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Generation of buffalo embryonic stem cell-like cells from *in vitro* produced, day 8 hatched and day 9 expanded blastocysts

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ABSTRACT

The present study was taken up to isolate ICM cells from *in vitro* fertilized blastocysts for generation of BuES cell-like cells. A total of 69 hatched (HBs) and 84 expanded blastocysts (ExBs) were used for the isolation of ICM cells. The total cell number of day 8 HBs (149.3 ± 2.06) was significantly more than that of the day 9 ExBs (123.6 ±1.72). The attachment and primary colony formation rate of HBs was significantly higher over the ExBs, and time required for the attachment of ICMs to the feeder layer and primary colony formation was significantly low in HBs. After continuous sub-passage of primary colony obtained from HBs and ExBs, the cells were grown up to 11 and 6 passages, respectively. BuES cell-like cells appeared healthy, proliferative and exhibited dome shaped morphology, expressed alkaline phosphatase and had a normal karyotype. BuES cell-like cells also expressed *OCT-4* and *NANOG* and differentiated in to neuron like cells in the presence of *all*-trans retinoic acid. These differentiated cells expressed NF-68 gene. Thus intact method for isolation of ICMs from HBs (day 8) proved to be a superior method for generation of BuES cell-like cells than enzymatic method for ExBs (day 9).

Key words: Characterization, Differentiation, Embryoid bodies, Feeder layer, Neuron cells

Embryonic stem (ES) cells are the pluripotent cells with the capacity of long-term propagation and broad differentiation, which were first isolated from the inner cell mass (ICM) of mouse blastocysts (Evans and Kaufman 1981). Beside ICMs, blastomeres obtained from different stages of embryos are used to create ES cells in a variety of mammals, however, isolated blastomeres have successfully generated immortal pluripotent ES cells only in mice (Wakayama *et al.* 2007). Attempts were also made to establish ES cell-like cell lines from blastomeres of 4–8 cells in pig (Li *et al*. 2003); morula in cattle (First *et al*. 1994), dog (Hatoya *et al*. 2006), pig (Chen *et al*. 1999), buffalo (Verma *et al*. 2007); and from ICM in cattle (First *et al*. 1994), dog (Hatoya *et al*. 2006), goat (Zhu *et al*. 2007), pig (Piedrahita *et al*. 1990) and buffalo (Verma *et al*. 2007, Anand *et al*. 2011). Furthermore in relation to the embryonic age, which may be a critical factor in determining successful derivation of ES cells, no report is available in domestic animals to generate ES cells from day 8 blastocycts. *Oct-4*, *Nanog*, *Sox2*, *Foxd3* and *Rex1 genes* are thought to be central to the transcriptional regulatory hierarchy (Kirchhof *et al*. 2000, Hart *et al*. 2004). Besides the expression of these markers, most definitive test of pluripotency is the formation of chimeras in mice.

The generation of ES cell lines in farm animal would be useful in the genetic engineering. Pluripotent stem cells were used for the production of chimeras or transgenic animals in bovine (Cibelli *et al*. 1998) and swine (Piedrahita *et al*. 1998). Buffalo pluripotent stem cells might be a better material for developing new strategies to improve the yields of transgenic and cloned embryos or animals for developing nations. Therefore the present study was taken up to isolate ICM cells from hatched and expanded blastocysts obtained on day 8 and 9, respectively, for generation of BuES cell-like cells. Simultaneously, we examined alkaline phosphatase (AP) activity, chromosomal integrity and the expression of *OCT-4*, *NANOG* and *18S rRNA* genes by RT-PCR in the derived BuES cell-like cells, and determined the differentiation potential of these cells *in vitro* by embryoid body (EB) formation and induced differentiation.

