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Effect of different concentrations of serum supplementation on proliferation of embryonic cells derived from early stage *in-vitro* buffalo embryos

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ABSTRACT

An attempt was made to study the effect of different culture condition on derivation of ES cell from IVF derived early stage embryos in buffalo. Early stage 16–32 cell stage IVF derived embryos were treated with proteinase-K for zona removal. The zona free embryos were cultured on mitomycin-c inactivated buffalo feeder cells in medium supplemented with different percentages of serum. It was observed that blastomere clump attached to the feeder cells within 24 h and multiplied. Supplementation of 20% FCS to the culture supplementation supported the multiplication of blastomere cells better than the supplementation of 10 or 15% serum. In some cases, there was formation of blastocyst like structure with distinct inner cell like mass although there were no trophectodermal cell at the border. When the inner cell like mass from the blastocyst like structure was cultured on inactivated feeder cells, no primary colony developed.

Key words: Buffalo, ES cells, Culture condition

Embryonic stem cell (ES) has been recognized as one of the most promising therapeutic tools for the next decade. The totipotent stem cells can be a better choice for reproductive cloning as well as making different body cells easily and efficiently. ES cells have been derived from different stages of embryos in different species (Evans *et al*. 1981, Thomson *et al*. 1998). However no cell lines could be made so far in buffalo (Verma *et al*. 2007, Huang *et al*. 2008). In buffalo inner cell mass from blastocyst has been proved to be the stage for formation of ES cell like colonies but they could not be sustained more than 6–10 passages (Huang *et al*. 2008). The failure of ES cell lines in this species may be due to lack of a proper culture media. In the present study, the effect of different percentage of serum supplementation was observed on the multiplication of blastomere cells in an attempt to derive ES cell line from very early stage *in vitro* derived buffalo embryos.

MATERIALS AND METHODS

Oocytes were aspirated from all visible non-atrectic follicles and the cumulus oocyte complex (COC) were isolated, evaluated and graded as per Kobayashi *et al*. (1994). Only excellent and good COC were cultured at 38.5°C, 5% CO_2 and 21% oxygen under humidified air (90%) for 24 h in CO_2 incubator in maturation medium. After maturation, the oocytes with good cumulus expansion were co-incubated with capacitated sperm. After 18 h of sperm oocyte co-incubation, the oocytes (presumptive zygotes) were washed 10–15 times in embryo development medium, viz. modified synthetic oviductal fluid (mSOF), and cultured in the same media supplemented with 0.8% BSA. The cleavage rate was recorded after 48–72 h of post insemination and further observations were made to monitor the development of embryos. The embryos of 16–32 cells were treated with protease-K for the dissolution of zona pellucida. After complete dissolution of zona pellucida, the clumped blastomere cells were cultured on mitomycin-c inactivated buffalo embryonic feeder in ES cell medium supplemented with different percentage of fetal bovine serum. The medium used were: (i) DMEM + LIF + ITS + ß-mercaptoethanol + 10% FBS—(group 1), (ii) DMEM++ LIF + ITS + ß mercaptoethanol +15% FBS (group 2), and (iii) DMEM++ LIF + ITS + ß-mercaptoethanol + 20% FBS (group 3). Seeing the multiplication of cells, the blastomere growth and establishment of embryonic stem cells, if any, were observed in each group.

RESULTS AND DISCUSSION

In the present study, different percentages (Table 1) of serum was tested for derivation of ES cell from early stage *in-vitro* derived buffalo embryos (Fig. 1A). In all groups,

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Table 1. Effect of serum on multiplication of blastomere cells of IVF derived buffalo embryos

Total ovaries	Culturable oocytes	16–32 cell stage	Media used	Details of stem cells clone
133	72	19	DMEM+ 10% FBS+LIF +ITS+β-mercaptoethanol	Clumped blastomere attached to feeder at D-1. Growth of blastomere clones arrested on D-3
48	30	8	DMEM(4 mg/ml)+ 15% FBS+LIF+ ITS+ß-mercaptoethanol	Growth of blastomere clones arrested on D-4
117	75	22	DMEM(4 mg/ml)+ 20% FBS+LIF+ITS +ß-mercaptoethanol	Growth of stem cell clones for a long period but could not make ES cell colonies on passage.

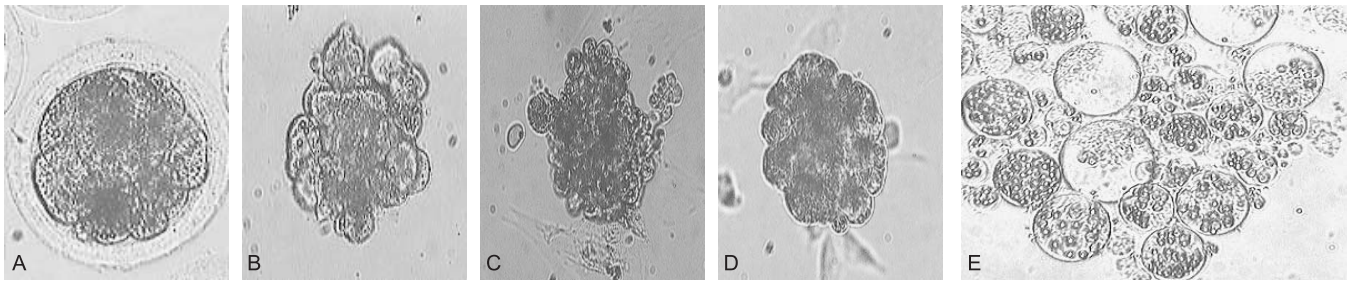

Fig. 1(A–E): A. Early stage buffalo embryos, 10×; B. zona free embryos (10×); C. after 3 days of culture in 20% FBS (32×); D. after 3 days culture with 10% FBS (10×)'; E. formation of tiny cells within blastomere and blastocyst with inner cell mass (20×).

the clumped blastomere was found attached to the feeder within 24 h (Fig. 1B). Within 3 days of attachment more than 100 cells were formed (Fig. 1C). However, some of the clumped blastomeres even after attachment did not grow further (Fig. 1D). After 3 days, there is the formation of tiny cells within each blastomere for which the blastomere size increased (Fig. 1E). There was also appearance of vacuole like structure in blastomere. In group 3, some blastomeres developed into blastocyst like structure having inner cell like mass at one end, which increased over the time (Fig. 1E) and the blastocysts were devoid of any trophectodermal outer cell lining. Unfortunately, when the inner cell like mass was isolated and put on inactivated feeder, no cell colony emerged. It was observed that 20% serum supported long term growth and multiplication of blastomere than 10 or 15% serum supplement. The blastomere latter multiplied but after 3 days the growth of the cells arrested.

So far no ES cell colonies have been derived in buffalo globally and attempt with different stage of buffalo embryos failed to derive true ES cell clones. However, proven pluripotent ES cell line have been derived in mice, and primate of different stages of IVF derived embryos (Martin 1981, Tesar 2005). It is surprising to note that buffalo blastomere or ICM did not grow after certain passage (Huang *et al*. 2008). The failure indicated that a specific media or culture conditions must be evolved so that ES cell lines can be derived from buffalo. Unfortunately the media used for ES cells derivations in buffalo is almost same for mouse. It is known that for growth of mouse embryo different media is required than that of bovine. Therefore, it is imperative that for ES cell derivation, a separate media should be made so that repeatable proven ES cell lines can be generated from different stages of buffalo embryos.

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