



## Identification and Characterization of totipotent and pluripotent stem cells from buffalo embryo and fetal placenta

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### Abstract

*The present study was carried out with the aim to isolate, culture and characterize the totipotent stem cells from in vitro derived embryos and pluripotent stem cells from a month-old fetus in buffalo. The oocytes were matured, fertilized and the embryo development upto 16 cell stage were recorded under 15% O<sub>2</sub>, 5% CO<sub>2</sub>, and 38.5 °C temperature and 90% humidity. The overall cleavage rate and morula development was 9.1 % and 4.6% respectively. The results indicated that blastomeres from 2 cell, 4 cell and 8 cell state embryo did not grow and degenerated. However, blastomere cells isolated from 16 cell stage embryos cleaved and formed clones. Pluripotent stem cells were derived from 30-40 days old buffalo fetus and cultured on buffalo fibroblast cell monolayer with RPMI-1640 and 15% FBS in the presence of LIF. The pluripotent stem cells and totipotent s were characterized and found positive for SSEA-1,4, EMA1 and alkaline phosphatase and negative for SSEA-3. From this result it was concluded that totipotent and pluripotent stem cells shall be identified differently and more specific markers needed to study before using these cells for specific reproductive technologies.*

### Introduction

Stem cell research has gained momentum in reproductive biology and medicine in recent years due to its importance in health and reproduction. A lot of knowledge gap is persisting which needs to be worked out in the field of stem cell research. Although, it has been known that totipotent cells capable of giving rise to the entire conceptus and would enhance our capacity to study early embryo development, and might enable more efficient generation of chimeric animals, totipotent stem cells marker identification is still ambiguous in

some species. Although there are couple of reports which are published in recent years where the pluripotency analysis of embryonic stem cells has been done<sup>1</sup>.

In most organisms, development commences following fusion between sperm and eggs to generate a zygote, which gives rise not only to a new individual but, theoretically at least, to an endless series of generations. In this way, germ cells provide the enduring link between all generations. The newly fertilized egg or zygote is therefore unique because no other cell has the

potential to develop into an entirely new organism. This property is referred to as "totipotency." Germ cells are unique as transmitters of both genetic and epigenetic information to subsequent generations, and they show many exceptional properties that are required to fulfill this potential. The oocyte also has the striking property of conferring totipotency on cell nuclei from somatic cells, such as a nerve cell when it is transplanted into the egg, a process referred to as cloning or nuclear reprogramming<sup>2</sup>. During development from a zygote onward, there is a progressive decline in totipotency of the newly dividing cells. In mammals, only the products of very early cell divisions retain totipotency in which each of the cells is, in principle, separately capable of generating a new organism. In animal sciences, the development of totipotent stem cells has been envisaged particularly for animal cloning to produce quality herds with shortest possible time<sup>3</sup>.

Here we did get the totipotent effect of cells at 16 cells stage from buffalo embryos. Thus in case of buffaloes very early stage markers for the totipotency needs to reveal for their use in production and therapeutic uses in proper directions.

### Material and Methods

All media and chemicals were obtained from M/S Sigma chemical company. Monoclonal antibodies against stage specific embryonic antigen (SSEA-1,3,4) and EMA-1 were obtained from Developmental Hybridoma Bank, IOWA. Secondary antibodies conjugated to HRP were obtained from Promega and FITC were obtained from UPSTATE, Newyork, USA. Chemicals for alkaline phosphatase staining were obtained from Sigma, USA.

### Collection of Ovaries

The ovaries were collected from local slaughter house in normal saline solution fortified with antibiotics at 30-35 C in a thermos flask within 2 hours of slaughter.

### Collection of Oocytes

Oocytes were aspirated from all visible non-atretic follicles with 18 gauge needle attached to 5 ml sterile plastic syringe with oocyte collection media. The Cumulus oocyte complexes (COCs) along with follicular fluid were pooled into a fresh 50 ml sterile plastic tube. The COCs were graded as per Kobayashi et al 1994.

### In Vitro Maturation of oocytes

Washed 25-40 COCs were cultured in 60 µL drop of maturation media covered with sterile mineral oil in 35 mm culture dish at 15% O<sub>2</sub>, 5% CO<sub>2</sub>, and 38.5 °C temperature and 90% humidity. Assessment of maturation was done as per Kobayashi et al, 1994<sup>4</sup>.

