)	2~4 cells embryo (mean $\pm$ SEM)	8~16 cells embryo (mean $\pm$ SEM)	Morula (mean $\pm$ SEM)	Blastocyst (mean $\pm$ SEM)
2,255	2,294	1.02	1,651 (70.93 $\pm$ 1.32)	1,160 (70.00 $\pm$ 0.36)	711 (42.91 $\pm$ 0.63)	345 (20.98 $\pm$ 0.53)

Embryonic stem cell culture over homologous feeder layers

Eighteen ICMs were cultured on each feeder and fetal fibroblast and granulosa cell monolayer showed 100% attachment with 10 ng, 20 ng and 30 ng of LIF whereas, only 5 (out of 18) ICMs could be attached (27.78%) to oviductal cell monolayer with 10 ng and 20 ng LIF and 6 (out of 18) ICMs showed attachment (33.33%) with 30 ng LIF (Table 2). None of the seeded ESCs could maintain their pluripotency at 10 ng LIF irrespective of the feeder and get spontaneously differentiated into cystic embryoid bodies (EBs). Mitomycin-C treated fetal fibroblast feeder could support 12 ICMs (66.67%) at 20 ng LIF and 15 ICMs (83.33%) at 30 ng LIF to form primary ESC colonies 6~7 days post ICMs seeding whereas, granulosa cell monolayer could form 61.11% (11/18) primary colonies at 20 ng LIF and 83.33% (15/18) primary colonies at 30 ng LIF. However, oviductal feeder was found to be very poor in supporting primary ESC colonies and could support only 2 primary colonies (11.11%) from 6 ICMs which were found attached to the oviductal feeder layer in the present

study (Table 2). The results indicated that the two homologous feeders, fetal fibroblast and granulosa cell feeder layer were equally effective in supporting the undifferentiated growth and maintenance of pluripotency of buffalo ESCs at 30 ng LIF. However, oviductal feeder was found to be very poor in supporting primary ESC colonies (Fig. 3A).

Embryonic stem cell culture over synthetic matrices

All the ICMs seeded onto synthetic matrices used for present study showed 100% attachment with 10 ng, 20 ng and 30 ng LIF but the time of attachment varied between 24~60 hrs for different matrices (Table 3). Similar to that of feeder layers, none of the ICMs cultured onto synthetic matrices could maintain the pluripotency at 10 ng LIF and spontaneously differentiated into cystic EBs. Synthetic matrix, fibronectin could support 55.56% and 77.78% ICMs while, collagen type I could form 50.00% and 66.67% of primary colonies at 20 ng and 30 ng LIF respectively. Matrigel supported 55.56% and 66.67% of primary colonies at 20 ng and 30 ng of LIF in the present study (Table 3). Amongst extracellular matrices, fibronectin

Fig. 2. Developmental stages of different homogenous feeder layers on different culture days. (A~C) Fetal fibroblast cells, (D~F) granulosa cells and (G~I) oviductal cells on days 0, 5 and 8 respectively showing uniform monolayer. Before *in vitro* culture of ESCs, different cell monolayers were treated with mitomycin-C. Scale bar: 50 μ m.

Table 2. Developmental potential of pluripotent stem cells derived from buffalo blastocysts produced *in vitro* over homogenous feeders with different concentrations of LIF

Feeder cells	Concentration of LIF used (ng/ml)	No. of ICMs used	No. of attachments	% of attachment	Time of attachment in hrs (mean $\pm$ SEM)	No. of primary colonies formed	% of primary colonies
Fetal fibroblast monolayer	10	18	18	100.00 ^a	72.67 $\pm$ 1.81 ^a	0	0.00
	20	18	18	100.00 ^a	70.67 $\pm$ 1.92 ^a	12	66.67 ^a
	30	18	18	100.00 ^a	70.67 $\pm$ 1.33 ^a	15	83.33 ^b
Granulosa cell monolayer	10	18	18	100.00 ^a	72.00 $\pm$ 1.68 ^a	0	0.00
	20	18	18	100.00 ^a	71.33 $\pm$ 1.81 ^a	11	61.11 ^a
	30	18	18	100.00 ^a	71.33 $\pm$ 1.18 ^a	15	83.33 ^b
Oviductal cell monolayer	10	18	5	27.78 ^b	86.40 $\pm$ 2.40 ^b	0	0.00
	20	18	5	27.78 ^b	86.40 $\pm$ 2.40 ^b	0	0.00
	30	18	6	33.33 ^b	84.00 $\pm$ 3.10 ^b	2	11.11 ^c

Different letters of superscript showing significant differences at the level ($p < 0.05$).

tin was found to be better than collagen type I and matrigel for growth of primary ESC colonies at 30 ng LIF (Fig. 3B).

