Effect of DNA modifying agents on *in vitro* development of interspecies cloned Cattle-Buffalo embryos

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(2016-V-324-M)

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Faculty of Veterinary Sciences and Animal Husbandry

Sher-e-Kashmir University of Agricultural Sciences &

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Master of Veterinary Sciences
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Dedicated to.....

Livestock rearing community whose perseverance makes our research meaningful.....

Sher-e-Kashmir
University of Agricultural Sciences and Technology of Kashmir
Division of Biotechnology, Shuhama Campus, Srinagar

Certificate – I

This is to certify that the thesis entitled, **"Effect of DNA modifying agents on *in vitro* development of interspecies cloned Cattle-Buffalo embryos"** submitted in partial fulfilment of the requirements for the award of the degree of **Master of Veterinary Sciences (Biotechnology)**, to the **Faculty of Veterinary Sciences and Animal Husbandry, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir** is a record of bonafide research work carried out by **Dr. Haris Rasool Beig (Regd. No. 2016-V-324-M)** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

It is further certified that information received during the course of investigation has duly been acknowledged.

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Chairman
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F. V. Sc & A. H. Shuhama

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We, the members of the Advisory Committee of **Dr Haris Rasool Beig (Regd. No. 2016-V-324-M),** a candidate for the degree of **Master of Veterinary Sciences (Biotechnology)** have gone through the manuscript of the thesis entitled, **"Effect of DNA modifying agents on *in vitro* development of interspecies cloned Cattle-Buffalo embryos"** and recommend that it may be submitted by the student in partial fulfilment of the requirements for the award of the degree.

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Certificate – III

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ABSTRACT

Somatic cell nuclear transfer is one of the most advanced and least exploited technology among assisted reproductive technologies. It has immense potential in the conservation of endangered species as well as revival of extinct species. However, on account of limited oocyte availability from such species, a modified approach of interspecies somatic cell nuclear transfer (iSCNT) has been devised. In addition, iSCNT also provides a window of study on nuclear reprogramming and embryonic stem cell production for clinical and research purposes. Somatic cell nuclear transfer embryos as a whole and iSCNT embryos, in particular, have low efficiency to develop into transferable quality embryos which has been attributed to abnormal reprogramming of the donor somatic cell mediated by DNA methylation and histone acetylation. Current investigation was carried out to determine the efficiency of iSCNT cloned Cattle-Buffalo embryo production using Hand Made Cloning (HMC) technique and to compare *in vitro* development of iSCNT cloned Cattle-Buffalo embryos following donor cell treatment with epigenetic re-modelling drugs or chromatin modifying agent like DNA demethylation agents and histone deacetylase inhibitors (HDAC) inhibitors i.e. 5-aza- 20-deoxycytidine (5-aza-dC) and Scriptaid. A total of 1591 abattoir derived buffalo ovaries in 16 replicates were used for the experiments. 3347 COC's were harvested upon aspiration of surface

follicles (size ranging from 2mm - 8mm). A total of 2378 (71.05%) COC's were of usable quality, therefore, selected for IVM. COC's with not less than2 layers of surrounding cumulus were selected for *in vitro* maturation (IVM). Three types of IVM media were used, IVM media supplemented with with 1) pFSH + β estradiol, 2) pFSH +β estradiol+Follicular fluid and 3) follicular fluid only. The maturation percentage as determined by cumulus expansion and polar body extrusion was found to be higher when IVM medium supplemented with pFSh + 17 βestradiol + Follicular fluid was used as compared with supplementation of Pfsh+17 βestradiol or follicular fluid only (94.07% ±3.65% v 86.25% ±4.86% and 79.42%±1.37% respectively) but higher maturation percentage observed in IVM medium supplemented with pFSh + 17 βestradiol + Follicular fluid was statistically non-significant (P>0.05).The percentage of iSCNT cloned Cattle-Buffalo embryos without any treatment of donor cells which cleaved on day 2 was 26.25%±0.95%. 2-4 cell stage, 8 cell stage and 16-32 cell stage embryo percentages obtained on Day 7 were 42.86%±10.13%, 33.33%±7.01% and 23.81%±8.96%, respectively. The percentage of cleaved embryos as compared to control, was found to be significantly higher in all treatment groups (26.25%±0.95% vs 35.29%±0.74%, 41.79%±3.23%, 35.53%±3.77%). Highest cleavage rates were found in Sriptaid treated group but was non-significant in comparison with 5-aza-dC and combination treatment group. Other developmental stages were comparable and did not show any significant variation between the various treatment groups or from the control group. The embryos did not progress beyond 32-cell stage in any treatment group.

Key words: Cleavage, Cloning, DNA demethylation agents, histone deacetylase inhibitors (HDAC) inhibitors, Interspecies Somatic Cell Nuclear Transfer (iSCNT), Nuclear reprogramming,

Signature of Student
Dated_____

Signature of Major Advisor
Dated_____

ACKNOWLEDGMENT

"In the name of Allah, the most beneficent, the most merciful"

Every process in this world is a result of fine synchronization of many events, persons and materials coming together for a reason. Similar is the case with this research, which would not have been possible save for the coming together of some souls.

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Haris Rasool Beig

Place: Shuhama, Srinagar

Dated :

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Chapter-1

INTRODUCTION

Animal Husbandry is occupation of a significant population in India. The livestock sector alone contributes almost 25.6% of Value of Output at current prices of total value of output in Agriculture, Fishing & Forestry sector. According to recent census, the overall contribution of Livestock Sector in total GDP is nearly 4.11%. Cattle contribute about 37.28% of total livestock population of India. Total Cattle population of India is 190.9 million with only 39.7 million i.e. 20.8 % crossbred/Exotic population (19th Livestock Census). The high percentage of cattle are non-descript with low milk yield. Compounding the problem is the threat of extinction to the local defined breeds e.g. Punganur (Reddy *et al.,* 2004), Vechur (Susamma, 1996).

Various strategies have been formulated to address these issues with dairy cattle like conservation of breeding tracts of indigenous species, AI programmes and Embryo transfer technology. Other modern reproductive biotechnologies or assisted reproductive techniques (ART) like *in vitro* fertilization (IVF), semen/embryo sexing and genome resource banking (GRB), cryopreservation of gametes/embryos can also be utilized. SCNT can be a part of *in situ* and *ex situ* conservation programs for many mammalian endangered species. Due to the lack of oocytes and surrogate animals, interspecies somatic cell nuclear transfer (iSCNT) is being extensively applied in reconstructed embryo research.

However little work on cattle Cloning has been undertaken in India, which could have acted as an adjunct to other strategies, far less as compared to cloning in buffaloes. To a large extent it could be due to difficulty in procuring cattle ovaries for oocyte collection due to legal and religious sanctions on cattle slaughter in most Indian states. Interspecies somatic cell nuclear transfer (iSCNT) appears to provide a way out for production of cloned bovine embryos as oocytes from buffalo ovaries could be used for production of iSCNT cattle embryos of High yielding Cattle as well as Indigenous endangered cattle breeds. Nearly 8.2

million buffaloes are slaughtered annually in registered slaughterhouses throughout India, thus buffalo ovaries are abundantly available (The Indian Buffalo-Overview of Meat Value Chain by Agriculture Division-Federation of Indian Chamber of Commerce and Industry FICCI). One of the studies aimed at determining whether oocytes from domestic buffalo species are capable of reprogramming somatic cells derived from different species such as cattle, goat and rat found that the domestic buffalo oocytes have reprogramming ability to support ruminant somatic cells till transferable quality embryos production (Selokar *et al.*, 2011).

The iSCNT entails the removal of oocyte metaphase II chromosomes followed by its replacement with a somatic cell nucleus derived from another species (Tecirlioglu *et al*., 2006). This technology was established for reasons including production of embryos from species from which oocytes are difficult to obtain or where their collection is under restricted control (Thongphakdee *et al*., 2008). In addition to its role in wildlife conservation and revival, it is also a valuable research tool for studying nuclear cytoplasm interactions, understanding the molecular and cellular mechanisms of nuclear reprogramming, producing embryonic stem cells (ESCs) for research and clinical medicine (Tecirlioglu *et al*., 2006).

The successful application of iSCNT for development of cattle-buffalo embryos will also act as a model to be applied for conservation of other related endangered ruminant species and endangered breeds of cattle.

Although many mammalian species have been cloned to date, the percentage of reconstructed oocytes that develop into normal, healthy offspring is remarkably low, often below 1% (Panarace *et al*., 2007). The poor outcome of somatic cell nuclear transfer (SCNT) is thought to be a consequence of incomplete reprogramming of the donor cell (Jeon *et al*., 2008).

In iSCNT also there have been limited live births which include Gaur (Lanza *et al*., 2000), Mouflon (Loi *et al*., 2001), African Wildcat (*Felis Silvestris lybica*) (Gomez *et al*., 2004), Banteng (*Bos javanicus)* (Sansinena *et al.,* 2005),

gray wolf (*Canis lupus*) (Kim *et al*., 2009), Coyotes (*Canis latrans*) (Hwang *et al*., 2012), Wild buffalo (https://icar.org.in/node/6041), Bactrian camel (*Camelus bactrianus*) (Wani *et al*., 2017), Bucardo **(***Capra pyrenaica pyrenaica***) -** an extinct wild goat (Folch *et al.,* 2009) was also successfully cloned, however, it died soon after its birth.

A range of different outcomes to the reprogramming process are possible viz (i) no reprogramming of the genome, resulting in immediate death of the NT (Nuclear transfer) embryo, (ii) partial reprogramming, allowing initial survival of the clones but resulting in an abnormal phenotype and/or lethality at various stages of development, (iii) faithful reprogramming producing normal animals. The phenotypes observed in nuclear clones suggest that complete reprogramming is the exception (Rideout *et al*., 2001).

Additionally, there are other issues, observed and addressed to varying degrees in iSCNT like little-known mechanisms of nuclear reprograming by the oocyte cytoplasm (Tecirlioglu *et al.*, 2006) and elimination of the accompanying donor cells' mitochondrial DNA (mtDNA) in favour of the recipient oocyte's genetically more divergent population (Jiang *et al.,* 2011). Thus, there is inheritance of maternal mitochondrial DNA e.g. in coyote-dog iSCNT (Hwang *et al.,* 2012) and defects in nucleolus formation (Cheong *et al.,* 2012). Nuclear-mitochondrial incompatibility seems to correlate with low electron transport chain (ETC) activity and extremely low blastocyst development of rhesus monkey-cow iSCNT embryos (Kwon *et al.,* 2016). Other problems encountered in iSCNT are embryonic centrosome dysfunction which might be caused by incompatibilities between ooplasm and somatic cell (Zhong *et al.*, 2007), births of morphologically abnormal offsprings.

Some of these problems may also be directly and indirectly related to abnormal reprogramming of the donor somatic cell mediated by DNA methylation and histone acetylation (Xiong *et al.,* 2013).

Addition of species compatible mtDNA and reprogramming factors improves developmental outcomes for iSCNT embryos (Jiang *et al.*, 2011).

Simonsson and Gurdon (2004) analysed the mechanism of activation of the stem cell marker gene oct4 by Xenopus oocytes using nuclear and DNA transfer from mammalian somatic cells. They found the demethylation of promoter DNA as a necessary step in the epigenetic reprogramming of somatic cell nuclei (Simonsson and Gurdon, 2004).

To improve cloning efficiency, several epigenetic re-modelling drugs or chromatin modifying agents like DNA demethylation agents and histone deacetylase inhibitors (HDAC) inhibitors such as 5-aza- 20-deoxycytidine (5-aza-dC) in cloned bovine blastocysts (Wang *et al*., 2011), Zebularine and Scriptaid in yak iSCNT cloned embryos (Xiong *et al*., 2013) . These treatments have been applied to somatic cells and consequent use of these treated cells as nuclear donors in somatic cell nuclear transfer and/or to the embryos during pre-implantation growth.

To date, there have been limited studies to demonstrate effect of DNA demethylation agents and HDAC inhibitor treatment of nuclear donor bovine fibroblasts for improving the developmental efficiency of iSCNT cattle-buffalo embryos. So, to fill this void, a study in this direction was proposed with the following objectives:

1) *In vitro* production of interspecies embryos from somatic cells of Cattle and Buffalo oocytes.
2) Study the effect of donor cell treatment with DNA demethylation agent (5-aza-dC) and HDAC inhibitor (Scriptaid), individually, on the developmental efficiency of iSCNT cloned bovine embryos.
3) Study the combined effect of donor cell treatment with DNA demethylation agent and HDAC inhibitor on the developmental efficiency of iSCNT cloned bovine embryos.

Chapter 2

REVIEW OF LITERATURE

2.1 Origin of SCNT/ Cloning

Producing exact replicas of animals or even humans has been fascinating to man. Cloning is the face of this fascination in science. Cloning may be defined as generation of any organism that is an exact copy of parent organism from where it is derived and its genetic material is identical between parents and offsprings. Somatic cell nuclear transfer (SCNT) concept was put forward by Spemann (1938) who proposed SCNT from adult differentiated cells. Gurdon, (1962) reported the use of nuclei from intestine of tadpoles to produce fertile adult frogs. "Dolly', was the first cloned animal produced from adult mammary gland cell (Wilmut *et al.*, 1997). This meant production of hundreds and thousands of elite animals from a single tested elite animal. The bovine/buffalo embryos offer several advantages with relatively late activation of its genome and late implantation compared to other animal's model like mouse (Verma *et al.,* 2012).

