CHAPTER – 5

Summary and Conclusions
5. SUMMARY AND CONCLUSIONS

Embryonic stem (ES) cells are a special kind of cells, are capable of undergoing unlimited number of symmetrical mitotic cell divisions without differentiation; maintaining stable diploid karyotype; are clonogenic i.e., a single ES cell can give rise to a colony of genetically identical cells or clones, which have the same properties as the original cell; are capable of either entering a path leading to a fully differentiated cell or remaining as stem cells. For the production of these cells, the inner cell mass (ICM) of blastocyst stage embryos is cultured on a suitable feeder layer. ES cell technology is expected to have immense importance for the production of transgenic farm animals. The currently available gene transfer methods are highly inefficient. Availability of ES cells in large numbers offers a means for efficient gene transfer since these cells can be genetically manipulated by using conventional recombinant DNA techniques for their further use in transgenesis. ES cells may also enable studies on gene targeting, the fundamental events in embryonic developmental, and production of therapeutic delivery systems.

ES cells in their undifferentiated state are characterized by a distinct morphology and the presence of a set of markers classified into intracellular and extracellular types. Since ES cells must possess the ability to differentiate to any cell type, spontaneous and directed differentiations are integral parts of their characterization. A thorough understanding of the factors or conditions for the long-term culture of ES cells, without compromising their pluripotency and a stable genetic make-up, is very important for the production and maintenance of ES cells from different species of farm animals. Because of their potential use for targeted gene manipulation, availability of ES cells from an important farm animal like buffalo can be immensely useful for other reproductive technologies like cloning and transgenesis. Very few reports are available in buffalo on the production of pearthenogenetically derived embryonic stem cell-like cells and their characterization by examination of intracellular or surface markers. Keeping this in view, the present study was carried out with the following objectives:
1) To compare different methods for production of parthenogenetic blastocysts and isolation culturing of the embryonic stem cells, 2) To study the expression of pluripotency associated markers in embryos and putative buffalo embryonic stem cells and 3) To examine the in vitro differentiation potential of cultured embryonic stem cells.

For the production of buffalo blastocysts in vitro, immature oocytes were collected from slaughterhouse buffalo ovaries by aspiration of surface follicles (2-8 mm diameter) and were classified into usable and unusable grades. Oocytes with homogeneous cytoplasmic granulation and ≥2 layers of unexpanded cumulus layers, which were considered to be of usable grade were subjected to in vitro maturation (IVM) by placing groups of 15-20 oocytes in 100 µl droplets of the IVM medium (TCM-199 + 10% FBS + 5 µg/ml pFSH + 1µg/ml estradiol 17-β + 0.81 mM sodium pyruvate + 5% buffalo follicular fluid) and culturing for 24 h in a CO\textsubscript{2} incubator (5% CO\textsubscript{2} in air) at 38.5°C. The oocytes were incubated with processed spermatozoa for 18 h in BO medium for in vitro fertilization (IVF), after which these were cultured for up to 8 days post insemination in in vitro culture (IVC) medium which consisted of modified Charles Rosenkrans medium with amino acids (mCR2aa) + 10% FBS.

Buffalo embryonic fibroblast feeder layers were prepared by taking skin biopsies from fetuses and their culture in DMEM + 20 % FBS + 50 µg/ml gentamicin sulphate. Monolayer fibroblasts were allowed to grow till confluence. The confluent monolayers were trypsinized, split and subcultured for cell multiplication. Cells from initial passages were cryopreserved in small aliquots for future use. To reduce variability, the passage number of feeder cells was kept constant. For the preparation of feeder layer, the fibroblast monolayers were inactivated by mitomycin-C treatment after which the monolayer was trypsinized. The feeder layer was prepared by seeding the harvested cells one day prior to the culture of ICMs. The ICMs isolated either mechanically from hatched blastocysts or enzymatically by trypsin treatment from expanded blastocysts after removal of zona pellucida by pronase treatment were seeded individually on mitomycin-C treated feeder layers. The culture medium was changed every day and further colonization of the cells was observed routinely. The primary colonies, obtained 2-4 days after seeding of ICMs were disaggregated mechanically and
the aggregates of cells were individually reseeded on new feeder layers. The colonies exhibiting typical morphological features of putative ES cells were subcultured until the cells remained in an undifferentiated state or when colony formation stopped.

The characterization of the putative buffalo ES cells was carried out at different passages by staining for alkaline phosphatase, examining the expression of intracellular markers like OCT4, FOXD3, SOX2, REX-1, NANOG and NUCLEOSTEMIN and surface markers like stage specific embryonic antigen (SSEA)-1, SSEA-3, SSEA-4, tumor rejection antigen (TRA)-1-60, TRA-1-81, CD9 and CD90 through immunofluorescence or RT-PCR. In vitro produced buffalo embryos at various developmental stages were also examined for the expression of some of these markers. The chromosomal integrity of the putative buffalo ES cells was analyzed by performing karyotyping. Different combinations of media and media supplements were compared for their efficacy in supporting the growth and maintenance of buffalo ES cells. For confirming the pluripotent status of the putative ES cells produced, these were subjected to culture by ‘hanging drop method’ in the absence of LIF and feeder layer support for the formation of embryoid bodies, which were then subjected to suspension culture for carrying out spontaneous or induced differentiation.

