CELL CYCLE SYNCHRONIZATION OF BUFFALO FIBROBLASTS AND EFFECT OF REPROGRAMMING TIMES ON DEVELOPMENTAL COMPETENCE OF INTRA AND INTER SPECIES HAND MADE CLONED EMBRYOS

THESIS SUBMITTED TO THE
NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF

MASTER OF VETERINARY SCIENCE
IN
DAIRYING
(ANIMAL BIOTECHNOLOGY)

BY

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Approved by:

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This is to certify that the thesis entitled,**"Cell cycle synchronization of buffalo fibroblasts and effect of reprogramming times on developmental competence of intra and inter species hand made clone embryos."** submitted by **Selokar Naresh Lalaji** towards the partial fulfilment of the award of the degree of **Master of Veterinary Science in Dairying (Animal Biotechnology)** of the **National Dairy Research Institute (Deemed University)**, Karnal (Haryana), India, is a bonafide research work carried out by him under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

Dated: 18/07/09

(S. K. SINGLA)
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THIS THESIS IS DEDICATED

TO

MY MATOSHREE

&

DADA

AND

DNS

WHOSE ENCOURAGEMENT, CONSTANT SUPPORT, CARE, VALUES AND LOVE WILL ALWAYS REMAIN IN ME FOREVER

“ They Alone Live Who Live For Others ”

-Vivekananda

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NARESH SELOKAR

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ABBREVIATIONS AND SYMBOLS

%	=	Percent
μg	=	Microgram
μl	=	Microlitre
μm	=	Micrometer
^{o}C	=	Degree Celsius
AC	=	Alternating current
A I	=	Artificial insemination
ANOVA	=	Analysis of variance
ART	=	Assisted reproductive technologies
BL	=	Blastocysts
BSA	=	Bovine serum albumin
BSA (FAF)	=	Bovine serum albumin (Fatty acid free)
buFF	=	Buffalo follicular fluid
CL	=	Corpus luteum
cm	=	Centimeter
COC	=	Cumulus-oocyte-complex
DC	=	Direct current
DIC	=	Differential Interference Contrast
6-DMAP	=	6-dimethyl amino purine
DMEM	=	Dulbecco's modified eagle's medium
DMSO	=	Dimethyl sulphoxide
DPBS	=	Dulbecco's phosphate buffered saline
EDTA	=	Ethylenediamine tetra-acetic acid
EG	=	Ethylene glycol
ES cells	=	Embryonic stem cells
ETT	=	Embryo transfer technology
FBS	=	Fetal bovine serum
FCS	=	Fetal calf serum
Fig.	=	Figure
FS	=	Flat surface
FSH-p	=	Follicle stimulating Hormone (Porcine)
G1	=	Gap phase between mitosis and DNA synthesis in

		cells
G2	=	Gap phase between DNA synthesis and mitosis in cells
GO	=	Arrested stage of G1
HMC	=	Handmade cloning
iHMC	=	Interspecies Handmade cloning
iSCNT	=	Interspecies somatic cell nuclear transfer
I.U.	=	International units
ICM	=	Inner cell mass
IVC	=	In vitro culture
IVF	=	In vitro fertilization
IVEP	=	In vitro embryo production
IVMFC	=	In vitro maturation fertilization and culture
IVM	=	In vitro maturation
kV/cm	=	Kilovolts per centimeter
LSD	=	Least significant difference
M+B	=	Morulae + Blastocysts
MD	=	Microdrop
mg	=	Milligram
ml	=	Millilitre
mm	=	millimeter
N	=	Number
NBF	=	Newborn Buffalo fibroblasts
MPF	=	Maturation promoting factor
MAPK	=	Mitogen activated protein kinase
M I	=	Meiosis I
M II	=	Meiosis II
NEBD	=	Nuclear envelope breakdown
PVA	=	Polyvinyl alcohol
TSA	=	Trichostatin-A
5’-aza-dc	=	5’-aza-deoxy cytidine
NT	=	Nucleus transfer
PA	=	Parthenogenetically activated
PGF2∞	=	Prostaglandin F2∞

PHA	=	Phytohemagglutinin
RVCL	=	Research Vitrocleave medium
SCNT	=	Somatic cell nuclear transfer
SE	=	Standard error
S	=	DNA synthesis phase of cell cycle
TCM-199	=	Tissue culture medium-199
T0	=	TCM-199 without FBS
T2	=	TCM-199 supplemented with 2% FBS
T10	=	TCM-199 supplemented with 10% FBS
T20	=	TCM-199 supplemented with 20% FBS
TC	=	Traditional cloning
TE	=	Trophectoderm cells
UV	=	Ultraviolet
V	=	Volts
WOW	=	Well of the Wells

ABSTRACT

The present study was carried out in buffalo, 1) To study the efficiency of different methods for synchronization of cell cycle in G1/G0 phase of cultured buffalo fibroblast. 2) To study the effect of reprogramming time on developmental competence of intra-species Handmade cloned buffalo embryos. 3) To study the efficacy of buffalo oocyte to support *in vitro* development of inter-species Handmade cloned embryos. Our results show that full confluency, combination of full confluency with serum starvation is as efficient as 10μM ROS, 20μM ROS, and 30μM ROS to synchronize cell cycle in G1-G0 phase. At concentration 30μM ROS, most cells synchronized in G1/G0 phase (95%) but cell survivability was low. During experiment all treatment groups of aphidicoline cells died after 24 hours of incubation hence these groups were not processed for further analysis. It became clear that maternally inherited factors present within the oocytes also have the extraordinary property to restore the totipotency to a differentiated somatic nucleus when transplanted into it. Our study indicated that the timing between fusion and activation has impact on genomic reprogramming. Higher blastocysts percentage (52.0 %) were obtained when holding time between fusion and activation was 6 hours as compare to other holding time considered during study. In case of interspecies cloning, cleavage rates were same for buffalo, cow, goat, and rat NT embryos and blastocyst rates were 51.4%, 3.5%, 2.2%, and 0% for buffalo, cow, goat, and rat NT embryos respectively. During our study, it was shown that cleavage rate did not depend upon source of donor nuclei but blastocyst rate were affected. Transferable quality blastocysts, having adequate cell number and inner cell mass cells, were produced and transferred into synchronized recipient buffaloes and cattle. Pregnancy was established (~3month) in three (50%) of the recipients out of six diagnosed through ultrasonography in case of buffalo while no pregnancy was established in case of cattle. Buffalo recipient failed to maintain pregnancy beyond 90 day.

सारांश

भैंस में यह अध्ययन किया गया कि

1. किस प्रक्रिया से अधिकतम फायब्रोब्लास्ट कोशिकाओं G0-G1 को स्थिति में लाया जा सकता है। 2. भैंस भ्रुणों के अधिकतम उत्पादन के लिए कितने घंटे रीप्रोग्रामिंग समय की आवश्यकता है। 3. भैंस के डिम्बों की अंतराजातीय हस्तनिर्मित क्लोनिंग की क्षमता देखी गई।

यह अध्ययन से पता चला है कि पूर्ण जमावडा और सिरम भूखमरी के साथ पूर्ण जमावडा, फायब्रोब्लास्ट कोशिकाओं को G0-G1 स्थिति में 10, 20, 30 माइक्रोमोलर रोस्काव्हिटिन के साथ बराबर कार्य कुशल है। यह देखा गया है कि सर्वाधिक कोशिकाएं 30 माइक्रोमोलर मात्रा में एक ही साथ G0-G1 स्थिति में पाए गए हैं। किन्तु मृत कोशिकाओं की संख्या अधिक है। चुने गए ऐफीडिकोलिन रसायन की मात्रा में अधिकतम कोशिकाएं मृत हो गई। इसीलिए इन कोशिकाओं को आगे अध्ययन नहीं किया गया।

यह देखा गया है कि 6 घंटे का मिलन और उत्तेजित मध्य का समय सर्वाधिक ब्लास्टोसिस्ट दर मिला है। आंतरजातीय भैंस, गांय, बकरी एवं चूहा की क्लोनिंग के दरार की अनुपात में विभिन्नता नहीं पाई गई। किन्तु ब्लास्टोसिस्ट दर में विभिन्नता है। समान जाति भैस, के ब्लास्टोसिस्ट को 6 पशु में डाला गया। इसमें से तीन पशुओं ने गर्भधारण किया। किन्तु व तीनों पशुओं का गर्भपात 90 दिन के भीतर हो गया।

CHAPTER – 1

Introduction

INTRODUCTION

India is having the largest and best buffaloes population of the world. Still, the percentage of elite animals is very little and there is an urgent need to enhance this number. Aimed at enhancing the buffalo productivity, the buffalo has been the subjected to different assisted reproduction techniques including artificial insemination (AI), gamete preservation, embryo transfer (ETT) and *in vitro* fertilization (IVF) etc., which are expected to lead to faster multiplication of elite animals. But very limited success had been achieved in this species due to their low reproductive efficiency. The use of recent embryo technologies, like an embryo cloning by nuclear transplantation in farm animals has been reported but to a limited success, especially in buffaloes. Somatic cell nuclear transfer technique (SCNT) provides many research avenues such as stem cell production, multiplication of genetically elite animals and conservation of endangered species. Traditional cloning is micromanipulation based technique which require skilled worker and economically expensive. The new Hand guided cloning (HGC) / handmade cloning (HMC) technique is more effective and economical than micromanipulation based nuclear transfer.

The cloning efficiency depends upon many factors and one of them is cell cycle stage of donor cell. Many studies have been reported that G1/G0 donor cells have better capability to undergo nuclear reprogramming in an unfertilized oocyte. Serum starvation and cell confluency is widely used to synchronize donor cells in G1/G0 stage, however serum starvation causes reduced cell survival, increase DNA fragmentation and apoptosis. It is unclear which cell cycle stage of donor is effective for reprogramming and production of cloned animals. Galli C *et al.,* 2003 achieved successful cloning with cell type other than the G0 stage. In fact many studies show that G1 stage donor nuclei gives rise to high cloning efficiency as compared to other cell cycle stage. The second major factor for developmental competence of cloned embryo is reprogramming time before activation during which

unidifferentiated genes of donor cells are modified into multipotent genes of embryo subsequently developing into whole animal. Several groups have shown that the duration of exposure of the donor nucleus to oocyte cytoplasm after NT affects in vitro development; however, the conclusions are mixed. Some reports have indicated that prolonged exposure to the oocyte cytoplasm before activation may be beneficial in promoting embryo development for bovine (Wells *et al.,* 1998, 1999). Another study reported that exposure of donor nuclei to cytoplasm for less than 30 min before activation yielded significantly lower blastocysts development than a 2-hour exposure (Liu *et al.,* 2001). Conversely, other research indicates that excessive exposure of the donor DNA to oocyte cytoplasm results in lower rates of in vitro development in cloned embryos (Akagi *et al.,* 2001). Most recently, Choi *et al.,* (2004) demonstrated that in vitro development of bovine NT embryos to blastocysts decreased as time in hold increased from 1 to 5 h.