2.2 Bypassing the tradition - Handmade Cloning

The standard procedure for animal cloning is by traditional somatic cell nuclear transfer. But this process is very inefficient as it does not produce viable offsprings in maximum no of attempts, further depending upon the species to be cloned and the recipient oocytes (Solter, 2000). It is expected that in near future, cloned embryos will be high in demands in basic science investigations and it will provide a source of embryonic stem cells for therapeutic cloning (Gurdon and Colman, 1999). Cloned embryo production methods with higher throughput are required to meet this demand. Currently, most Nuclear Transfer (NT) protocols are micromanipulation based which are laborious, cost-intensive and require high technical skills.

The standard procedure of SCNT includes the following four steps i.e. Removal of genetic material (enucleation) of oocytes, Transfer of the donor cells (or nuclei) into nucleus free oocyte, Activation of the reconstructed embryo and subsequently, the cloned embryos are cultured *in vitro* for a period and when these reach the optimal stage for transfer and then these cloned embryos are transplanted into a mother recipient animal.

Handmade cloning is new alternative technique to that of traditional micromanipulator based SCNT. It does not require micromanipulators because the manipulations required for both enucleation and nucleus transfer are performed by hand. The HMC technique includes manual bisection of zona-free oocytes, selection of cytoplasts by staining or protrusion cone quided, and the simultaneous fusion of the somatic cell with two cytoplasts to produce a cloned embryo. HMC is a rapid and efficient technique that suits large-scale NT programs. It requires less expertise and time than traditional NT methods and the cost of equipment is significantly less. Production efficiency is high and embryo quality, in terms of pregnancy rates and live births, is not compromised. Although HMC has been developed particularly for bovine NT, the technique is applicable to other species. The method may become a useful tool for both experimental and commercial somatic cell cloning because it allows for standardization of procedures and provides the possibility of automation. (Vajta *et al*,2006)

2.3 Interspecies somatic cell nuclear transfer (iSCNT)

Some of the proposed utilities of cloning are saving endangered or reviving extinct animals and ESC (Embryonic stem cells) production, however these objectives seem to hit a hurdle at the very first step – the non-availability or restricted access to oocytes which are required in thousands for cloning experiments. A potential solution is iSCNT which uses oocytes from some other related and widely available specie as oocyte donors.

Interspecies somatic cell nuclear transfer (iSCNT) involves the transfer of a nucleus or cell from one species into the cytoplasm of an enucleated oocyte from

another. Once activated, reconstructed oocytes can be cultured in vitro to blastocyst, the final stage of preimplantation development. An extensive range of interspecies embryos have been developed for different purposes like ESC development, nuclear reprogramming studies and wildlife conservation and de-extinction (Tecirlioglu *et al.,2006)*. Interspecies cloned embryos of different species developed in-vitro are given in the table below

S No.	**Somatic cell donor**	**Oocyte donor**	**Reference**
1.	Argali (*Ovis ammon*)	Domestic Sheep (*Ovis aries*)	(White *et al.*, 1999)
2. *	Giant panda (*Ailuropoda melanoleuca*)	Rabbit	(Chen *et al.*, 1999)
3.	Gaur bull (*Bos gaurus*)	Domestic cows.	(Lanza *et al.*, 2000)
4.	Mouflon(*Ovis orientalis musimon*)	Domestic sheep (*Ovis aries*)	(Loi *et al.*, 2001)
5.	African Wildcat (*Felis silvestris lybica*)	Domestic cat cytoplasts.	(Gomez *et al.*, 2004)
6.	Banteng (Bos javanicus)	Cow (*Bos taurus or Bos indicus*)	(Sansinena *et al.*, 2005)

7. *	Leopard cat (*Prionailurus bengalensis*)	Domestic cat (*Felis silverstris catus*)	(Yin *et al*. 2006)
8. *	Camel and Tibetan Antelope	Rabbit	(Zhoa *et al*., 2006)
9.	Ibex (*Capra ibex*)	Goat (*Capra hircus*)	(Wang *et al*., 2007)
10. *	Human	Rabbit	(Ji *et al*., 2007)
11. *	Marbled cats (*Pardofelis marmorata*)	Domestic cat and rabbit	(Yun *et al*., 2008)
12. *	Wood bison (*Bison bison athabascae*)	Domestic cattle (*Bos taurus*)	(Kumar *et al*., 2009)
13.	Gray wolf (*Canis lupus*)	Domestic dog	(Kim *et al*., 2009)
14. *	Bucardo **(***Capra pyrenaica pyrenaica***)**	Goat (*Capra hircus*)	(Folch *et al*., 2009)
15. *	Asian Elephant	Rabbit	(Sathanawongs and Jarujinda 2010)
16. *	Rhesus monkey (*Macaca mulatta*)	Cow (*Bos taurus*)	(Kwon *et al*., 2011)

17. *	cattle, goat and rat	Domestic Buffalo (*Bubalus bubalis*)	(Selokar *et al*. 2011)
18. *	Korean raccoon (*nyctereutes procyonoides koreensis*)	Pig	**(Cheong *et al.* 2012)**
19.	Coyotes	Domestic Dog	(Hwang *et al*. 2012)
20.	Wild Buffalo (*Bubalus arnee*)	Buffalo (*Bubalus bubalis*)	(Priya *et al*. 2014)
21. *	Tibetan antelope (*Pantholops hodgsonii*)	Bovine, ovine and caprine oocytes.	(Guanghua *et al*. 2014)
22. *	Goat	Sheep	(Khan *et al*. 2014)
23. *	Tiger (*Panthera tigris*)	Domestic cat (*Felis silvestris*)	(Moro *et al*. 2015)
24.	Bactrian camel *(Camelus bactrianus*)	Dromedary camel (*Camelus dromedarius*)	(Wani *et al*., 2017)
25. *	Asiatic cheetah (*Acinonyx jubatus*	Domestic cat	(Moulavi *et al*. 2017)

	venaticus)		

S no.'s marked star (*) are inter genera or inter higher taxon nuclear transfers.

The success of offspring production with iSCNT has however been achieved with only a limited number of species which include Gaur (Lanza *et al*. 2000), Mouflon (Loi *et al*., 2001), African Wildcat (*Felis silvestris lybica*) (Gomez *et al*., 2004), Banteng (*Bos javanicus)* (Sansinena *et al*., 2005), Bucardo **(***Capra pyrenaica pyrenaica***) -** an extinct wild goat (Folch *et al*., 2009), gray wolf (*Canis lupus*) (Kim *et al*., 2009), Coyotes (*Canis latrans*) (Hwang *et al*., 2012), , Wild buffalo (NDRI Karnal, 2015), Bactrian camel (*Camelus bactrianus*) (Wani *et al.*, 2017), .The cloning of other extinct species like woolly mammoth is on table. (Loi *et al*., 2011)

2.3.1 Applications of Interspecies somatic cell nuclear Transfer

It has been proposed that iSCNT will act as a de-extiction tool as well as an adjunct to other conservation techniques being utilized for endangered species. Bucardo **(***Capra pyrenaica pyrenaica***) -** an extinct wild goat was sucessfully cloned and a live offspring delivered, however it could not survive (Folch *et al*., 2009).Some endangered species have also been cloned, however the number remains quite less.

In human embryonic stem cell production also, this technique is quite useful as restricted human oocyte availability is a great constraint. The use of nonhuman oocytes for reprogramming could be of immediate value as a tool for the production of human-nuclear-transfer-derived embryonic stem cells (NTESCs) from individuals suffering from such late onset diseases as diabetes, Parkinson's disease, and Alzheimer's disease, among others. In turn, these cells could be used to develop new treatments in vitro.(Beyhan *et sal.,* 2007)

2.3.2 Challenges to Interspecies somatic cell nuclear Transfer

Although many mammalian species have been cloned to date, the percentage of reconstructed oocytes that develop into normal, healthy offspring is

remarkably low, often below 1% (Panarace *et al*., 2007). The poor outcome of somatic cell nuclear transfer (SCNT) is thought to be a consequence of incomplete reprogramming of the donor cell (Jeon *et al*. 2008).

A range of different outcomes to the reprogramming process are possible viz (i) no reprogramming of the genome, resulting in immediate death of the (Nuclear transfer) NT embryo, (ii) partial reprogramming, allowing initial survival of the clones but resulting in an abnormal phenotype and/or lethality at various stages of development, (iii) faithful reprogramming producing normal animals. The phenotypes observed in nuclear clones suggest that complete reprogramming is the exception.(Rideout *et al*., 2001)

Additionally, mechanisms of nuclear reprograming by the oocyte cytoplasm are little-known (Tecirlioglu *et al*., 2006). Elimination of the accompanying donor cell's mitochondrial DNA (mtDNA) in favour of the recipient oocyte's genetically more divergent population (Jiang *et al*., 2011) and nuclear-mitochondrial incompatibility correlating with low electron transport chain (ETC) activity and extremely low blastocyst development e.g. in rhesus monkey-cow iSCNT embryos (Kwon *et al*., 2016) is also observed.

There are certain other problems encountered in iSCNT like embryonic centrosome dysfunction which might be caused by incompatibilities between ooplasm and somatic cell (Zhong *et al*., 2007), births of morphologically abnormal offsprings and the clones' inheritance of maternal mitochondrial DNA e.g. in coyote-dog iSCNT (Hwang *et al*., 2012) and defects in nucleolus formation (Cheong *et al*., 2012).

Some of these problems may also be directly and indirectly related to abnormal reprogramming of the donor somatic cell mediated by DNA methylation and histone acetylation (Xiong *et al*., 2013).

To add to all this are the ecological challenges. Even cloned organisms from frozen cells are not be identical to the extinct organism with which they share their nuclear genome. For example, in SCNT, the mitochondria that are present in the enucleated egg cell are passed on to the developing offspring. Mitochondria

have their own genome that encodes for genes involved with cellular metabolism, which is fundamental to life. The products of these mitochondrial genes interact with the products of genes encoded by the nuclear genome. This interaction may therefore affect the phenotype of the clone (Burton *et al*., 2006). Perhaps, more importantly, however, are gene–environment interactions that will necessarily differ from those experienced by the extinct species. The clones will develop within the eggs and uteri of a different species, whose diet, environment and even genes (Bird 2007; Li, Zheng & Dean 2010; Teh *et al*. 2014; Dosch 2015) influence the developmental process and resulting phenotype. When the animal is born, it will be raised by a surrogate species, with different behaviours and social structures, which will affect its phenotype. It will live in an environment that is different from that which persisted in the past, and consume a different diet than was consumed by other members of its species. It will have a different microbiome, different stressors and, ultimately, a different epigenome than the donor of the somatic cell to which it is genetically identical.

In the majority of ongoing de-extinction projects, the goal is to create functional equivalents of species that once existed: ecological proxies that are capable of filling the extinct species' ecological niche.(Shapiro 2017)

2.4. Somatic Cell Nuclear Transfer (SCNT) Reprogramming

Epigenetic modification stands for change in gene expression with same DNA sequence. Nuclear reprogramming is defined as the set of epigenetic changes that are important for normal embryo and placental development. DNA methylation and histone acetylation are the key mechanisms of this process. In context to mammalian SCNT, reprogramming is the process that enables somatic cell nucleus to adopt the role of zygotic nucleus (Eckardt *et al.,* 2004). The transition from the differentiated somatic cell to the embryonic stage through SCNT requires activation energy to reprogram the resultant zygote to a pluripotent state (Bonasio *et al.,* 2010; Mikkelsen *et al.,* 2008). It is known that SCNT can reprogram a differentiated somatic cell to a totipotent state because

cloned animals are born with all tissue types (Kikyo *et al*., 2000; Wade and Kikyo, 2002). However, the main cause of developmental failure in cloned animals is incomplete nuclear reprogramming. In cattle, several studies have shown that DNA/Histone methylation, Histone acetylation (Bourchis *et al.,* 2001; Dean *et al.,* 2001; Santos *et al.,* 2003) and gene expression (Daniels *et al.,* 2000, 2001) are abnormal in SCNT embryos as compared to in vivo and *in vitro* generated counterparts.

2.4.1 Epigenetic modifications: DNA methylation and histone modifications

DNA methylation and covalent modifications of the core histones are the two most important epigenetic controls of gene expression during early embryogenesis and gametogenesis. The methylation pattern in genomic DNA is characterized by the presence of methylated cytosines on the bulk of DNA, while the unmethylated ones are located mainly within particular regions termed CpG islands. The mammalian genome contains 30,000–40,000 CpG islands. These can be located around the promoters of housekeeping genes, but are also associated with tissue-specific genes (Antequera, 2003). One of the characteristics of CpG islands is that most cytosine residues are unmethylated, with the exception of those associated with imprinted or X-linked genes (Razin and Cedar, 1994). The correct pattern of DNA methylation of cytosines in CpG dinucleotides is required for normal mammalian development, and plays a role in X chromosome inactivation and in the allele-specific expression of imprinted genes (Li and Jaenisch, 1993; Beard *et al*., 1995). DNA methylation is catalyzed by a number of DNA methyltransferases (DNMTs). DNA methyltransferase 1 (DNMT1) is the maintenance enzyme responsible for methylation of hemimethylated CpG dinucleotides after DNA replication (Bestor, 1992). DNMT3a and DNMT3b are required for *de novo* methylation *in vivo* and, thus, are needed to establish new DNA methylation patterns during development (Hsieh, 1999; Okano *et al*., 1999). The oocyte-specific isoform DNMT1o (Howell *et al*.,2001) is responsible for maintaining, but not establishing, maternal imprints.