In present study, the usable quality oocytes activated with ethanol for parthenogenetic embryo production, the cleavage rate 77.80±1.47, 48.64±3.74 and 75.34±0.71 when these cleaved oocytes were cultured, the blastocyst production rate 25.93±0.90, 7.01±1.02 and 33.93±0.39 was observed in mCR2, mSOF and RVCL, respectively. The cleavage rate was significantly higher (P<0.05) when the activated oocytes cultured in mCR2 and RVCL then SOF, while the blastocyst production rate was highest in RVCL then mCR2 and lowest in mSOF and they were significantly differ (P<0.05). The usable quality oocytes activated with calcium inophore for parthenogenetic embryo production, the cleavage rate 62.76±0.98, 45.95±0.94 and 68.37±0.51 and when these cleaved oocytes were cultured, the blastocyst production rate 16.63±1.10, 6.15±0.45 and 18.16±0.93 was observed in mCR2, mSOF and RVCL, respectively. The cleavage rate was significantly higher (P<0.05) when the activated oocytes cultured in mCR2 and RVCL then SOF, while the blastocyst production rate was
highest in RVCL then mCR2 and lowest in mSOF and they were significantly differ (P<0.05).

The usable quality oocytes activated with electric pulse for parthenogenetic embryo production, the cleavage rate 46.89±1.87, 30.20±0.65 and 56.97±2.37 and when these cleaved oocytes were cultured, the blastocyst production rate 8.17±0.66, 3.36±0.31 and 13.30±0.29 was observed in mCR2, mSOF and RVCL, respectively. The cleavage rate was highest in RVCL and lowest in SOF and they were significantly differ (P<0.05). When these activated oocytes cultured in mCR2 and RVCL then SOF, while the blastocyst production rate was highest in RVCL then mCR2 and lowest in mSOF and they were significantly differ (P<0.05).

The nonusable quality oocytes activated with ethanol for parthenogenetic embryo production, the cleavage rate 24.95±2.29, 19.39±0.63 and 38.57±2.57 and when these cleaved oocytes were cultured, the blastocyst production rate 1.23±0.54, 0.39±0.24 and 3.81±0.40 was observed in mCR2, mSOF and RVCL, respectively. The cleavage rate was significantly higher (P<0.05) when the activated oocytes cultured in RVCL then mCR2 and SOF, while the blastocyst production rate was also significantly higher (P<0.05) in RVCL then mCR2 and mSOF.

The nonusable quality oocytes activated with calcium inophore for parthenogenetic embryo production, the cleavage rate 23.60±0.27, 22.35±0.72 and 34.36±1.02 and when these cleaved oocytes were cultured, the blastocyst production rate 1.58±0.19, 0.61±0.25 and 4.06±0.15 was observed in mCR2, mSOF and RVCL, respectively. The cleavage rate was significantly higher (P<0.05) when the activated oocytes cultured in RVCL then mCR2 and SOF, while the blastocyst production rate was highest in RVCL then mCR2 and lowest in mSOF and they were significantly differ (P<0.05).

The nonusable quality oocytes activated with electric pulse for parthenogenetic embryo production, the cleavage rate 20.18±1.37, 18.95±0.55 and 31.11±0.48 and when these cleaved oocytes were cultured, the blastocyst production rate 0.98±0.43, 0.59±0.24 and 2.10±0.23 was observed in mCR2, mSOF and RVCL, respectively. The cleavage rate was significantly higher (P<0.05) when the
activated oocytes cultured in RVCL then mCR2 and SOF, while the blastocyst production rate was significantly higher (P<0.05) in RVCL then mCR2 and mSOF. When the putative buffalo ES cells were subjected to alkaline phosphatase staining at every alternate passage, the cells stained red, indicating a high expression of alkaline phosphatase, as long as they were cultured on feeder layers in the presence of LIF. In contrast, the fetal fibroblast feeder layer cells, which were used as controls remained unstained. The alkaline phosphatase expression was lost after the differentiation of putative ES cells, as indicated by a change in their morphological appearance. The putative buffalo ES cells did not exhibit the expression of SSEA-1 and SSEA-3, whereas, SSEA-4 was strongly expressed in these cells at different passages. Both TRA-1-60 and TRA-1-81 were also found to be expressed at different passages in the putative buffalo ES cells although the expression detected was weak. The putative buffalo ES cells were found to exhibit a weak expression of CD9 and CD90 also.