Extinction of wild animals is part of natural process of evolution and is irreversible, but is now occurring at much higher rate due to human activity such as habitat destruction, hunting competition with introduced herbivores (Holt and Pickard, 1999). In case of domesticated species, extinction has been due to intensive selection and cross breeding and market demand. According to species survival commission in 2006, 1528 animal species are critically threatened, including 162 species of mammals. The major aim of animal conservation is to maintain ecological diversity. The habitat maintenance is one of best way to conserve biodiversity (Loi *et al.,* 2001). *In situ* conservation strategies are able to maintain live population of animal in their adaptive environment but these efforts are some time insufficient for small populations. Hence *ex situ* conservation strategies are being developed for establishing viable population through cryopreservation of animal genetic resources like gametes, embryos, cells/tissue samples and DNA (Andrabi S.M.H. *et al.,* 2007).

Modern reproductive biotechnologies/assisted reproductive techniques (ART) including artificial insemination (AI), embryos transfer (ETT), in vitro embryos production (IVF), cryobiology, semen/embryos sexing, reproductive cloning, genome resource banking are valuable tools for *in situ* and *ex situ* conservation programs for endangered mammalian species. Major obstacle for applying ART is low availability of oocytes and lack of knowledge of reproductive physiology. Somatic cell nuclear transfer has been suggested as potential technique to become an integral part of wild life conservation programs, specifically interspecies cloning in which oocyte of domestical animal could combine with somatic cell from close related wild counterpart.

Keeping in view the lack of information on use of hand made cloning for production of interspecies cloned embryos in any species and effect of reprogramming timing on developmental competence of hand made cloned buffalo embryos and there is no report available on synchronization of cultured buffalo cells for increasing cloning efficiency, the present investigation was taken up with following objective.

1. **To study the efficiency of different methods for synchronization of cell cycle in G1/G0 phase of cultured buffalo fibroblast.**

2. **To study the effect of reprogramming time on developmental competence of intra-species Handmade cloned buffalo embryos.**

3. **To study the efficacy of buffalo oocyte to support *in vitro* development of inter-species Handmade cloned embryos.**

Chapter- 2

Review of Literature

REVIEW OF LITERATURE

2 .1 HISTORICAL OVERVIEW

First cloning experiment on animal was started by Hans Driesch in 1891 who separated the blastomeres of two cell embryos mechanically by shaking them in seawater. Eleven years latter, Spemann carried same experiment in amphibians in 1938 demonstrated that nuclei of new salamanders are pluripotent upto the 8-cell stage. Nicholas and Hall (1942) demonstrated that one blastomere is totipotent in a two-cell embryo. They derived progeny in the rats resulting from one of the two-cell embryo following destruction of second blastomere. Briggs and King (1952) showed that oocyte receiving blastula nuclei in *R. pipiens* could be reared to maturity. Research in cloning of adult animals began in 1950s and resulted in dogma that offspring could be produced from NT using non-differentiated totipotent cells of the germline, but not from differentiated somatic cells (reviewed by Di Bernardino, 1997). Eight sets of twin mice were successfully produced from the morulae of the mice by bisecting the morulae into two halves (Moustafa and Hahn, 1978). The principle of deriving the offspring from morulae was independently applied by Ozil *et al.* (1982) and Williams *et al.* (1982) to produce identical twins in cattle.

The first attempt to clone mammals was in mice, where Illmensee and Hoppe (1981) produced three mice from surgical transplantation of nuclei from blastocyst-inner cell mass (ICM) into recently fertilized mouse eggs, although the results of the studies were, not repeatable. McGrath and Solter (1983) showed that the cloning could be carried out by fusion of the transferred nuclei rather than merely injecting a somatic cell nucleus into an enucleated oocyte. This procedure led to a superior cell-survival rate compared to microinjection alone, and was used in most subsequent studies dealing with animal nuclear transfer.

The next milestone in the history of cloning research was the use of electrofusion to introduce 8-16-cell stage donor blastomeres into the enucleated metaphase II oocytes, in case of sheep were produced by Willadsen (1986). This method was elegant and highly efficient. The

method first involved the removal of the genetic material from a recipient cell using a unique noninvasive approach. Eventually sheep were produced from embryonic cell stages and the inner cell mass of blastocysts (Smith and Wilmut, 1989), and *in vitro* cultured cells from inner cell mass (Campbell *et al.,* 1996; Wilmut *et al.,* 1997). Goats were also cloned and recloned from embryonic blastomeres cultured *in vitro* (Yong and Yuqiyang, 1998).

In cattle, the first cloned calves involved the blastomere nuclear transfer followed by electrofusion of donor nuclei to enucleated metaphase-II bovine oocytes. The resulting nuclear transplanted embryos (blastocyst stage) were transferred to the oviducts of sheep for some days before being transferred into recipient females (Robl *et al.,* 1986).

Since birth of first lamb from culture fibroblasts, the interest in cloning research has exploded. At present somatic cell cloning, has been successful in a number of farm animals (sheep, cow and pig etc.), experimental animal models (mice, rabbits, ferrets etc) and endangered animals (gaur, mouflon). Table 2.1 provides the list of important milestones in the history of nuclear transfer. Efforts are continuing to improve the efficiency of animal cloning through modifying technical steps (as in handmade cloning or zona-free nuclear transfer), modified methods for culturing cloned embryos (Vajta *et al.,* 2000; Ribas *et al.,* 2005) and introducing novel mechanisms to enhance nuclear reprogramming (Collas, 1998; 2006; Betthauer *et al.,* 2000; Cavalleri, *et al.,* 2006).

At present nucleus transfer is being used in mammals as valuable tool in embryological studies and a method for the multiplication of elite animals. Most of the reports available regarding cloned animals are mentioned in Table 2.2. Animal cloning is important in maintaining the biodiversity of the domesticated endangered breeds of the livestock species, and conservation of the endangered wild animals (Wells *et al.,* 1997). In addition, nucleus transfer is considered as an important tool for basic research for the investigation of cell biology and nuclear reprogramming of the mammalian genome. The bovine embryo offers several advantages with relatively late activation of its genome and late implantation compared to other animals model like mouse. NT method has

provided a much simpler and more efficient method for multiplication of the superior germplasm and production of transgenic animals (First and Prather, 1991; Fulka *et al.,* 1996; 1997; Chan *et al.,* 1998; Cibelli *et al.,* 1998; Stice *et al.,* 1998; Forsberg *et al.,* 2002). So far, animal cloning using differentiated somatic cells from adult mammals has been achieved for number of species, about 150 cloned animals all over the world are produced from differentiated somatic cells. However, the number of cloned animals is still negligible compared to the impact of other reproductive biotechnologies such as AI, ETT. For instance nearly 5 million embryo transfers were performed every year in cattle from *in vitro* or *in vivo* produced embryos (Colleau *et al.,* 1998). Somatic cell cloning has great promise in future but the limitations due to low pregnancy rates and calf survival restrict its current use on large scale. Presently, somatic cell cloning is still in the research phase. Considerable work needs to be done for improvement of survival rates and evaluate variations in results before cloning can be commercialized. The second phase will be small-scale commercialization of the technology to multiply animals of high value. As efficiency and the quality of embryos improve, cryopreservation will become feasible and large number of embryos will be sold in straws like as semen.

Table 2.1 Some of the milestones of NT technology.

Key event	References
First nuclear transfer in frogs	Briggs *et al.,* 1952
Nuclear transfer in rabbit	Bromhall, 1975
Lambs cloned from blastomeres	Willadsen, 1986
Calves cloned from ICM cells	Sims and First, 1994
Sheep cloned from embryonic cell line	Campbell *et al.,* 1996
First mammal cloned from adult cell (“Dolly”)	Wilmut *et al.,* 1997
Transgenic sheep produced by nuclear transfer	Schnieke *et al.,* 1997

Table 2.2 Species in which cloning has been successful

Species	Donor cell type	References
Lambs	Blastomeres	Willadsen, 1986
Cattle	Blastomeres	Prather *et al.,* 1987
Pigs	Blastomeres	Prather *et al.,* 1989
Calves	ICM cells	Sims and First, 1994
Sheep	Embryo	Campbell *et al.,* 1996
Sheep	Fetal	Wilmut *et al.,* 1997
Sheep (Dolly)	Adult	Wilmut *et al.,* 1997
Cattle	Fetal	Cibelli *et al.,* 1998
Cattle	Adult	Kato *et al.,* 1998
Mouse	Adult	Wakayama *et al.,* 1998
Mouse	Embryo	Wakayama *et al.,* 1999
Goat	Fetal	Baguisi *et al.,* 1999
Pig	Adult	Polejaeva *et al.,* 2000
Gaur	Adult	Lanza *et al.,* 2000
Mouflon	Adult	Loi *et al.,* 2001
Rabbit	Adult	Chesne *et al.,* 2002
Zebra fish	Embryo	Lee *et al.,* 2002
Rat	Fetal	Zhou *et al.,* 2003
Mule	Fetal	Woods *et al.,* 2003
Horse	Adult	Galli *et al.,* 2003
Dog	Adult	Lee *et al.,* 2005
Buffalo	Fetal	Shi *et al.,* 2007
Buffalo	New born	Shah *et al.,* 2009 (report)

2.2 HANDMADE CLONING

Animal cloning by traditional nuclear transfer is now a standard procedure for cloned animal production in most of the laboratories in the world. But it is, however, still very inefficient process and fails to produce viable offspring in about 95-99% of attempts, further depending upon the species to be cloned and the recipient oocytes (Solter, 2000). It is expected that the demand for cloned embryos and animals in commercial agriculture and basic science investigations will increase. In near future, cloned embryos will provide a source of autologous embryonic stem (ES) cells for therapeutic cloning (Gurdon and colman, 1999), not only for human applications, but also in veterinary science applications. Cloned embryo production methods with higher throughput are required to meet this demand. Currently most NT protocols are micromanipulation based which is laborious, cost-intensive and require high technical skills.

The present and almost exclusively used technique for oocyte enucleation and reconstruction was originally introduced by Willadsen (1986) for blastomere cell cloning. With slight improvement to make enucleation more accurate (Westhusion *et al.,* 1992), this technique was subsequently adapted without much significant changes for somatic cell nuclear transfer (Wilmut *et al.,* 1997). A remarkable modification allowing direct injection of the donor nucleus into the cytoplast has been successful in mouse (Wakayama *et al.,* 1998) and in some domestic species (Trounson *et al.,* 1998; Galli *et al.,* 2003). The first attempt of zona free nucleus transfer done by Tatham *et al.* (1995), who enucleated zona-free bovine oocytes with density gradient centrifugation, and fused them with blastomeres of precompaction stage embryos. However, the reconstructed embryos did not reach the blastocyst stage, probably due to low efficiency in each step of the procedure.

Successful attempt to exclude micromanipulators from the mammalian nuclear transfer procedure was that of Peura *et al.* (1998), resulting in healthy offspring even from fourth generation cloning (Peura *et al.,* 2001). The hand made cloning (HMC) technique was developed without the requirement for micromanipulators using the zona free oocytes and embryonic cells (Peura *et al.,* 1998) and then somatic cell (Vajta *et al.,*

2001). For restoring the original volume, two cytoplasts were fused with one blastomere, using alternating current to establish proper alignment and cell to cell contact. Reconstructed embryos were then cultured in drops to avoid clumping and blastocysts were produced from them. As the method did not require the use of micromanipulators, an entirely new way of performing nuclear transfer had been developed. Several calves have been reported to born using this technique (Booth *et al.,* 2001b; Oback *et al.,* 2003; Tecirlioglu *et al.,* 2003; French *et al.,* 2004).