DNMT3L, a protein that by itself has no DNMT activity, colocalizes with DNMT3a and DNMT3b and is thought to be essential for establishing methylation imprints in the female germ line (Bourc'his *et al*., 2001; Hata *et al*., 2002). Although the murine DNMT3L gene is both expressed and essential for imprint establishment during murine oogenesis; during human development, transcripts of the human DNMT3L gene are only detected after fertilization (Huntriss *et al*., 2004), suggesting that the mechanisms and/or the timing of imprint establishment may differ in humans. To date, no data are available regarding DNMT3L expression in bovine preimplantation development.

As in the case of DNA methylation, histone modifications are also important for the regulation of chromatin structure and gene expression. The core histones that form the nucleosomes are subject to many covalent modifications, such as methylation and acetylation. For instance, five lysine residues can be methylated in histone H3 (Lachner *et al*., 2003). Methylation at lysine residues K4 and K9 plays opposing roles, with K4 methylation being associated with transcriptionally active chromatin and K9 methylation with inactive chromatin (Lachner and Jenuwein, 2002). Methylation of histone H3 at lysine 9 (H3-K9) mediates heterochromatin formation by forming a binding site for HP1 (heterochromatin protein 1), and also participates in silencing gene expression at euchromatic sites. ESET, G9a, SUV39-h1, -h2, and Eu-HMTase are all members of a group of histone methyltransferases that catalyse H3-K9 methylation in mammalian cells. In active genes, histones H3 and H4 are hyperacetylated due to the activity of histone acetyltransferases (HATs), whereas in silent genes they are under acetylated (Clarke *et al*., 1993; O'Neill and Turner, 1995; Grunstein 1997). Histone deacetylation is carried out by histone deacetylases (HDACs).

It is not yet clear whether DNA methylation is the cause or the effect of transcriptional repression (Park and Pfeifer, 2003). Results of a recent study suggest that *de novo* DNA methylation and histone deacetylation might proceed simultaneously to produce gene silencing (Santoro *et al*., 2002).

2.4.2 SCNT epigenome

The cloning of mammals by somatic cell nuclear transfer (NT) requires epigenetic reprogramming of the differentiated state of donor cell to a totipotent, embryonic ground state (Gurdon and Colman, 1999). It means that the donor cell must cease its own program of gene expression and assume an expression program typical of a zygotic genome. If the reprogramming process operates inefficiently, the resulting epigenetic anomaly affects embryonic development (Bestor, 1998). Poor epigenetic reprogramming in early cleavage embryos entails dysregulation of gene expression, and the accumulated action of abnormally expressed genes in cloned fetuses can disrupt their normal development (Humpherys *et al*., 2001).

Recently several studies were done to understand the chromatin remodeling in cloned embryos to improve the SCNT technique. Suteevun *et al.* (2006), examined the expression profile of genes involved in DNA and histone modifications in swamp buffalo (*Bubalus bubalis*). The expression levels of all the DNA modifying genes (DNMTs and HDACs) were higher in the SCNT embryos at 8-cell and blastocyst stages as compared with IVF embryos. The DNA methylation has been studied in cloned embryos from many species. All the species except pig exhibited global DNA hypermethylation (Dean *et al.,* 2001; Kang *et al.,* 2001). The methylation pattern observed in cloned bovine embryos differs from both *in vitro* and *in vivo* produced control embryos and abnormalities in DNA methylation levels are quite variable among individual clones (Kang *et al.,* 2001). The phenomenon of hypermethylation has been found in both cattle and mice hence proving the fact that cloned Embryos exhibit a state of hypermethylation (Bourc'his *et al.,* 2001; Dean *et al.,* 2001; Kang *et al.,* 2001). Many studies have reported that the aberrant levels of DNMTs mRNA in cloned embryos may contribute to the abnormalities seen in embryos and fetal development (Dean *et al.,* 2001; Yang *et al.*, 2007). In particular, Cho *et al.,*

(2007) found that hypermethylation in the placenta of a cloned cat may be responsible for the low success rate in cloned felid species.

The first investigation of the transcription of *HAT1* and *HDAC1*, which are involved in histone acetylation, in IVF and cloned embryos in felid species, was done by Imsoonthornruksa *et al*. (2010). Higher transcription of the DNA methylation genes and lower transcription of the histone acetylation genes were observed in cloned embryos as compared with IVF embryos. The results demonstrate that the felids' donor nucleus could not be completely reprogrammed and so the re-establishment of embryonic totipotency was not achieved. The problem of incomplete nuclear reprogramming and the low blastocyst rate in iSCNT are not solved yet. If CpG methylation/histone acetylation is the basis for epigenetic based cellular memory and ensuring function of a differentiated cell, then approaches aimed at altering global levels of methylation/acetylation in an attempt to restore developmental and differentiation potential should be beneficial to improve developmental competence of SCNT generated reconstructs.

2.5 Strategies to improve Embryo development rates

2.5.1 Oocyte Extracts

X. R. Xiong *et al*. (2012) carried out a study aimed to evaluate the effect of pre-treatment of donor cell with oocyte extracts on the early developmental competence of yak iSCNT embryos. Yak fibroblasts were reversibly permeabilized with streptolysin O, and then treated with yak oocyte extracts (YOE) or bovine oocyte extracts (BOE) prior to iSCNT. The 8-cell and blastocyst formation increased significantly compared with the control group when donor cells were pre-treated with YOE or BOE. The relative expression level of embryo-specific genes TBP1 and Mash2 were also up-regulated both in the blastocysts of the YOE and BOE groups. In addition, the methylation level of pluripotency-specific genes (Oct4 and Nanog) in the blastocysts of the YOE and BOE groups were similar to that of its IVF counterpart (53.1%, 48.8% vs. 40.1%; 24.8%, 26.5% vs. 35.9%). Their results suggested that pre-treatment of donor cells with

oocyte extracts can improve nuclear-cytoplasmic reprogramming; thus representing a novel way to improve the efficiency of yak iSCNT (Xiong *et al*., 2012).

2.5.2 Mitochondrial DNA

Cloned Embryos often arrest during the early stages of preimplantation development; fail to reprogramme the somatic nucleus; and eliminate the accompanying donor cell's mitochondrial DNA (mtDNA) in favour of the recipient oocyte's genetically more divergent population. This last point has consequences for the production of ATP by the electron transfer chain, which is encoded by nuclear and mtDNA. Using a murine-porcine interspecies model, the importance of nuclear-cytoplasmic compatibility on successful development was investigated Y jiang *et al*. (2011). In this experiment, murine fetal fibroblasts were transferred into enucleated porcine oocytes, which resulted in extremely low blastocyst rates (0.48%); and failure to replicate nuclear DNA and express Oct-4, the key marker of reprogramming. Using allele specific-PCR, peak levels of murine mtDNA were detected at 0.1460.055% of total mtDNA at the 2-cell embryo stage and then at ever-decreasing levels to the blastocyst stage. Furthermore, these embryos had an overall mtDNA profile similar to porcine embryos. Porcine oocytes were then depleted of their mtDNA using 10 mM 29,39-dideoxycytidine and transferred murine somatic cells along with murine embryonic stem cell extract, which expressed key pluripotent genes associated with reprogramming and contained mitochondria, into these oocytes. Blastocyst rates increased significantly (3.38%) compared to embryos generated from non-supplemented oocytes (P,0.01). They also had significantly more murine mtDNA at the 2-cell stage than the non-supplemented embryos, which was maintained throughout early preimplantation development. At later stages, these embryos possessed 49.99% murine mtDNA. They also exhibited an mtDNA profile similar to murine preimplantation embryos. Overall, these data demonstrate that the

addition of species compatible mtDNA and reprogramming factors improves developmental outcomes for iSCNT embryos (Y Jiang *et al*. 2011)

2.5.3 Use of Chromatin modifying agents

Wittayarat *et al*. (2013) reported that the iSCNT cat and parthenogenetic bovine embryos were treated with various concentrations of TSA (0, 25, 50, or 100nM) for 24 h, respectively, following fusion and activation. Treatment with 50nM TSA produced significantly higher rates of cleavage and blastocyst formation (84.3% and 4.6%, respectively) of iSCNT embryos than the rates of non-TSA–treated iSCNT embryos (63.8% and 0%, respectively). Similarly Opiela *et al*. (2017) conducted an investigation which showed that TSA-dependent epigenomic modulation of nuclear donor MSCs highly affects both the in vitro developmental capability and the cytological quality of inter-species (porcine/bovine) cloned embryos. The developmental competences to reach the blastocyst stage among hybrid (porcine/bovine) nuclear-transferred embryos that had been reconstructed with bovine ooplasts and epigenetically modulated porcine MSCs were maintained at a relatively high level. These competences were higher than those noted in studies by other authors, but they were still decreased compared to those of intra-species (porcine) cloned embryos that had been reconstituted with porcine ooplasts and either the cell nuclei of epigenetically transformed MSCs or the cell nuclei of epigenetically non- transformed MSCs. (Opiela *et al*., 2017)

Ding *et al*. (2008) also reported that treatment of both donor cells and early cloned embryos with a combination of 10nM 5-aza-dC and 50nM TSA significantly improved the ability to develop into blastocyst (Ding *et al*., 2008).Furthermore, Wang *et al*. (2011) showed that this treatment also significantly enhanced the development of NT bovine embryos *in vivo*, thereby dramatically increasing the cloning efficiency (number of surviving calves at 60 days of birth/number of recipient cows) from 2.6% to 13.4%.

Xiong *et al*. (2013) reported on the cell proliferation and viability of Zebularine or Zebularine plus Scriptaid treatments, there was no significant difference between treated groups and the control group, indicating that 20 μM Zebularine and 0.5 μM Scriptaid have little cytotoxicity on yak fibroblasts. Moreover, treatment of both donor cells and early embryos with epigenetic modification drugs had no effect on the cleavage rates, but the ability of cloned embryos to develop into blastocysts was significantly improved after the treatment. Moreover, the total cell number of cloned embryos significantly increased after treatment with epigenetic modification drugs. In addition, the cryosurvival rate of vitrified–thawed cloned blastocysts derived from early embryos treated with 0.5μM Scriptaid was significantly higher than that of control group (Xiong *et al*., 2013).

Sun *et al*. (2012) reported increased cleavage rate of human nuclear transfer embryos after 5-aza-2'-deoxycytidine treatment (Sun *et al*. 2012). Jeon *et. al*. (2008) concluded that S-adenosylhomocysteine (SAH) induces global DNA demethylation that partially reactivates the X inactived chromosome (Xi) , and that a hypomethylated genome may facilitate the nuclear reprogramming process (Jeon *et al*. 2008).

2.6 Work done on iSCNT in India

Priya *et al*. (2014) developed wild buffalo embryos by interspecies somatic cell nuclear transfer (iSCNT) through handmade cloning using wild buffalo somatic cells and domestic buffalo (Bubalus bubalis) oocytes. They observed the the cleavage (92.6 ± 2.0% vs 92.8 ± 2.0%) and the blastocyst rate (42.4 ± 2.4% vs 38.7 ± 2.8%) was not significantly different between the intraspecies cloned embryos produced using skin fibroblasts from domestic buffalo and interspecies cloned embryos produced using skin fibroblasts from wild buffalo (Priya *et al*. 2014)

Khan *et al*. (2014) investigated the effect of three different culture media on the development of Handmade cloned intraspecies (goat-goat) and interspecies

(goat-sheep) embryo reconstructs. They found Research vitro cleave media (RVCL) to yield higher cleavage and morula-blastocyst development in intraspecies and interspecies nuclear transfer groups compared with G1. G2 and modified synthetic oviductal fluid (mSOFaaci).

2.7 Work done on Reprogramming in India

Panda *et al*. (2012) reported that treatment of Handmade cloned (HMC) embryos with 500 or 1000 nmol/L scriptaid significantly increased the cleavage rate (91.3 ± 2.8 and 91.9 ± 2.0%, respectively) than that of controls (80.7 ± 2.4%). Blastocyst rate was significantly higher following treatment with 1000 nmol/L (54.1 ± 5.0%) than that with 500 nmol/L scriptaid (42.6 ± 2.9%) which, in turn, was higher than that of the controls (38.0 ± 2.6%). Also, HMC embryos treated with 500 or 1000 nmol/L scriptaid had higher cell number (339.9 ± 1.4 and 343.4 ± 2.4, respectively) than the untreated embryos (150.7 ± 2.0). These results demonstrate that scriptaid treatment improves the developmental potential of HMC buffalo embryos, but compromises that of zona-free Parthenogenetically Activated embryos.

Saini *et al*., 2015 reported cells of fibroblast origin are easier to reprogram than those of epithelial origin, when used in interspecies SCNT, and cloning efficiency, epigenetic status, and gene expression pattern vary among cells having different origin although they may be from the same tissue.

Chapter 3

MATERIALS & METHODS

3.1 Plastic ware and Glassware

All the glassware used in the present investigation was made of high-grade pyrex glass. The glassware, wherever used, were thoroughly cleaned, rinsed with ultrapure water and then heat sterilized at 250˚C for 4 hours. Disposable 35 mm x 10 mm cell culture Petri dishes, 15 and 50 ml centrifuge tubes, 100 mm x 100mm square Petri dishes with 13 mm grid and capillary pipettes (10 µl, 20 µl capacity; Unopette®) were purchased from Becton, Dickinson and Co., Lincoln Park, New Jersey, United States of America (USA) or from Nunc Rosklide, Denmark. Disposable plastic syringes were non-toxic and non-pyrogenic obtained from Becton, Dickinson and Co., Disposable 18-gauge hypodermic needles were from Dispovan (Hindustan Syringes and Medical Devices Ltd. Faridabad, India) whereas the 0.22 and 0.45 µm filters were from Millipore Corporation, Bedford, MA, USA. Autoclavable disposable tips for micropipettes were obtained from Labware, USA.

