Selection of appropriate isolation method based on morphology of blastocyst for efficient derivation of buffalo embryonic stem cells

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Abstract The efficiency of embryonic stem cell (ESC) derivation from all species except for rodents and primates is very low. There are however, multiple interests in obtaining pluripotent cells from these animals with main expectations in the fields of transgenesis, cloning, regenerative medicine and tissue engineering. Researches are being carried out in laboratories throughout the world to increase the efficiency of ESC isolation for their downstream applications. Thus, the present study was undertaken to study the effect of different isolation methods based on the morphology of blastocyst for efficient derivation of buffalo ESCs. Embryos were produced in vitro through the procedures of maturation, fertilization and culture. Hatched blastocysts or isolated inner cell masses (ICMs) were seeded on mitomycin-C inactivated buffalo fetal fibroblast monolayer for the development of ESC colonies. The ESCs were

analyzed for alkaline phosphatase activity, expression of pluripotency markers and karyotypic stability. Primary ESC colonies were obtained after 2–5 days of seeding hatched blastocysts or isolated ICMs on mitomycin-C inactivated feeder layer. Mechanically isolated ICMs attached and formed primary cell colonies more efficiently than ICMs isolated enzymatically. For derivation of ESCs from poorly defined ICMs intact hatched blastocyst culture was the most successful method. Results of this study implied that although ESCs can be obtained using all three methods used in this study, efficiency varies depending upon the morphology of blastocyst and isolation method used. So, appropriate isolation method must be selected depending on the quality of blastocyst for efficient derivation of ESCs.

Keywords Buffalo embryonic stem cell · Pluripotency markers · Alkaline phosphatase · Karyotype

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Introduction

Embryonic stem cells (ESCs) are pluripotent cells derived from the inner cell mass (ICM) of blastocyst, an organization of cells formed during early embryogenesis. ESCs possess the unique ability of self-renewal that confers indefinite maintenance of the

undifferentiated state *in vitro* while retaining capacity to generate derivatives of three embryonic germ layers that precede the formation of all tissues of the developing fetus. As ESCs can differentiate potentially into all kinds of cells in the body, they promise an exciting potential as a source of cells for regenerative medicine for treating injuries and degenerative diseases (Lindvall and Kokaia 2005), congenital diseases (Passier and Mummery 2003) and drug discovery to assess the teratogenic potential of pharmaceutical compounds (Pouton and Haynes 2007). ESCs play an important role in understanding the mechanism of early embryonic development and differentiation, particularly in research dealing with genetic control of early development (Rathjen et al. 1999). They are valuable tools for gene targeting (Zwaka and Thomson 2003), cloning (Saito et al. 2003), production of chimeras (Piedrahita et al. 1998) and transgenic animals (Saito et al. 2003). Owing to their ability to transfer their genome to the next generation, ESCs are expected to be one of the most useful tools for manipulating germlines of domestic animals (Saito et al. 2005).

Since 1981, when ESCs were first established from mouse (Evans and Kaufman 1981; Martin 1981), several attempts have been made to obtain ESCs/ESC-like cells from various domestic animals and isolation of ESCs from blastocysts have been reported from species like cattle (Mitalipova et al. 2001), sheep (Dattena et al. 2006), pig (Wheeler 1994), rabbit (Giles et al. 1993), horse (Saito et al. 2002) and buffalo (Verma et al. 2007). The derivation of ESCs from the inner cell mass (ICM) of pre-implantation blastocysts has become a procedure undertaken by researchers all over the world. Blastocyst quality is one of the most important factors determining the efficiency of derivation of ESCs. However, not all embryos develop into good expanded blastocysts, which are characterized by large, distinct ICMs. In some cases, the embryos possess smaller ICMs. In many cases, blastocysts also possess poorly defined ICMs (Kim et al. 2005). Therefore, different methods, depending on the morphology of the blastocysts and the appearance of their ICMs could be adapted for efficient derivation of ESCs.