MATERIALS AND METHODS

In vitro *production of blastocysts and total cell number:*

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Buffalo ovaries were obtained from a nearby abattoir and transported to the laboratory in an insulated container in 0.9% normal saline containing antibiotics 400 IU /ml penicillin and 500 mg/ml streptomycin at 32–37°C within 4–5 h. Oocytes were collected by aspiration of surface follicles (2–8 mm diameter) with a 19-gauge disposable needle attached to a 10 ml syringe containing TCM-199 and 0.6% BSA. The compact cumulus oocyte complexes with an unexpanded cumulus mass having <4 layers of cumulus cells with homogenous evenly granular ooplasm were used for *in vitro* maturation, fertilization and culture (Kumar *et al.* 2007). The total cell number (TCN) of HB and ExB obtained on days 8 and 9 post insemination respectively, was determined by staining. For this, the embryos were washed several times with DPBS and then transferred to slides containing a micro drop of 1 mg/ml Hoechst 33342 and 10% glycerol, prepared in DPBS, for 10 min. The number of nuclei was counted using a fluorescence microscope (excitation wavelength, 330–380 nm; barrier filter, 420 nm).

Preparation of fetal fibroblast feeder layers and their cryopreservation: Buffalo fetal fibroblasts feeder layers were prepared as per Kumar *et al*. (2011).

Isolation and culture of inner cell mass cells: The isolation of ICMs from ExBs, zona pellucida was removed by treatment with 1.0% pronase for ~6 min. Zona free blastocysts were treated with 0.25% trypsin-EDTA and observed under a zoom stereomicroscope until the trophoblasts became loose, and dispersed to release the ICM. For HBs, they were seeded as such onto feeder layers, were allowed to attach and proliferate. On the basis of morphological appearance of the colony, ICM was separated from trophectoderm with the help of fine needle and pasture pipette. The isolated ICMs were washed and seeded again onto mitomycin-C treated feeder layer individually. The ICM cells were cultured in 200 µl of ES medium in 4-well plate in 5% CO_2 at 38.5°C and examined at interval of 24 h with ES medium being replaced every day. The ES medium was DMEM supplemented with 20% FBS, 2 mM L-glutamine, 1000 IU/ml leukemia inhibitory factor (LIF), 1X nonessential amino acids and 50 mg/ml gentamycin sulphate. Subculture of ES cell colonies was performed by mechanically cutting out the colonies and dividing it into pieces with the aid of 2 fine glass needles under a zoom stereomicroscope. The primary colony formation rate; primary colony formation time in days, maximum number of passages and colony morphology were recorded carefully.

Alkaline phosphatase (AP) staining: AP staining was performed as per Kumar *et al.* (2011). The cells that stained red, which is an indicator of AP, were considered to be the BuES cell-like cells.

Karyotyping: Chromosomal integrity of BuES cell-like cells was analyzed by performing karyotyping. The BuES cells were incubated with 0.1µg/ml colcemid for 4 h at 37 °C. The colonies were washed, trypsinised and resuspended in freshly prepared 68 mM KCl and incubated for 30 min at 37°C. They were washed and fixed in chilled fixative (3:1, methanol: glacial acetic acid) for 20 min at room temperature and centrifuged at 200g for 10 min. The pellets were resuspended in 5 ml of chilled fixative for another 10 min and centrifuged again. The metaphase spreads were prepared by dropping the cells onto ice cold glass slides and stained with 2% Giemsa for 5–6 min. The glass slides were rinsed and observed under oil immersion (1000×) using a compound microscope.