3.2 Chemicals, Cell Culture Media and Supplements

The culture media used in the present study, which included tissue culture medium-199 (TCM-199), Dulbecco's phosphate buffered saline (DPBS) and the additives which included bovine serum albumin (BSA), porcine follicle stimulating hormone (FSHp) and antibiotics (gentamicin, penicillin and streptomycin) were purchased from Sigma Chemical Co., St. Louis, MO, USA. Various additives like sodium pyruvate and L-glutamine were also purchased from Sigma Chemical Co. All the cell culture media were in the form of ready-to-use liquid media. Mineral oil, enzymes (Hyaluronidase, Pronase and Trypsin) and other chemicals used were also from Sigma Aldrich Chemicals, unless otherwise indicated. Most of the chemicals used were either embryo culture tested or of cell

culture grade. Fetal bovine serum (FBS; Canadian Origin) was from Hyclone (Logan, Utah, US).

3.3 Equipment

3.3.1 Microscopy

i) Zoom stereomicroscopes

Low magnification zoom stereomicroscopes (OLYMPUS, Japan, Model SZX 7 and SZX 10) were used for searching the aspirated oocytes, evaluating the topography and quality of the oocytes and embryos, manipulating zona-free oocytes, electrofusion etc.

ii) Inverted microscope

An inverted microscope (OLYMPUS, Japan, Model 1X71) was used for the examination of oocytes and embryos at various stages during *in vitro* culture. The microscope with the light source at the top and a long working distance condenser allowed oocytes and embryos in the dishes to be viewed and photographed whenever needed. The microscope was equipped with a warm stage attachment, so that optimum temperature conditions could be maintained during working. The microscopes were equipped with programmable still photography and video recording facilities.

3.3.2 CO2 incubator

A Thermo Fisher Scientific (USA, Model 3131) make CO_2 incubator, with facility to maintain humidified environment, adjustable incubation temperature and CO_2 concentrations was used for the culture of somatic cells, oocytes and embryos. The incubator was attached with a data recorder for continuous real time monitoring of the incubation parameters during prolonged embryo culture.

3.3.3 Electrofusion machine

The electrofusion machine model used for fusion of somatic cells and recipient oocytes was BLS CF-150B from BLS, Budapest, Hungary, capable of

providing regulated AC and DC voltages required for electrofusion. The fusion chamber was made of BLS microslide 0.5mm gap, model GSS-500.

3.4 Laminar Flow Cabinet

Experiments including cleaning, processing and culturing of oocytes and somatic cells, *in vitro* produced embryos and cloning procedures were carried out in Laminar flow cabinet (CLEANAIR Laminar Flow Systems, India), which served the purpose of eliminating microbial contamination and ensuring the safety of the operator. UV irradiation and thorough cleaning of working places with ethanol (70% v/v) was used to maintain hygienic and sterile environment throughout the experiments.

3.3.5 Centrifuge

Refrigerated Centrifuge (REMI R-12C) with facilities to adjust centrifugation speed, time and temperature was used for centrifugation of the tissue explants, washing of the cells etc. as and when needed

3.3.6 Vortex Shaker

Vortex shaker (SPINIX, from REMI) was used for the dissociation of cumulus cells for denuding the *in vitro* matured oocytes etc.

3.4 Biological Material

3.4.1 Ovaries

The ovaries served as the source of immature oocytes and cumulus cells during the present investigation. The buffalo ovaries were collected from abattoir (New Delhi, India). Buffalo ovaries were collected from abattoir and then washed 3-4 times with warm isotonic saline (32-37˚C) containing 400 IU/ml penicillin and 500 μg/ml streptomycin and transported to the laboratory within 6 h.

3.4.2 Ear tissue for fibroblast cell culture

Ear tissues were aseptically obtained from Cattle reared at Mountain livestock research institute (MLRI) Manasbal in sterile Dulbecco's phosphate-

buffered saline (DPBS) and due consideration was given to animal ethics and welfare.

3.5 METHODOLOGY

3.5.1 Composition of Different Media

1. Isotonic Saline

Component	Quantity 500ml
Sodium Chloride	4.5 g
Penicillin	400IU/ml
Streptomycin	250 mg
Distilled water	500 ml

2. Oocyte searching medium

Component	Quantity 50 ml
TCM-199	50 ml
BSA	150 mg

3. Oocyte washing medium

Component	Quantity 10 ml
TCM-199	8.5 ml
FBS	1.5 ml
Sodium pyruvate	0.8 mM
L –glutamine	0.68 mM
Gentamicin	50 µg/ml

4. Maturation medium

Component	Quantity 1 ml
Washing medium	1 ml
FSH	5 µg/ml*
Estradiol β-17	1 µg/ml *
Buffalo Follicular Fluid	10%*

Entries marked Star(*) were varied in different groups

5. T0 Medium

Component	Quantity 10 ml
HEPES modified M-199	10 ml
L-glutamine	2.0 mM
Sodium Pyruvate	0.2 mM
Gentamicin	50 µg/ml

FBS is supplemented as per requirement, percentage of FBS is denoted by number following T, for example T2 denotes T0 supplemented by 2% FBS

6. Activation Medium

1 mg/ml stock of calcium ionophore kept in Dimethyl Sulphoxide, and stored at 4˚C in small amber coloured bottle with tightly closed cap. 2µl of stock solution is added to 1 ml of M-199 supplemented with 20% FBS.

7. Embryo culture medium

Component	Quantity 5 ml
RVCL (Research vitro cleave *media*)	5 ml
Fatty acid free BSA	10 mg/ml

3.5.2 Collection and In vitro maturation of oocytes

Buffalo ovaries will be collected from abattoir and washed three times with warm isotonic saline (32–37°C) containing 400 $IUmL^{-1}$ penicillin and 500 μgmL^{-1} streptomycin and transported to the laboratory within 6 hours in insulated boxes. In laboratory ovaries were rinsed 2-3 times, trimmed to remove extra tissue and washed again.

Oocytes from follicles (2 to 8 mm in diameter) will be aspirated with 19-gauge needle attached to a 10-mL syringe. The aspiration medium consisted of M-199 containing 0.3% bovine serum albumen (BSA). The oocytes were searched under zoom stereo microscope at around 20X magnification. The oocytes were then washed four to six times with the washing medium, which consisted of M-

199 supplemented with 10% fetal bovine serum (FBS), 0.68 mM L-glutamine, 0.8 mM sodium pyruvate and 50 μgmL^{-1} gentamicin. Cumulus–oocyte complexes (COCs) having a compact and unexpanded cumulus mass with equal to or greater than three layers of cumulus cells and homogenous granular ooplasm were used for in-vitro maturation (IVM).

After washing thrice with IVM medium (M-199 supplemented with 10% FBS, 5 μgmL^{-1}pFSH, 1 μgmL^{-1} estradiol-17β, 0.8 mM sodium pyruvate and 50 μgmL^{-1} gentamicin), groups of 15–20 COCs were cultured in 100μL droplets of IVM medium, overlaid with sterile mineral oil in 35-mm Petri dishes, and cultured for 21h in a humidified CO_2 incubator (5% CO_2 in air) at 38.5°C.

3.5.3 Preparation of recipient cytoplasts

COCs with expanded cumulus were transferred into a 1.5mL microcentrifuge tube containing 500 μL Hyaluronidase (0.5 mgmL$^{-1)}$ in T2 (where T denotes HEPES modified M- 199 supplemented with 2.0 mM L-Glutamine, 0.2 mM Sodium pyruvate, 50 µgmL^{-1} gentamicin and the following number denotes 2% FBS) and incubated for 1 min at 38.5°C followed by vortexing (3 min). Completely denuded oocytes with evenly granular cytoplasm were selected and incubated in Pronase (2.0 mgmL−1 in T containing 10% FBS) for 8 min at 38.5°C. Oocytes with completely digested zona pellucida were transferred into T20 (T containing 20% FBS) and incubated at 38.5°C for 30 min or until a prominent protrusion cone was easily visible. Protrusion cone bearing oocytes were transferred (five to eight each time) into a 35-mm dish containing 4 mL T20 with 2.5µgmL−1 Cytochalasin B and manually bisected using microblade in such a way that the protrusion cone remains with the smaller half. After bisection, the larger demicytoplasts without protrusion cone were transferred into T20 and incubated for 10–15 min at 38.5°C so as to regain spherical shape.

3.5.4 Establishment of Somatic cell Cultures

Ear skin biopsies were taken from ear pinna aseptically from high yielding pedigree jersey calves from Mountain livestock research institute (MLRI)

Manasbal in sterile Dulbecco's phosphate-buffered saline (DPBS). Norms of ethical handling of animals were followed strictly. Tissues were washed thoroughly with sterile Ca^{2+} and Mg^{2+} free DPBS. After removing the skin along with hair follicles the remaining tissue was cut into small pieces and cultured in 12 well cell culture plates containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% FBS. The explants were continuously observed for proliferation of fibroblasts. When cells achieve 80% confluence, they were rinsed, trypsinized, and recultivated.

Cell culture stocks were prepared to be cryopreserved. The cryopreservation media consisted of DMEM (Dulbecco's Modified Eagle Medium): FBS (Fetal bovine serum): DMSO (Dimethyl sulfoxide) in ratio of 6:3:1.Cryovials were preserved by Slow freezing upto -80^0C followed by plunging into liquid nitrogen.

3.5.5 Preparation of Donor cell for Nuclear transfer

The confluent cells at 5^{th} to 10^{th} passage were allowed to grow further 3 days to achieve over-confluence. These Cell culture flasks containing fibroblasts from same animal were randomly divided into four groups based on treatment with different reprogramming agents

1) 10 nM 5-aza- 20-deoxycytidine (5-aza-dC) for 24 hours (group A) ,
2) 0.5µM Scriptaid for 24 hours (group B) ,
3) 0.5µM Scriptaid+ 10 nM 5-aza-dC (group C)for 24 hours and
4) without any treatment (control group, group D).

These cells will then be immediately used as **nuclear donors** in iSCNT to assess the effect of using these cells on developmental competence of iSCNT embryos.

3.5.6 Pairing and fusion

The enucleated demi-cytoplasts were immersed in Phytohemagglutinin (0.5 mgmL−1 in T2) for 3–4 sec and transferred into T2 containing low density donor cells. Each demicytoplast were then allowed to attach to a single, rounded,

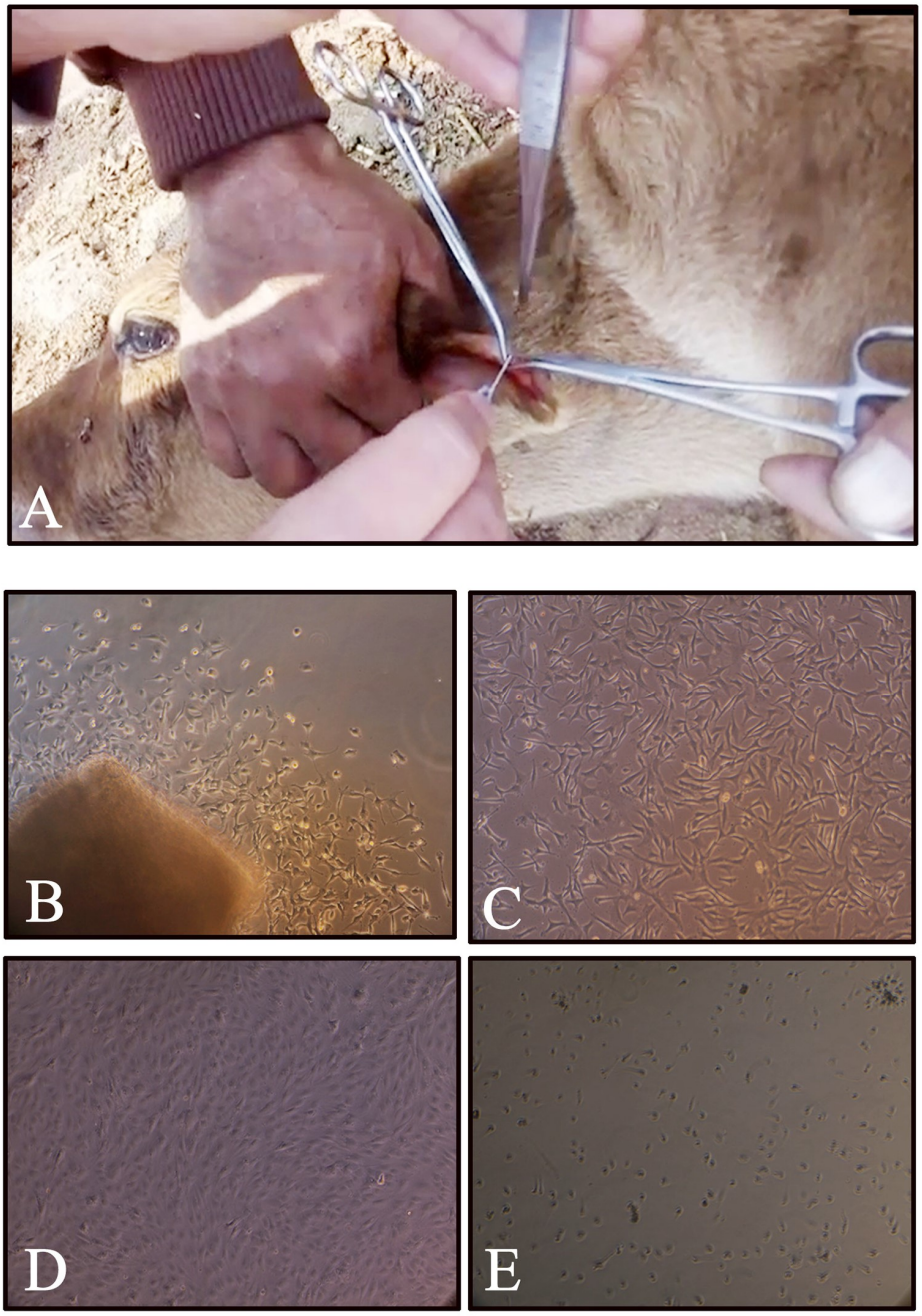

Plate No.1: (A) Collection Of Ear Tissue For Primary Cell Culture (B) Cells Spreading Out Of Tissue Explant (C) A monolayer of cattle fibroblasts at 70% confluence (D) A Confluent Monolayer Of Cattle Fibroblasts (E) Fibroblasts rounding off after trypsinisation before use as nucleus donor.

medium sized cell by gently rolling the demicytoplast over it. The couplets (demicytoplast–donor cell pairs) were then transferred to fusion medium (0.3M D-Mannitol, 0.1 mM $MgCl_2$, 0.05 mM $CaCl_2$ and 1 $mgmL^{-1}$ Polyvinyl alcohol) for equilibration.

