Effects of Growth Factors on Establishment and Propagation of Embryonic Stem Cells from Very Early Stage IVF Embryos and Their Characterization in Buffalo

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Background and Objectives: Although ES cells have been derived from very early stage embryos in different species, but, so far ES cell line could be derived from early stage *IVF* embryos in buffalo. The present experiment was carried out to study the effects of different growth factors on attachment, formation of ES cell colonies, their extent of passaging and relative expression of pluripotency marker in these colonies in buffalo.

Methods and Results: For this, 8~16 cell stages zona free *IVF* embryos were cultured with different culture condition viz. Control, Media-I: (Control+SCF), Media-II: (Control+SCF+bFGF) and Media-III: (Control+SCF+bFGF+IGF1). A total of 25 number of embryos were cultured in each medium. The efficiency (%) of blastomere attachment, % stem cell colony formation were recorded and number of passaging were evaluated in each culture condition. The results indicated that the efficiency of embryonic blastomere attachment, % stem cell colonies formation and propagation were significantly higher when medium was supplemented with growth factors viz. SCF, bFGF and IGF-1 (Media-III) than when supplemented with either SCF or SCF+bFGF. The expression of pluripotent genes viz Oct4, Nanog, FoxD3 and KLF4 were significantly higher ($p < 0.005$) when medium was supplemented with three growth factors.

Conclusions: It can be concluded that when 8~16 cell stages zona free *IVF* embryos of buffalo was cultured on feeder, the % of blastomere attachment, % of ES cell colony formation and their further propagation were higher in ES cell medium supplemented with SCF+bFGF+IGF1 which may be due to high expression of pluripotent stem cell markers.

Keywords: Growth factors, Embryonic stem cell like cells, Buffalo

Introduction

Since, the discovery of the embryonic stem (ES) cells in mice in early eighties (1, 2), a number of stem cell lines have been derived in different species including human

being. Providing specific culture conditions, ES cells can be maintained in undifferentiated state for a prolonged period of time (3-6). An important characteristic of ES cells is their ability to undergo a spontaneous differentiation in vitro (3, 7-9) forming a variability of progenitors and differentiated cells. The ability of ES cells to differentiate into any tissue represents an enormous therapeutic potential to treat different diseases. Further, ES cell can be used for embryo cloning and the remarkable characteristic of ES cells to contribute germ line transmission has paved the way for generating transgenic animals more efficiently (10). Apart from inner cell mass (ICM), ES cells have been derived from very early stage

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embryos in different species including human being (11-15). ES cell derived from early stage embryos may have added advantage than that of blastocyst derived ES cells not only in therapeutics but also in reproduction like cloning and transgenic animal production as these cells are more primitive than ICM derived ES cells. This is because the stage of embryonic development at which ES cells are derived seems crucial to their subsequent function in experimental models and cell therapy (13, 16). In buffalo, attempts have been made to derive ES cell from ICM of *IVF* as well as parthenogenetically derived embryos (17-20).

However, there is no stem cell line from early stage buffalo embryos and meager efforts are currently being made to derive ES cell line from very early stage embryos in buffalo. In our laboratory, attempts have been made to derive ES cells from early stage *IVF* derived embryos but the percentage of attachment on feeder layer and number of colonies formation was not only less but also the ES cell colonies formed did not contain sufficient number of cells even after prolong culture and the colonies could not be propagated (21, 22). In these studies no extra supplementation of growth factors except leukemia inhibitory factor (LIF) were added in the culture medium. Therefore, the present study was conducted to see the effect of supplementation of different growth factors in ES cells culture medium on efficiency of blastomere attachment, formation of ES cell like colonies, their propagation and characterization in buffalo.