Buffalo is a prime dairy animal in India. Buffalo's contribution to dairy and agriculture sector makes itself an ideal livestock species to harness this futuristic technology. Hence, this study was taken up to establish an effective approach to generate buESCs.

Materials and methods

Except where otherwise indicated, all chemicals and media used for *in vitro* embryo production and ESC culture were procured from Sigma Chemicals Co. (St. Louis, MO, USA), which were of embryo culture grade and endotoxin tested. Primary antibodies (Mouse Polyclonal IgG) against SSEA-4, TRA-1-81 and fluorescein isothiocyanate (FITC) and Texas red conjugated secondary antibodies (Goat Anti-mouse IgG) were procured from Millipore (Molsheim, France).

In vitro embryo production

Buffalo ovaries were obtained from local abattoir and transported to the laboratory in 0.9 % sterile saline supplemented with 50 µg/ml gentamycin sulphate at 35–37 °C within 2–3 h. Ovaries were washed 4–5 times with physiological saline solution and oocytes were aspirated from all visible ovarian follicles of 3–8 mm diameter in washing medium [Tissue culture medium-199 (TCM-199) + 5.0 mM NaHCO₃ + 5 mM Hepes + 2 % Fetal bovine serum (FBS)] using a 18-gauge needle fitted to a 5 ml syringe and kept undisturbed in an incubator at 37 °C. After 15–20 min, supernatant was discarded and oocytes were recovered from sediment under a stereozoom microscope (Olympus, Tokyo, Japan).

In vitro maturation (IVM)

Compact cumulus oocytes complexes (COCs) with an unexpanded cumulus having more than 5 layers of cumulus cells and evenly granular homogenous ooplasm were selected for *in vitro* maturation. Selected COCs were washed five times in maturation medium (TCM-199 supplemented with 10 ng/ml epidermal growth factor (EGF), 10 % FBS, 0.5 µg/ml follicular stimulating hormone (FSH), 5.0 µg/ml luteinizing hormone (LH), 1 µg/ml 17-β-estradiol, 0.68 mM L-glutamine, 0.25 mM sodium pyruvate and 50 µg/ml gentamycin sulphate). 10–15 oocytes were kept for maturation in 60–70 µl microdrops of maturation medium overlaid with mineral oil in a 35 mm culture dish for 24 h at 38.5 °C and 5 % CO₂ in air with 95 % relative humidity.

In vitro fertilization (IVF)

The mature oocytes with expanded cumulus cells were subjected to in vitro fertilization in glucose-free Tyrode-albumin-lactate-pyruvate (TALP) supplemented with 0.2 mM sodium pyruvate, 6 mg/ml fatty-acid-free bovine serum albumin (BSA), 10 mg/ml heparin and 50 µg/ml gentamycin sulphate. A single frozen buffalo semen straw (0.25 ml) was thawed at 37 °C for 30 s and washed in Fertilization-TALP (FT) medium by centrifugation at 55 g for 10 min. The supernatant was aspirated out and the sperm pellet obtained after centrifugation was resuspended in FT medium to a final concentration of 2×10^6 spermatozoa/ml. Ten to fifteen COCs were introduced into 50 µl droplets of processed buffalo spermatozoa and kept in an incubator with 5 % CO₂ at 38.5 °C with maximum humidity for 18 h.

In vitro culture (IVC) of embryos

After 18 h co-incubation of sperm and oocytes, the presumptive zygotes were washed 10–15 times in embryo development medium (EDM) comprising 3 mg/ml BSA, 0.25 mM sodium pyruvate with 1 % essential and nonessential amino acids, 0.68 mM L-glutamine and 50 µg/ml gentamycin sulphate. The presumptive zygotes (10–15 per 50 µl droplet) were cultured without FBS for 48 h post insemination, afterwards embryos were cultured in EDM supplemented with 10 % FBS at 38.5 °C and 5 % CO₂ in air for development until blastocyst stage.