The couplets and the remaining demicytoplasts were then transferred away from the positive and negative poles, respectively, of the fusion chamber. A single step fusion protocol was followed wherein a demicytoplast and a couplet were picked using a fine pulled capillary pipette having an inner diameter of 100–120 μm. Initially, the couplet will be expelled and aligned with an AC pulse (6 volts) using BTX Electrocell Manipulator 200, so that the somatic cell faced the negative electrode and immediately after alignment the demicytoplast was introduced into the fusion chamber close to the somatic cell. As soon as the somatic cell was sandwiched between the demicytoplasts, single DC pulse (3.36 kVcm−1 for 4 μs) was applied. The triplets were then incubated in T20 (for rounding up) for 4 h at 38.5°C.

3.5.7 Activation

The reconstructed oocytes were then activated (approx. 29h after the start of maturation) by incubating in T20 containing 5µM Calcimycin for 5 min at 38.5°C. After washing thrice in T20 the oocytes were then incubated individually in 5 μL droplets of respective culture medium containing 2 mM 6-Dimethylamino purine (6-DMAP) covered with mineral oil and in CO_2 incubator at 38.5°C for 4h.

3.6 Embryo culture

Research Vitro Cleave medium (containing NaCl, KCl, KH2PO4, MgSO4, NaHCO3, CaCl2, sodium pyruvate, sodium lactate, D-glucose, gentamicin, L-glutamine, and EDTA in purified water) supplemented with 1% FAF-BSA was used. The reconstructed embryos were cultured (10–15 embryos per well) in 400 μL of media on a flat surface in each well of a 4 well dish, covered with 400 μL mineral oil and kept undisturbed in CO_2 incubator for 7 days.

3.7 Statistical Analysis

The data obtained from the experiments was analysed using software package SPSS 20.0 (IBM SPSS Statistics). The differences between means were analysed by one-way ANOVA followed by Fisher's LSD test and significance was determined at $P \leq 0.05$.

Chapter – 4

EXPERIMENTAL FINDINGS

Interspecies cloning presents ample opportunities for research with potential conservational, biomedical and agricultural applications. However, the efficiency of interspecies cloning remains below optimum. Abnormal reprogramming of the donor somatic cell mediated by DNA methylation and histone acetylation is deemed to be an important factor in low efficiency of cloning procedures. The aim of the present study was to determine the efficiency of cloned cattle embryos from use of somatic cells treated with different reprogramming agents viz 5-aza-2'-deoxycytidine (5-aza-dC) (Group A), Scriptaid (Group B), Scriptaid + 5-aza-dC (Group C) and without any treatment (Control group, Group D).Various experiments carried out for achieving this included:

i) Establishment and maintenance of adult skin fibroblast somatic cell cultures for use as nuclei donor.

ii) Comparison of different supplemented constituents of *in vitro* maturation (IVM) media with respect to maturation.

iii) *In vitro* production of interspecies cattle-buffalo embryos by zona-free (HMC) method without any treatment of donor cells (Control group, Group D).

iv) Study effect of using reprogramming agent treated somatic cells as nuclear donors viz 5-aza- 2'-deoxycytidine (5-aza-dC) (Group A), Scriptaid (Group B), Scriptaid + 5-aza-dC (Group C) and compare it with control group.

4.1 Establishment of somatic cell cultures:

4.1.1 Primary culture of Skin Fibroblast cells

Ear skin biopsies taken from jersey calves and seeded in culture flasks started proliferating 2-5 days after initiation of culture. These primary cell cultures

attained 70-80% confluence in 7-12 days' time. Upon sub culturing, the cells formed a confluent monolayer within 48-72 hours of seeding. The cells after 5th passage were used as nuclei donor. The remaining stock of cells was cryopreserved at different passages which served as replacement stock for future use.

4.1.3 Cell viability after treatment with 5-aza-dC and Scriptaid

For assessment of cell viability of the somatic cells, the cells treated with reprogramming agents were harvested and were stained with Trypan blue. The dead and live cells were counted by using a haemocytometer. The Cell viability following treatment was thus checked

4.2 Harvest of Cumulus Oocyte Complex's (COC'S) from abattoir derived buffalo ovaries

COC's were harvested from abattoir derived sheep ovaries by puncturing method. The COC's thus harvested were graded on the basis of presence of cumulus layers, homogenous and evenly granular ooplasm (Table 1).

A total of 1591 abattoir derived buffalo ovaries in 16 replicates were used for the experiments. 3347 COC's were harvested upon aspiration of surface follicles (size ranging from 2mm - 8mm). Thus, a harvest ranging from 1.46 to 2.68 with an average of 2.07 COC's per ovary was obtained. 1399 COC's (41.80%) were of Grade-A and 979 (29.25%) COC's were of Grade-B, while as 969(28.95%) COC's were of Grade-C and Grade-D. A total of 2378 (71.05%) COC's were of usable quality, therefore, selected for IVM. Grade-C and Grade-D COC's were not selected for IVM.

Table 1: Recovery rate and gradation of COC's

Expt. No.	No. of ovaries aspirated	No. of COC's collected	No. of COC's per ovary	Grade A	Grade B	Grade A+B	Grade C+D
1	52	105	2.02	43 (40.95%)	18 (17.14%)	61 (58.10%)	44 (41.90%)
2	108	240	2.22	87 (36.25%)	89 (37.08%)	176 (73.33%)	64 (26.67%)
3	92	186	2.02	78 (41.94%)	50 (26.88%)	128 (68.82%)	58 (31.18%)
4	58	102	1.76	38 (37.25%)	25 (24.51%)	63 (61.76%)	39 (38.24%)
5	102	149	1.46	56 (37.58%)	41 (27.52%)	97 (65.10%)	52 (34.90%)
6	90	141	1.57	75 (53.19%)	35 (24.82%)	110 (78.01%)	31 (21.99%)
7	96	180	1.88	80 (44.44%)	58 (32.22%)	138 (76.67%)	42 (23.33%)
8	101	173	1.71	62 (35.84%)	63 (36.42%)	125 (72.25%)	48 (27.75%)
9	107	201	1.88	93 (46.27%)	57 (28.36%)	150 (74.63%)	51 (25.37%)
10	63	147	2.33	65 (44.22%)	47 (31.97%)	112 (76.19%)	35 (23.81%)
11	98	241	2.46	78 (32.37%)	107 (44.40%)	185 (76.76%)	56 (23.24%)
12	149	400	2.68	175 (43.75%)	137 (34.25%)	312 (78.00%)	88 (22.00%)
13	151	380	2.52	189 (49.74%)	102 (26.84%)	291 (76.58%)	89 (23.42%)
14	120	210	1.75	67 (31.90%)	53 (25.24%)	120 (57.14%)	90 (42.86%)
15	103	240	2.33	76 (31.67%)	54 (22.50%)	130 (54.17%)	110 (45.83%)
16	101	252	2.50	137 (54.37%)	43 (17.06%)	180 (71.43%)	72 (28.57%)
Total	1591	3347	2.07	1399 (41.80%)	979 (29.25%)	2378 (71.05%)	969 (28.95%)

4.3 *In vitro* maturation

For *in vitro* embryo production, immature buffalo oocytes are generally matured for 22 hours. In our study also, oocytes were matured for 22 hours. Only A and B grade COC's with an unexpanded cumulus of not less than two layers were used for in vitro maturation. Mature COC's exhibited a characteristic cytoplasmic granulation and a uniform cumulus expansion. First polar body extrusion was used as indicator of effective maturation.

Table 2:Effect of Supplementation of IVM media with 1) pFSh+ β estradiol, 2) follicular fluid only and 3) pFSh+ β estradiol + Follicular fluid

IVM Medium	No. of COC's	Percent Matured (n)
Supplemented with pFSH + 17 β estradiol	1040	86.25% [a] ±4.86% (897)
Supplemented with follicular fluid only	413	79.42% [a] ±1.37% (328)
Supplemented with pFSh + 17 β estradiol + Follicular fluid	590	94.07% [a] ±3.65% (555)

Figures quoted as percent mean ± s.e.m (n).
Values having same superscripts along columns, differ non-significantly (P<0.05)

Supplementation of IVM media with 1) pFSH + β estradiol, 2) pFSH + β estradiol + Follicular fluid and 3) follicular fluid only was done to study effect of these substances in improving maturation percentages. The results were obtained as follows

The maturation percentage as determined by cumulus expansion and polar body extrusion was found to be higher when IVM medium supplemented with

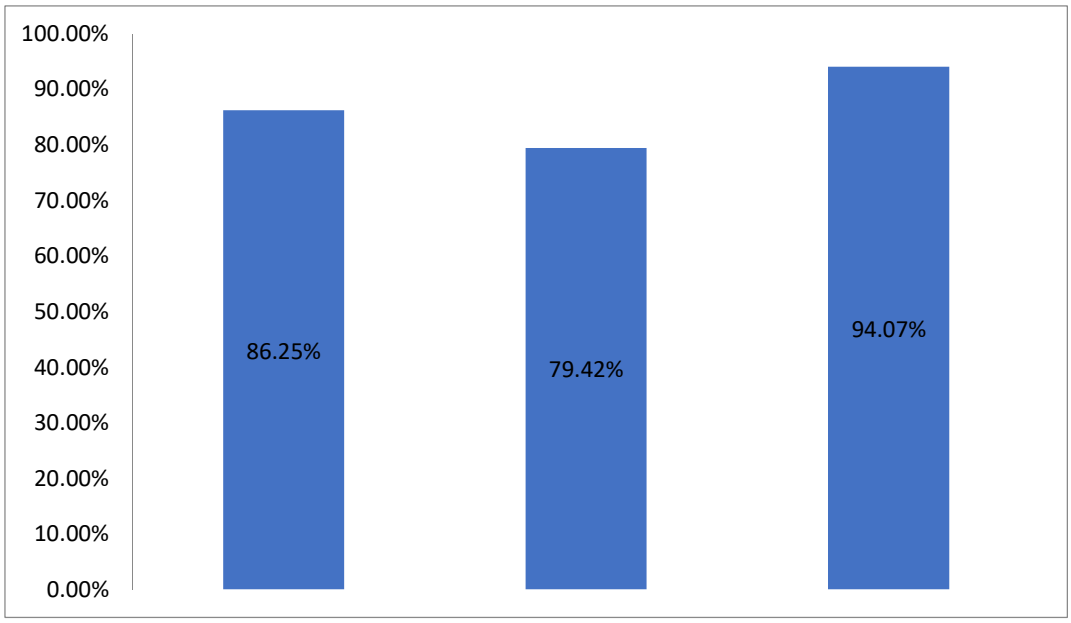

Figure 1:Effect of Supplementation of IVM media with 1) pFSH+ β estradiol, 2) follicular fluid only and 3) pFSH+ β estradiol + Follicular fluid