### Sperm preparation for In Vitro Fertilization

One straw of frozen semen was thawed in warm water for 1 min and was emptied and sperms were prepared for fertilization using the TALP media and concentration was adjusted to 4-5x10<sup>6</sup> ml.

### In Vitro Fertilization of Matured Oocytes

After maturation, the oocytes were washed several times in media for removal of expanded cumulus cell mass. After washing, oocytes were transferred into 50 µL of processed spermatozoa. The droplet were covered with warm paraffin oil and placed in CO<sub>2</sub> incubator at 38.5 C, 5% CO<sub>2</sub>, 15% O<sub>2</sub> and 90% relative humidity.

### In Vitro Culture of fertilized oocytes

After 18h of sperm oocytes co-incubation, the oocytes were washed 10-15 times in embryo development medium (modified synthetic oviductal fluid) and cultured in same media.

### Isolation and culture of totipotent stem cells from Early Stages Buffalo embryos

The embryos obtained upto different cell stages were made zona free with the help of 0.5% protease and immediately after the zona dissolution, protease activity was stopped with fetal bovine serum to avoid the damage of isolated blastomeres. After 2-3 washing, blastomere cells were cultured on mitomycin C-inactivated<sup>4</sup> oviductal cell monolayer in the presence of RPMI-1640 and 15% FBS, supplemented with LIF at

38.5 °C, 5% CO<sub>2</sub> , and 90% humidity in a CO<sub>2</sub> incubator for a period of 10-20 days.

#### **Isolation and culture of pluripotent stem cells from Early developing buffalo fetus:**

The asynchronous gravid genital tract of pregnant buffaloes (30-40 days old), having well developed corpus luteum were brought to the laboratory from the slaughterhouse. After thorough washing , the gravid uterus was incised and the developing fetus with allantoic membrane was taken in a sterile petridish. Cell suspension was created and washed 2-3 times with sterile DPBS and finally washed with RPMI-1640. The washed cell were then cultured in tissue culture bottle as well as six well plate on mitomycin-C inactivated buffalo fibroblast cell monolayer in RPMI-1640 (This was cultured at our end)having the same incubation conditions as above for 20 days.

The freshly isolated cells were taken as droplet on the grease free slides and allowed the droplet to dry in air. The slides were kept at 4 °C in refrigerator for 2-3 days until used for characterization for alkaline phosphatase activity and also for the presence of SSEA-1, 3, 4 and EMA -1.

#### **Characterization of Totipotent and pluripotent stem cells**

Alkaline phosphatase activity of totipotent and pluripotent stem cells were determined by Moore and Piedrahita<sup>5</sup>. The immunocytochemistry of totipotent and pluripotent stem cells was done as per the method of Kuhhlozer<sup>6</sup>.

### **Results**

#### **In vitro Maturation, Fertilization and Culture of buffalo oocytes**

In the Present study, a total no of 1340 buffalo ovaries were collected from local slaughterhouse from which 1080 COCs were recovered . The culturable quality oocyte was 0.85 per ovary, as per Table 1.

The average maturation rate varied from 66% to 87%. The cleavage and development of morulae stage embryo under 15% oxygen tension in the month of March was 8.27 and 2.4% respectively.

In month of April, the cleavage rate was 5.6% and morula was 3.1%. Similarly the month of May cleavage rate was 8.18% and morulae were 5.4%. When all the data was pooled throughout the study, the overall cleavage rate was 9.1% and development of morulae (Upto 16 cell stages) was 4.6% (Table 2.), (Figure 1 (a, b,c,d,e)

#### **Isolation and Culture of Totipotent stem cells**

In the present study, blastomere cells were separated from 2, (Figure 1e) 4 (Figure 1,f),8 (Figure 1g) and 16 (Figure 2 h) cell stage *in vitro* produced buffalo embryos and these isolated cells were cultured on mitomycin-C inactivated oviductal cell monolayer in the presence of LIF in RPMI-1640 with 15% FBS. The results indicated that when blastomere cells isolated and cultured from 2 and 4 cell stage embryos, they did not multiply even after 10 days of culture. Similarly, blastomere cells of 8 cell stage embryos when cultured, although there was cleavage in the initial stage but started degenerating after few days of culture. However when blastomere cells isolated and cultured from 16-cell stage embryos were formed out of which nine embryos used, from two cleaved and form clones of cells.