Preparation of feeder layer

Pregnant uteri were collected from local abattoir to obtain fetus (approx. 40 days old) for the preparation of feeder layer. Fetus was washed properly with Dulbecco's Phosphate buffer saline (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 flask and cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose, supplemented with 10 % FBS, 2 mM L-glutamine and 50 µg/ml gentamycin in a CO₂ incubator (5 % CO₂) at 38.5 °C. The explants were removed after 24 h and the fetal fibroblasts were allowed to grow till confluency. Primary fetal fibroblast monolayers were

passed by incubating in 0.25 % trypsin–EDTA for 5–8 min at 37 °C and were used as feeder layer for ESC culture after third passage. Feeder layer was inactivated with mitomycin-C (10 µg/ml) for 3 h and washed thoroughly (4–5 times) with DPBS prior to ESC culture.

Isolation and primary culture of buESCs

Three different methods were compared for the derivation of buESCs based on the quality of the expanded blastocysts. Blastocysts hatched on day nine of in vitro culture were used in this study. The buESCs were derived either by culturing intact hatched blastocysts or ICMs isolated either by manual or enzymatic method. In whole-embryo culture method, intact hatched blastocysts were cultured as such along with trophoctodermal cells in uncoated cell culture plates or onto passaged mitomycin-C (10 lg/ml) inactivated buffalo fetal fibroblast monolayer and were cultured in ESC medium composed of DMEM supplemented with 20 % FBS, 1,000 IU/ml murine leukemia inhibitory factor (mLIF), 1 % non-essential amino acids, 0.1 mM β-mercaptoethanol, and 2 mM L-glutamine. In the manual method, the ICMs of the hatched blastocysts were separated out from the cells of trophectoderm mechanically with the aid of two fine glass needles under a zoom stereomicroscope (Olympus SZ 40), whereas in the enzymatic method the zona-free blastocysts were transferred to a 0.25 % trypsin–EDTA solution and observed under a zoom stereomicroscope until the trophectodermal cells (TE) became loose, and were shed from the ICM by gentle pipetting. Isolated ICMs were then individually seeded on mitomycin-C (10 µg/ml) treated feeder layers and cultured in ESC medium. The culture medium was replaced after 2 days initially till attachment of cells and daily afterwards. Further colonization of the cells was observed routinely under an inverted microscope (Olympus).

Characterization of embryonic stem cell

For characterization, buESCs were assessed for morphology, alkaline phosphatase (AP) activity, expression of mRNA transcripts for transcription factors (OCT-4, NANOG, and SOX-2), cell surface antigens (SSEA-4, and TRA-1-81) and genetic stability.

AP staining

The buESCs were cultured for 5 days prior to analyzing AP activity. The buESCs were fixed with 4 % paraformaldehyde in DPBS for 1–2 min avoiding overfixation. Fixative was aspirated and cells were rinsed with 1× Rinse Buffer (20 mM Tris–HCl, pH 7.4, 0.15 M NaCl, 0.05 % Tween-20). Each well was overlaid with (0.5 mL for a well of a 24-well plate) stain solution (Fast Red Violet, Naphthol AS-BI phosphate solution and water in a ratio 2:1:1) to cover the colonies properly. Plate was incubated in dark at room temperature for 15 min. Subsequently staining solution was aspirated from wells and cells were rinsed with 1× Rinse Buffer and covered with DPBS to prevent drying and AP activity was documented in the colonies (Table 1).

Reverse transcription–polymerase chain reaction (RT–PCR) analysis

Expression of pluripotency markers *OCT-4*, *NANOG* and *SOX-2* was assessed in primary buESC colonies. Reverse transcription was carried out using FastLane cell cDNA kit (Gien, Valencia, CA, USA) according to manufacturer's instructions. Briefly, buESCs were collected by scraping and pelleted at 200 g for 5 min. Cells were briefly washed with wash buffer to remove cell-culture medium, extracellular material released by living cells, and intracellular material released by any dead and lysed cells. The cultured cells were then lysed for 5 min using lysis buffer. Genomic deoxyribonucleic acid (gDNA) elimination reaction was carried out in a total volume of 14 µl using 2 µl of gDNA wipeout Buffer (7×), 4 µl of FastLane lysate and 8 µl of RNase-free water. After thorough mixing and brief centrifugation, reaction mixture was incubated at

