Full Length Article

Establishment of Embryonic Stem Cell like Cell Colonies from *In-vitro* Produced Buffalo Compact Morulae

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ABSTRACT

*The study was aimed at establishing the embryonic stem cells (ESC) like cell colonies from in-vitro produced buffalo (Bubalus bubalis) compact morula (CM). A total of 33 CM were subjected for zonalysis using pronase and embryonic cells were derived by three different methods viz., **T 1**: Intact CM; **T 2**: Disaggregation of CM by gentle pipetting and **T 3**: Slicing of CM using Bard Parker blade. The efficiency of establishment of ESC-like cell colonies in the three groups were 66.7, 83.6 and 6.7 per cent respectively. There were no significant differences on the day of attachment and initiation of primary colony formation between T1 and T2 groups. However, the primary colonies established significantly earlier (Day 3.8) in T2 group than in T1 group (Day 4.8). In both the groups, the primary colonies spread out vigorously exhibiting rapid proliferation rate. The ESC-like cell colonies of T1 and T2 groups survived upto four passages. Immunostaining of CM, primary and passaged ESC-like cell colonies revealed distribution of Oct-4 protein. The study demonstrated successful establishment of ESC-like cell colonies from pipette dissociated blastomeres of in-vitro produced CM.*

Key Words: Buffalo, Compact morula, Embryonic stem cell colonies

INTRODUCTION

Embryonic stem cell (ESC) is a powerful tool to understand the mechanisms that control early developmental processes and substantial attention is being given for their potential in regenerative medicine. The stage of embryonic development at which ESCs are derived may be crucial to their subsequent establishment into colonies and cell lines (Ito et al., 1996). Successful attempts have been made in mouse, bovine and human to produce pluripotent ESC lines after the culture of blastomeres obtained from morulae (Eistetter, 1989; Strelchenko, 1996; Strelchenko and Verlinsky, 2004). However, such attempts to produce ESC-

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like cells from morulae in buffaloes remained unsuccessful (Verma *et al.,* 2007 and Huang *et al.,* 2010).

We hypothesized that compact morula would be a more appropriate stage to derive ESC like cells, when blastomeres are not committed for differentiation, but has more lineage towards undifferentiated ICM than morula. An analogous cell aggregation occurs in the morula stage embryos prior to formation of blastocyst, which could be visually identified as compact morula (CM). Hence, the present investigation is attempted to derive ESCs from blastomeres of *in-vitro* produced buffalo CM.

MATERIALS AND METHODS

All the chemicals used for *in vitro* production of embryos and culture were procured from Sigma Aldrich (USA) unless otherwise mentioned specifically.

In-vitro production of compact morulae

Ovaries from sexually mature buffaloes (*Bubalus bubalis)* were collected irrespective of their age, breed and stage of oestrous cycle from Chennai Corporation abattoir and utilized for the study. In the laboratory, the extra-ovarian tissues were trimmed off and the ovaries were washed in running tap water and normal saline. All the visible follicles (>2 mm diameter) were aspirated using a sterile 18 or 20G hypodermic needle attached to a 10 ml disposable syringe. The collected follicular fluid was transferred to 90mm gridded petridish and screened for oocytes. All the cultureable quality oocytes i.e., oocytes enclosed in a compact cumulus mass of ≥ 3 layers with evenly granulated cytoplasm were selected and subjected for *in-vitro* production of embryos. The *in-vitro* maturation, *in-vitro* fertilization and *in-vitro* embryo co-culture were performed as described by Satheshkumar *et al.* (2017). Embryo development was observed for nine days post-insemination and embryos that attained the CM stage were utilized for the study.

Preparation and inactivation of buffalo fetal fibroblast feeder layer

Buffalo fetuses (around 60 days old) were collected from slaughtered animals and used for preparing buffalo fetal fibroblast feeder layer (BFF) as described by Verma *et al.* (2007). The feeder layer was inactivated with mitomycin C (10µg/ml) for two hours (Munoz *et al.,* 2008), after which the feeder layer was washed off and replaced with ESC media.