Materials and Methods

In vitro embryo generation

Buffalo ovaries of unknown reproductive status were collected from local slaughterhouse and carried to the laboratory in normal saline solution (NSS, 0.85% NaCl) fortified with gentamycin (50 μ g/ml) in a thermo flask at 30~35°C within 2 hours of slaughter. Oocytes were aspirated from all the visible non atretic surface follicles (2~8 mm diameter) in containing oocyte collection medium (OCM) and searching of oocytes were carried out under stereo zoom microscope (Bausch and Lomb). The cumulus oocytes complexes (COCs) were evaluated and graded by the standar methods (23). The processed COCs were cultured in 50 μ l droplets (20~25 oocytes/droplet) of maturation media (TCM-199 with HEPES media, 10% FBS, 10% buffalo follicular fluid, 0.5 μ g/ml FSH, 10 μ g/ml LH, 0.5 μ g/ml estradiol) in 35 mm sterile Petri dish. The droplets were covered with warm nontoxic mineral oil and cultured in CO₂ incubator with an atmosphere of 5% CO₂,

21% O₂, 37°C and 95% relative humidity (RH) for 24 hours. After maturation, the oocytes were first treated with 1mg/ml hyaluronidase (Sigma) in OCM followed by pipetting to remove the expanded cumulus cells. After partial removal of cumulus cells, the oocytes were washed several times with sperm TALP (24) and finally with fertilization TALP media and transferred to 50 μ l drop of fertilization TALP media. To this, 20 μ l of capacitated spermatozoa were added and the droplets were covered with warm mineral oil and finally incubated in CO₂ incubator at 37°C, 5% CO₂, 21% O₂ and 95% relative humidity. After 18~20 hours of sperm-oocyte co-incubation, the presumptive zygotes were taken out of fertilization drops, washed 8-10 times in modified synthetic oviductal fluid (mSOF) (25) and cultured further in 100 μ l drop of the same media. The embryo developments were assessed by observing the cleavage rate after 48~72 hours of *in vitro* culture (IVC).

Development of embryonic stem cell colonies on buffalo monolayer

For derivation of embryonic stem cell colonies, blastomere of 8~16 cell staged *IVF* embryos were removed from IVC drops and put into warm PBS for washing. After washing, embryos were shifted to proteinase-K (0.02%) drops and the thinning or dissolution of zona pellucida was observed under zoom stereo microscope. After thinning or dissolution of zona pellucida, the proteinase-K activity was neutralized by addition of media containing FBS (20% FBS in DMEM media). The clumped blastomere cells were washed in 4~5 drops of stem cell culture media (composition given below) and kept in CO₂ incubator for growth. The undissociated clumped blastomere were cultured in wells on mitomycin-C inactivated buffalo fibroblast monolayer at 37°C, 5% CO₂ and 90% relative humidity in CO₂ incubator in different media containing different growth factors. The good quality ES colonies were isolated from the culture with different media, washed in PBS and stored at -80°C in PBS for molecular studies. When the clumped blastomere divided and formed 200~300 cells, the colonies were eluted by 0.25% trypsin EDTA (Gibco, 25200) solution and further cultured in respective culture condition. For the development of embryonic stem cell colonies following media were used; Control media (CM): Knockout DMEM supplemented with 10% FBS, LIF (40 ng/ml), ITS (0.3%), NEAA (0.5%); Media-I: Knockout DMEM supplemented with 10% FBS, LIF (40 ng/ml), ITS (0.3%), NEAA (0.5%), SCF (stem cell factor) (20 ng/ml); Media-II: Knockout DMEM supplemented with 10% FBS, LIF (40 ng/ml), ITS (0.3%), NEAA (0.5%), SCF (stem cell factor) (20

ng/ml), bFGF (Basic fibroblast growth factor) (4 ng/ml) and Media-III: Knockout DMEM supplemented with 10% FBS, LIF (40 ng/ml), ITS (0.3%), NEAA (0.5%), SCF (20 ng/ml), bFGF (4 ng/ml), IGF -1 (insulin like growth factor) (100 ng/ml).

Immunocytochemistry of embryonic stem cell colonies

The immunocytochemistry of embryonic stem cell colonies were done for the stem cell markers, Oct-4, Nanog, SSEA-1, TRA 1-60, TRA 1-80 as per the methods followed by the National Institutes of Health (NIH) 2009 resource for stem cell research, using fluorescent isothiocyanate (FITC) conjugated secondary antibody.