42 °C for 5 min to eliminate genomic DNA contamination. Reaction was reverse transcribed in a final volume of 20 µl using 1 µl (200 units) of Quantiscript reverse transcriptase (RT), 4 µl of Quantiscript RT buffer, 1 µl (0.5 µg/ml) of RT primer mix and 14 µl of lysate. Reaction mixture was incubated at 42 °C for 30 min followed by inactivation of Reverse Transcriptase at 95 °C for 3 min. The resulting cDNA was stored at –20 °C. Following first strand synthesis, the second strand was PCR amplified using gene specific primers (Table 2) using heated lid XP Thermal Cycler (Bioer technology Co., Ltd., Tokyo, Japan). PCR amplification was carried out in a total volume of 25 µl of 1× PCR Buffer (200 mM Tris HCl pH 8.4, 500 mM KCl), 1.5 mM MgCl₂, 0.2 units of Taq DNA polymerase and 10 pM of each primer with thermal profile consisting of initial denaturation cycle at 95 °C for 3 min and subsequent 35 cycles of PCR with denaturation at 95 °C for 45 s, annealing at temperature specific for individual gene (Table 2) for 30 s, extension at 72 °C for 1 min and final extension at 72 °C for 10 min. Reference β-actin gene was included as an endogenous control to evaluate the quality of cDNA. Non template control was run with every PCR amplification program to check contamination or PCR carryover. PCR products were run on 1.5 % agarose gel containing ethidium bromide (0.5 µg/ml) along with DNA ladder for determining their tentative molecular sizes and visualized on a gel documentation system (Alpha Imager-2200, Alpha Innotech Corporation, San Leandro, CA, USA).

Immunostaining of buESC colony

To localize the proteins of pluripotency markers (SSEA-4 and TRA-1-81), undifferentiated buESCs colonies were processed for immunocytochemistry

Table 1 Comparison of methods used for the derivation of primary buESCs colonies based on the quality of the expanded blastocysts

Derivation methods	Size and morphology of ICM			
	Large and distinct	Small but distinct	Indistinguishable	Total
1. Manual method	26/40 (65.0) ^{A, a}	32/64 (50.0) ^{A, a}	1/16 (6.3) ^{A, b}	59/120 (49.2) ^A
2. Enzymatic method	16/28 (57.1) ^{A, a}	13/48 (27.1) ^{B, b}	1/18 (5.6) ^{A, b}	30/94 (31.9) ^B
3. Whole-embryo culture method	8/22 (36.4) ^{B, a}	8/25 (32.0) ^{B, a}	7/23 (30.4) ^{B, a}	23/70 (32.9) ^B
Total	50/90 (55.6) ^a	53/137 (38.6) ^b	9/57 (15.8) ^c	112/284 (39.4)

^{A, B, C} Values within the same column with different superscript letters differ significantly ($P < 0.05$, Chi square test)

^{a, b, c} Values within the same row with different superscript letters differ significantly ($P < 0.05$, Chi square test)

Table 2 Oligonucleotide primers and conditions used for gene specific RT–PCR amplifications

Gene identity	Primer sequence	Annealing temperature (°C)	Fragment size (bp)	Reference sequence
OCT-4	F-5'-AGGATCACACTAGGATATAC-3' R-5'-CCGAAGAGTACAGAGTAG-3'	63	1102	JF898834.1
NANOG	F-5'-TTTTCCTCCTCCTTCAACTCA-3' R-5'-CATATCTTCAGGCTGTATgTT-3'	59	929	JF898835.1
SOX-2	F-5'-ATGTACAACATGATGGAGACG-3' R-5'-TCACATGTGCGAGAGGGGC-3'	61	966	GQ451841.1
β-actin	F-5'-GGAGGCCCCCTCTGAACCCCAA-3' R-5'-TTCTCCTTGATGTCACGAACGA-3'	59	327	DQ661647.1