The expression of some transcription-based markers was detected by immunofluorescence staining. It was found that the putative buffalo ES cells expressed OCT4, FOXD3 and SOX2 strongly. Besides these, the expression of two other transcription-based pluripotency markers i.e., *REX-1* and *NUCLEOSTEMIN* was examined by RT-PCR. Both these markers were found to be expressed by the putative buffalo ES cells. When karyotyping was performed at passage-3 and passage-6, the putative buffalo ES cells of all the groups were found to show a normal diploid chromosome number.

The ability to form embryoid bodies by ES cells is taken as a measure of their pluripotency since embryoid bodies contain all the three germ layers- ectoderm, mesoderm and endoderm. For the formation of embryoid bodies, colonies of putative buffalo ES cells were separated from the feeder layer and were mechanically split into small clumps of around 600-800 cells. The clumps were cultured for 2-3 days in hanging drops in ES cell culture medium without LIF. When the embryoid bodies were harvested from the hanging drops on Day 3 of culture, both types of embryoid bodies formed solid mass and cystic type. The harvested embryoid bodies were transferred to bacteriological dishes for further culture in suspension for additional 3 days, during the course of which they increased in size. In the cystic embryoid bodies, the number of the fluid filled
cavities also increased besides their size. The embryoid bodies were characterized by examining lineage-specific gene expression by RT-PCR. When these EBs cultured further on gelatin coated dishes, they differentiated spontaneously to several types of cells such as epithelial cell-like cells, neuron-like cells etc.

The cells obtained from harvested embryoid bodies were found to express \textit{NF-68} and \textit{CYTOKARETIN} specific for ectodermal lineage; bone morphogenic protein-4, \(\alpha\)-skeletal actin and \textit{MSX1} specific for mesodermal lineage and \(\alpha\)-fetoprotein, \textit{GATA-4} and hepatocyte nuclear factor-4 specific for endodermal lineage. The formation of embryoid bodies containing cells that expressed genes of lineages of all the three germ layer cells confirmed that the ES cells produced in the present study were indeed pluripotent.

For carrying out directed differentiation, the embryoid bodies were placed in suspension for an additional 3 days and after which they were transferred to gelatin coated tissue culture dishes or 4-well plates in culture medium containing the stimulating agent i.e., retinoic acid at concentrations of \(10^{-8}\) M. After 2 days, the embryoid bodies were found to have adhered and had started to grow at the periphery with the outgrowths at their periphery assuming neuron cell-like morphology. These cells were examined for the expression of \textit{NF68}, which are marker for neuron cells by RT-PCR.

Cell-cell interactions promoted by aggregation initiate to a spontaneous differentiation of embryoid bodies to all three germ layers i.e., ectoderm, mesoderm and endoderm. In the present study, when these embryoid bodies were cultured on gelatin coated dishes, they spontaneously differentiated to several types of cells such as neuron-like cells, epithelial cell-like cells, muscles like cells and fibroblast like cells depending on the duration of culture. The epithelial cell-like cells were characterized by expression of \textit{CYTOKARETIN 18}, which are specific for epithelial cell.

In conclusion, the results of the present study suggest that:

- The A+B grade/ usable quality oocytes have good cleavage rate in ethanol and calcium inophore then the electric pulse.
• The A+B grade/usable quality oocytes have better blastocyst development rate in RVCL and mCR2 medium then mSOF medium

• The C+D grade/ unusable quality oocytes have better development rate in RVCL when activated with ethanol and calcium inophore.

• The buffalo embryos of different development stages produced parthenogenetically expressed the markers of pluripotency.

• Surface markers like alkaline phosphatase, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, CD9 and CD90 are expressed by buffalo embryonic stem cells.

• SSEA-1 is not expressed by the putative buffalo PGES cells.

• Putative buffalo parthenogenetically derived embryonic stem cells also express transcription-based markers like OCT4, SOX2, NANOG, FOXD3, REX-1, cMYC, TELOMERASE and NUCLEOSTEMIN.

• PGES cells after viability test produce colony of PGES cells and expressing markers of pluripotency

• PGES cells maintain normal karyotyping.

• Embryoid bodies formed by spontaneous differentiation of putative buffalo ES cells contain cells from all three germ layers i.e., ectoderm, mesoderm and endoderm, as confirmed by expression of markers specific to these cell types.

• PGES cells directed to differentiate in neuron like cells, as confirmed by expression of specific marker.

We suggest that artificial parthenogenesis represents an alternative source for deriving pulripotent lines of buffalo and other large animal species particularly in human that could also be considered ethically acceptable. In our opinion, the uniparental origin and asymmetric imprinting of parthenogenetic cell lines make them a valuable tool for studies addressed to a better understanding of the mechanisms driving early embryo development as well as imprinting. Our results suggest that the cell line generated from parthenogenetically produced blastocyst maintaining the properties of ES cells, and can be used as an in vitro model to study the effects of imprinting on cell differentiation and as an invaluable material for extensive molecular studies on imprinted genes. These cells can be easily transected for production of transgenic animals such as, disease resistant animal, high milk yielding animal in future.
BIBLIOGRAPHY