Comparative gene expression analysis of embryonic stem cell colonies

Comparative gene expression of Oct-4, Nanog, FoxD3 and Klf4 of the stem cell colonies were studied using Real Time PCR. From cell colonies stored frozen from different cultures were washed carefully thawed in PBS and RNA was isolated using mini RNA kit (Zymo research, Cat No. R1005 & R1006). The cDNA was synthesized from total RNA using reverse transcriptase kit (Bio-Rad, USA, 170-8891). The relative expression of genes was performed with Bio-Rad CFX96 (operated by C1000TM QPCR software) thermal cycler with Eva Green QPCR master mix (Bio-Rad, USA, 172-5201). Each PCR reaction was put in triplicate in a total volume of 20 μ l, which contained forward and reverse primers (10 pmol/ μ l) 1 μ l each, template cDNA 2 μ l and 10 μ l QPCR master mix and rest Nuclease-free PCR grade water (6 μ l). Nuclease free filter tips were used for taking the individual reaction components and preparation of reaction mixture. No template control (NTC) was put for either gene quantification or for checking the contamination in the reaction components other than the cDNA. The PCR condition was as follows, initial denaturation at 95°C for 5 min, denaturation at 95°C for 30 sec, annealing at 60°C (Oct 4, Klf4 and Fox D-3) and annealing at 55°C (Nanog) for 30 sec and gradual increment from 70°C to 95°C @ 0.5 degree per min and lastly per 5 sec. The amplification and denaturation data were taken for further calculations. The relative expression of genes was calculated by modified standard method based on Ct values (26).

Details of primers used for quantitative reverse transcription-polymerase chain reaction are as follows (Table 1).

Statistical analysis

All the experimental data including relative expression of pluripotent genes were analyzed by Sigma Software

(SPSS-16.0) using one way ANOVA.

Results

Development of embryonic stem cell colonies on buffalo fibroblast monolayer

The results indicated that the percentage and time taken for attachment and colony formation (Table 2, Fig. 1) was comparatively higher in culture medium supplemented with three growth factors viz SCF, bFGF and IGF-1 (Medium-III) as compared to medium without any growth factor supplementation (control) or with supplementation of only SCF (Medium-I) or SCF+ IGF1 (Medium-II). The attachment of colonies was also faster in culture Medium-III. The ES cell colonies could be propagated only up to 3rd passage in Medium-3 than any other medium tested (Table 2).

Characterization of embryonic stem cells

The ES cell colonies developed in different culture condition was positive for TRA-1-80, Oct-4, Nanog and alkaline phosphatase (Fig. 2). The colonies also expressed pluripotent genes like Oct4, Nanog, FoxD3, Klf4 in all the culture condition (Fig. 3).

Effect of growth factors on relative expression of pluripotent genes in embryonic stem cells colonies

The relative expressions of pluripotent genes in differ-

Table 1. Details of primer used in the study

Primer	Primer sequence (5_-3_) forward and reverse	Annealing temp. (°C)	Product size (bp)
β -actin	F 5'-TCC AAG GCG ACG TAG CAG AG -3' R 5'-CGC ACC ACT GGC ATT GTC AT- 3'	60	227
Oct-4	F 5'-GAA AGA CGT CCG AGT GTG GT-3' R 5'-TCC CCC TGT GAA AGG AGA C-3'	60	125
Nanog	F 5'-GTC CCG GTC AAG AAA CAA AA -3' R 5'-TGC ATT TGC TGG AGA CTG AG- 3'	55	107
Klf4	F 5'-GGA AGT TTG CCC GCT CAG AT-3' R 5'-GCC TCT TCA TGT GTA AGG CG-3'	60	129
FoxD3	F 5'-GCT GAC TCT GAG CGG CAT-3' R 5'-TCC TCG GAC TGA GGG TCC A-3'	60	200

Table 2. Effect of supplementation of growth factors on establishment and propagation of ES cell in buffalo

Media used	No. of embryo cultured	No. of embryo attached (% attachment)	Time taken for attachment	No. of primary colonies formed (% of P ₀ colonies formed)	P ₁	P ₂	P ₃
Control media	25	08 (32)	72~96 hrs	16 (64)	√	√	×
Media-I	25	08 (32)	72~96 hrs	17 (68)	√	√	×
Media-II	25	11 (44)	72~96 hrs	21 (84)	√	√	×
Media-III	25	12 (48)	44~48 hrs	22 (88)	√	√	√

Control media: knockout DMEM supplemented with 10% FBS, LIF (40 ng/ml), ITS (0.3%), NEAA (0.5%); Media-I: Control+SCF; Media-II: Control+SCF+bFGF; Media-III: Control+SCF+bFGF+IGF-1, P₀: primary ES colonies; P₁: first passage; P₂: second passage; P₃: third passage.