Although buffalo is important animal for milk and beef production specifically in Asian country, there is only a single report of a viable clone in this species (Shi *et al.,* 2007). In the early attempts on micromanipulation and cloning investigations on buffalo oocytes and embryos using traditional nuclear transfer, Singla *et al.,* (1997a) reported development of cloned 32-cell embryos from buffalo blastomeres. Parnpai *et al.* (1999) reported the production of blastocysts using fetal fibroblasts as the nucleus donor. In another study it was shown that fetal fibroblasts and granulosa cells had the same ability to be reprogrammed after transfer into the enucleated oocytes (Parnpai *et al.,* 2000). Recently, it has been reported that flat surface culturing system gives better result than microdrop and well of well culture system in case of buffalo (Shah.*et al.,* 2008), also reported first viable buffalo calf produced through hand made cloning using new born donor nuclei (Shah *et al.,* 2009 news report)

2.3 INTERSPECIES NUCLEAR TRANSFER

Somatic cell nuclear transfer (SCNT) is valuable for the production of genetically identical animals and offers the possibility of preventing the extinction of wild species. However, owing to the limited availability of oocytes from wild animals, the cloning of endangered species requires the use of donor oocytes from a related domestic species. Space availability, small number of individuals, behaviour, nutrition, health and welfare and reproductive failure has a major effect on traditional captive breeding programs (Lasley *et al*. 1994). SCNT can provide unique opportunities for overcoming these limitations and enabling the distribution of genetic material, thereby promoting the multiplication and improvement of the genetic make-up of captive-bred species. With the development of NT

techniques, there have been numerous efforts using interspecies nuclear transfer to preserve endangered species (Loi *et al.,* 2001; Vogel, 2001) and to investigate the interactions between recipient cytoplasm and donor cells (Dominko *et al.,* 1998 1999a, 1999b), and overcome the shortage of recipient oocytes (Kitiyanant *et al.,* 2001; Saikhun *et al.,* 2002). Interspecies nuclear transfer was proven successful with the birth of a cloned mouflon created by transferring a mouflon granulosa cell into an enucleated sheep oocyte (Loi *et al.,* 2001) and viable gaur (Lanza, R.P., *et al.,* 2000b). Saikhun *et al.* (2002) introduced buffalo oviductal cells, cumulus cells and fetal fibroblasts into enucleated bovine oocytes and achieved 14-34% blastocyst rates. Lu *et al.* (2005) showed that the embryo reconstructed by interspecies NT of adult fibroblasts between cattle and buffaloes can develop to blastocysts, but bovine cytoplasts directed embryonic development more effectively than buffalo cytoplasts, regardless of the donor cell species. iSCNT embryos developed to the blastocyst stage using rabbit oocyte with remarkably high efficiency. Depending on the donor species— these include cat, ibex, panda, camel, antelope, macaque, and human—6%–33% of iSCNT embryos developed into blastocysts (Wen *et al.,* 2005). These numbers put the efficiency of iSCNT using rabbit oocytes within or above the range of successful intraspecies SCNT blastocyst development frequencies (Dinnyes *et al.,* 2001; Chesne *et al.,* 2002). It is intriguing to speculate that the rabbit oocyte may be more efficient at supporting preimplantation development as bovine oocyte. Important studies on interspecies cloning are mention in following tables.

Table 2.3 Interspecies cloning using rabbit recipient cytoplast.

Recipient oocyte	Donor nuclei	Embryonic stages	Live born	References
Rabbit	Cow	Blastocysts	No	Techakumphu M. *et al*.2005
Rabbit	Goat	Blastocysts	No	Jiang Y. *et al.*2005
Rabbit	Cat	Blastocysts	No	Wen DC. *et al.*2005
Rabbit	Monkey	Blastocysts	No	Zhou Q. *et al.,* 2004
Rabbit	Chicken	Blastocysts	No	Liu SZ.*et al.,* 2004
Rabbit	Human	Blastocysts	No	Chung Y.*et at.*2009

Table 2.4 Interspecies cloning using porcine recipient cytoplast.

Recipient oocyte	Donor nuclei	Embryonic stages	Live born	References
Pig	Whale	4 cells	No	Ikumi S. *et al.*2004
Pig	Tiger	Blastocysts	No	Hashem *et al.*2007

Table 2.5 Interspecies cloning using bovine recipient cytoplast

Recipient oocyte	Donor nuclei	Embryonic stages	Live born	References
Cow	Sheep	Blastocysts	No	Dominko T. *et al*., 1999
Cow	Pig	Blastocysts	No	
Cow	Monkey	Blastocysts	No	
Cow	Rat	2 cells	No	
Cow	Goral	Blastocysts	No	Oh B.C. *et al.,* 2006
Cow	Yak	Blastocysts	No	Li Y. *et al.,* 2007
Cow	Camel	16 cells	No	Zhou H. *et al*., 2006
Cow	Horse	Blastocysts	No	Li GP. *et al* ., 2003
Cow	Dog	Blastocysts	No	Murakami M. *et al*., 2005
Cow	Chicken	Blastocysts	No	Kim TM. *et al*., 2004
Cow	Human	Blastocysts	No	Lanza R. *et al*., 2000a
Cow	Gaur	Blastocysts	Yes	Lanza R. *et al*., 2000b
Cow	Buffalo	Blastocysts	No	Saikhun J. *et al*., 2002

Table 2.6 Interspecies cloning using Ovine /Caprine recipient cytoplast.

Recipient oocyte	Donor nuclei	Embryonic stages	Live born	References
Sheep	Cow	16 cells	No	Hamilton HM. *et al*., 2004
Sheep	Pig	4 cells	No	Hamilton HM. *et al*., 2004
Sheep	Camel	Morula	No	Zhou H. *et al*., 2006
Sheep	Mouflon	Blastocysts	Yes	Loi P. *et al*., 2001
Goat	Ibex	Blastocysts	No	Jiang Y.*et al*., 2005

2.4 FACTORS AFFECTING THE EFFICIENCY OF CLONING

Cloning efficiency is defined in term of birth of healthy adult animals but often compromises the development of reconstructed embryos. One of the most difficult challenges faced is low efficiency and high developmental abnormalities including various clinical and pathological abnormalities and cloning syndromes (Hill *et al.,* 1999; Renard *et al.,*

1999; Kubota *et al.,* 2000). Currently, the efficiency for nuclear transfer is between 0–10 %, i.e., 0–10 live births after transfer of 100 cloned embryos. It has been proposed that low cloning efficiency may be largely attributed to the incomplete reprogramming of epigenetic signals. Remodeling of donor chromatin by the recipient cytoplasm after nuclear transfer is frequently associated with deregulation of specific genes. Cloning by the present method is very inefficient owing to the extraordinary demands placed on the oocyte cytoplasm in reprogramming a donor cell nucleus rather than a sperm nucleus.

2.4.1 RECIPIENT OOCYTE

The oocyte is the unique and complex cell that reprograms the donor nucleus and is one of the factor that affect the results of cloning. Preparation of the donor nucleus for reprogramming also has important implications for proper development. The oocyte maturation process is a crucial step for the generation of quality oocytes capable of being fertilized and undergoing normal embryonic development upto blastocysts after *in vitro* fertilization (Wang *et al.,* 1997). Failure of blastocysts to implant and abnormal fetal development may also stem from inappropriate oocyte maturation (Moor *et al.,* 1998). Interactions occurring between the recipient cytoplasm and the donor nucleus are decisive for initiation of cloned embryo development (Fulka *et al.,* 2001). Identification and then selection of the most suitable recipient oocyte cytoplasts will improve cloning efficiency.

Maturation promoting factor (MPF) activity during oocyte maturation is maximal at both metaphase-I (MI) and metaphase-II (MII) (Campbell *et al.,* 1996). The rate of blastocyst formation by embryos reconstituted with MI oocytes was significantly lower than that of embryos reconstituted with MII oocytes in porcine. This result suggests that MII oocytes rather than MI oocytes are more appropriate recipients for production of differentiated cell-derived cloned embryos in mammals (Miyoshi *et al.,* 2003). MII-arrested oocytes (Stice *et al.,* 1998) and activated oocytes (Campbell *et al.,* 1996; Baguisi *et al*., 1999; Tani *et al.,* 2001) have been used as recipients in most somatic cell nuclear transfer

studies. However, after reduction of maturation/meiosis/mitosis-promoting factor (MPF) activity, preactivated recipients cytoplast are suitable for production of cloned embryos derived from blastomere (Barnes *et al.,* 1993; Stice *et al.,* 1994; Bordignon and Smith, 1998). In contrast, there is only one report of cloned animals being produced from embryos reconstructed by transferring differentiated cells into preactivated or partially activated recipient oocytes (Baguisi *et al.,* 1999). Conclusively, a high level of MPF activity is required for reprogramming a donor nucleus from a differentiated cell. However, in general, oocyte maturation is asynchronous and therefore in any population oocytes will reach MII at different time points and therefore have differing levels of MPF and MAPK activities (Campbell *et al.,* 2007). The use of methods to synchronize oocyte maturation coupled with the stabilization or increase of MPF and MAPK activities may overcome such differences and result in a more uniform population of oocytes for use as cytoplast recipients.

The presence of the chromosomes in MII stage is important for the transfer of donor nucleus into it. According to a majority of authors, the highest proportion of MII oocytes occurs after 24 h of incubation (Singh and Majumdar, 1992; Yadav *et al.,* 1997), however, investigations by Gasparrini, (2002) revealed a shorter time for IVM in buffalo species. Most of the buffalo oocytes (70%) at 15 and 16 h reached MII and 87% of oocytes were found in MII at 19 hours after IVM (Neglia *et al.,* 2001).

2.4.2 DONOR CELL

2.4.2.1 DONOR CELL-CYCLE STAGE

Dolly was created from a donor cell induced to quiescence G0 phase by serum deprivation (Wilmut *et al.,* 1997) and since then many offspring have been produced using cells that were expected to be quiescent in G0 phase. Cibelli *et al.,* (1998) proposed that G0 is not necessary for embryonic development and calves could be produced from actively dividing fibroblasts. In other studies in which serum starvation versus serum supplementation were directly compared, evidences were found that both quiescent and proliferating somatic donor cells can be fully reprogrammed after nuclear transfer and result in viable offspring

(Zakhartchenko *et al*., 1999; Hill *et al.,* 2000; Roh *et al.,* 2000; Kasinathan *et al.,* 2001a, 2001b; Wells *et al.,* 2003). However, it is still debatable which cell cycle stage, result in the best cloning efficiency. Interestingly, Zakhartchenko *et al.* (1999) observed a positive effect of serum starvation on the efficiency of nuclear transfers using bovine fetal fibroblasts. Similar observations were noted by Hill *et al*. (2000) who reported that serum starvation of adult donor cells did not improve development rates of cloned embryos to blastocyst, but when fetal cells were serum-starved, there was a significant increase in their blastocyst development. Conversely, Roh *et al*. (2000) found that fetal transgenic lines did not differ in blastocyst development with or without serum starvation or confluence.