Developmental competence of embryonic stem cells on homologous feeders and synthetic matrices

ICMs seeded onto fetal fibroblast and granulosa cell

monolayer showed non-significant difference ($p > 0.05$) for the time taken to get attached at all the concentrations of LIF which varied between 62~84 hrs after seeding ICMs. However, a significant difference ($p < 0.001$) was shown for the attachment time as compared to ICMs cultured onto synthetic matrices. Post seeding ICMs took significantly ($p < 0.001$) lesser time for the attachment to all

Fig. 3. ICMs cultured over the different confluent homogenous feeder monolayer (A) and different extracellular matrices (B) with different concentrations of LIF. ESCs colonies over (a~c) fetal fibroblast monolayer, (d~f) granulosa cell monolayer, (g~i) oviductal cell monolayer, (i~iii) collagen type-1, (iv~vi) fibronectin and (vii~ix) matrigel with 10, 20 and 30 ng/ml LIF are showing different developmental behavior on 8 days of culture respectively. ESCs at 10 ng of LIF showing differentiated colony as cystic EBs (arrows) formation while, 20 and 30 ng of LIF maintaining pluripotency of ESCs over different homogenous monolayers and extracellular matrices. Scale bar: 80 μ m.

Table 3. Developmental potential of pluripotent stem cells derived from buffalo blastocysts produced *in vitro* on synthetic matrices with different concentrations of LIF

Synthetic matrices	Concentration of LIF used (ng/ml)	No. of ICMs used	No. of attachments	% of attachment	Time of attachment in hrs (mean $\pm$ SEM)	No. of primary colonies formed	% of primary colonies
Fibronectin	10	18	18	100.00	41.33 $\pm$ 1.45	0	0.00
	20	18	18	100.00	42.00 $\pm$ 1.75	10	55.56 ^a
	30	18	18	100.00	39.33 $\pm$ 1.89	14	77.78 ^a
Collagen type I	10	18	18	100.00	42.67 $\pm$ 1.74	0	0.00
	20	18	18	100.00	42.33 $\pm$ 1.72	9	50.00 ^a
	30	18	18	100.00	42.67 $\pm$ 1.74	12	66.67 ^a
Matrigel	10	18	18	100.00	42.00 $\pm$ 1.46	0	0.00
	20	18	18	100.00	41.33 $\pm$ 1.45	10	55.56 ^a
	30	18	18	100.00	40.00 $\pm$ 1.68	12	66.67 ^a

The figures having different superscripts within the columns and between the columns differ significantly at level ($p < 0.05$).

the synthetic matrices, it varied between 24~60 hrs. However, non-significant difference ($p > 0.05$) was observed for the attachment time amongst all the synthetic matrices i.e. fibronectin, collagen type I and matrigel (Table 4). Although, ICMs seeded onto oviductal cell monolayer showed comparatively higher time for attachment (84~96 hrs) at different concentrations of LIF.

Developmental efficiency was found numerically higher for ESCs cultured on fetal fibroblast and granulosa feeder layers (83.33%) followed by fibronectin (77.78%) at 30 ng

LIF. Oviductal feeder was found to be least efficient, showing only 11.11% developing ESCs at 30 ng LIF. Development of ES cells onto either feeder layers or synthetic matrices does not supported by 10 ng/ml LIF (Table 5). Amongst all, oviductal feeder was found to be poorest for the culture of ES cells.

Characterization of pluripotent stem cells derived from *in vitro* produced buffalo embryos

Primary embryonic stem cell colonies were found pos-

Table 4. Comparison for developmental potential of pluripotent stem cells derived from buffalo blastocysts produced *in vitro* on homogeneous feeders and synthetic matrices with different concentrations of LIF

Concentration of LIF used	Time of attachment in hrs (mean $\pm$ SEM)					
	Feeder layers			Synthetic matrices		
	Fetal fibroblast monolayer	Granulosa cell monolayer	Oviductal cell monolayer	Fibronectin	Collagen type I	Matrigel
10 ng/ml	72.67 $\pm$ 1.81 ^a	72.00 $\pm$ 1.68 ^a	86.40 $\pm$ 2.40 ^b	41.33 $\pm$ 1.45 ^c	42.67 $\pm$ 1.74 ^c	42.00 $\pm$ 1.46 ^c
20 ng/ml	70.67 $\pm$ 1.92 ^a	71.33 $\pm$ 1.81 ^a	86.40 $\pm$ 2.40 ^b	42.00 $\pm$ 1.75 ^c	42.33 $\pm$ 1.72 ^c	41.33 $\pm$ 1.45 ^c
30 ng/ml	70.67 $\pm$ 1.33 ^a	71.33 $\pm$ 1.18 ^a	84.00 $\pm$ 3.10 ^b	39.33 $\pm$ 1.89 ^c	42.67 $\pm$ 1.74 ^c	40.00 $\pm$ 1.68 ^c

The figures having different superscripts within the columns and between the columns differ significantly at level ($p < 0.05$).