Fig. 1. Primary ES cell like cell colony formed from 8~16 cell stage IVF derived embryos in buffalo (Scale bar 20 μ m).

ent culture conditions and passages have been presented (Figs. 3~5). The relative expressions of pluripotent genes in different culture conditions were compared. It was observed that the primary ES cell colonies formed in presence of three growth factors (Medium-III) had significantly ($p < 0.005$) higher expression of pluripotent genes viz Oct-4, Nanog, Klf4 and FoxD3 as compared to other Mediums (Fig. 3). Further, it was observed that addition of all three growth factor (s) increased the expression of all the pluripotent genes (Fig. 3). When the primary ES cell like colonies were passaged successively (P1 and P2), the expression of pluripotent genes remained

higher in Medium-III as compared to other culture condition (Figs. 4, 5). In Medium-III, the ES cell colonies could be propagated for another passage (P3) while in other culture condition the ES cell like cells stopped growing following second passage (P2) (Table 2).

Discussion

So far no ES cell line could be generated from very early stage IVF derived buffalo embryos. In the present study, the percentage of blastomere attachment on feeder, the ES cell colony formation and propagation was very low in

Fig. 2. Immunocytochemistry of ES colonies cell in buffalo (Scale bar 20 μ m).

Fig. 3. Relative expression of development related genes in embryonic stem cell clone in different culture media on buffalo fibroblast monolayer (P0) on buffalo fibroblast monolayer. P₀: without passage; abcd means bearing different subscripts in a column differ significantly ($p < 0.005$).

control media (Table 2). However, these ES formation and propagation were significantly higher in medium supplemented with SCF, bFGF and IGF1 (Medium-III). When the stem cell media was supplemented with SCF only, the time for attachment of embryonic cells, percentage of establishment of primary colonies were same, but the colony size and further propagation was not improved as compared to control media (Table 2). It is not known that whether buffalo embryonic stem cells expresses c-kit as it is well known that the role of SCF for lineage maintenance and cell renewal depends on the cells that express c-kit (27). Further, the activity of SCF on stem cells depends on species (9), as it has been shown that hSCF binds to murine c-kit with a very low affinity and stim-

Fig. 4. Relative expression of development related genes among different media in passage-I (P1) on buffalo fibroblast monolayer. P₁: primary passage; abcd means bearing different subscripts in a column differ significantly ($p < 0.005$).

ulates the proliferation/survival of murine c-kit⁺ cells only at very high concentrations (2, 28). The failure of any positive effect of SCF in the present study might be that the SCF which was used was of human origin and may not have worked in bovine species as well as the dose might have been very less even if the c-kit ligand is present.

When basic fibroblast growth factor (bFGF) was added in the medium along with SCF, the percentage of primary ES cells colony formation was increased which might be due to mitogenic effect of this factor. Fibroblast growth factor from bovine pituitary gland shown to have a mitogenic effect on many cells types (29). It has been also observed that certain growth factors promote the survival of primordial germ cells (PGCs) *in vitro* (30-35), possibly through anti-apoptotic activities. It has also been shown

Fig. 5. Relative expression of development related genes among different media in passage II (P2) on buffalo fibroblast monolayer. P2: secondary passage; abcd means bearing different subscripts in a column differ significantly ($p < 0.005$).

that bFGF improves cell human ES cells adhesion on cell monolayer (36), growth and maintained undifferentiated state of cattle ES-like cells derived from inner cell mass for several passages (37). Human ES cells are also routinely cultured on feeder layers of mouse or human fibroblasts in medium that is supplemented with bFGF (38).