Reverse transcription polymerase chain reaction (RT-PCR): For the isolation of total RNA, the cells were washed with ice cold PBS, 20–50 µl of cold cell lysis buffer was added and the reaction mixture was incubated in a thermal cycler at 75°C for 10 min. The cell lysate was treated with DNase-I at 37°C for 30 min to degrade genomic DNA and then heated at 75°C for 5 min to inactivate DNase-I. The cell lysate (10 µl) was used for making cDNA using random primer. The PCR cycle included heating to 94°C for 2 min, followed by repeated cycles of 94°C for 30 sec, annealing temperature 55–60°C for 30 sec, and extension at 72°C for 45 sec. this cycle was repeated 33–35 times with final extension for 72°C for 10 min. The following primers were used for PCR: for OCT-4 (accession no. AF022987), 5-GTTCTCTTTGGAAAGGTGTTC-3 and 5-ACACTCGGACCACGTCTTTC-3; for NANOG (accession no. DQ487022), 5-GGGAAGGGTAATGAGTCCAA-3 and 5-AGCCTCCCTATCCCAGAAAA-3; for NF-68 (accession no. NM174121), 5-AGGAAGATGCTGAGGAAGCA-3 and 5-GTTGACCTGATTTCGGGAGA-3; for 18S-rRNA (accession no. AF176811), 5-GAGAAACGGCTACCACATCC-3 and 5-GGACACTCAGCTAAGAGCATCG-3; for ACTIN (accession no. K00622/BC016624), 5-TGAACCCTAAGGCCAACCGTG -3 and 5-TGTAGCCACGCTCGGTCAGG -3. A –ve RT reaction (i.e. without MMLV enzyme) was set with every batch of cDNA preparation to check genomic DNA contamination and a –ve PCR without template was also set. Either 18S rRNA or ACTIN was amplified at each stage as a house keeping marker gene. Feeder cells and ES cells were used as negative control for ES cells and differentiated cells respectively. Amplifications yielded products of 341 bp (OCT-4), 211 bp (NANOG), 183 bp (NF-68), 337 bp (18S rRNA) and 268 bp (ACTIN).

Embryoid body formation and induced differentiation in vitro: For evaluation of differentiation capacity of BuES cell-like cells *in vitro*, the P-4 and P-9 BuES cell-like cells were disaggregated into small clumps using 2 fine needles. The dissociated single cells or small clumps were transferred to suspension culture dishes containing ES medium without LIF and were cultured in the absence of feeder layer. The cells were fed every alternate day by tilting the plate, allowing cells to settle, and carefully removing the medium. After 10–

12 days, the EBs were formed which later converted to cystic EBs. Furthermore, BuES cell-like cells were cultured in the presence of 10^{-6} M *all*-trans retinoic acid to evaluate its differentiation into neurons and for its confirmation, check the expression of NF-68 gene through RT-PCR.

Statistical analysis: Data were analyzed using SYSTAT 7.0 after arcsine transformation. Differences between mean % were analyzed by one-way ANOVA followed by Fisher's LSD test for pair wise comparison of means. The determination of total cell number of HBs on day 8 and ExBs on day 9 post insemination were analyzed by unpaired t-test.

RESULTS AND DISCUSSION

HBs (69) and ExBs (84) were obtained on day 8 and 9 post insemination, respectively, and were used for the isolation of ICMs. The TCN of day 8 HBs (149.3 ± 2.06) was significantly more (P<0.01) than that of the day 9 ExBs (123.6 ±1.72). The primary colony obtained from HBs and ExBs through intact culture and enzymatic method, respectively, is shown in Fig. 1. The attachment rate and primary colony formation rate of HBs was significantly higher (P<0.01) over the ExBs, and time require for the attachment and primary colony formation was significantly low (P<0.01) in HBs (Table 1). After continuous sub passage of primary colony obtained from HBs, the cells were able to grow up to 11 passages whereas cells survived only 6 passages derived from ExBs. Our observation suggests that the source of ICM is an important criteria for ES cell derivation in buffalo where HBs obtained on day 8 had significantly higher (P<0.01) attachment rate and primary colony formation rate, obtained in relatively shorter time period in comparison to the ExBs collected on day 9. This observation prompts towards better ICM compaction, presence of more viable ICM or higher ICM cell number leading to an increased efficiency of BuES cell-like cell derivation from day 8 HBs compared to day 9 ExBs. Further superiority of HBs over ExBs could be because of 2 more reasons. Firstly, since the HBs were seeded intact on the feeder layers without being exposed to any proteolytic enzyme, the damage to the ICM cells could be expected to be minimal. Secondly, since HBs are at a stage of development, which is more advanced than ExBs, they possess a higher number of ICM cells that could have given them an edge over the ExBs. In earlier studies too, the isolation of ICMs either from morula, early stage of blastocyst or late stage of blastocyst gave an unsatisfactory result as compared to HB in various species (Chen *et al*. 1999,Verma *et al*. 2007). Establishment of pig ES-like cell cultures was decidedly greater (21 vs 0%) from recently HBs than from late-HBs (Chen *et al*. 1999) probably because the early HBs had fewer trophectoderm cells and more prominent ICM cells. Traditionally, ICMs were isolated from blastocysts by immunosurgery (Solter and Knowles 1975, Yadav *et al*. 2005), but it is not a method of choice because of the need of