Kasinathan *et al.* (2001a, 2001b) found that a high degree of confluence was more effective than serum starvation for arresting cells in G0, and G1 cells could be obtained using a "shake-off" procedure. No differences in *in-vitro* development were observed between embryos derived from the high-confluence cells (G0) or from the "shake-off" cells (G1). It was concluded that, in nuclear transfer the donor cell cycle stage is important particularly effecting late fetal development, and that actively dividing G1 cells support higher development rates than cells in other stage of cell cycle. A high nuclear transfer success rate was obtained by Cho *et al*. (2002) who subjected donor cells to serum starvation and found no improvement in blastocyst development from adult donor cells, but resulted in a 27.3% calving rate. Wells *et al.* (2003) have reported that serum starvation synchronization into G0 resulted in a significantly higher percentage of viable calves at term than did synchronization in early G1 or late G1. They suggested that it might be necessary to coordinate donor cell type and cell cycle stage to maximize overall cloning efficiency. Das *et al.* (2003) have also used growing fibroblast cells for cloning studies in goat. Various chemical synchronizers has been be used by many researchers to synchronize cells in various phase of cell cycle, include roscovitine which is reversible inhibitor of cdk2 kinase that synchronize cell in G0/G1 phase (Kues WA *et al.,* 2000). Roscovitine-treated adult somatic cells enhanced survivability of cloned calves (Gibbons j *et al.,* 2002). The other chemical synchronizer also improved cloning efficiency such as aphidicoline, by Collas *et al.,* 1992.

For nuclear transfer in Buffalo, Saikhun *et al.* (2004) while using serum fed or starved fetal fibroblast cells as nucleus donor, observed higher blastocyst rates (35%) in serum starved than serum fed (21% only) group. The quiescence induced by serum starvation makes cells more preferable to nuclear reprogramming through nuclear envelope breakdown (NEBD) and the condensation of the chromosome, thus allowing oocyte cytoplasmic factors to access easily to the DNA. These nuclei would undergo DNA replication and subsequent cleavage faster than in the case of serum fed donors, consequently resulting in higher rates of blastocyst development. Sub confluent (Suteevun *et al.,* 2006), serum starved (Simon *et al.,* 2006), metaphase arrested, cytochalasin-B treated (Meena and Das, 2006) or Aphidicolin (reversible inhibitor of mammalian DNA polymerase which blocks cells at G1 to S phase transition) treated (Shi *et al.,* 2007) donor cells have been used for the production of cloned blastocysts in buffalo species.

Till date it remains unclear which cell cycle stage; G0, G1, G2/M or M phase imparts higher nuclear transfer efficiency. This question will continue to be debated until large-scale nuclear transfer studies can be conducted.

2.4.2.2 DONOR CELL TYPE

Theoretically, all the cells in an individual possess an identical genome and, therefore, any cells of animal can be reprogrammed following nuclear transfer. Investigators have evaluated and used different somatic cells, such as embryonic stem cells, blastomeres and other somatic cells for the production of cloned animals.

During early phase, all cloned animals were derived from somatic cells that were produced from the cells of female reproductive system such as mammary gland cells (Wilmut *et al.,* 1997), cumulus and granulosa cells, (Kato *et al.,* 1998; Wakayama et 1998; Wells *et al.,* 1999), and oviductal cells (Kato *et al.,* 1998), raising the question whether male cells could be cloned and reprogrammed in the same way. Male mice were cloned from tail tip cells and there was no significance difference in the developmental rates of embryos derived from male or female nuclei in cattle (Kato *et al.,* 2000). Kato and colleagues (2000) compared the

efficiency of nuclear transfer with skin, kidney, gut and muscle cells from female bovine fetuses, as well skin, heart, kidney cells etc.

Many somatic cell types, including embryonic cells (Campbell *et al.,* 1993), fibroblasts (Kato *et al.,* 1998), mammary gland cells (Wilmut *et al.,* 1997), cumulus granulosa cells (Wakayama *et al.,* 1998), fibroblast cells from skin and internal organs, oviduct cells (Kato *et al.,* 2000), sertoli cells (Ogura *et al.,* 2000), blood leukocytes (Galli *et al.,* 1999; Hochedlinger and Jaenisch, 2002), mural granulosa cells (Wells *et al.,* 1999), germ cells (Bordignon *et al.,* 2003), embryonic stem cells (Eggan *et al.,* 2002), liver cells (Brem and Kuhholzer, 2002a) and colostrum-derived mammary gland epithelial cells (Kishi *et al.,* 2000) have been successfully utilized as nucleus donors. However, it is still unclear which type is the most efficient for nuclear transfer into oocytes (Kato *et al.,* 2000).

These results showed that donor cell type could significantly affect embryo development *in vitro* as well as *in vivo.* No differences were detected in the cleavage rates of embryos from different cell types. Cumulus cells produced the highest rate of blastocyst development and resulted in 6 full-term cloned calves. Cumulus cells could be the most effective somatic cells as nucleus donor with highest cloning efficiency and resulting in the least number of abnormalities in cloned animals. (Tian *et al.,* 2003).

Kato *et al*. (2000) compared efficiency of cloning using various somatic cell types from adult, newborn, and fetal female and male donor cattle. The percentage of blastocysts that developed from each of the donor cell types was not significantly different, except for the extreme case of fetal muscle cells compared with adult male liver cells. Similarly, no differences among embryos derived from fetal and adult bovine fibroblasts with regard to fusion, cleavage, and blastocyst formation rates were detected (Nakajima *et al.,* 2000). Similar results were obtained using various cell types derived from mice of different strains, sexes and ages (Wakayama and Yanagimachi, 2001). A clear consensus, however, has not yet been reached as to the superior somatic cell type for nuclear transfer.

In buffaloes, cloned embryos have been produced using serum starved (Simon *et al.,* 2006) or cytochalasin-B treated (Meena and Das, 2006) fetal skin fibroblast cells but the efficiency of blastocyst formation was less. Blastocysts were produced and pregnancies upto 90 days established from cloned buffalo embryos reconstructed with serum fed and starved buffalo fetal fibroblast cells (Saikhun *et al.,* 2004). Shah *et al.,* 2009 concluded that newborn fibroblast and cumulus cells are more efficient in nuclear reprogramming of hand made cloned embryos than adult and newborn fibroblasts and newborn cells are efficient in pregnancy establishment than adult and fetal fibroblasts.

2.4.2.3.DONOR CELL AGE

Studies carried on the cloning efficiency of fibroblast cells from donors of different ages indicated that the cells from fetuses and newborn animals were more efficient in nuclear transfer. However, when cells from adult animals were used, little changes were observed in the cloning efficiency of cells from cattle varying in age from 2 to 16-years-old. Similarly, Renard (1999), Hill *et al.* (2000) and Wakayama and Yanagimachi (2001) also reported that development rates of somatic cell cloned embryos remained similar regardless of donor age. However, Kato *et al.* (2000) noted that clones derived from adult cells frequently aborted in the later stages of pregnancy, and calves developing to term showed a higher number of abnormalities than did those derived from newborn or fetal cells. Forsberg *et al.* (2002) transferred a large number of cloned embryos in cattle. They also concluded that, in general, embryos cloned from fetal cells produced higher pregnancy and calving rates than those from adult cells. The studies of Kubota *et al.* (2000) concluded that fibroblasts of aged animals remain competent for cloning and prolonged culture did not affect the cloning competence of adult somatic donor cells.

In conclusion, it appears that cells from fetus, as well as aged adults, can lead to blastocyst development of cloned embryos. Nevertheless, fetal cells may be better than adult cells in producing healthy live births. This might be due to the fact that the somatic cells of adult animals have accumulated more genetic mutations, are more

terminally differentiated than fetal cells, and are thus more likely to fail at full term development.

Fibroblast cells derived from adult buffaloes have been used as nuclei donors for the production of SCNT buffalo embryos (Lu *et al.,* 2005; Suteevun *et al.,* 2006). Fibroblast cells from newborn animal, which are reported to have lesser chromosomal abnormalities than adult cells, have also been used for SCNT in buffalo previously at Embryo biotechnology lab. NDRI (Amit, 2006; Birbal, 2006).

2.4.2.4 DONOR CELL EPIGENETIC MODIFICATION

Histone acetylation and DNA methylation are nonheritable modifications of the chromatin with out changes in gene sequences (epigenetic signals). These epigenetic modifications are believed responsible for the derivation of various cell types with the same genetic makeup. In natural reproduction, relatively low levels of DNA methylation exist in the gametes, which are further de-methylated during early embryo development (Mayer *et al.,* 2000; Oswald *et al.,* 2000). With nuclear transplantation, the somatic donor nucleus carries the specific epigenetic modifications of its tissue type, which must be erased during nuclear reprogramming. Therefore, the levels of epigenetic modification existing in donor cells may affect their reprogramming ability following nuclear transfer. A discrepancy in the donor cell's susceptibility to reprogramming has been observed between different cell types, resulting in differences in *in-vitro* and *in-vivo* development of cloned embryos. Therefore, treating donor cells with chemical agents to remove some epigenetic marks prior to nuclear transfer may improve the ability of the donor cells to be fully reprogrammed by the recipient cytoplast, e.g Trichostatin-A (TSA) and 5-aza-deoxy-cytadine (5-aza-dC) (Enright *et al.,* 2003b) have been found to increase histone acetylation and decrease DNA methylation, respectively. These changes have been associated with increased levels of gene expression.

2.5 NUCLEAR REPROGRAMMING

Nuclear reprogramming can be loosely defined as a set of epigenetic changes (those not involving a change in genomic DNA

sequence) required for a nucleus to define the developmental fates. Classic examples of reprogramming include the cloning of animals from adult somatic cells (Wilmut *et al.,* 1997, Cibelli *et al.,* 1998a), the derivation of embryonic stem (ES) cells after transplantation of a somatic cell nucleus into an enucleated oocyte (Cibelli *et al.,* 1998b; Wakayama and Yanagimachi, 2001) and reprogramming terminally differentiated fibroblasts to express T-cell specific functions (expression of T cell receptors, interleukin-2 receptors etc.) using certain specific cell extracts (Hakelien *et al.,* 2002). During nuclear transfer process, it is the oocyte that changes the fate of the donor nucleus from its original status (the terminally differentiated cells like skin cells, granulosa or cumulus cells) similar to that of a zygotic nucleus. In the context of mammalian cell cloning, the term reprogramming refers to the processes that enable somatic cell nucleus to adopt the role of zygotic nucleus (Eckardt and McLaughin, 2004).

Essentially, the success of cloning from differentiated cells rests in the ability of a cell nucleus to retain and re-transcribe the complete array of messages (gene expression) previously turned on and turned off with cell differentiation, as well as ability of a properly timed and metaphase-II oocytes to completely erase the differentiation repertoire of the donor cell. It is generally believed that low cloning efficiency is due to inadequate nuclear reprogramming which in turn is the result of a number of events and factors including the method of activation and type of donor cell used. In natural reproduction, relatively low levels of DNA methylation exist in male and female gametes, which are further demethylated during early development. Differentiated somatic cells and embryos cloned by NT have been shown to have much higher levels of DNA methylation than either gametes or early embryos, due to tissue differentiation.

Moreover, reprogramming of the genome is a highly complex process involving the timely activation and deactivation of the genes leading to a predetermined proteome expression. For instance, proper activation of the early developmental related genes (like Oct-4) is essential for early as well as immediate peri-implantation survival of the embryo (Bortvin *et al.,* 2003). Improper nuclear reprogramming of the donor

nucleus in the oocyte is thought to be the major reason of failure in the cloning process.