#### **Development of Pluripotent Stem cells**

In the present study, stem cell like cells were isolated from early developing buffalo fetus of 30-40 days old. The cells were cultured on mitomycin-C inactivated fibroblast cell monolayer in RPMI-1640 supplemented with LIF at conditioned mentioned above. The cells took two to three days for attachment to the fibroblast cell feeder layer. After 20 days of culture, the cells form different clones of cells.

#### **Characterization of Stem cells**

##### **Totipotent stem cells**

In the present study, identification of totipotent stem cells were done after 10-20 days of culture of blastomere cells isolated from 8-16 cell stage *in vitro* produced buffalo embryos. Although the blastomere cells isolated from 8 cell stage embryo did not form clones of cells, they showed positive staining for SSEA-4. The results indicated that the blastomere cells maintained stem cell

characteristics till 10 days of culture. Further when blastomere cells from 16 cell stage embryos were cultured and showed the positive staining for SSEA4, SSEA2, SSEA-1 and EMA-1 and alkaline phosphatase (Figure 2 i, j, k,)

**Pleuriotent stem cells**

The freshly isolated cells from early developing fetus showed positive staining for SSEA-1, 4, EMA-1 and alkaline phosphatase. However they

did not stain for SSEA-3 showing that those are stem cells. The cells were further cultured on mitomycin-c inactivated fibroblast cell monolayer for a period of 20 days in RPMI-1640 with 15% FBS supplemented with LIF and they were stained with differen stem cell specific cell markers viz SSEA-1, 3,4, EMA1 and alkaline phosphatase activity. (Figure 3 l-u)

**Table 1:** Culturable oocyte recovery per ovary in buffalo

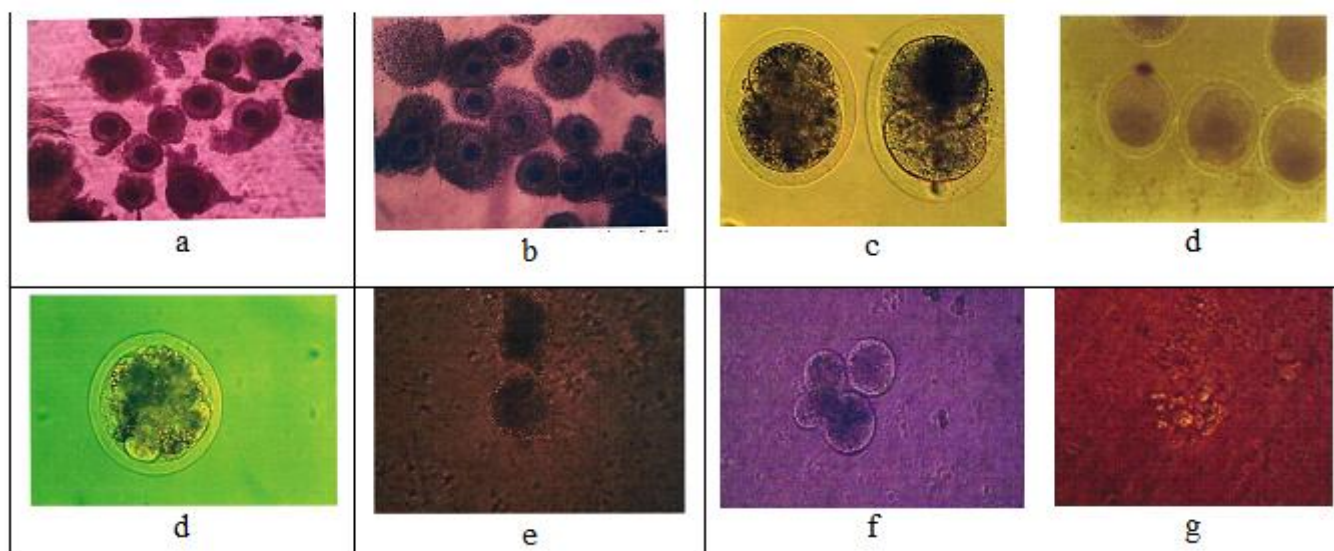
No. of Ovaries	Total number of oocytes aspirated	Percentage of oocytes harvested (Excellent and Good)	No. of Culturable oocytes/ovary
1340	1080	85.07	0.85