When the ES cell medium was supplemented with another growth factor, IGF-1 (Media- III), the attachment of blastomere was very quick and numbers of primary ES cell colonies were significantly higher with larger size of the colony than any other media (Table 2). IGF-1 helps in mitosis of cells different types of cells, an anti-apoptotic agent, a potent survival factor (35), promote cell mitogenesis and proliferation in early embryonic developmental stages of embryo (38) and its combination along with other bFGF4 might have positive effect on cell growth and proliferation (Fig 1, Media III). Surprisingly even with different growth factors, the established primary ES cell colonies could not be propagated beyond three passages.

In the present study, ES cell colonies formed was tested positive for TRA 1-80, Oct-4, Nanog and alkaline phosphatase (Fig. 2). Stem cell colonies derived from 8 cell stage bovine IVF embryos expressed SSEA-1 and other pluripotent markers (12) and in another study observed that ES cell colonies derived from early stage pre-implantation murine embryos also expressed all the pluripotent markers as of ICM derived ES cells (13).

The expression of pluripotent genes were almost similar

between Control and SCF supplemented medium (Media II) (Fig. 3). However, the expressions of pluripotent genes like Oct4, Nanog, Klf4 and Foxd3 (Fig. 3) were enhanced when all the growth factors were in the stem cells medium. This might be also another reason that the ES cell colony formation in this medium was better than other medium (Table 2) and one more passaging could also be made (Table 2). It is known that growth factors like SCF, FGFs, IGFs etc are supplemented in stem cells medium in different species and in different concentration (6, 39), work in different pathways for enhancement of clonal expansion and maintenance of pluripotency in stem cells of different species including human being. Stem cell factor, IGFs, LIF, FGF etc maintain pluripotent gene expression/increase the expression of pluripotent genes through different pathways when added exogenously. Human ES cells have been derived and maintained by using increase doses of fibroblast growth factor (6). However, little is known about these pathways in domestic animal and particularly in buffalo. This might have been the reason in failure of derivation of true ES cell lines in ungulates (40, 41).

In the present study, even after addition of three growth factors viz SCF, bFGF and IGF1, the ES cell could not be propagated beyond passage three. The results indicated that further modification of culture medium need to be done for generation of buffalo ES cell line from early stage IVF derived embryos. Although stem cell lines have been derived easily in mice, human from early stage embryos (42), but in many cases required special procedures/intervention in culture medium for generation of ES cell lines (36, 43). As on today the total understanding of blastomere behavior of bovine embryos and especially of buffalo has not been elucidated. Our personal experiments showed that the expression of pluripotent genes in buffalo embryos during extended culture decreases (44). Similar observation has been reported in cattle (45). In one report, it has been shown that extra dosed of FGF only helped in establishment of human embryonic stem cells (6). Further, the present result may also indicated that the main reasons hampering the derivation of ES line from early stage IVF derived embryos in buffalos may be the incompetence of the starting material ie., embryo stage itself, breed differences, the culture condition used for derivation of ES cell line and limited knowledge about the growth factor (s) requirement. It has been observed that in mice, specific strain is most permissive for derivation of ES cell lines than other strain of mice irrespective of stage of embryos. A frequency of 10~30% ES cell line derivation has been reported for SV129 strain of mice

while only 2.0 and 2.96% have been reported for C57BL/6 and BALB/c strain, respectively. ES cells derived from C57BL/6 blastocysts are more difficult to propagate in vitro, generate chimeras less efficiently, and contribute less frequently to the germline than ES cell lines from the 129 strains (46, 47). Further, a report showed that the establishment of ES cells differs with culture condition (48).

The study indicated that ES cell medium supplemented with SCF, bFGF and IGF-1 was found to be better for percentage of blastomere attachment on feeder, percentage of primary ES cell like colony formation and further propagation than medium supplemented with either SCF or bFGF alone. The expression of pluripotent genes in ES cell like cell colonies viz Oct4, Nanog, FoxD3 and KLF4 were significantly also higher when medium was supplemented with three growth factors (Medium-III) than any other medium used. However, even after adding these factors the stem cell colonies could not be propagated beyond passage 3. Further, work is needed to deduce a better culture condition with more combination of growth factors in different dosed which would support unlimited passage of ES cells generated from early stage *IVF* derived embryos for making a stem cell line in buffalo.

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Potential conflict of interest

The authors have no conflicting financial interest.

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