In cattle, several studies have shown that methylation (Bourchis *et al.,* 2001; Dean *et al.,* 2001; Santos *et al.,* 2003) and gene expression (Daniels *et al.,* 2000, 2001) are abnormal in NT embryos when compared to *in vivo*-and *in vitro*-generated counterparts. Using bisulfite-sequencing technology, Kang and co-workers (Kang *et al.,* 2001a) found that the patterns of genomic demethylation in repetitive sequences of the cloned donor genome were similar between cloned and IVF pig embryos. These findings contrast with the maintenance of hypermethylation of satellite sequences observed in bovine cloned embryos up to blastocyst stage (Kang *et al.,* 2001a, b). Recently, Enright and coworkers (2003a), showed that histone- acetylation levels in cells changed with respect to the stage of cell, cell type and numbers of cell passages, suggesting that histone acetylation could be a factor in improper nuclear reprogramming in NT embryos. Epigenetic abnormalities caused by the NT process, however, are not passed on to the offspring of cloned animals, as shown in mice (Tamashiro *et al.,* 2002). A recent idea that may increase efficiencies in nuclear reprogramming during the NT process is the exposure of donor cells to remodeling factors through *in vitro* systems before NT is initiated (Alberio *et al.,* 2003). The addition of *Xenopus laevis* egg extracts was shown to successfully inhibit transcription, which has been hypothesized to facilitate nuclear reprogramming (Alberio *et al.,* 2003). In the same line, Sullivan and coworkers (2004) have recently introduced the concepts of *in vitro* nuclear remodeling and chromatin transfer. In this novel system, permeabilized donor cells were exposed to a mitotic cell extract *in vitro*, followed by transfer of condensed chromosomes into enucleated oocytes prior to activation. There is evidence that this treatment initiates remodeling of mammalian somatic nuclei *in vitro* prior to cloning procedures. Although chromatin transplantation was successfully used to generate live cloned calves (Sullivan *et al.,* 2004), the superiority of this new cloning procedure over the traditional nuclear transfer technique has not yet been demonstrated.

From experiments, it became clear that maternal inherited factors contained within the oocytes also have the extraordinary property to restore the totipotency to a differentiated somatic nucleus when transplanted into it. This transformation of differentiated cell to a totipotent state is most widely understood meaning of reprogramming. Extensive epigenetic reprogramming of the genome also occurs in the germ cell line and during the development, which is essential for generating the totipotent zygote. Because the reprogramming factors normally play a significant role during early development, it is important to determine their role in this context, and how these factors act on somatic nuclei restoration of totipotency or pluripotency.

2.6 *IN VITRO* EFFICIENCY OF HANDMADE CLONED EMBRYOS

In cattle, the HMC system based on random bisection is capable of producing approximately 50% blastocyst rates using the WOW system, among the highest described for somatic cell cloning (Vajta *et al.,* 2004). The reported fusion rate in the two cytoplast plus one somatic cell sandwich system was 94% (Tecirlioglu *et al.,* 2003) compared with the 67% that has been achieved with fusion of one cytoplast to a somatic cell (Oback and Wells, 2003). In porcine nuclear transfer, the development rate to blastocysts is 1% to 11%. Efficiency is comparable with 5% and 6% achieved in zona free cloning by Booth *et al.* (2001a) and Kragh *et al.* (2004). According to few available data, higher fusion rates for zona free embryos are accompanied by higher cleavage per reconstructed embryo and higher blastocyst per attempted fusion. Regarding blastocyst quality, the limited number of observations indicate that the only difference between zona free and traditional cloning may be the slightly higher cell number in the embryos derived from the zona free system (Vajta *et al.,* 2003; Kragh *et al.,* 2004) for cattle and pig, respectively.

2.7 EMBRYOS PRODUCTION IN BUFFALO

In vitro embryo production (IVEP) through a combination of the techniques of in vitro maturation, fertilization and culture (IVMFC) has been successfully used for producing morulae and blastocysts in buffalo (For reviews see Palta and Chauhan, 1998; Nandi *et al.,* 2002). Pregnancies (Madan *et al.,* 1994b) and birth of live offspring (Chauhan *et*

al., 1997a) have been reported following the transfer of in vitro produced buffalo embryos to suitably synchronized recipients. Protocols are now available for IVEP in buffalo (Madan *et al.,* 1994b; Chauhan *et al.,* 1997a, b, c, d; 1998a, b, c, d). Shah *et al.,* 2008, 2009 work was carried out on production of blastocysts through handmade cloning in buffalo.

Establishment of pregnancies through transfer of cloned blastocysts to recipients has been reported by a few workers in the world are mention in Table 2.7.

Table 2.7 Pregnancies and births reported after transfer of cloned embryos to recipient buffaloes

Donor cell	Embryos transferred	Recipients	Pregnant (>60 d)	Births	References
Fetal Fibroblast	-	12	3	0[A]	Saikhun *et al.,* 2004
Fetal Fibroblast	-	6	0	0	Simon *et al.,* 2006
Fetal Fibroblast	32	16	3	3[B]	Shi *et al.,* 2007
Granulosa cells	10	5	1	1[C]	Shi *et al.,* 2007
Fetal fibroblasts	18	6	1	#	Shah *et al.,* 2009
Newborn fibroblast	27	9	2	@	Shah *et al.,* 2009

[A] Pregnancies did not survive beyond 90 days
[B] One calf died after 20 minutes of birth. One pregnancy yielded premature dead twins.
[C] Calf died on day 14 after birth.
Calf born on june06, 2009
@ One live birth reported but calf died on day 6 after birth.

Chapter- 4

Materials and Methods

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Plasticwere and Glassware

All the glassware used in the present investigation was made of high-grade pyrex glass and was thoroughly cleaned, rinsed with Ultra pure water and then heat sterilized at 250°C for 4 h. Disposable Pasteur pipettes were from Labco, Ambala, India. The plasticware, disposable 35 mm x 10 mm cell culture Petri dishes, 15 and 50 ml Falcon tubes, 100 mm x 100mm square Petri dishes with 13 mm grid (searching dishes) and capillary pipettes (10 µl, 20 µl capacity; Unopette®), four well, six well culture plates were purchased from Becton, Dickinson and Co., Lincoln Park, NJ, USA or from Nunc Rosklide, Denmark. Disposable non-toxic and non-pyrogenic plastic syringes were from Sigma Aldrich Chemicals (Norm-Ject, Henke-Sass Wolf GmbH, Germany). Disposable 19-guage 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, and USA. Autoclavable disposable tips for micropipettes were obtained from Labware, USA. For embryos transfer, French straws (0.25 ml) were from IMV, L'Aigle, France.

3.1.2 Chemicals, Cell Culture Media and Supplements

The culture media used in the present study for culture of oocytes/embryos and cells, which included tissue culture medium-199 (TCM-199), Dulbecco's modified eagle's medium (DMEM), Dulbecco's phosphate buffered saline (DPBS) and the supplements which included bovine serum albumin (BSA), sodium pyruvate, L glutamine, fatty acid free BSA, porcine follicle stimulating hormone (FSHp) and antibiotics (gentamicin, penicillin and streptomycin) were purchased from Sigma Chemical Co., St. Louis, MO, USA. All the cell culture media were in the form of ready-to-use liquid media. Mineral oil, enzymes (Hyaluronidase, Pronase E, and Trypsin-EDTA), Phytohaemagglutinin

(PHA-L), propidium iodide (PI) and other chemicals used were also from Sigma Chemical Co., unless otherwise indicated. Most of the chemicals used were embryo culture tested or of cell culture grade. Fetal bovine serum (FBS) was from Hyclone (Logan, Utah, US).

3.1.3 Equipments

3.1.3.1 Microscopes

i) Zoom stereomicroscope

Low magnification zoom stereomicroscopes (NIKON, Japan, Model SMZ-2T) were used for searching, evaluating the morphology, quality of the oocytes, and culture of primary tissue explants.

ii) Inverted microscope

An inverted microscope (NIKON, Japan, Model TMD) was used for the examination of cell cultures, monitoring the cell health, morphological characteristics and growth of the cultured cells. The inverted microscope was also equipped with UV fluorescence and differential interface contrast (DIC) attachment, which helped in micromanipulation of the oocytes and capturing the images of the cell cultures and *in vitro* produced embryos. The microscopes were equipped with programmable still photography and video recording facilities.

iii) Compound microscope

A compound microscope (NIKON, Model MICROPHOT-FXA) with a movable slide holding stage and photography facilities was used to count cells in the culture, differentiate between viable and non-viable cells, examining the cell cultures for morphological evaluations and capturing the images of karyotypes of the cells at different passages.

3.1.3.2 CO_2 incubator

A Thermo Forma Scientific (Marietta, Ohio, USA, Model 3131) 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.

3.1.3.3 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 minimizing the incidences of 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.

3.1.3.4 Centrifuge

Refrigerated Centrifuge (Sigma 3K30, Germany) with facilities to adjust centrifugation speed, time and temperature was used for centrifugation of chemicals, trypsinized cells and washing of the cells etc. as and when needed.

3.1.3.5 Vortex Shaker

Rapid vortexing was required for dissociating the cumulus cells for denuding the *in vitro* matured oocytes, vortex shaker was used for the dissociation of cells after trypsinized the tissue pieces for establishing primary cell cultures of various somatic cells etc.

3.1.3.6 Electrofusion Equipment

Electrofusion machine (BTX ELECTRO CELL MANIPULATOR 200, San Diego, CA), with facilities for manual as well as automatic controlled electrical inputs and outputs was used for generating the electrical pulses i.e. alternating current (AC) and direct current (DC) pulses for dielectrophoresis and membranes fusion, respectively. An Optimizer (BTX make) was connected in series with the Electrofusion machine to analyze the actual output pulse parameters provided by the fusion machine. Electrofusion also served to activate the fused triplets through electro-activation.

3.1.3.7 Flow cytometric analyzer

The BD FACSCalibur (BD Biosciences 2350 Qume Drive San Jose, CA USA 95131) multicolor flow cytometer has been designed specifically to support a wide range of applications. The BD FACSCalibur system combines unique dual-laser technology, an automated sample loader option, and powerful software to provide the high throughput necessary to meet productivity requirements of clinical laboratories and basic research laboratory. The machine provided cell sorting, cell concentration, cell cycle analysis, clonal selection of homogenous population, and immunocytochemistry. Along with machine, cell quest software for data analysis was provided.

3.1.4 Biologicals

3.1.4.1 Ovaries and oocytes

The buffalo ovaries were obtained from a nearby abattoir (New Delhi). The ovaries served as the source of immature oocytes, follicular fluid and cumulus cells during the present investigation. The mature oocytes were processed for cloning.

3.1.4.2 Follicular Fluid of buffalo

For the collection of follicular fluid, buffalo ovaries were obtained from Delhi slaughterhouse and transported to the laboratory at $3^{o}C$ within 3-4h of slaughter. Follicular fluid was aspirated from all visible non-atretic healthy surface follicles (2 to 8 mm in diameter) with a 19-gauge needle. The cellular debris were removed by centrifugation at 3,000 rpm for 30 min. The supernatant was carefully collected and filtered through 0.45-µm-membrane filter. 500 µl aliquots (one aliquot required at a time) were prepared in 600 µl microfuge tubes and stored at - $20^{o}C$ until further use.