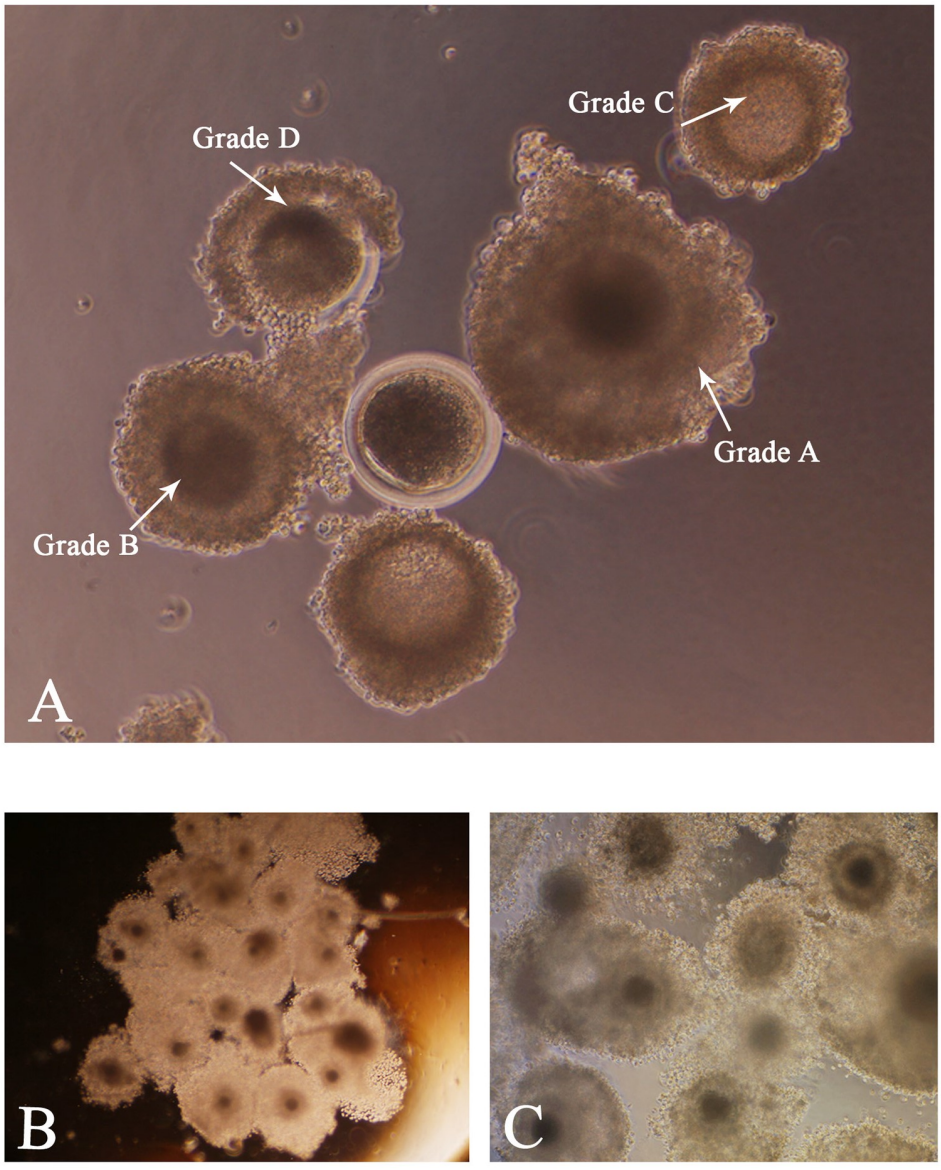

Plate No.2: Different Grades Of oocytes (A) In vitro matured oocytes after 22 hours of incubation in IVM medium at 38.5°C, showing cumulus expansion at 100x(B) and 200x (C).

pFSh + 17 β estradiol + Follicular fluid was used as compared with supplementation of Pfsh+17 β estradiol or follicular fluid only (94.07% ±3.65% v 86.25% ±4.86% and 79.42%±1.37% respectively) but higher maturation percentage observed in IVM medium supplemented with p FSh + 17 β estradiol + Follicular fluid (94.07% ±3.65%)was statistically non-significant (P>0.05). (Table-2, Fig.1)

4.4 *In vitro* Embryo production

4.4.1 Efficiency of in vitro cloned buffalo embryo production by iHMC (zona-free) method:

Cloned zona-free iHMC cattle-buffalo embryos were produced by iHMC technique which involved enucleation by protrusion cone guided manual bisection. Overall, four trails of zona-free sheep embryo production by HMC were conducted and 80 embryos/reconstructs were produced. This group served as the control group.

The percentage of embryos which cleaved on day 2 was 26.25%±0.95%. 2-4 cell stage and 8cellstage and 16-32 cell stage embryo percentages obtained on Day 7 were 42.86%±10.13%, 33.33%±7.01% and 23.81%±8.96%, respectively. (Table-3, Fig.2).

4.4.2 Effect of 10 nM of 5-aza- 2'-deoxycytidine (5-aza-dC) (group A) treatment of nuclei donor somatic cells on i*n vitro* cloned buffalo embryo production

The percentage of embryos which cleaved on day 2 was 35.29%±0.74% 2-4 cell stage and 8 cell stage and 16-32 cell stage embryo percentages obtained on Day 7 were 41.67%±0.82%, 29.17%±4.10% and 25.00%±5.59%, respectively. (Table-3, Fig.2).

4.4.3 Effect of 0.5μM of Scriptaid (group B) treatment of nuclei donor somatic cells on in vitro cloned buffalo embryo production

The percentage of embryos which cleaved on day 2 was 41.79%±3.23%. 2-4 cell stage and 8 cell stage and 16-32 cell stage embryo percentages obtained

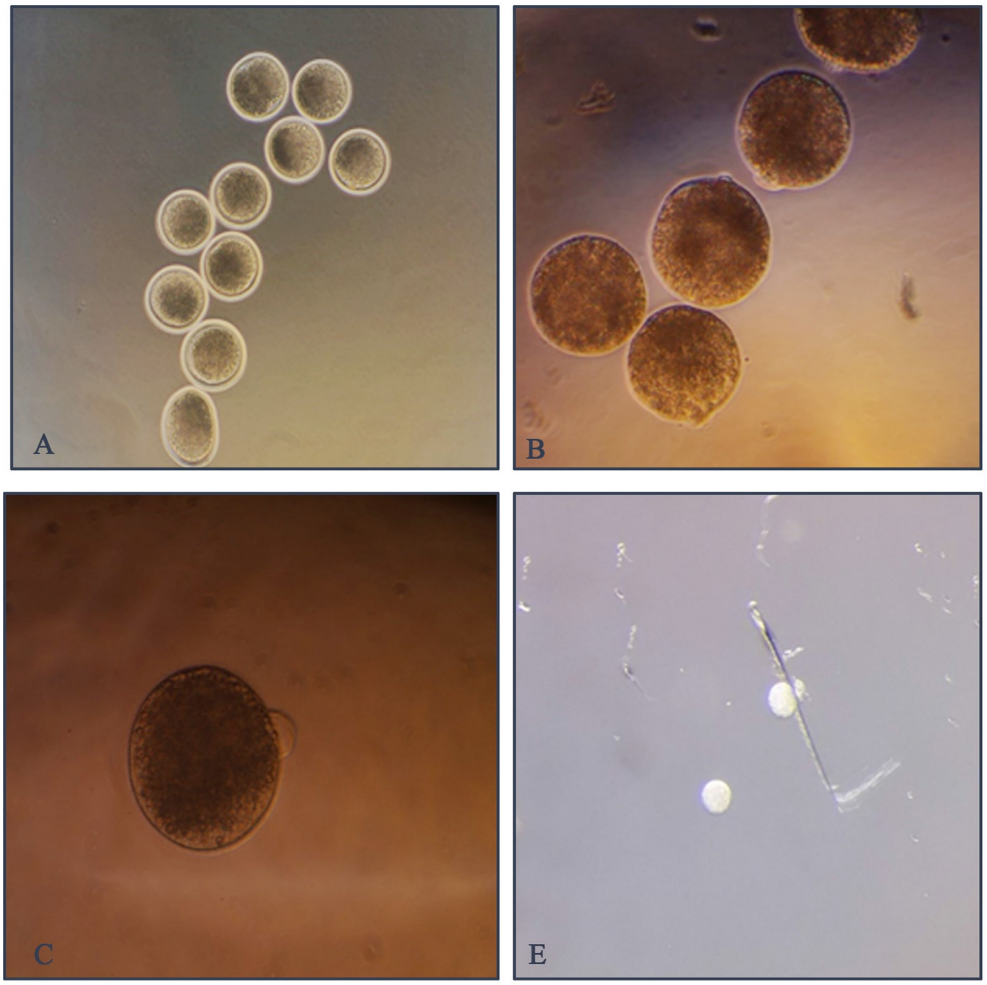

Plate No.3: Hyaluronidase treated COC's(A) Pronase treated COC's(B) Oocyte With Prominent Protrusion Cone(C) Oocyte with protrusion cone guided bisection(D)

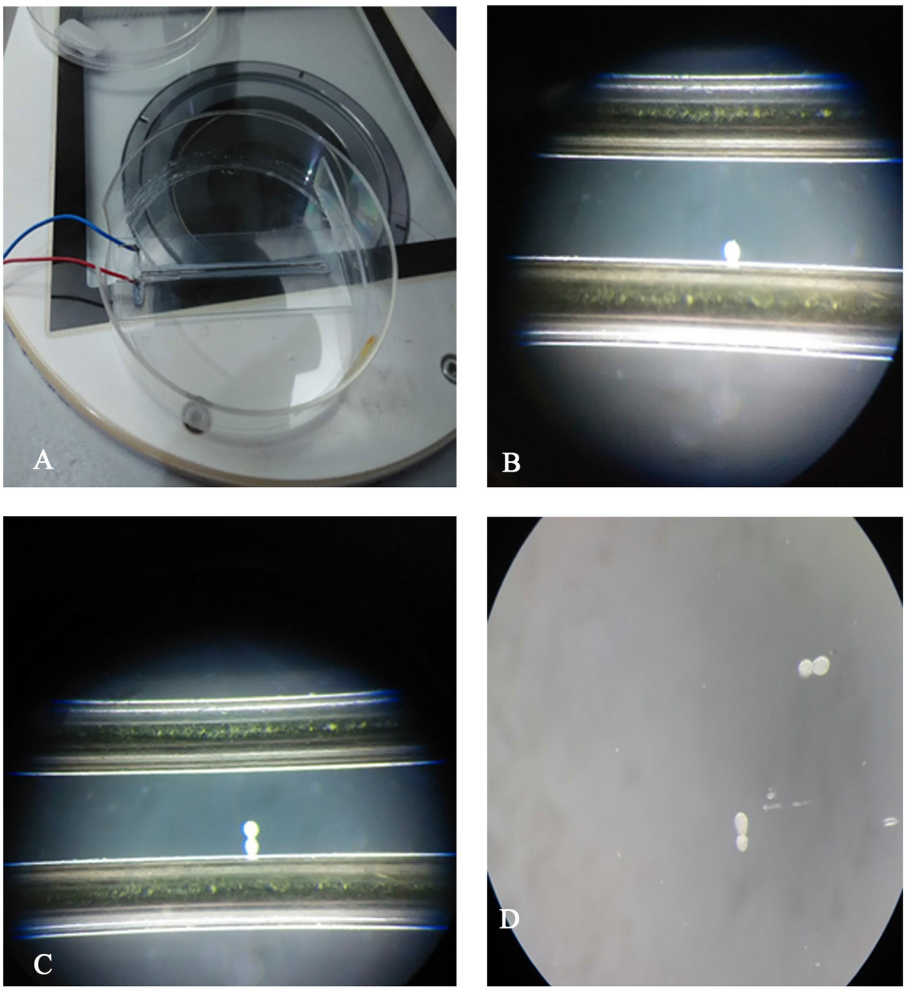

Plate No.4: Fusion Chamber(A) Demicytoplast with Attached Somatic cell(B) Demi-cytoplast and a couplet attached to electrode(C) Recon-structs 10 mins after fusion(D)

on Day 7 were 42.86%±4.36%,35.71%±3.60% and 21.43%±5.59%, respectively. (Table-3, Fig.2).

4.4.4 Effect of 0.5µM of Scriptaid + 10 nM of 5-aza-dC (group C) treatment of nuclei donor somatic cells on in vitro cloned buffalo embryo production

The percentage of embryos which cleaved on day 2 was 35.53%±3.77%. 2-4 cell stage and 8 cell stage and 16-32 cell stage embryo percentages obtained on Day 7 were 44.44%±5.51%,29.63%± 4.50% and 22.22%±4.33% respectively. (Table-3, Fig.2).

Table 3: Effect of using Chromatin modifying agent treated fibroblast on *in vitro* development of interspecies cloned cattle-buffalo embryos

Treatment group	No. Activated	Cleavage	2-4 Cell stage	8 Cell stage	16-32 cell stage
Control	80	26.25%[a] ±0.95% (21)	42.86%[a] ±10.13% (9)	33.33% [a] ±7.01% (7)	23.81% [a] ±8.96% (5)
5-aza-dC	68	35.29%[b] ±0.74% (24)	41.67%[a] ±0.82% (10)	29.17% [a] ±4.10% (7)	25.00% [a] ±5.59% (6)
Scriptaid	67	41.79%[b] ±3.23% (28)	42.86%[a] ±4.36% (12)	35.71% [a] ±3.60% (10)	21.43% [a] ±5.59% (6)
Combo	76	35.53%[b] ±3.77% (27)	44.44%[a] ±5.51% (12)	29.63% [a] ± 4.50% (8)	22.22% [a] ±4.33% (6)

Figures quoted as percent mean ± s.e.m (n).
Percentage of different embryonic stages developed have been calculated out of total number of cleaved embryos.
Values having different superscripts along columns, differ significantly (P<0.05).