**Table 2:** Cleavage rate (%) and production of different stages of IVF embryos in buffaloes.

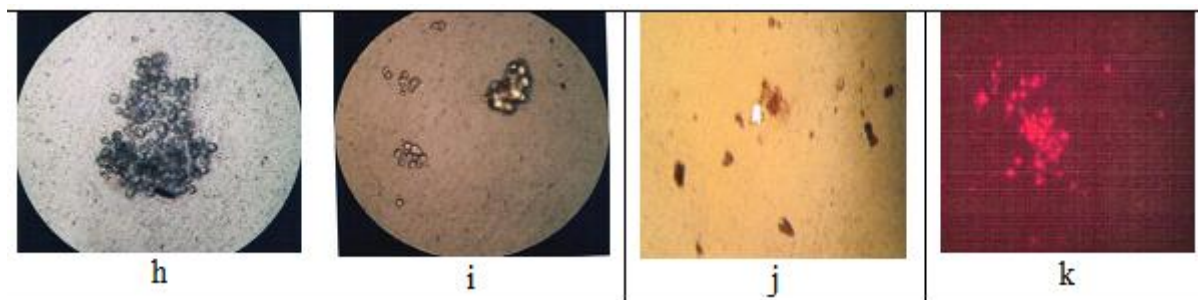
Group	No of Culturable oocytes	No. of matured oocytes (Cumulus expansion)	No of oocyte cleaved	No of Morula
I (March)	342	234 (68%)	29 (8.27%)	9 (2.5%)
II (April)	408	329 (80.63%)	23 (5.6%)	13 (3.1%)
III (May)	320	289 (87.57 %)	27 (8.18%)	18 (5.4%)
Overall average	360	284 (77.77 %)	26 (9.1%)	13 (4.5%)

**Table 3:** Number of embryos used for blastomere separation and their culture for development of totipotent stem cell clones in buffalo.

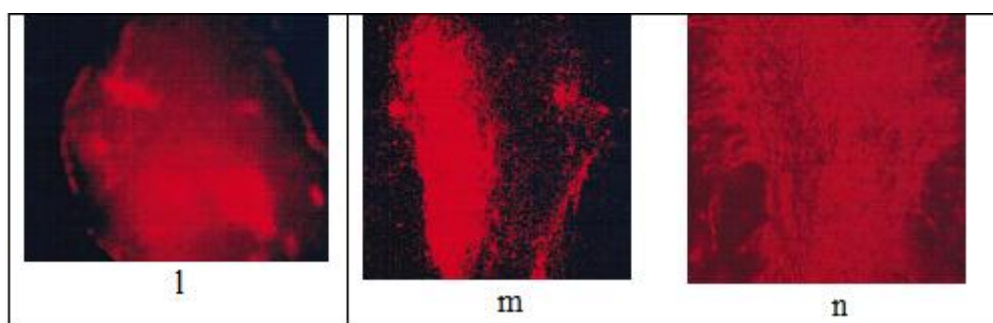
Embryos at different stage	No of embryos cutured	No of blastomere cleaved	No. of clones formed
2-cell	8	-	-
4-cell	11	-	-
8-cell	10	-	-
16-cell	9	2	2



**Figure 1** a. Showing immature compact cumulus oocytes complexes (COCs) after collection prior to culture (20X). b. Showing expanded cumulus oocytes complexes after 24 hr of maturation (40X). c. Showing 2-cell cleaved embryo after culture for 48 hrs (40X). d. Showing 8-cell cleaved embryo after 2-days of culture (40X). e. Showing 16-cell cleaved embryo after 4 days of culture (40X). f. Showing isolated blastomere from 2- cell embryo after protease treatment prior to culture (40X). g. Showing cultured blastomere cells from 8-cell stage embryos. Some of the cells started degenerating. (20X)



**Figure 2** h. Showing colonies of cells isolated from blastomere of 16 cell embryo cultured for 20 days. i. Showing isolated blastomere cells from 16-cells embryos after culture stained with SSEA-1 bind with peroxidase (20X). (20X). j. showing isolated blastomere cells from 16-cell embryo after culture stained with alkaline phosphatase (20X). k. Showing isolated blastomere cells from 16 cell embryo after culture stained with SSEA-1 bind with FITC (20X).