3.1.4.2 Somatic Cell Cultures

For somatic cell cultures, ear skin samples were obtained by biopsy from adult females Murrah buffaloes, Sahiwal cattles, Goats (AB) and Wister rat (tail tip). Newborn female and male cattle calves

were available at the Cattle Yard, NDRI, Karnal, and fetus obtained from the slaughterhouse were used to establish fetal cell cultures. The laboratory animals were available in small animal house at NDRI.

3.2 Method

3.2.1 Preparation of different media

The composition of various media used in the present study has been provided in ANNEXURE-I.

3.2.2. Collection and classification of oocytes

Buffalo ovaries were collected from Delhi slaughterhouse immediately after slaughter. These were washed 4-6 times with isotonic saline (32-37°C) containing 400 I.U/ml penicillin and 500μg/ ml streptomycin. The washed ovaries were then put in a thermos flask containing saline and antibiotics. The collected ovaries were transported to the laboratory within 4 h of slaughter. In the laboratory, the ovaries were rinsed twice with saline and trimmed to remove the extra tissue and washed properly (5-6 times) with warm saline containing antibiotics.

Oocytes were collected by aspiration of surface follicles (2-8 mm diameter) with a 19-gauge needle attached to a 10 ml syringe containing the aspiration medium (TCM-199 + 0.3% BSA+ 50 µg/ml gentamycin sulfate). The contents of the syringe, which included the aspirated oocytes, follicular fluid, granulosa cells and debris, were transferred in 15 mL centrifugation tube for settling the oocyte and then settled material transferred to 100 mm × 100 mm square Petri dishes (Searching dishes) and the oocytes were searched under a zoom stereomicroscope at around 20 X magnification. The oocytes were then shifted to 35 mm Petri dishes containing the washing medium (TCM-199 + 10% FBS + 0.81 mM sodium pyruvate + 50 μg/ml gentamicin sulfate).

The collected oocytes were graded on the basis of their morphology as described below

Grade A: Compact cumulus-oocyte-complexes (COCs) with an unexpanded cumulus mass having ≥4 layers of cumulus cells, and with homogenous evenly granular ooplasm. (Fig 1A)

Grade B: Compact cumulus-oocyte-complexes (COCs) with an unexpanded cumulus mass having ≥2 layers of cumulus cells, and with homogenous evenly granular ooplasm.

Grade C: Oocytes partially or wholly denuded or with expanded or scattered cumulus cells or with an irregular and dark ooplasm.

Oocytes of only Grades A and B were used for the study

3.2.3 In vitro maturation of oocytes

The oocytes were washed 4-6 times with the washing medium (TCM-199 + 10% FBS + 0.68 mM L-glutamine + 0.81 mM sodium pyruvate + 50 μg/ml gentamycin sulfate), then 2-3 times with the IVM medium (TCM-199 + 10% FBS + 5 μg/ ml pFSH + 0.68 mM L-glutamine + 0.81 mM sodium pyruvate + 1 μg/ml estradiol-17β + 50 μg/ml gentamycin sulfate). For *in vitro* maturation, groups of 15-20 COCs were placed in 100 μl droplets of the IVM medium, overlaid with sterile mineral oil in 35 mm Petri dishes and cultured for 24 h in a humidified CO_2 incubator (5% CO_2 in air) at 38.5°C.

3.2.4 Preparation of recipient cytoplasts

The recipient cytoplasts were prepared as described previously (Vajta et al., 2006) with minor modifications. Various steps involved in the preparation of enucleated cytoplasts are elaborated as below.

3.2.4.1 Preparation of zona free Oocytes

Oocytes with expanded cumulus (Fig.1B) were transferred into 1.5mL microcentrifuge tube containing 500μL Hyaluronidase (0.5mg/mL) in T2 (where T denotes TCM-199 supplemented with 2.0 mM L-Glutamine, 0.2mM Sodium pyruvate, 50μgmL^{-1} gentamicin and the following number denotes percentage of FBS) and incubated for 1 min at 38.5ºC. It was followed by a gentle pipetting using 1 ml micro tip

and a subsequent vortexing at a high speed for 2-3 min. in order to remove cumulus mass. The contents of the tube were transferred to a 35 mm dish containing T2 and completely cumulus free oocytes (Fig.1C) with evenly granular cytoplasm were selected and transfer in another 35mm dish containing T2 media and washed twice in fresh T2 for removal of residual cumulus cells.

The polar body containing oocytes were selected by observation under the high power of zoom-stereomicroscope. For polar body observation the oocytes were gently rolled with the help of fine and blunt glass capillary under the microscope. Polar body containing oocytes were collected with the help of a fine pulled and fire-polished glass micro tip (20 µl capacity) attached to Unopette and transferred to another T2 containing dish.

Denuded and polar body containing oocytes were transferred to a 60 mm dish containing Pronase (2.0mg/mL in T10) and incubated for 10 minutes at 38.5ºC with a periodic gentle rotation of the dish. Oocytes with completely digested zona pellucida were transferred into a dish containing T20 so as to stop the activity of the enzyme. Zona-free oocytes were washed twice in T20 and incubated in same medium at 38.5ºC for 0-30 minutes for a prominent protrusion cone was easily visible under the zoom-stereomicroscope.

3.2.4.2 Enucleation of oocyte

Bisection of the oocytes was performed using a method on basis of protrusion cone guided Bisection, which avoids the use of Hoechst 33342 stain and subsequent selection under UV illumination. Protrusion cone bearing oocytes (Fig.1E) were transferred (8-10 each time) into 35 mm dish containing 3mL T20 and 2.5µg/mL Cytochalasin B and manually bisected using microblade (Fig.8 B) in such a way so that the smaller half (1/3) bears the protrusion cone and the larger half (2/3) is enucleated (Fig.1F). The bisected half-oocytes without protrusion cone were transferred into T20 and incubated for 10-15 minutes at 38.5ºC so as to regain spherical shape.

3.2.5 Establishment of somatic cell cultures

3.2.5.1 Fibroblast Primary Cell Cultures

Skin biopsies were taken aseptically from the ear pinna or tail tip of the healthy animals. The margin of the ear pinna was shaved using a razor and wiped with alcohol. Skin tissue was pinched off from the ear pinna of the animals and the wound was dressed. Norms regarding the ethical treatment of animals during the whole operation were strictly followed. The tissue was held in sterile DPBS and transported to the laboratory for further processing. The tissues were then washed, thoroughly, with Ca^{++} and Mg^{++}-free DPBS. The tissue was transferred into a cell culture dish containing 2ml DMEM with 10% FBS, and skin along with the hair follicles was removed on both surfaces. The remaining tissue was minced into small pieces (about 1 mm in size) using a sterile surgical blade. The tissues were washed again in culture medium (DMEM containing 10% FBS) for three times, and seeded into 25 mm^2 tissue culture flask without addition of any medium (the small amount of medium sticking to the tissue was sufficient to nourish them till their attachment). The flasks were incubated for 4-8 hours and observed at different intervals so as not to allow them to dry out completely. Upon attachment of tissues, culture medium (DMEM containing 20%FBS) was added to the flasks that were then incubated in a CO_2 incubator. Tissue explants were regularly observed for proliferation of fibroblasts from them (Fig.2A), and were removed aseptically when a sufficient number of cells had proliferated and formed a monolayer on the cell culture dishes. The monolayer was treated as primary cell culture of respective cells. After reaching 60-70% confluence, the fibroblast cells were sub-cultured by partial trypsinization.

3.2.5.2 Sub-culturing the cells

When a monolayer of the cells (60-70% confluent) was formed, the cells were ready for subculture. The cells were washed with Ca^{++}

and Mg^{++} free DPBS for 5 min. After decanting the supernatant, the cells were overlaid with Trypsin-EDTA (0.25%) and incubated for a 5-8 minutes, till the cell monolayer started separating from each other and the cell culture flask surface. In the primary cell cultures, the monolayer was trypsinized till the separation of different cells from each other. This was in view of the fact that in primary cell cultures, morphologically different cells had established. A selected population of the cells was required to be chosen at this stage. The passaged or subcultured cells were observed for in vitro growth for about 12h. The dead cells were removed and anchored cells were allowed to grow further.

3.2.5.3 Maintenance of somatic cell cultures

Once the somatic cell cultures were established, the representative cell culture stocks were preserved by cryopreservation. The cells were allowed to grow *in vitro* to exponential phase of growth. Cell culture was checked for healthy growth, contamination and presence of specific morphological characteristics for skin fibroblast cells. The cells were harvested by trypsinizing the cell monolayer in order to preparing the cell suspensions. The cell suspension at a final concentration of 10^6 - 10^8 cells / ml was prepared in the holding medium. Cells were preserved by slow freezing method and subsequently stored at -196^oC.

3.2.5.4 Cryopreservation by Slow Freezing

After centrifugation, a trypsinized cell was washed with freezing medium (15% Glycerol + 30% FBS + 55% DMEM). Cell pellets were resuspended in 1 ml of medium and transferred to a Cryovial (Corning). The Cryovial was subjected to slow freezing in Cryo 1^oC freezing container (Mr. Frosty) at -80^oC for 2-3 hours (a slow and constant cooling rate equivalent to -1^oC per minute is achieved by this method). When the vial attained -80^oC temperature, it was plunged into liquid nitrogen. This was done quickly, as the cells deteriorate rapidly, if temperature rises above -50 ^oC.

When required, the vials were removed from liquid nitrogen, thawed in warm water (35 ^{o}C) by holding the vial and vigorously shaking and transferred into the complete medium previously equilibrated in CO_2 incubator.

The survival of the cells was evaluated by thawing after 7 days and culturing the frozen thawed cells in DMEM supplemented with 10% FBS. Quantitative survivability of the cells was determined by staining the frozen thawed cells with Trypan blue (0.4%) whereas the qualitative survivability was evaluated by allowing the frozen thawed cells to grow *in vitro*.

3.2.6 Evaluation of Chromosomal Stability of Somatic Cells

Cells were subjected to chromosomal analysis according to the method cited by Dyban (1983) with some modifications. Actively growing cells (growing in exponential phase of cell growth) in culture medium (DMEM with10% FBS) were treated with colcemid (0.1mg/ ml) for 4 h. The cells were then washed with ice chilled calcium and magnesium free DPBS for 5 min. Individual cells suspension was prepared by trypsinizing the confluent cells. Traces of trypsin-EDTA were removed by washing the cells through centrifugation (1500 X g, 5 min.). The cell pellet was suspended in hypotonic solution of KCL (0.75 mM) for 30 min at room temp. Hypotonic treated cells were pelleted by centrifugation (1500X g, 5 min.) and fixed in 10 ml of freshly prepared ice-chilled fixative (3:1 methanol/ glacial acetic acid) for 20 min at 4°C. The supernatant was removed by centrifugation and the cell pellet was resuspended in 2-3 ml of fresh chilled fixative so as to get sufficient number of cells.

The metaphase spreads (of the chromosomes) were prepared by dropping the cells suspension from 2-3 ft height onto the ice-cold glass slides. The spreads were allowed to air dry for 5 min. Chromosomes were stained with 10% Giemsa stain and observed under oil immersion (1000 X) using a compound microscope (Nikon, Microphot-FXA, Japan). The images were captured and processed.