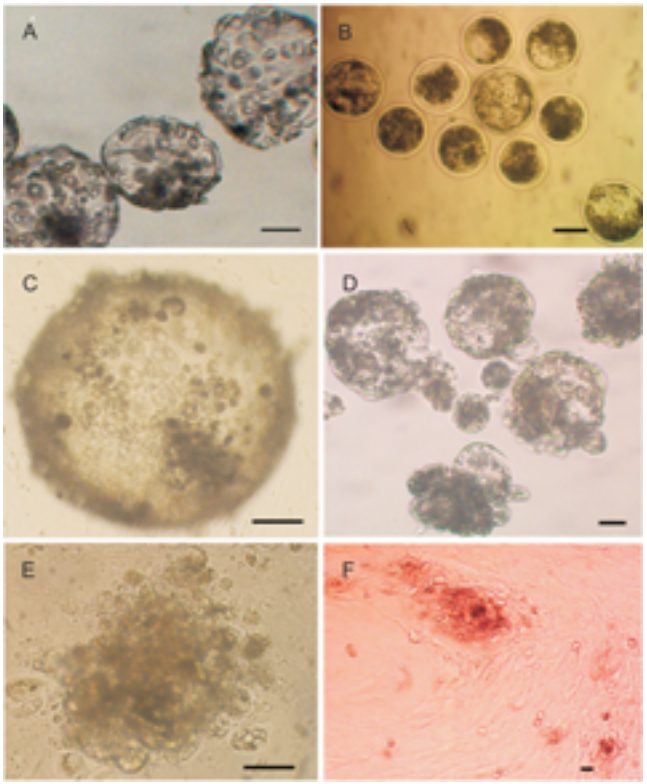

Fig. 1. Formation of primary colony after isolation of ICM through intact culture of HBs and enzymatic treatment of ExBs. A group of *in vitro* produced HBs collected at day 8 (A), and ExBs collected at day 9 post-insemination (B), HB seeded intact on buffalo fetal fibroblast feeder layer for isolation of ICM (C), ICM isolated by enzymatic treatment from ExBs (D), primary colony of BuES cells from intact method of HB (E), and from enzymatic method of EB (F). Bar =50 µm (A, C-F), and 100 µm (B).

Table 1. Isolation and culture of embryonic stem cell-like cells from buffalo HBs and ExBs obtained on day 8 and 9 post-insemination

Source material	Blastocysts seeded (n)	Attachment rate (%)	Attachment time (days)	Primary colony formation rate (%)	Primary colony formation time (days)	Number of passages up to which cells survived
Hatched blastocysts	69	72.5 ± 5.75[a]	2.4 ± 0.11[a]	61.6 ± 6.29[a]	4.2 ± 0.22[a]	11
Expanded blastocysts	84	42.8 ± 4.16[b]	3.5 ± 0.13[b]	35.2 ± 4.64[b]	5.1 ± 0.28[b]	6

Values are mean ± SEM.Values with different superscripts within column differ significantly at (P<0.01).