**Figure 3.** l. Cultured pluripotent stem cells stained with SSEA-1 after 20 days of culture with FITC fluorescent binding indicates the stem cells (40x). m. Cultured pluripotent stem cells stained with SSEA-4 after 20 days of culture with FITC fluorescent binding indicates the stem cells (40X). n. Cultured pluripotent stem cells stained with EMA-1 after 20 days of culture with FITC fluorescent binding indicates the stem cells (40X).

## Discussion

### IVFMC of bufflo oocytes

In the present experiments, cleavage and development of morulae stage embryo under 15% oxygen tension varied from month to month. When all the data was pooled throughout the study, the overall cleavage rate was 9.1% and development of morulae upto 16 cell stage was 4.6%. The cleavage rate after post insemination varied from laboratory to laboratory<sup>7</sup>. Cause of low cleavage rate may be due to environmental temprature which is one of the factors for low cleavage rate obtained at higher temperature<sup>8</sup>. Studies have confrimed that better climatic conditions increases the fertility rate<sup>9</sup>.

In the present study, the maturation of oocytes was considered on the basis of cumulus expansion, which does not indicate real nuclear

maturation of oocytes. However, proper maturation of oocytes is a prerequisite for successful fertilization learding to embryo development. Gasparini<sup>10</sup> reported higher cleavage rate and blastocyste development in mSOF. But in his study, When mSOF was used, the incubation environment was adjusted by lowering the oxygen tention to 7%. In the present experiment, the oxygen level was maintained by 15%. Higher oxygen tension may damage the embryo development by increasing the level of reactive oxriations ygen species (ROS) in the cells and this may be one of the reasons for lower transferable embryo production in mSOF medium. Recent report suggest that the optimal oxygen tension in embryo culture may depend on the stage of development<sup>11</sup>.

In the present experiments, semen from different bulls was used. Considerable variations have been reported among different buffalo bulls in terms of ability of their spermatozoa to fertilize oocytes in vitro<sup>12</sup>.

#### **Isolation and culture of totipotent stem cells**

In the present study, blastomere cells were separated from 2, 4, 8, and 16 cell stage in vitro produced buffalo embryos and these isolated cells were cultured in mitomycin-C inactivated oviductal cell monolayer in the presence of LIF in RPMI-1640 with 15% FBS. During our experiments we have observed that 2, 4 and 8 cells stage embryos did not form any clones but for 16 cells stage embryos were formed out of nine 2-cleaved and formed clones of cells.

It has been reported that successful embryonic cell lines have usually been derived from outgrowth of epiblast cells of inner cells mass in different species. There is evidence that late stage ICMs with higher cell counts more consistently develop to cell lines. Thus our might be the first case to show in buffalo for deriving the cell line from 16 cell stage embryo for studying the totipotent markers.

Although totipotent cell lines could be made from 8 cells IVF embryos in bovine and human<sup>13,14</sup>. It showed that early stage embryonic blastomere cleavage culture is hardly possible which reflected in the present study.

#### **Development of Pluripotent stem cells**

In the present study, stem cell like cells were isolated from early developing fetus of 30-40 days old. The isolated cells were cultured on mitomycin-c inactivated fibroblast cell monolayer in RPMI-1640 supplemented with LIF at 38.5 °C, 5% CO<sub>2</sub>, 15% O<sub>2</sub> and maximum humidity in CO<sub>2</sub> incubator. The cells took 2-3 days for attachment to the fibroblast cell feeder layer for culture of fetal stem cells. For culture of stem cells, several feeder cells are currently being used. Previously, fetal fibroblast cells have been used as feeder layer for stem cells culture<sup>15,16</sup>. Further, it has been reported that stem cells could be cultured without feeder cells by using fibroblast

conditioned media or cell free media supplemented with LIF<sup>17,18</sup>.