F Forward, *R* reverse, *bp* base pair

following steps of fixation and blockage of non-specific binding as described below. Briefly, after washing twice with 1× Rinse Buffer, buESCs were fixed in 4 % paraformaldehyde in PBS at room temperature (37 °C) for 15–20 min. Non-specific binding was blocked by incubating cells with donkey serum (1:10 dilution in PBS) for 30 min at 37 °C. The buESCs were then incubated with primary antibodies (1:20 dilution in PBS) for 1 h at 37 °C followed by incubation with secondary antibodies (1:200 dilution in PBS) conjugated with FITC/Texas Red for 1 h at 37 °C. Every step was followed by proper washing (2–3 times) with 1× Rinse Buffer. Expression of individual pluripotency marker was documented under fluorescent microscope (IX 71 Olympus) using suitable filters.

Karyotype analysis of buESCs

Karyotype analysis was performed on primary buESCs according to the method described by Dyban and Sorokin (1983) with slight modifications. Briefly, the cell colonies were incubated in culture medium supplemented with 0.1 mg/ml colcemid for 4 h at 38.5 °C. The cells were then washed, trypsinized and pelleted by centrifugation at 200 g for 8 min. Cell pellet was resuspended in 2 ml of hypotonic solution (0.56 % KCl and 0.5 % Na citrate) for 30 min at 38.5 °C. After pelleting of cells again by centrifugation, the supernatant was removed and cells were fixed in freshly prepared chilled Carnoy's solution (3:1 methanol/glacial acetic acid) for 10 min at –20 °C. Fixative was added drop-wise to a final volume of 10 ml. Cell suspension was centrifuged at 200 g for 8 min and supernatant was decanted off. Pellet was resuspended in 5 ml of fresh ice-chilled fixative and

fixed for another 10 min and then centrifuged again. The metaphase spreads were prepared by dropping the cells onto ice cold glass slides. Chromosomes were stained with 10 % Giemsa for 5–6 min and observed under oil immersion using a compound microscope (Nikon, Microphot-FXA, Tokyo, Japan) and photographed for analysis.

Statistical analysis

Different methods for derivation of buESCs were compared in terms of the colony formation rates by Chi square (χ^2) analysis. A value of $P < 0.05$ was considered to be statistically significant.

Results

In vitro embryo production

Out of the 4333 cleaved oocytes, a total of 894 embryos reached to blastocyst stage and 284 hatched giving cleavage rate, blastocyst percent and hatching percent of 69.36 ± 1.32 , 21.16 ± 0.53 and 31.17 ± 0.73 , respectively. Only hatched blastocysts (Fig. 1a) were used for the isolation of buESCs.

Preparation of feeder layer

On day five fetal fibroblast grew to confluency. Fetal fibroblast monolayers showed uniform growth after passaging. Passaged cells attained confluency within 48 h of reseeded. Mitomycin-C (10 µg/ml) inactivated confluent cells (Fig. 1b) after third passage were used as feeder layer for buESC culture. Mitomycin-C

treated cells remained attached to the surface of cell culture plates and grew in size without division.

Isolation and primary culture of buESCs

A total of 284 hatched blastocysts were used to compare three distinct methods of buESCs derivation based on the quality of the expanded blastocysts (Fig. 2a–c). Both manually and enzymatically isolated ICM cells attached to feeder layer (Fig. 2d, e) within 2 days of culture whereas, intact hatched blastocyst (Fig. 3f) took comparatively longer time (3–4 days) for attachment. Primary colonies were obtained after 5–7 days of seeding them on mitomycin-C-treated fetal fibroblast monolayer (Fig. 2g–i). The establishment of buESC colonies from both high-quality blastocysts possessing large distinct ICMs and blastocysts possessing somewhat smaller but distinguishable ICM was determined to be significantly ($P < 0.01$) more successful when the manually isolated ICM culture method was used compared to the enzymatically isolated ICM culture method or the whole-embryo culture method (Table 1). The enzymatically isolated ICM culture method was found to be better for the establishment of buESC colonies from high-quality blastocysts possessing large ICMs. Colony formation rate did not differ significantly ($P > 0.05$) for the type of blastocysts when the whole-embryo culture method was used. The whole-embryo culture method was found to be the method of choice for derivation of buESC colonies from blastocysts in which the ICM and trophoectoderm were poorly defined. Neither the manually isolated ICM culture method nor the enzymatically isolated ICM culture