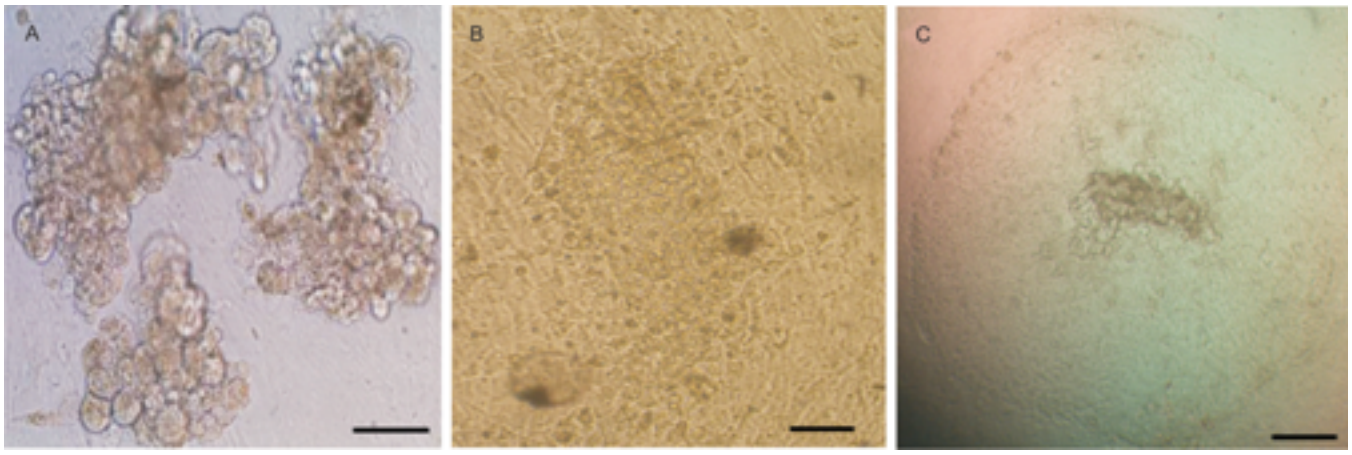

Fig. 2. Morphology of BuES cell-like cells at different passages of *in vitro* culture. Colony of BuES cells appear compact and dome shaped (A), at passage 4 (B) and at passage 10 (C). Bar =50 µm (A), 100 µm (B and C).

species-specific antiserum for binding to the outer layer of trophectoderm cells and time consuming procedure involved more labor. So, in this study, we adopted a simple and efficient method for isolation of ICMs from HBs i.e. intact culture of blastocyst on feeder layer followed by separation of ICM on the basis of difference in morphological appearance which probably gave pure quality of ICMs. The similar kind of method was used in ES cell derivation from parthenogenetic buffalo embryos (Sritanaudomchai *et al*. 2007), porcine IVF embryos (Miyoshi *et al*. 2000) and cattle embryos (Talbot *et al*. 2007). Upon comparison of intact blastocyst culture method with the widely used enzyme treatment method, we observed a better efficiency of former in terms of BuES cells derivation suggesting that lysis of trophectoderm cells was not completed and that the reaction of the enzyme might eventually interfere with the derivation of ES cells. Our such results correspond with the previous result by Shiue *et al*. (2006) where primary ES cell colonies could not be identified easily and trophectoderm colonies were still established near pluripotent ES cells after using enzyme treatment method for isolating ES cells in porcine.

The morphology of BuES cell-like cells derived either from HBs or ExBs was same. The BuES cell-like cell colonies appeared healthy, proliferative and showed tight or compact cells, had high nucleus to cytoplasm ratio with dome shape and a clear margin (Fig. 2A), and maintained at different passages of *in vitro* culture (Fig. 2B and 2C). During later passages the cells colonies lost their integrity, compactness and floated in culture medium indicating sign of degeneration (Fig. 3A and 3B). The expression of AP was positive in BuES cell-like cells colonies grown on buffalo fibroblast feeder layer cells (Fig. 3C). In previous study, the morphology of BuES cell-like cells grown on buffalo fetal fibroblast was similar to our study (Verma *et al*. 2007), and closely resembled porcine ES cells (Gerfen and Wheelar 1995). The proliferative potential of derived BuES cell-like cells was confirmed by the expression of AP as also observed previously in buffalo (Verma *et al*. 2007, Huang *et al*. 2010, Anand *et al*. 2011), sheep (Dattena *et al*. 2006) and dog (Hatoya *et al*. 2006). This however is contrary to observation in cattle where the ES cell–like cells have been reported to lose AP expression after 18–22 h of culture (Mitalipova *et al*. 2001).