Derivation of embryonic stem cell like cells

CM were zonalysed by incubating in 0.5 per cent pronase and were subjected to three protocols for the derivation of embryonic cells viz., **T1:** The zona free CM were seeded intact (n= 12 intact CM); **T2:** The blastomere cells of the zona free CM were dissociated by repeated pipetting with a narrow bore pipette (n= 44 cell clusters) and **T3:** The zona free CM were placed individually in 5 µl PBS media and sliced mechanically using No.11 Bard Parker blade (n= 30 sliced cells). The intact CM / cell clusters/ slices (Figure - 1) were washed in ESC medium and seeded over the mitomycin inactivated feeder layer. A total of 18 trials were conducted with six trials in each group.

Seeding and culturing of embryonic cells

The intact CM/cell clusters/slices of CM seeded onto the inactivated feeder layer (Day 0) were cultured at 38.5ºC in a humidified atmosphere of 5% CO_2 in air. The cells were monitored regularly at 24 h interval. Once the cells were found attached to the feeder layer the ESC medium was changed every two days. The cell clusters were observed for attachment, formation and establishment of primary colonies as described by Tesar (2005). When got established, the primary colonies were dissociated mechanically as described by Verma et al. (2007) and reseeded to another culture plate of mitomycin C treated feeder layer. Further passages were performed in the same procedure, every four to five days until the ESC like cell colonies lose their characteristic attachment and establishment.

Characterization of Embryonic stem cell colonies

Morphological characterization

The ESC-like cell colonies were identified and monitored based on their morphological characteristics as described by Chen *et al.* (1999) and Li-Wang *et al.* (2005). The colonies which have flat, polygonal/round shaped cells with clear border and faster proliferation rate were considered as typical ESC-like cell colonies.

Fluorescence Antibody Technique

One representative primary and passaged colony in each trial was retrieved mechanically along with some accompanying layer of BFF and then subjected for Fluorescence antibody technique (FAT) against *Oct-4* protein to confirm the property of stemness by the method described by Peura *et al.* (2007). The derived ESC like cell colonies were fixed with 4% Paraformaldehyde (Merck UN 2213) in a glass slide and incubated in primary antibody (Anti Oct-3/4 (N-19)-Goat IgG; Santacruz Biotechnology-SC-8268) and secondary antibody rhodamine conjugate (Anti-Goat IgG, Donkey-Rhodamine Conjugate; Millipore AP 18-0R). The cell colonies were observed under fluorescence microscope at a wave length of 520-560 nm after adding DAPI /antifade (Millipore S1173). The fluorescence of reddish yellow colour was considered positive for stemness characteristics.

Statistical analysis

The data on various parameters of establishment of ESC-like cell colonies were compared and analysed between different treatment groups by student t-test (Snedecor and Cochran, 1994)

RESULTS AND DISCUSSION

A total of 1420 oocytes were retrieved from 865 buffalo ovaries with a mean recovery rate of 1.7 ± 0.1 oocytes per ovary. Out of the 562 (39.6%) cultureable quality oocytes, 33 (5.9 ± 0.8 %) CM were produced.

The characteristics of primary ESC like cell colonies established in all the three treatment groups (T1, T2 and T3) were presented in Table 1 and Fig.1 (a, b and c). When zona free intact CM (T1) were seeded, two variable modes of establishment of primary colonies were observed. In the first mode, the intact embryos initially got dissociated and then

attached to the BFF layer by Day 5 and the resultant atypical ESC colony got detached by Day 7. These colonies did not establish further on passaging. In the second mode, primary ESC-like cell colonies were established and the data from these colonies were considered for further statistical analysis. The overall establishment rate of 66.67 per cent in the present study was much higher than 36.4 per cent reported by Zhang et al. (2006) while intact CM were cultured for establishing human ESC colonies. Eistetter (1989) suggested that the variability in establishment of ESC colonies when cultured intact might be due to over exposure of cells to the differentiation stimulating influences.