was particularly successful in the establishment of buESC colonies from blastocysts in which the ICM and trophoectoderm were indistinguishable. The primary ESC colonies were formed in 49.2 % of ICMs obtained from hatched blastocysts by the manual method, which was significantly higher ($P < 0.01$) than that for the enzymatic (31.9 %) or the whole-embryo culture method (32.9 %) as shown in Table 1.

Characterization of embryonic stem cell

Morphology of buESC and buESC colony

Morphologically, the cells in the blastocyst-derived primary colonies were densely packed and had a high nucleus to cytoplasm ratio. Colonies were dome shaped, with a clear border (Fig. 2g–i).

AP staining

Primary buESC colonies (Fig. 3a) were positive for AP staining (Fig. 3b). No staining was observed for feeder cells which acted as negative control.

Immunostaining of buESC colony

Pluripotency of the buESC was demonstrated by expression analysis of cell surface markers: SSEA-4 and TRA-1-81. The buESCs expressed SSEA-4 (Fig. 3c, d) and TRA-1-81 (Fig. 3e, f). These markers were localized to the cell surface and matrix between individual buESCs as expected for detection of the high molecular weight glycolipid and proteoglycan

Fig. 1 **a** Bright field micrographs of in vitro produced buffalo blastocysts hatched on day nine of in vitro fertilization showing hatched blastocysts (HdBs), empty zona pellucida (EZ), inner

cell mass (ICM) and trophoectoderm (TE) ($\times 200$). **b** Morphology of a mitomycin-C treated fully confluent buffalo fetal fibroblast monolayer ($\times 100$)

Fig. 2 Bright field micrographs showing morphology of hatched blastocysts and inner cell masses used for derivation of buESCs. Blastocysts with **a** large, **b** small, and **c** indistinguishable inner cell masses ($\times 400$). **d** Manually and **e** enzymatically isolated inner cell mass cells and **f** Intact hatched

blastocyst on mitomycin-C treated confluent buffalo fetal fibroblast monolayer ($\times 200$). Primary buESC colonies (**g–i**) on buffalo fetal fibroblast monolayer on day five of culture ($\times 600$)

antigens detected by these antibodies. The staining was specific to buESC colonies as buffalo fetal fibroblasts cells did not take the stain.

Reverse transcription–polymerase chain reaction (RT–PCR) analysis

1.5 % agarose gel electrophoresis of ethidium bromide-stained RT–PCR products revealed the amplification of 1102, 966 and 929 bp amplicons of *OCT-4* (Fig. 4a), *SOX-2* (Fig. 4b) and *NANOG* (Fig. 4c) gene transcripts, respectively. *OCT-4*, *SOX-2* and *NANOG* gene transcripts were observed in primary colonies of buESCs, but not in buffalo fetal fibroblast feeder cells.

Chromosomal stability of buESCs

Cytogenetic analysis of buESCs in primary colonies revealed karyotypes with normal diploid number ($2n = 50$) of chromosomes (Fig. 4d). Although the limit of resolution precluded the unequivocal identification of normal chromosomes, aneuploidies were not detected.