In addition to this, chromosomal status of BuES cell-like cells analyzed at passage 5 and 10 was found to be normal with number of chromosomes being 2n=50 (Fig. 3D). We also preformed the RT-PCR for assessing the expression of *OCT-4* and *NANOG* genes in AP positive colonies of BuES cell-like cells. We found that the expression of *OCT-4* (Fig. 4A) and *NANOG* (Fig. 4B) were positive in BuES cell-like

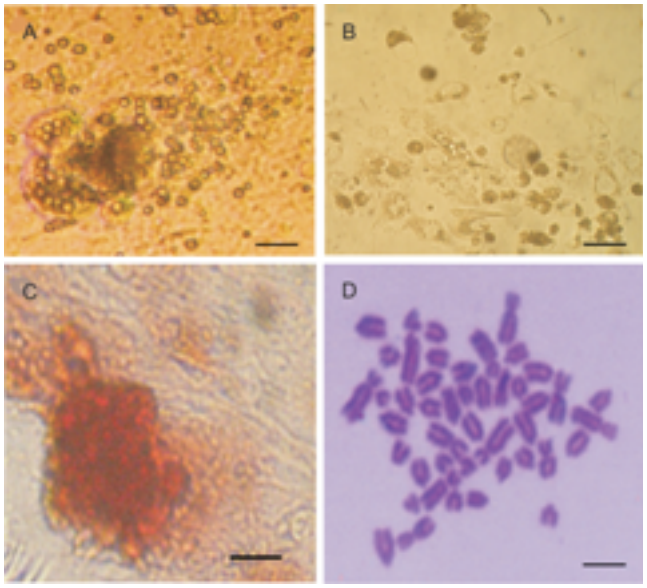

Fig. 3. Degenerated colonies of BuES cell-like cells showing floating appearance (A) and lack of compactness and scattered cells in culture (B), BuEs cell-like cells showing alkaline phosphatase expression (C) and exhibited a normal karyotype at passage 10 (D). Bar =100 µm (A-D).

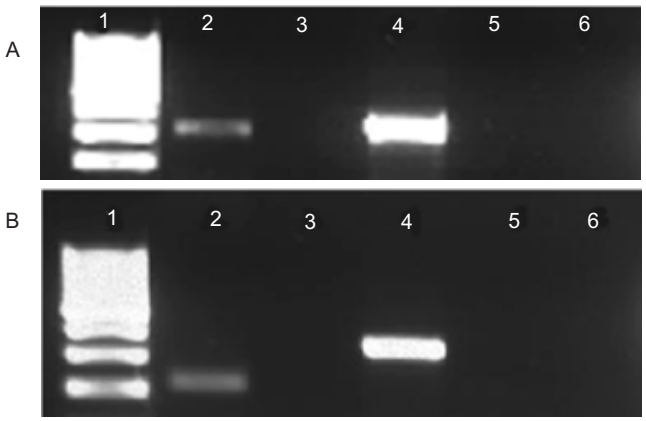

Fig. 4. RT-PCR analysis of gene expression in BuES cell-like cells. Expression of *OCT-4* (A) and *NANOG* (B) shown in lane 2 whereas lane 1. Marker (100 bp); 3. Feeder cells; 4. 18-S rRNA (337 bp); 5. –ve RT; and 6. –ve PCR.