The cells at 5^{th} and 10^{th} passages before using them as nucleus donors as well as in routine cultures were evaluated for their chromosomal stability from their karyotype (Fig.9 A, B and C). Similarly, the frozen thawed cells were allowed to grow to exponential stage of cell growth, and evaluated for chromosomal profile.

3.2.7 Synchronization of donor cell

Cells that are used in cloning, are synchronized in G1/G0 by various treatment such confluency, serum starvation, roscovitine treatment and combination of serum starvation with confluency. To check efficiency of synchronization, cells were analyzed by flow cytometry by propidium iodide staining. The staining protocol was followed according to Riccardi C *et al.,* 2006 with some modification. Suspended cells in 1-2 x 10^6 /ml of phosphate buffer solution (PBS) and centrifuged at 200g for 5 min. at room temperature followed by removal of PBS. Cells were fixed by adding 1 ml of 70% ice chilled ethanol and kept for 30 min. and ethanol removed by centrifugation at 400g for 5 min. and after that single PBS washing was given at room temperature. Cell suspension was subjected to DNase free RNase treatment (1mg/ml) for 30 min. followed by staining with DNA staining solution (50μg of PI with 1% triton X-100) and incubated at least for 30 min., and then cells were analyzed for cell cycle in flow cytometry by using 488-nm laser line for excitation. Collected at least 20,000 events per sample. Cells were gated on forward light scatter verses side light scatter such that only viable cells with out debris were analyzed. DNA histogram plot for red fluorescence was created using Cell Quest Program and the percentage of cells existing with in various phase of the cell cycle was calculated by program with same algorithm in all samples.

3.2.8 Preparation of Donor Cells for Nuclear Transfer

The confluent cells at 5^{th} to 10^{th} passage were used as donor nuclei. As a result of confluency, majority of the cells were expected to synchronize in G1 stage of cell cycle. Culture medium of the cells was

removed by aspiration and the cells were washed with calcium and magnesium free DPBS for 5 min. After removing DPBS the cells were subjected to trypsinization using Trypsin-EDTA solution. The dissociating cells were then harvested in T20 medium and centrifuged to get a loose cell pellet. The pellet was resuspended with T20 and mixed by pipetting to get single cell suspension in a 1.5 ml tubes and held at room temperature. The cells were ready for use as nucleus donors.

3.2.9 Pairing and electrofusion of donor cells and Cytoplasts

The enucleated demi-oocytes from T20 were transferred in Phytohaemagglutinin (0.5mg/mL in T2) for 3-4 seconds and transferred into T2 containing low density donor cells prepared and stored during the 10-15 minutes incubation interval for rounding of demi-oocytes. Each demi-oocyte was then allowed to attach to a single (Fig.3 A), rounded, medium sized cell by gently rolling the demi-oocyte over it. The couplets (demi-oocyte-donor cell pairs) were transferred to fusion medium (Annexure) for equilibration. Electrofusion of the couplets and demi-oocytes was then carried out by double step method.

3.2.9.1 Single step fusion

The couplets and the remaining demi-oocytes were then transferred to southern and northern parts, respectively, of the fusion chamber (BTX microslide 0.5mm gap, model 450; BTX, San Diego, CA) (Fig.8 A), containing the fusion medium. A single step fusion protocol was followed wherein a demi-oocyte and a couplet were picked using a fine pulled capillary pipettes (Unopette® Becton Dickinson, NJ, USA) having an inner diameter of 100-120µm (Fig. 4D). Initially, the couplet was expelled and aligned with an A.C. pulse (4 Volts) using BTX Electrocell Manipulator 200 (BTX, San Diego, CA, USA), so that the somatic cell faces the negative electrode. Immediately after alignment, another demi-oocyte was introduced into the fusion chamber close to the somatic cell. As soon as the somatic cell was sandwiched between the demi-oocytes (Fig.3B), D.C. pulse

was applied. The triplets were then incubated in T20 (for rounding up and subsequent reprogramming) for 6 h at 38.5ºC.

3.2.10 Activation of reconstructed oocytes

The reconstructed oocytes were then activated (~27-29 hours after the start of maturation) by incubating in T20 containing 5 µM Calcimycin A23187 for 5 min. at 38.5ºC. The oocytes were washed thrice in T20 and were then incubated, individually in 5µL droplets of respective culture medium containing 2mM 6-Dimethylaminopurine (6-DMAP) and covered with mineral oil in CO_2 incubator, at 38.5ºC for 4 h. Reconstructed embryos were removed from 6-DMAP and washed five times in embryo culture medium.

3.2.11 *In Vitro* Culture of Embryos

In order to evaluate the developmental competence of reconstructed and PA embryos different culture systems were used.

Flat Surfaces (FS)

For this system, 400 µL of culture medium was added to each well of a 4 well dish overlaid with 400 µL mineral oil. Reconstructed and PA embryos were gently placed (10-15 per well) along the periphery of the well at a distance from each other so as to avoid aggregation. The dish was handled carefully so as not to displace the embryos and incubated undisturbed at 38.5ºC in CO_2 incubator for 7 days.

3.2.12 Assessment of embryo development and determination of blastocyst cell number

Cleavage and blastocyst rates of embryo development were recorded on day 3 and 7 of IVC and percent development of each stage calculated. For examining the health of the embryos, the total cell number of trophectoderm (TE) and inner cell mass (ICM) of day 8 blastocysts was determined by differential staining, as described by Giraldo A M *et al.,* (2008). Briefly the day 8 blastocysts were incubated in DPBS containing 10µg/ml of Hoechst 33342 for 30 min. fallowed by permeabilization with 0.04% triton X100 for 3 min, embryos were

counter stained with 25µg/mL propidium iodide (PI) for 5 min. Stained embryos were mounted onto a glass microscope slide in drop of 25 % glycerol, gently covered by cover slip and visualized for cell counting. The number of trophectoderm (TE; nuclear stain with PI) and inner cell mass cells ((ICM; nuclear stain with H33342) was determined using an epifluorescent microscope equipped with UV filter cube (Fig.9 D)

3.2.13 Cryopreservation of Embryos

Blastocysts were cryopreserved for future transfer to recipient animals. Cryopreservation was carried out by the process of Vitrification method described by Du *et al.,* (2007) with minor modifications. Briefly, healthy blastocysts having good cell number and distinct ICM, were transferred into equilibration solution (ES) consisting of 7.5% ethylene glycol (EG) and 7.5% dimethylsulfoxide (DMSO) in T0 and 20% FBS at 39^{o}C for 5 to 15 min. After an initial shrinkage, blastocysts regained their original volume. Blastocysts were then transferred into a dish containing vitrification solution (VS) consisting of 15% EG, 15% DMSO and 0.5M sucrose dissolved in T20. After incubation for 20–30 s, blastocysts were loaded into French straws and plunged into liquid nitrogen. The process from exposure in VS to plunging was completed within 1min.

For thawing, the straws were removed from liquid nitrogen and immersed directly in 37^{o}C water bath. The blastocysts were equilibrated in thawing solution (1.0M sucrose dissolved in T20) for 1min, and then transferred to dilution solution (0.5M sucrose dissolved in T20) for 3 min. Subsequently, blastocysts were incubated twice for 5 min in the washing solutions (T20). Survival of vitrified blastocysts was determined according to re-expansion rates after 18–24h recovery in culture medium (RVCL).

3.2.14 Synchronization of Recipient animals for embryo transfer

i) Natural Heat

Murrah buffaloes and cattle available at the cattle yard, NDRI, who had come naturally in heat, were used as recipients for the

transfer of cloned embryos. These animals were left unbred and were subsequently examined for the presence of corpus luteum (CL) on day 7 after the exhibition of heat. Animals having the CL on either ovary and free from any uterine infections were selected for embryo transfer through non-surgical method (Fig.8 E & F).

ii) Single injection of $PGF_{2\infty}$

In order to synchronize the animals in accordance with the production of cloned embryos, the cycling buffaloes possessing a corpus luteum were injected with $PGF_{2\infty}$ (Lutalyse, 5 ml or 25 mg; intramuscularly) or its analogue (Cloprostenol sodium, 500 µg; intramuscularly). Estrus was exhibited at about 72 hours after the treatment.

3.2.15 Loading of embryos in French straws

Transferable quality day 7 blastocysts having a good total cell number and inner cell mass (ICM) cells were rinsed (5-6 times) in sterile DPBS containing 0.4% BSA (FAF) in 35 mm culture dishes. After rinsing, the blastocysts were aseptically loaded (one or two blastocysts in each straw) into 0.25 ml French straws using a unopette. The embryo loaded straws were temporarily kept in an incubator till their transfer into recipients.

3.2.16 Embryo transfer to recipients

Embryos were transferred non-surgically to recipient buffaloes and cattle on day 7 after the exhibition of estrus. The recipients were checked for the presence of CL and ruled out any possibility of infection through history of natural/induced heat. An epidural injection of local anesthetic, Lignocaine hydrochloride was given before transfer of embryos to each recipient so as to decrease the straining and allow successful transfer. Two embryos were transferred into the ipsilateral uterine horn to the ovary containing the CL, whereas, a single embryo was transferred into the contra lateral uterine horn in case of buffaloes and single embryo transfer in ipsilateral uterine horn in case of cattle.

3.2.17 Monitoring and Diagnosis of Pregnancies

Pregnancies were monitored by ultrasonography after one-month of transfer and the results of each animal recorded on ultrasonograph. The observation was repeated at one to two month intervals to assess the development and viability of the fetus.

3.2.18 Experimental Design

3.2.18.1 Efficiency of different methods for synchronization of cell cycle in G1/G0 phase of cultured buffalo fibroblast.

Cultured buffalo fibroblasts were synchronized in G1/G0 by various methods. The cultured fibroblasts were seeded in 25cm^2 flask (1x 10^5 cell/flask). After 24 h in culture, fibroblast cells were exposed to following treatments (1) Cells cultured at 60-70% confluency (cycling cells), (2) Cells cultured to 100% confluency and then cultured for additional 3-5 days (contact inhibition), (3) Cells cultured in serum starved condition for 24 h. (4) Cells cultured to 100% confluency with serum starvation for 24 h. (5) Cyclic cells (60-70% confluency) were treated with Roscovitine at different concentrations such as 20,40,60,μM. (6) Cyclic cells (60-70% confluency) were treated with Aphidicoline at 2,4,6,μM dose. In all above experiments, cells were cultured under same conditions (5% CO_2 in air and high humidity at 37 ^{0}C) and at same passaged level.

3.2.18.2 Effect of reprogramming time on developmental competence of intra-species Handmade cloned buffalo embryos.

The reconstructed embryos were incubated for various reprogramming time such as 0, 2,4,6,8, hours after fusion in order to optimize the reprogramming time for higher rate of blastocysts. Each experiment was repeated for 5 times.

3.2.18.3 Efficacy of buffalo oocyte to support *in vitro* development of inter-species Handmade cloned embryos.

A cultured skin fibroblasts somatic cells from adult and new born cattle, adult goat and adult rat (Wister) were used for donor nuclei in somatic cell nuclear transfer in order to study efficiency of buffalo oocyte to support in vitro development of inter species cloned embryos.

3.2.19 Statistical Analysis

The data was analyzed using SYSTAT 6.0 (SPSS Inc. Chicago, IL, USA) after arcsine transformation. The differences between means were analyzed by one way ANOVA followed by Fisher's LSD test. Significance was determined at $P<0.05$.