Discussion

For derivation of ESCs two methods are commonly used, one is the direct plating of intact hatched

Fig. 3 Alkaline phosphatase and immunocytochemical staining of buESCs for the expression analysis of pluripotency related markers. **a** Unstained buESC colony, **b** the buESCs colony showing (stained red) alkaline phosphatase activity, **c** A buESC colony under bright light **d** epifluorescence exhibiting expression of SSEA-4, **e** A buESC colony under bright light **(f)** epifluorescence exhibiting expression of TRA-1-81 ($\times 200$). (Color figure online)

blastocysts on a feeder layer and the other one is the plating of isolated ICMs. Efficiency of derivation of ESCs however, depends on the quality of blastocyst and isolation method used. In the present study, we have reported and described three distinct methods namely, the manual method, the enzymatic method and the whole-embryo culture method for the derivation of buESCs. BuESCs could be obtained by all three methods but efficiency varied depending on the quality of the blastocyst and the isolation method used. The best results were obtained when ESCs were isolated from blastocysts with large and distinct ICMs. As the number of cells in ICM of hatched blastocyst is higher than those of expanded blastocysts, these cells will have more chance to develop and form primary ESC colonies. So, in our study we have used only hatched blastocysts. Working with human embryos,

Stojkovic et al. (2004) reported that cells isolated from day-8 human blastocysts, which possess significantly more ICM cells than day-6 blastocysts, resulted in a higher number of colonies.

The manual method was found to be more efficient than the enzymatic and the whole-embryo culture methods for derivation of buESC colonies when high-quality blastocysts possessing large and distinguishable ICMs and blastocyst exhibiting normal morphology but a somewhat smaller ICM were used. The enzymatic method was comparable to the manual method but superior to the whole-embryo culture methods for derivation of buESC colonies. However, neither the manual nor the enzymatic culture method was particularly successful in the establishment of buESC lines from blastocysts in which the ICM and trophoctoderm were poorly defined. The manual and

Fig. 4 RT-PCR analysis of β -actin, OCT-4, SOX-2 and NANOG gene transcripts in primary buESCs and karyotypic analysis of primary buESCs. **a** Lane M-100 base pair DNA ladder, Lane 1-OCT-4 gene transcript in primary buESC colonies, Lane 2- β -actin gene transcript in primary buESC colony, Lane 3-Fibroblast feeder cells (negative control). **b** Lane M-100 base pair DNA ladder, Lane 1-SOX-2 gene transcript in primary buESC colonies, Lane 2- β -actin gene transcript in primary buESC colony, Lane 3-Fibroblast feeder cells (negative control). **c** Lane M-100 base pair DNA ladder, Lane 1-NANOG gene transcript in primary buESC colonies, Lane 2- β -actin gene transcript in primary buESC colony, Lane 3-Fibroblast feeder cells (negative control). **d** Metaphase spread showing euploid number of chromosomes in primary buESCs ($\times 1000$)

enzymatic methods are quite efficient in the establishment of colonies from hatched blastocysts, which harbor large and distinct ICMs. However, among blastocysts, there are also ones with smaller or indistinct ICMs. In these cases, the manual and enzymatic methods tend to result in the loss of the ICM when the lysed trophoectodermal layer is removed during the ICM isolation step. To overcome these limitations, the whole-embryo culture methods can be used as alternatives. When dealing with blastocysts harboring indistinct ICMs, the whole-embryo culture method is most frequently used. Kim et al. (2005) reported similar findings in derivation of human ESCs. Verma et al. (2007) reported the mechanical method to be better than the enzymatic method for derivation of buESC.

The criteria most commonly used to appraise cultured cells as ESCs are morphological features, AP activity, expression of molecular markers

characteristic to ESCs and normal chromosome number. The buESCs were found to form dome shaped colonies with a clear border, which were similar in morphology to earlier reports describing the colonies of mouse (Evans and Kaufman 1981; Martin 1981), bovine (Wang et al. 2005) and buffalo ESC colonies (Huang et al. 2007).