Table 5. Primary colony formation (percentage) by pluripotent stem cells derived from buffalo embryos produced *in vitro* on homogenous feeders and synthetic matrices with different concentrations of LIF

Concentration of LIF used	Feeder layers			Synthetic matrices		
	Fetal fibroblast monolayer	Granulosa cell monolayer	Oviductal cell monolayer	Fibronectin	Collagen type I	Matrigel
10 ng/ml	0/18 (0.00)	0/18 (0.00)	0/18 (0.00)	0/18 (0.00)	0/18 (0.00)	0/18 (0.00)
20 ng/ml	12/18 (66.67) ^a	11/18 (61.11) ^a	0/18 (0.00)	10/18 (55.56) ^a	9/18 (50.00) ^a	10/18 (55.56) ^a
30 ng/ml	15/18 (83.33) ^b	15/18 (83.33) ^b	2/18 (11.11) ^c	14/18 (77.78) ^b	12/18 (66.67) ^b	12/18 (66.67) ^b

The figures having different superscripts within the columns and between the columns differ significantly at level ($p < 0.05$).

itive for AP staining with or without feeder layers. Immunolocalization for surface antigens and nuclear marker in primary ESC colonies cultured for 7~9 days onto different feeders and synthetic matrices was found positive for SSEA-4 and TRA-1-60 and Oct-4 (Fig. 4). cDNA synthesized from 3 primary ESC colonies grown on each feeder (except oviductal feeder where only 2 primary ESC colonies were used for cDNA synthesis) and synthetic matrices were used for RT-PCR analysis. RT-PCR products after gene amplification were confirmed by running 1.5% agarose gel. Sharp bands for Oct-4 gene of 541 bp were seen for ESCs cultured onto the feeder layers and synthetic matrices. Housekeeping gene β -actin was included as an endogenous control to evaluate the quality of cDNA synthesis. Single band of 327 bp was obtained in all ESC colonies developed with and without feeder (Fig. 5).

Discussion

Buffalo ESCs, like mouse and human ESCs are traditionally cultured onto homologous or heterologous (mouse embryonic fetal fibroblast) feeder layer, which allow their continuous growth in an undifferentiated state. In this study, we examined feeder and feeder free culture system with three different doses of LIF to find out better supportive culture condition for the buffalo ES cell growth and

development.

In the present study, 20 and 30 ng/ml LIF were found effective to maintain the ESCs in undifferentiated state in both feeder and feeder-free culture conditions indicated that dependency of ESCs on LIF for maintaining pluripotency. However, neither feeder layers nor synthetic matrices could develop primary colonies with 10 ng LIF, suggested that an optimal concentration of LIF is required to prevent differentiation of buffalo ES cells. Interestingly, maintenance of pluripotency in human (13) and bovine (14, 15) ES cell lines does not exhibit a clear dependency on LIF further suggested that human and bovine ESCs require different signals to maintain self-renewal and pluripotency. However, in buffaloes (1, 12, 16) supplementation of 20 ng/ml LIF in ES cells culture medium was found able to maintain their pluripotency regardless of the presence or absence of feeder cells.

Several studies have reported that the culture of mice (17), rhesus monkey (9) and human (6, 18) ESCs on homologous feeders implying that homologous cells can serve as feeder cells for prolong undifferentiated growth of ESCs. In the present study, two homologous feeders, fetal fibroblast and granulosa cell feeder layer were found equally effective to support the undifferentiated growth and maintenance of pluripotency in buffalo ESCs. However, oviductal feeder was found to be very poor in supporting primary ESC colonies, the possible reason

Fig. 4. Characterization of ESCs over fetal fibroblast feeder layer and fibronectin matrix. Alkaline phosphatase showing positive red colonies of ESCs (A, B), Oct-4 showing positive colonies (texas red) red fluroscence (C~F), TRA-1-60 (G~J) and SSEA-4 (K~N) positive colony showing green fluroscence (FITC) over fetal fibroblast monolayer and fibronectin matrix with 30 ng LIF respectively. Sacle bar: 100 μ m.