### **Characterization of Stem cells**

#### **Totipotent stem cells**

In the present study identification of totipotent stem cells were done after 10-20 days of culture of blastomere cells isolated from 8-16 cell stage of in vitro produced buffalo embryos. Although the blastomere cells isolated from 8 cell stage embryo did not form clones of cells, they showed positive staining for SSEA-4. The results indicated that blastomere cells maintained stem cell characteristic till 10 days of culture. Further, when blastomere cells from 16 cell stage embryos were cultured in RPMI-1640 with 15% FBS supplemented with human LIF, out of nine only blastomere of two embryos cleaved and formed the clones. Further these clones showed positive staining for SSEA-1, SSEA-4 and activity for alkaline phosphatase (Figure 2 h,i,j,k)

Very meager reports are available on the staining of totipotent stem cells derived from early stage embryos. Mitalipova<sup>13</sup> observed that cell lines developed from 8-16 cell stage precompacting embryos showed positive staining for SSEA-1,3,4 and presence of c-kit receptors. Strelchenko<sup>14</sup> described the original technique for derivation of ES cell lines from 8 cell stage human morulae. So far literature lacks the events about the successful establishment of totipotent stem cell line from two cell stage embryo in large animals.

The failure of establishment of cell lines may also be attributed to the lack of addition of certain growth factors, viz stem cell factors, interleukins, etc. Most attempts to culture bovine.

Stem cell research has gained momentum in reproductive biology and medicine in recent years due to its importance in health and reproduction. A lot of knowledge gap is still persisting which needs to be worked out in the field of stem cell research. Totipotent stem cells marker identification is still ambiguous. Although there are couple of reports which are published in recent years where the SSEA-1, SSEA-4, and alkaline

phosphatase were used for characterization and showed positive reports for embryonic stem cells<sup>1</sup> thus report agrees with our data stating these are common markers for identification stem cells from buffalo embryos.

#### **Immunocytochemistry of pluripotent stem cells**

The isolated pluripotent stem cells showed strong alkaline phosphatase staining. Further these cells were stained with embryonic stem cell markers like SSEA-1, 3,4 and EMA-1. These cells were positively stained for SSEA-1, (Fig), 4 and EMA-1 (Fig). However isolated cells did not stain with SSEA-3. After 20 days of culture when the cell colonies were stained, they were shown strong alkaline phosphatase activity. These cell colonies show positive staining for SSEA-1, 4 and EMA-1. However, the cultured cells also did not show positive staining for SSEA-3.

The present study indicated that fetal stem cells could be maintained in the culture at least for a period of 20 days. When the stem cells are cultured, property of stem cells in culture is maintained till the cells express the intrinsic factor oct-4 and continuously receive the extrinsic signals from cytokine leukemia inhibitory factors. If no such factors are added, or when the LIF is withdrawn during the culture, then the stem cells spontaneously differentiated to form different body cells<sup>18</sup>. It has been reported that LIF support a totipotent state comparable to early embryonic cells that coexpress embryonic and extraembryonic determinants<sup>19</sup>. In the present study, the fetal cells were cultured with human leukemia inhibitory factors and thus many of the cell colonies did not differentiate and maintained stem cell characteristics. LIF used in the present study was of human origin. It is known that LIF of mouse origin shares high sequence similarity with bovine LIF.

In the present study, the isolated and cultured pluripotent stem cells of fetal origin stained with internationally accepted stem cell markers (SSEA1, SSEA4, EMA-1 and alkaline phosphatase) and has been used by many researchers<sup>20,21</sup>.

#### **Conclusion**

In conclusion, the present study indicated that stem cells clone could be developed in vitro from early stage embryos in buffalo and in vitro culture they maintained stem cell characteristics at least for 20 days. Similarly, pluripotent stem cells have been isolated and cultured from developing pluripotent fetal stem cells and totipotent embryonic stem cells from 8 cell and 16 cell stage in vitro produced buffalo embryos stained with widely accepted stem cell markers via SSEA-1,4, and EMA-1 (pluripotent cells tested only). Further both the type of cells did not show any activity for alkaline phosphatase

Although we would need several studies to come up with totipotency and pluripotency establishment in buffaloes but of this study will study is providing base towards empowering our efforts in animal biotechnology to increase the production and in the field of medicine in the future by using the pluripotent and totipotent stem cells and their application in the field.

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