Table – 1. Characteristics of Primary Embryonic Stem Cell like cell colonies in different groups

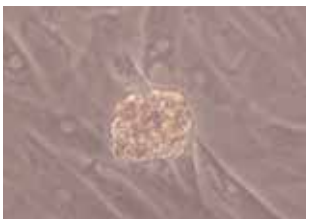
S.No	Treatment Groups	No. of Cells/ Clusters/ Slices seeded	No. of Primary Colonies formed	Day of initial attachment (Mean ± SE)	Day of initiation of colony formation (Mean ± SE)	Day of establishment of primary colony (Mean ± SE)
1.	**T 1**	12	8 (66.7 ± 21.2) ^{a}	1.0 ± 0.0 ^{a}	2.3 ± 0.3 ^{a}	4.8 ± 0.3 ^{b}
2.	**T 2**	44	36 (83.6 ± 7.6) ^{b}	1.0 ± 0.0 ^{a}	2.2 ± 0.2 ^{a}	3.8 ± 0.3 ^{a}
3.	**T 3**	30	2 (6.7 ± 0.0) $^{@}$	1.0 ± 0.0	2.0 ± 0.0	--
	Significance		**	NS	NS	*

Percentage inparanthesis (Mean ± SE)
Values within columns with different superscripts differ significantly
*: Significant ($P \leq 0.05$) ** : Significant ($P \leq 0.01$)
NS : Not significant ($P > 0.05$) @: Statistically not comparable

When pipette dissociated embryonic cells (T2) were seeded onto the BFF, their efficiency of attachment and establishment of primary ESC like cell colonies was 81.8 per cent. The present finding was higher than that reported (61.0 %) by Strelchenko and Stice (1994), who disaggregated bovine morulae using trypsin (0.25%) or pronase (0.5%). The reduced response reported by these researchers might be due to the damage caused by the enzymes to the zonalysed embryos as suggested by Ito et al. (1996). The characteristics of formation, establishment and proliferation rate of primary colonies corroborated with the reports of Eistetter (1989), who utilised the pipette dissociated blastomeres of mouse morulae.

None of the sliced cells (T3) established as colonies in concurrence with the findings of Huang et al. (2010). They also disaggregated the buffalo morulae into several pieces using pin head, similar to the method followed in the present study and found that none of the isolated cells formed colonies. Verma et al. (2007) suggested that the irreparable damage caused to the blastomeres during slicing might be the cause for non-establishment of colonies.

Figure -1. Characteristics of ESC-like cell colonies derived by different methods

T1			
	Day 0- Zonalysed CM (200x)	**Day 1- Attachment of intact CM (400x)**	**Day 5 – Establishment of ESC-like cell colony (200x)**
T2			
	Day 0 - Pipette dissociated CM (200x)	**Day 1 – Attachment of cell cluster (400x)**	**Day 3 – Establishment of ESC-like cell colony (200x)**
T3			
	Day 0- CM slices (200x)	**Day 1 – Attachment of cells (400x)**	**Day 3 – Detachment of colony (400x)**

Thus it was concluded that establishment of colonies was better ensured when pipette dissociated blastomeres of CM were utilized and there was a high inconsistency when seeded intact or sliced. Similarly First et al. (1994) also expressed that morula derived cell lines have not usually established without disaggregating the cells.