Fig. 5. mRNA expression profile of Oct-4 gene (541 bp) in ESCs cultured over homogenous feeders and extracellular matrices. Lane 1: ESCs over fetal fibroblast monolayer; Lane 2: ESCs over granulosa cell monolayer; Lane 3: ESCs over oviductal cell monolayer; Lane 4: ESCs over fibronectin; Lane 5: ESCs over collagen type-I; Lane 6: ESCs over matrigel. M=DNA ladder (50 bp).

could be that oviductal cells were no longer stable after mitomycin-C inactivation and showed detachment after 4~5 days of treatment. Because of their unstability after mitomycin-C treatment, it was difficult to maintain the

oviductal feeder and the ESCs throughout the study period. Mitomycin-C is a cytotoxin commonly used in cancer therapy and it gets inserted between the strands of DNA, prevents DNA replication, thus preventing cell division. Species difference might also one of the cause which produced some harmful substances after mitomycin-C treatment that leads to their detachment. In our study, even the sub-lethal dose (10 μ g/ml) of mitomycin-C was found to be detrimental for oviductal cells which are in contrast to the several studies in other species like monkeys (9) and humans (7) where oviductal cells successfully supported the growth of ESCs. In comparison to oviductal feeder, fetal fibroblast feeder and granulosa feeder was found to be most stable after mitomycin-C treatment.

Extracellular matrices (fibronectin, collagen type-I and matrigel) were tested for the growth and development of buffalo ESCs. Amongst extracellular matrices, fibronectin

was found better than collagen type I and matrigel for growth of primary ES cell colonies suggested that due to the glycoprotein nature, fibronectin possesses membrane-spanning receptor proteins called integrins which play a major role in cell adhesion and maintaining the proliferative ability of buffalo ES cells. To date, no published data is available to discuss the present findings in detail, however, Thein-Han et al. (19) reported that 3D porous chitosan scaffolds at different degree of deacetylation (% DD) supported buffalo ESCs and showed chitosan was cytocompatible. Moreover, extracellular matrices (1, 20, 21) as well as plastic surface (3) and 3D porous natural polymer scaffolds (22) were successfully used for culturing human and buffalo ESCs. This comparative study between feeder and feeder free culture system showed that developmental efficiency of ES cells was found highest onto fetal fibroblast and granulosa feeder layers (83.33%) followed by fibronectin (77.78%) with 30 ng of LIF. Oviductal feeder was found to be least efficient, only 11.11% with 30 ng LIF. Fibronectin and matrigel showed 55.56% efficiency while collagen type I reflected 50.00% efficacy with 20 ng LIF for ESC development. However, neither feeder layers nor synthetic matrices could develop primary colonies within 10 ng LIF. Amongst all, oviductal feeder was found to be poorest to develop ESC colonies.

Morphologically, ICM-derived undifferentiated primary colonies were densely packed with clear border on all the feeders and synthetic matrices while differentiated colonies showed cystic EBs at their periphery. Our findings were similar to Verma et al. (11) and Huang et al. (12), where they observed compactly packed dome shaped colonies in buffalo ESCs. Cells cultured in these conditions maintained all the characteristics of ES cells. After prolonged culture of more than 10 passages the cells remained undifferentiated, as demonstrated by the colony and single cell morphology and by the expression of alkaline phosphatase, nuclear (Oct-4) and surface markers (SSEA-1 & 4; TRA-1-60 & TRA-1-81) typical of undifferentiated ES cells. Kitiyanant et al. (23) and Sritanadomchai et al. (16) have also reported immunolocalization of SSEA-4 and TRA-1-60 in buffalo ESCs. Presence of SSEA-1 has not been detected in human ESCs (24), however in pig (25), dog (26) and sheep (27) ESCs express SSEA-1. Contradictory reports are available in bovines on the presence of SSEA-1 in ESCs, some studies showed its presence (14, 28) while others did not (29). SSEA-3 and SSEA-4 are expressed in 2 to 8-cell embryo and morula while, there is loss of expression in ICM of mouse embryo (30). However, these are expressed in ICM of human blastocyst and human ESCs (24). TRA-1-60 and TRA-1-81

were originally identified on human EC cells and ESCs (24) and have also been reported in buffalo (16, 23) ESCs. It has been well documented that the expressions of these surface markers change both with development and differentiation *in vitro* (31).

It can be concluded from the present study that buffalo ESCs require LIF to maintain the pluripotency, 30 ng/ml of LIF along with 10 ng/ml of bFGF was found to be the best combination for the development of ESCs irrespective of the use of feeder layers or synthetic matrices. Fetal fibroblast and granulosa cell feeder layers were found equally effective to support the undifferentiated growth and maintenance of pluripotency in buffalo ESCs at 30 ng LIF. Amongst synthetic matrices, fibronectin could show best proliferation of ESCs with 30 ng LIF. However, culture conditions need to be optimized to improve the developmental potential of ESCs under feeder-free conditions. The use of an extracellular matrix is advisable especially for the clinical applications of stem cells as it may eliminate the risk of transmitting pathogens from ESCs.

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Potential conflict of interest

The authors have no conflicting financial interest.

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