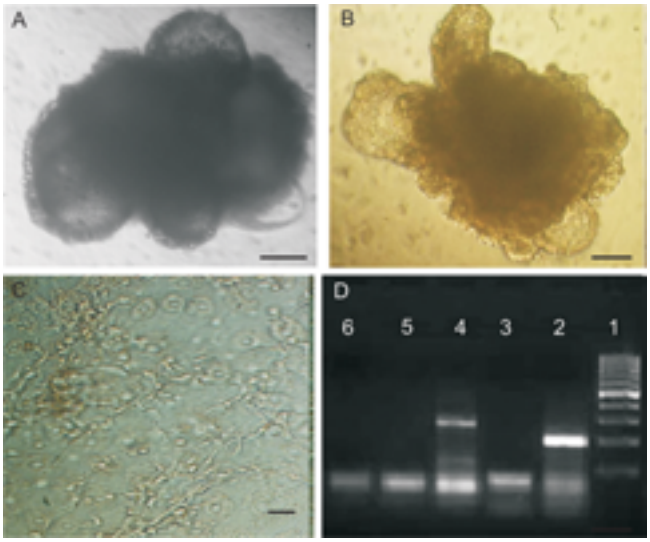

Fig. 5. *In vitro* differentiation of BuEs cell-like cells. The BuES cells cultured in suspension in the absence of LIF and feeder cells formed EB (A), cystic EB (B), induced differentiation of BuES cells into neuronal cells in the presence of *all*-trans retinoic acid (10^{-6} M) and (C) and characterization of induced neurons by expression of NF-68 gene (D), where lane 1. Marker (100 bp); 2. NF-68 (183bp); 3. –ve RT; 4. ACTIN (268 bp); 5. fibroblast cells; 6. BuES cells. Bar = 50 µm (A, B) and 100 µm (C).

cells derived from both HBs and ExBs. These markers were used as indicator of ES-like cells among mammals (Yadav *et al*. 2005, Hatoya *et al*. 2006, Verma *et al*. 2007, Anand *et al*. 2011), as they have essential roles in early development and are required for the propagation of undifferentiated ES cells in culture (Chambers *et al*. 2003, Hay *et al*. 2004). Furthermore, to determine the pluripotent ability of BuES cell-like cells, cells were cultured in suspension they formed EBs (Fig. 5A) within 10–12 days in absence of LIF and feeder cells. These EBs increased in size with increased period in culture leading to formation of cystic EBs (Fig. 5B). The formation of EBs was observed in almost all species like dog (Hatoya *et al*. 2006), sheep (Dattena *et al*. 2006), bovine (Yadav *et al*. 2005) and buffalo (Chauhan *et al*. 2006, Verma *et al*. 2007). However, the BuES cell-like cell colonies growing on buffalo fetal fibroblast were separated and disaggregated into small pieces using 2 fine needles and cultured in the presence of *all*-trans retinoic acid containing media, after 10–12 days of culture, these cells differentiated to neuron like cells (Fig. 5C) having long, slender bipolar cells in appearance, which were found to express *NF-68* gene (Fig. 5D) and hence proved to be neurons indeed. The treatment of ES cells with retinoic acid has been reported to promote the expression of neural genes while repressing mesodermal genes (Bain *et al*. 1996). Our result is similar to that earlier reported in mouse (Lang *et al*. 1989) and to our knowledge, this is the first report to document the induced differentiation of BuES cells to neurons and their characterization by expression of NF-68 gene.

The results of present study showed that *in vitro* produced day 8 HBs and intact method were superior for generation of buffalo ES cells over ExBs obtained on day 9 and enzymatic method. The generated ES cells were maintained *in vitro* undifferentiated for prolonged period of time and many characteristic feature of BuES cell-like cells were demonstrated by assessing the morphology of cells, chromosomal stability, analysis of pluripotent marker expression like AP, *OCT-4*, *NANOG*, formation of EBs, cystic EBs and by subjecting to induced differentiation to neuronal lineage which was further confirmed by neuron specific marker expression.

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