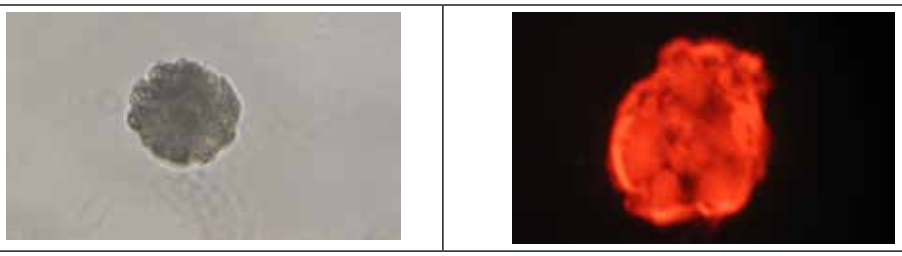
ESC-like cell colonies were found to be flat, polygonal shaped cells with clear border and easily distinguishable from the feeder layer. The typical ESC-like cell colonies were also found to spread out vigorously in a faster rate. In both the T1 and T2 groups, the ESC-like cell colonies successfully survived upto four passages. Verma et al. (2007) and Huang et al. (2010) who utilized *in-vitro* produced CM also reported failure in passaging the colonies.

The pluripotency of ESCs was reported to be maintained by transcription factor, *Oct-4*, a product of the *Pou5f1* gene and has been widely accepted as a pluripotent cell marker. Mitalipov et al. (2003) stated that embryonic expression of *Oct-4* initiated at the 4- to 8-cell stage with strong nuclear localization of the protein in all blastomeres during the morula stage. In the present study, immunostaining of CM (Fig. 2a) revealed

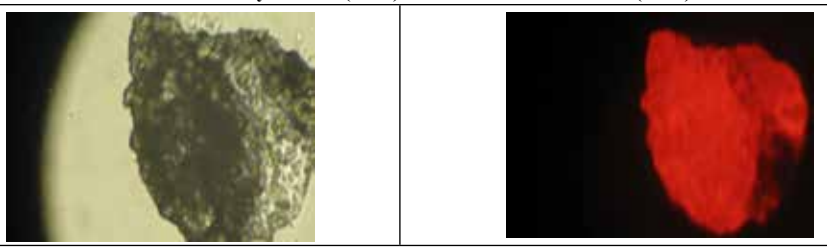
expression of *Oct-4* protein confirming their pluripotent nature, and thus supporting our view for utilizing the CM stage embryo for development of ESC like cell colonies. The primary and passaged ESC-like cell colonies specifically expressed *Oct-4*, where as the BFF feeder layer cells around the colony served as negative controls and did not expressed the marker signal (Fig. 2b and 2c). It was observed that primary colonies expressed intense fluorescence, while the colonies of fourth passage exhibited moderate fluorescence indicating the probable loss of pluripotent nature. These expression patterns ascertained the status of pluripotency in the cultured ESC-like cells derived from CM. Similarly, Strelchenko and Verlinsky (2004) opined that morula derived human ESC lines have expressed the ESC specific markers, including *Oct-4* same as that of the ESCs derived from blastocysts.

Based on the findings, it was concluded that buffalo ESC-like cell colonies could be successfully established from CM and the derivation of embryonic cells from pipette dissociated CM was found to be highly efficient and consistent in establishing colonies.

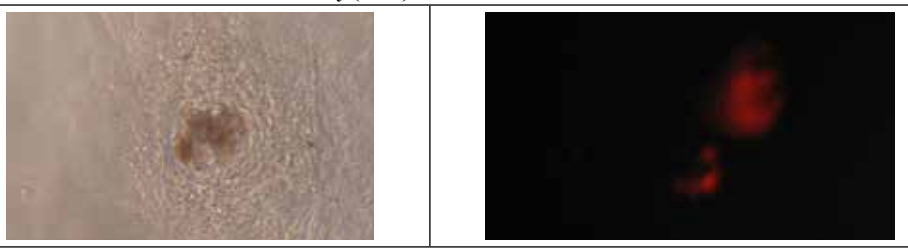
Figure – 2. Immunostaining against *Oct-4* of CM and ESC-like cell colonies



a. Zonalysed CM (200x) and Immunostained CM (400x)



b. Fixed ESC-like cell primary colony along with BFF (400x) and Immunostained ESC-like cell primary colony (400x) – Intense fluorescence



c. ESC-like cell colony from fourth passage (200x) and Immunostained ESC-like cell passaged colony (200x) – Moderate fluorescence

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