Embryonic stem cells express high levels of AP and loss of this activity has been identified as earliest indicator of a modified differentiation status. The buESCs established in this study were positive for AP activity. A high level of AP activity has been reported in pluripotent ESCs of other species like sheep (Dattena et al. 2006; Notarianni et al. 1991), rabbit (Graves and Moreadith 1993), pig (Notarianni et al. 1990; Wheeler 1994), cow (Roach et al. 2006; Stice et al. 1996), human (Thomson et al. 1998), buffalo (Huang et al. 2007; Verma et al. 2007), cat (Yu et al. 2008) and dog (Vaags et al. 2009).

A number of intracellular and extracellular markers have been used for monitoring the pluripotency of ESCs in many species. As per results obtained in our study, ESCs isolated from buffalo blastocysts expressed mRNA transcript for *OCT-4*, *NANOG*, *SOX-2* and cell surface antigens for SSEA-4 and TRA-1-81.

Embryonic stem cells require a coordinated network of transcription factors for maintenance of pluripotency. Among these OCT-4, NANOG and SOX-2 interact together and are central to the transcription regulatory hierarchy that specifies ESC identity (Chambers et al. 2003; Hart et al. 2004; Lee et al. 2004). All these transcription factors were strongly expressed in buESCs derived in this study. Our finding is similar to reports on expression of OCT-4 in other animal species like cattle (Wang et al. 2005; Yadav et al. 2005), goat (He et al. 2006; Pawar et al. 2009) and dog (Hatoya et al. 2006). SOX-2, an HMG domain-containing transcription factor is essential for pluripotent cell development (Avilion et al. 2003). The expression of SOX-2 has also been detected in human (Boyer et al. 2005), rhesus monkey (Navara et al. 2007) and pig (Hall 2008) ESCs. Repression of SOX-2 commonly results in trophoblast differentiation in mESCs (Ivanova et al. 2006; Masui et al. 2007) and hESCs (Fong et al. 2008) indicating its critical role in maintaining pluripotency. NANOG is a divergent homeobox transcription factor identified as a pivotal regulator of pluripotency in ESC (Chambers et al. 2003; Mitsui et al. 2003). Expression of NANOG has been reported in the ESCs of mouse (Chambers et al. 2003; Mitsui et al. 2003; Wang et al. 2003), monkey (Hart et al. 2004), human (Hatano et al. 2005) and goat ESCs (He et al. 2006). Our study confirms the expression of SSEA-4 on buESCs in conjunction with previous report (Huang et al. 2010). Among other animal species, sheep ESCs have been reported to express SSEA-4 (Dattena et al. 2006), while conflicting reports are available in cattle for the presence of SSEA-4 in ESCs. Gjørret and Maddox-Hyttel (2005), Mitalipova et al. (2001) and Wang et al. (2005) found bovine ESCs to be SSEA-4-positive, whereas Saito et al. (2003) reported that bovine ES-like cells do not express SSEA-4. The expression pattern of TRA-1-81 on buESCs, confirms the findings of Anand et al. (2011) and Sritanaudomchai et al. (2007) who reported their strong expression on ESCs. Expression of TRA-1-81 has also been reported on the

ESCs of other farm animals like cattle (Munoz et al. 2008), sheep (Bao et al. 2011), goat (Dutta et al. 2011; He et al. 2006) and horse (Guest and Allen 2007).

These results suggested that buESCs express pluripotency gene makers like ESCs/ES-like-cells isolated from other species. It has been well documented that the expression of these markers is related to the pluripotent state of ESCs and it changes both with development and differentiation in vitro. Expressions of these markers confirm the pluripotent state of the buESCs isolated in this study.

Cytogenetic analysis of primary buESCs revealed karyotypes with euploid number of chromosomes. However, some researchers have found that ESCs tend to have unstable karyotypes and acquire aneuploidies (Mitalipova et al. 2001). The buESCs isolated in this study maintained a stable karyotype.

Conclusions

In conclusion, the results of the present study demonstrated that selection of appropriate isolation technique depending on the quality of blastocyst is of utmost importance for improving efficiency of buESC derivation.

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Conflict of interest The authors indicate no potential conflicts of interest.

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