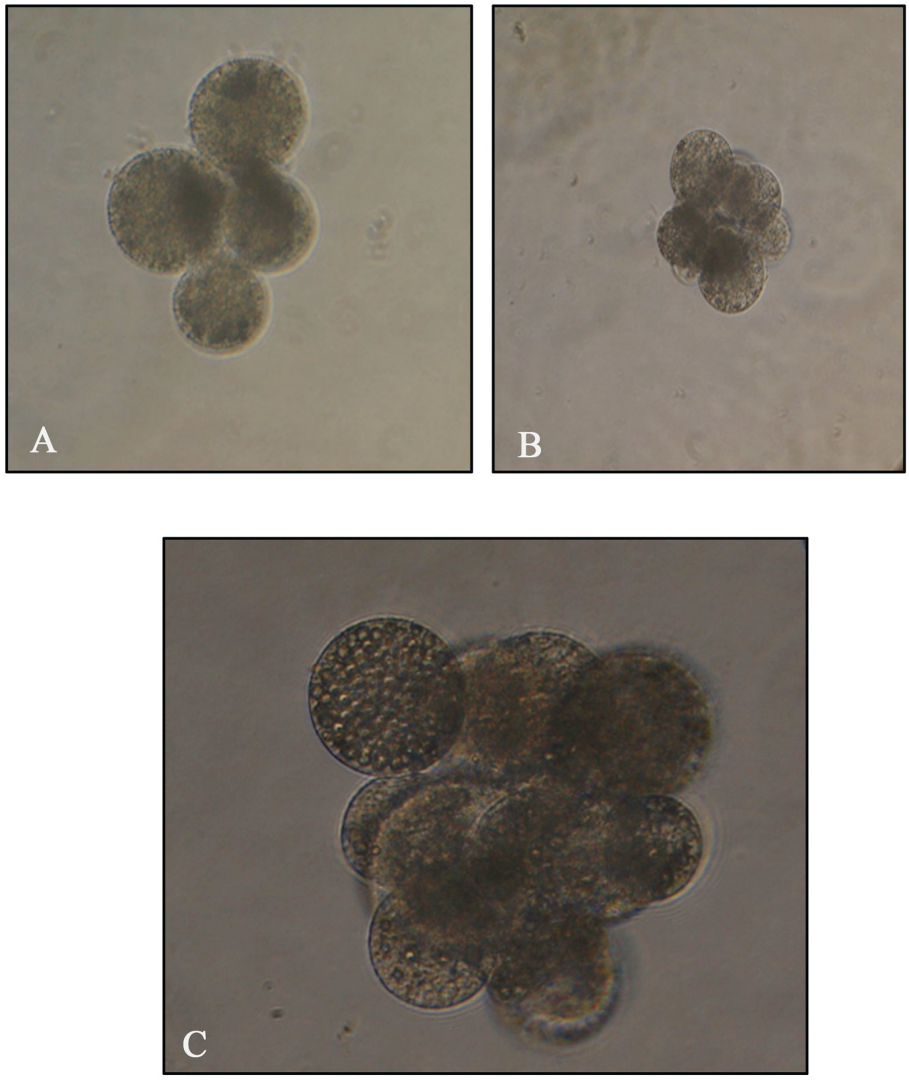

Plate No.5: Embryos in diferrent stages (A) 4 cell Stage (B) 8 cell Stage and (C) 16-32 cell Stage

4.6.2 Comparison of *in vitro* development of embryos from different nuclei donor cell treatment groups viz 5-aza- 2'-deoxycytidine (5-aza-dC) (Group A), Scriptaid (Group B), Scriptaid + 5-aza-dC (Group C) and without any treatment (Control group, Group D).

The *in vitro* development of zona-free (HMC) interspecies cattle-buffalo embryos with different nuclei donor cell treatment was compared. The IVM oocytes available during the experiment were divided into four groups.

The percentage of cleaved embryos was found to be significantly higher in all treatment groups (26.25%±0.95% vs 35.29%±0.74%, 41.79%±3.23%, 35.53%±3.77%). Highest cleavage rates were found in Scriptaid treated group but was non-significant in comparison with aza-dc and combination treatment groups. Other developmental stages were comparable and did not show any significant variation between the various treatment groups or from the control group. The embryos did not progress beyond 32 cell stage in any treatment group and thus no blastocysts were formed during this study.

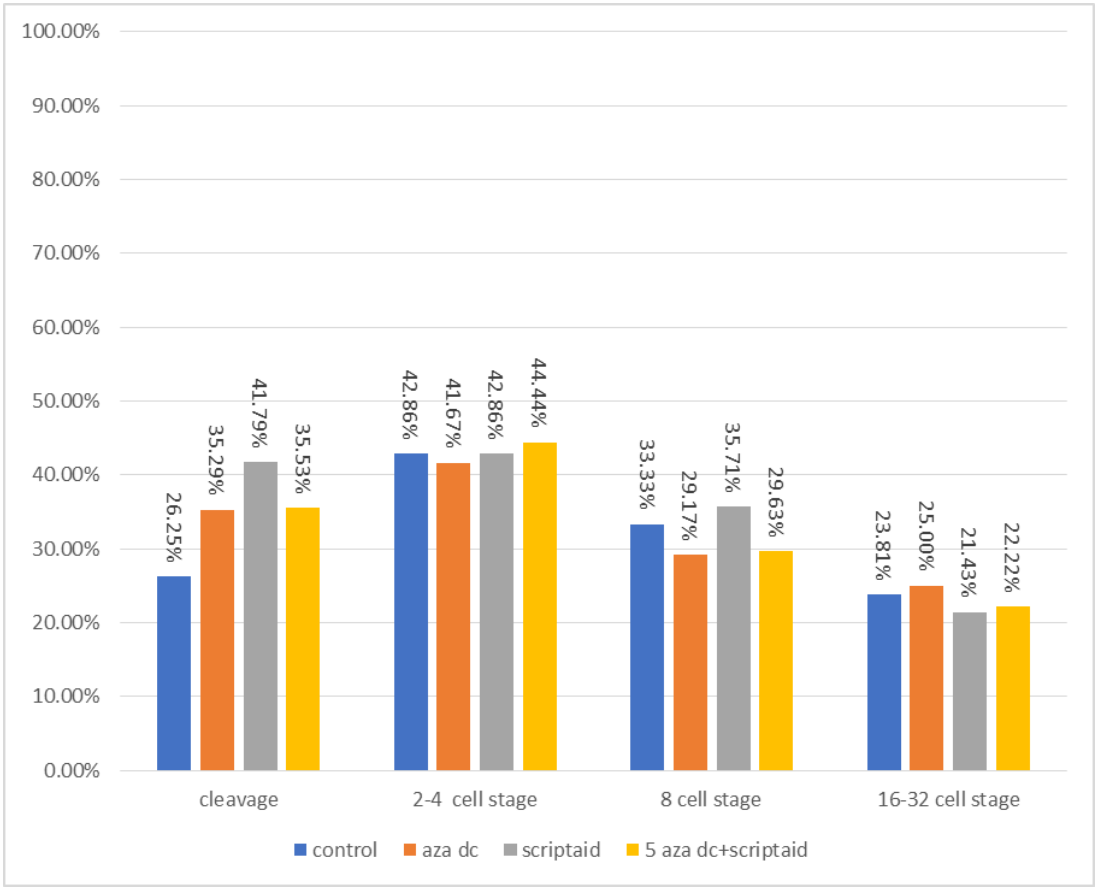

Figure 2:*in vitro* embryo development from different nuclei donor cell treatment groups viz 5-aza- 2'-deoxycytidine (5-aza-dC), Scriptaid, Scriptaid + 5-aza-dC and without any treatment (Control group).

Chapter 5

DISCUSSION

Somatic cell nuclear transfer (SCNT) is one of a few assisted reproductive techniques that failed to achieve adequate efficiency to enable large-scale commercial use. Although many mammalian species have been cloned to date, the percentage of reconstructed oocytes that develop into normal, healthy offspring remains remarkably low, often below 1% (Panarace *et al.,* 2007). The rates are still lower for iSCNT.

The factors that probably contribute to the low level of efficiency in cloning include laboratory to laboratory variation, oocyte source and quality, methods of embryo culture, donor cell type, possible loss of somatic imprinting in the nuclei of the reconstructed embryo, failure to reprogram the transplanted nucleus adequately, and the failure of artificial methods of activation that are supposed to mimic the normal events accompanying fertilisation.

In spite of its low efficiency, experimental animal cloning is being widely conducted in laboratories throughout the world because of the promise it holds in the fields of therapeutic cloning and endangered breed and species preservation (Faber *et al*., 2003; Denning and Priddle, 2003 and Paterson *et al*., 2003), as well as in the production of transgenic animals and for propagation of elite germplasm (Vajta and Gjerris, 2006) especially in developing countries.

The interspecies cattle embryo production has been studied on limited scale. It however presents a good adjunct to other conservational techniques for endangered native breeds of India as well as a source of elite animals for high production.

Keeping in view the above facts, the current study was conducted to determine the efficiency of interspecies cattle-buffalo embryo production and also to compare *in vitro* embryo development of embryos produced from nuclear donor cells treated with chromatin modifying agents. The results obtained in our study are discussed as under:

5.1 Harvest and Grading of COC's

5.1.1 Harvest of COC's

Recovery of COC's is a crucial and significant step in *in vitro* procedures related to IVM and subsequent procedures involved in *in vitro* embryo production either by in vitro fertilisation or by nuclear transfer. Various methods of harvesting of COC's from abattoir derived ovaries have been used by different workers which involves follicle aspiration under vacuum pressure, slicing of ovaries and follicle puncturing. The harvest obtained is usually highest with slicing followed by aspiration and puncturing.

In the present study aspiration technique was used and that is reported to be the best technique (Das *et al*., 1996; Mehmood *et al*., 2011). The aspiration technique opted in the present study retrieved competent oocytes with a less amount of tissue debris. The debris in the culture medium may have an adverse effect on the oocytes' maturation and subsequent development of poor quality of embryos *in vitro*. Another reason for considering this technique is retrieval of superior quality COC's from surface follicles because these follicles reside most competent oocytes with proper development while the oocytes located in the deeper cortex are in developing stage (Sharma *et al*.,2016). Das *et al*. (1996) also reported that slicing the ovaries released oocytes from two sources: surface follicles and those in deeper cortical stroma and this factor accounted for increased yield of oocytes after slicing.

In our study recovery ranging from 1.46 to 2.68 with an average of 2.07 COC's per ovary was obtained. It was higher than Das *et al*. (1996) who had retrieval rate of 1.7 by aspiration method. Rao and Mahesh (2012) had superior retrieval of 2.38 ± 0.19 per ovary by aspiration method.

5.1.2 Oocyte grading

In the present study all the oocytes retrieved were graded based on their morphology i.e. number of layers of cumulus cells and ooplasm character, and then classified as Grade A, B, C and D. Grade A and B oocytes were used for

further maturation and they were considered as culturable oocytes. Grade C and D were discarded and were considered as non-culturable oocytes. Mahesh *et al*. (2014) reported poor quality oocyte recovery is lowest in aspiration than puncture.

In present study 1399 COC's (41.80%) were of Grade-A and 979 (29.25%) COC's were of Grade-B, while as 969(28.95%) COC's were of Grade-C and Grade-D. A total of 2378 (71.05%) COC's were thus of usable quality, therefore, selected for IVM. Sharma et al. (2016) had comparable mean recovery rate of culturable oocytes as 1.35 ±0.06.

Higher recovery rates for culturable oocytes than that observed in present study were 1.68, 1.61 and 1.94 by Raza *et al*. (2001); Rao and Mahesh (2012) and Mahesh *et al*. (2014) respectively. On percent basis the higher percent of culturable grades of oocytes obtained were 78.82% and 77.67% by Prabhakar *et al*. (2012) and Mahesh et al. (2014) respectively.

In percentage terms lower percent so obtained for culturable oocytes than that observed in present study were 56.19% by Jamil *et al*. (2008) and on grade oocyte per ovary basis the lower recovery rates obtained for culturable oocytes than that observed inpresent study were 0.67 and 1.02 by Jamil *et al.* (2008) and Sharma *et al.* (2013) respectively.

Sharma et al. (2016) found the percent of grade A and B oocytes as 37.10±0.67 and 23.74±0.62 (respectively) i.e. the percentage of culturable oocytes were 61.40% which is lower than that observed in our study. Raza *et al.* (2001) reported lower percent of oocytes of grade A (20.62%) and B (15.62%) while Kulasekhar *et al*. (2012) reported slightly higher percent of grade A (42.57%) but lower grade B (20.92%) oocytes.

However, Mahesh et al. (2014) graded oocytes in to three categories and reported that good, fair and poor oocyteswere 43.0, 34.67 and 22.33%, respectively.

5.2 Invitro maturation

Oocyte maturation is the first and most critical step towards successful in vitro embryo production. Buffalo embryos has been gaining attention for its research and commercial application ever since the birth of the first buffalo calf through in-vitro fertilization of buffalo oocytes (Totey *et al.,*1992).

The culture medium and selection of protein supplements and hormones for IVM plays an important role in the subsequent maturation rate and embryonic development following IVF (Bavister *et al.,* 1992).

Maturation of bovine oocytes in follicular fluid at 100% levels has been found to inhibit maturation (Ayoub *et al.,*1993, Bevers *et al.,*1992, Sirrard *et al.*, 1998). However, addition of follicular fluid at levels of 10 to 20% has been observed to enhance oocyte maturation and fertilization rates and subsequent embryonic development in the case of bovine, porcine and ovine oocytes (Elmileik *et al*., 1995, Rath *et al*.,1995 and Sun *et al.,*1994). All these studies, however, employed an IVM culture medium which was supplemented with gonadotropins, estradiol and a serum source.

In our study maturation percentage as determined by cumulus expansion and polar body extrusion was found to be higher when IVM medium supplemented with pFSh + 17 β estradiol + Follicular fluid was used as compared with supplementation of pFSH+17 β estradiol or follicular fluid only (94.07% ±3.65% v 86.25% ±4.86% and 79.42%±1.37% respectively) but higher maturation percentage observed in IVM medium supplemented with pFSh + 17 βestradiol + Follicular fluid (94.07% ±3.65%) was statistically non-significant (P>0.05).

Ravindranatha *et al*.,2003 reported comparable maturation percentages of 86.0% with medium consisting of TCM-199 + FBS (10%) + pFSH (10 µg/ml). Chauhan *et al*. (1997) reported lower percentages with pFSH supplememented and BuFF (Buffalo Follicular Fluid) supplemented media (74% & 67% respectively).

Chauhan *et al.* (1997) reported maturation rates of oocytes matured in the presence of 20 or 40% BuFF were not significantly different from those of oocytes matured in the presence of FBS + FSH-P. It suggested that buffalo follicular fluid has substance(s) which promote cumulus expansion as well as nuclear and cytoplasmic maturation. Buffalo follicular fluid, therefore, has the potential of being used as an IVM medium supplement for the production of buffalo morulae and blastocysts. This is in consonance with our results.

Rate of maturation was lower when 17*β*-Estradiol was not added to IVM media, although the difference was not significant, implying the positive role which 17β-Estradiol plays in maturation of oocyte as reported by Pawshe *et al*. (1996) and Izquierdo *et al*. (1999).

5.3 Production of interspecies cattle embryos

The percentage of cleaved embryos in our study was 26.25%±0.95% in control group with no chromatin modifying treatment. No blastocysts were formed and embryo growth arrested at 32 cell stage.

The cleavage rates were lower than those observed by lu *et al.* (2005) who observed 45.3 % cleavage when Bovine (*Bos indicus*) fibroblasts were used as donor cells in buffalo oocytes. 4.5% blastocyst rates were also reported in the same study. The experiment however used *Bos indicus* as compared to *Bos Taurus* used in current study. Selokar et al (2011) also reported higher cleavage rates and blastocyst formation rates of 5%.

Tecirlioglu *et al* (2006) reported that only a small number of iSCNT heterokaryons (28%) cleaved in Murine-Bovine iSCNT. Similarly, no blastocysts were formed when Bontebok fibroblasts and bovine oocytes were used (Matshikiza *et al.,*2004).

The variation in *in vitro* development of embryos produced through both zona-included and zona-free cloning technique could be attributed to various factors. It has been observed that results of cloning experiments vary greatly between different species, different lab set ups and even between replications of

experiments under same conditions due to the fact that cloning involves oocyte retrieval from ovaries of different and mostly unknown sources and there is high variability in developmental competence of oocytes which greatly fluctuates the rates of development (Vajta *et al*., 2005; Lagutina *et al*., 2007).

As the taxonomic distance between donor and recipient species increases, the blastocyst production decreases. Whether this is because of a low efficiency of buffalo oocyte in their ability to reprogram somatic cells from *Bos Taurus* needs further investigation.

5.3.1 Effect of chromatin modifying agents

There have been attempts to improve epigenetic reprogramming of donor cells and cloned embryos, including treatment of donor cells and early embryos with epigenetic modification drugs to enhance the developmental potential of cloned embryos by altering epigenetic marks in the nucleus.

The percentage of cleaved embryos in our study was found to be significantly higher as compared to control, in all treatment groups (26.25%±0.95% [Group D] vs 35.29%±0.74%[Group A], 41.79%±3.23%[Group B], 35.53%±3.77%[Group C]). The treatment groups (A, B, C) showing non-significant difference among themselves. However other developmental stages did not differ significantly from control or among themselves.

Highest cleavage rates among treatment groups were found in Sriptaid treated group (41.79%±3.23%) but was non-significant in comparison with 5-aza-dC and combination treatment groups. Scriptaid is a novel HDAC inhibitor, which has a relatively higher histone acetylation activity and lower cellular toxicity compared with TSA (Zhao *et al.,* 2009). It has been reported that Scriptaid treatment could significantly increase the in vitro and in vivo development of cloned embryos (Van Thuan *et al.,* 2009).

DNA methylation is another critical component of the epigenetic status of donor nucleus. The methylation status of the donor cell nucleus can strongly influence the efficiency of nuclear transfer–derived blastocysts. That is possible

because the DNA methylation status of donor nucleus affects the gene expression in early cloned embryo. However, the pattern of DNA methylation is abnormal in cloned embryos (Bonk *et al.,* 2008), and abnormal DNA hypermethylation is believed to be associated with the low success rate of SCNT (Simonsson and Gurdon, 2004). Therefore, previous attempts to improve SCNT efficiency were restricted to changing the DNA methylation level of the donor nucleus using demethylating drugs (Enright *et al.,* 2005).

There is growing evidence suggesting that the developmental defects in cloned embryos correlated with the epigenetic modifications to the chromatin of donor cells and proper handing with epigenetic modification drugs can improve the epigenetic patterns (Kishigami *et al*., 2006). Substantial improvement in SCNT has been recently achieved by treatment of donor cells or (and) cloned embryos with some epigenetic remodeling drugs, e.g. TSA, VAP, butyric acid, scriptaid or 5-aza-2'-deoxycytidine (Kishigami *et al.,* 2006 ,Shi *et al.,*2008 , Enright *et al*.,2003 ,li *et al.,*2008 ,Miyoshi *et al*.,2010 ,Zhoa *et al*.,2009). Similarly, in pigs, treatment with 500 nM scriptaid increased the cloning efficiency from 0 to 1.3% (Zhoa *et al.,*2009). However, not all studies have reported improved development competence of cloned embryos following treatment of somatic donor cells or embryos with epigenetic remodelling drugs. Thus varying results from such experiments are observed as discussed below.

At gene level, Lee *et al.* (2010) reported that treating cat cells with TSA before SCNT significantly increased the H3K9ac level of the donor nucleus. Go´mez *et al*. (2011) showed that pre-treated donor cells with TSA improved the pluripotent gene expression but not embryo viability; Sangalli *et al.* (2012) treated bovine fibroblasts with 5-aza-dC and TSA, which increased histone acetylation. Likewise, wang *et al.,* 2011 observed that acetylation in H3K9 of yak fibroblasts was significantly increased when they were treated with Zebularine alone or Zebularine plus Scriptaid for 24 h. However, Shi *et al*. (2003) found that sodium butyrate (NaBu) has no obvious effect on the histone acetylation levels of donor cells. Travers *et al*. (2002) suggested that the histone acetylation levels of human

CD34+ hematopoietic progenitor cells are not significantly changed after TSA treatment. Wang *et al.* (2011) commented that inconsistent results may due to the different sensitivities of the distinct cells to drugs; also, the treatment programs in those studies are inconsistent

With regards to embryo quality, Enright *et al.,*2003 reported that donor cells pre-treated with 0.01 mM 5-aza-dC alone resulted in a non-significant increase in blastocyst rate and quality (Enright et al.,2003). In rabbits, Meng *et al* (2009) reported TSA-treatment had no effect on *in vitro* and *in vivo* development competence of cloned rabbit embryos; all offspring from TSA-treated embryos died within 19 d after birth, whereas four pups in the TSA-untreated group grew into adulthood . Wu et al (2008) also reported that cells treated with 50 ng/mL of TSA resulted in significantly lower blastocyst development (9.9%) compared with the control group (20%).

Kishigami *et al*. (2006) found that treating early cloned embryos with TSA significantly improved the *in vitro* developmental competence and full-term development of cloned mice embryos. Zhao *et al.* (2009) reported that Scriptaid treatment significantly improved the *in vitro* and *in vivo* development of cloned pig embryos. Treatment of both donor cells and early cloned embryos with AZA and TSA significantly improved the ability to develop into blastocysts and the birth rate of cloned bovine (Wang *et al.,* 2011b).

Xiong *et al.* (2013) chose yak fibroblasts after treatment with Zebularine plus Scriptaid as donor nuclei for iSCNT, and treated early cloning embryos with Scriptaid alone. The blastocyst formation rates were significantly higher than the controls (group iSCNT). In addition, donor cells and early cloned embryos treated with epigenetic modification drugs significantly increased the capability of cryosurvival of cloned blastocysts than in the untreated group. Perhaps the increased acetylation and decreased methylation levels of the donor nucleus facilitate nucleus reprogramming and subsequently enhance the developmental competence of cloned embryos. Oct-4 and Sox-2 were expressed appropriately after nuclear transfer.

Ding *et al.,* 2008 reported that treatment of donor cells and early cloned embryos with a combination of two epigenetic modification drugs (0.01 µM 5-aza-dC and 0.05 µM TSA) significantly increased *in vitro* development of cloned bovine embryos. Although the mechanism underlying how the combination treatment improves cloning efficiency remains unknown, they inferred that there was an interaction between 5-aza-dC and TSA; perhaps a lower level of methylation and a higher level of histone acetylation changed the chromatin configuration, facilitated the reprogramming of transferred nucleus, and subsequently enhanced development of cloned embryos.

Xiong *et al*. (2013) noted that discrepancies may have been due to differences in the type, application (concentration, timing) of these epigenetic remodelling drugs in various species. Selection of the appropriate epigenetic remodelling drugs and optimization of their application (concentration, timing) in different species might be the key in improving the success rate in animal cloning.

Chapter 6

SUMMARY AND CONCLUSION

The present study on *in vitro* development of interspecies cattle-buffalo embryos produced by zona-free cloning technique was carried out using slaughter house buffalo ovaries . A total of 1591 abattoir derived buffalo ovaries yielding 3347 COC's were used. A total of 2378 (71.05%) COC's were of usable quality, therefore, selected for IVM. The investigation evaluated the efficiency of iSCNT cloned Cattle-Buffalo embryo production using Hand Made Cloning (HMC) technique and also compared *in vitro* development of iSCNT cloned Cattle-Buffalo embryos following donor cell treatment by epigenetic re-modelling drugs or chromatin modifying agent like DNA demethylation agents and histone deacetylase inhibitors (HDAC) inhibitors i.e. 5-aza- 20-deoxycytidine (5-aza-dC) and Scriptaid.

The results obtained in the present investigation are summarised as:

i) A total of 1591 abattoir derived buffalo ovaries in 16 replicates were used for the experiments. 3347 COC's were harvested upon aspiration of surface follicles (size ranging from 2mm - 8mm). A total of 2378 (71.05%) COC's were of usable quality, therefore, selected for IVM. COC's with not less than 2 layers of surrounding cumulus were selected for *in vitro* maturation (IVM).

ii) 1399 COC's (41.80%) were of A grade and 979 (29.25%) COC's were of B grade, while as 969 (28.95%) COC's were of C and D grade. A total of 2378 (71.05%) COC's were of usable quality.

iii) Ear skin biopsies taken from jersey calves and seeded in culture flasks started proliferating 2-5 days after initiation of culture. These primary cell cultures attained 70-80% confluence in 7-12 days' time. Upon sub culturing, the cells formed a confluent monolayer within 48-72 hours of seeding. The cells after 5th passage were used as nuclei donor.

iv) The maturation percentage as determined by cumulus expansion and polar body extrusion was found to be higher when IVM medium supplemented with pFSh + 17 βestradiol + Follicular fluid was used as compared with supplementation of pFSH+17 βestradiol or follicular fluid only (94.07% ±3.65% v 86.25% ±4.86% and 79.42 % ±1.37% respectively) but higher maturation percentage observed in IVM medium supplemented with pFSh + 17 βestradiol + Follicular fluid (94.07% ±3.65%) was statistically non-significant (P>0.05).

v) 80 in vitro cloned interspecies cattle-buffalo embryos were produced by HMC. The percentage of embryos which cleaved on day 2 was 26.25%±0.95%. 2-4 cell stage and 8 cell stage and 16-32 cell stage embryo percentages obtained on Day 7 were 42.86%±10.13%, 33.33%±7.01% and 23.81%±8.96%, respectively.

vi) 68 embryos were produced from 10 nM of 5-aza- 2'-deoxycytidine (5-aza-dC) (group A) treated donor somatic cells. The percentage of embryos which cleaved on day 2 was 35.29%±0.74% 2-4 cell stage and 8 cell stage and 16-32 cell stage embryo percentages obtained on Day 7 were 41.67%±0.82% , 29.17%±4.10% and 25.00%±5.59%,respectively.

vii) 67 embryos were produced from 0.5µM of Scriptaid (group B) treated nuclei donor somatic cells. The percentage of embryos which cleaved on day 2 was 41.79%±3.23%. 2-4 cell stage and 8 cell stage and 16-32 cell stage embryo percentages obtained on Day 7 were 42.86%±4.36%,35.71%±3.60% and 21.43%±5.59%, respectively.

viii) 76 embryos were produced from 0.5µM of Scriptaid + 10 nM of 5-aza-dC (group C) treated nuclei donor somatic cells. The percentage of embryos which cleaved on day 2 was 35.53%±3.77%. 2-4 cell stage and 8 cell stage and 16-32 cell stage embryo percentages obtained on Day 7 were 44.44%±5.51%, 29.63%± 4.50% and 22.22%±4.33% respectively.

ix) The percentage of cleaved embryos was found to be significantly higher in all treatment groups (26.25%±0.95% vs 35.29%±0.74%, 41.79%±3.23%,

35.53%±3.77%). Highest cleavage rates were found in Sriptaid treated group but was non-significant in comparison with aza-dc and combination treatment groups.

x) Other developmental stages were comparable and did not show any significant variation between the various treatment groups or from the control group. The embryos did not progress beyond 32 cell stage in any treatment group and thus no blastocysts were formed during this study.

CONCLUSION

- Higher maturation percentage is observed in IVM medium supplemented with pFSh + 17 βestradiol + Follicular fluid however it was statistically non-significant ($P>0.05$).
- Donor cell treatment with Chromatin modifying agents significantly improves cleavage rates in iSCNT cattle-buffalo embryos.
- Chromatin modifying agents did not cause any significant change in developmental efficacy of other stages.

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CERTIFICATE

Certified that all the corrections/amendments as suggested by External Examiner **Dr. Khalid Majeed Fazili, Professor, Department of Biotechnology, University of Kashmir, Hazratbal, Srinagar** during viva voce examination held on **03-06-2019** have been incorporated in the manuscript entitled **"Effect of DNA modifying agents on *in vitro* development of interspecies cloned Cattle-Buffalo embryos"** submitted by **Dr. Haris Rasool Beig (2016-V-324-M)**

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