Chapter- 5

Results and Discussion

RESULTS AND DISCUSSION

The present study was carried out to establish procedure of interspecies hand made cloning using buffalo oocyte recipient cytoplast and donor nuclei from adult cattle, adult goat, adult rat (wister) and improve the efficiency of buffalo hand made cloning by optimizing reprogramming times for genomic reprogramming of differentiated somatic cells into undifferentiated embryonic cells. The synchronization of buffalo fibroblast in G0-G1 stage of cell cycle also improves the efficiency of cloning. Once interspecies cloning technique is standardized in domesticated animal, it will be applicable in conservation of wild endangered animal population.

4.1 ESTABLISHMENT OF SOMATIC CELL CULTURES

4.1.1 Primary cultures of Skin Fibroblast cells

Ear skin biopsies were taken from adult buffaloes, cattle, goat and tail of rat and seeded in culture flasks started proliferating after 6-8 days of primary culture (Fig.2 A). These primary cell cultures attained 60-70% confluence with in 1-2 weeks. Cattle and goat skin fibroblasts were observed to proliferate faster as compared to buffalo fibroblasts. It was observed that rat fibroblasts were not able to maintain for longer duration in culture. Upon sub culturing, the cells formed a confluent monolayer within 48-72 hours of seeding (Fig.2 C, D, E&F). The cells grew nicely till 10 to 15 passages except rat fibroblasts and stock of cells was cryopreserved at different passages for replacement in future.

4.1.2 Rat fibroblasts culture

Rat fibroblast cells were found to grow conveniently only till 3-4 passages and on subsequent passages, showed retarded proliferation and heterogeneous cell size. So these cells were used at 2-3 passages for nuclear transfer.

4.2 Synchronization of cell cycle of skin fibroblasts in buffalo

There are different factors that affect *in vitro* development of reconstructed embryos following nuclear transfer. The primary importance is the synchronization between the cell cycle of the donor nuclei and the recipient cytoplast. For successful reprogramming of the donor nucleus, it must be in G0 or G1 when transferred to metaphase II arrested oocytes having greater amounts of maturation promoting factor (MPF). This strict synchrony will allow chromosomes to condense properly and will enhance the correct ploidy in the resulting embryo. In an attempt to improve cloning efficiency, donor cells undergoing different treatments and synchronization efficiency was analyzed by flow cytometry. Common methods to induce a quiescent (G0) status of donor cells for nuclear cloning are serum starvation for several days or culture to confluence. The importance of serum starvation was highlighted in the paper that announced the birth of Dolly (Wilmut *et al.,* 1997). However, pregnancies and live calves have been reported from fetal fibroblasts without serum starvation (Cibelli *et al.,* 1998; Vignon *et al.,* 1998), indicating that serum starvation is not essential for the success of nuclear transfer. Furthermore, Cibelli *et al.,* (1998) suggested that actively dividing cells in the M phase support the development as similar fashion as blastomeres. In our study, cultured buffalo fibroblasts were synchronized in G1/G0 by various methods, initially the culture fibroblasts were seeded in 25cm^2 flask (1x 10^5 cell/flask). After 24 h in culture, fibroblast cells were exposed to following treatments such as: (1) cells cultured at 60-70% confluence (cycling cells), (2) cells cultured to 100% confluence and then cultured for additional 3-5 days (contact inhibition), (3) cells cultured in serum starve condition for 24 h. (4) cells cultured to 100% confluency with serum starvation for 24 h. (5) cyclic cells (60-70% confluence) were treated with Roscovitine at different concentrations such as 20,40,60,μM. (6) cyclic cells (60-70% confluence) were treated with

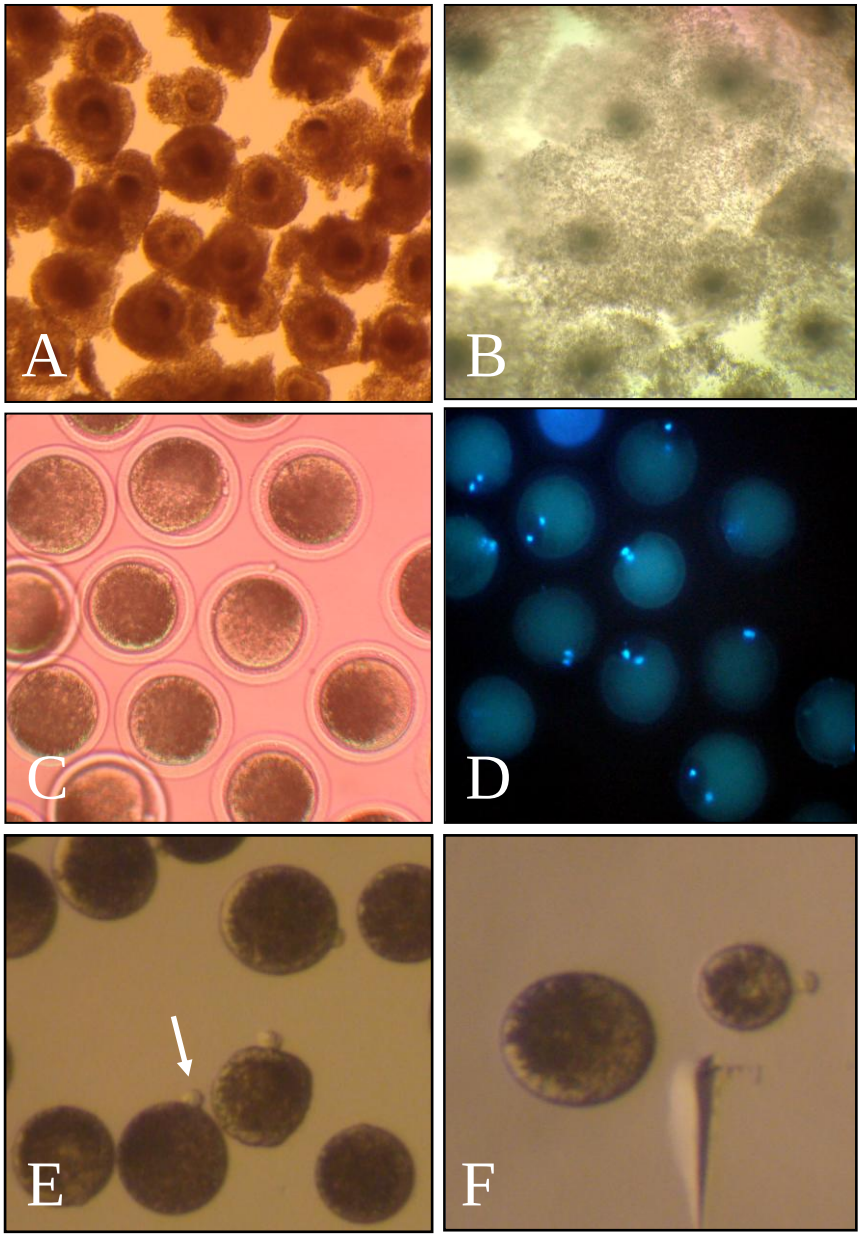

Fig.1

A) Immature oocytes
B) Mature oocytes with cumulus expansion
C) Denuded oocytes
D) **Oocyte showing metaphase plate after H33342 staining (UV)**
E) **Zona free oocytes showing protrusion cone (arrow)**
F) **Protrusion cone guided bisection of oocyte**

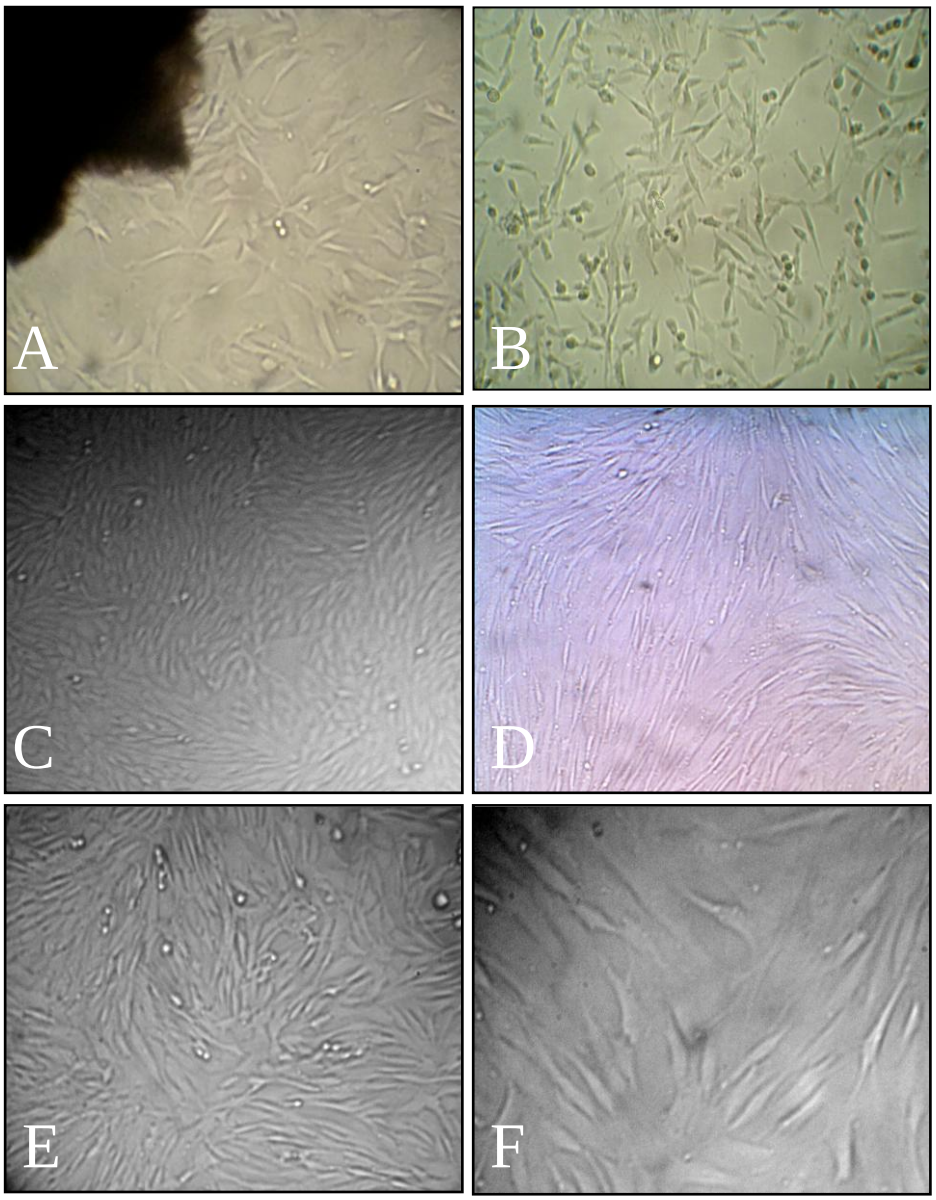

Fig.2

A) Primary explant out growth from buffalo skin tissue
B) Growing cells of buffalo fibroblasts
C) Full confluent fibroblast cells of cattle
D) Full confluent fibroblast cells of buffalo
E) Full confluent fibroblast cells of goat
F) Full confluent fibroblast cells of rat

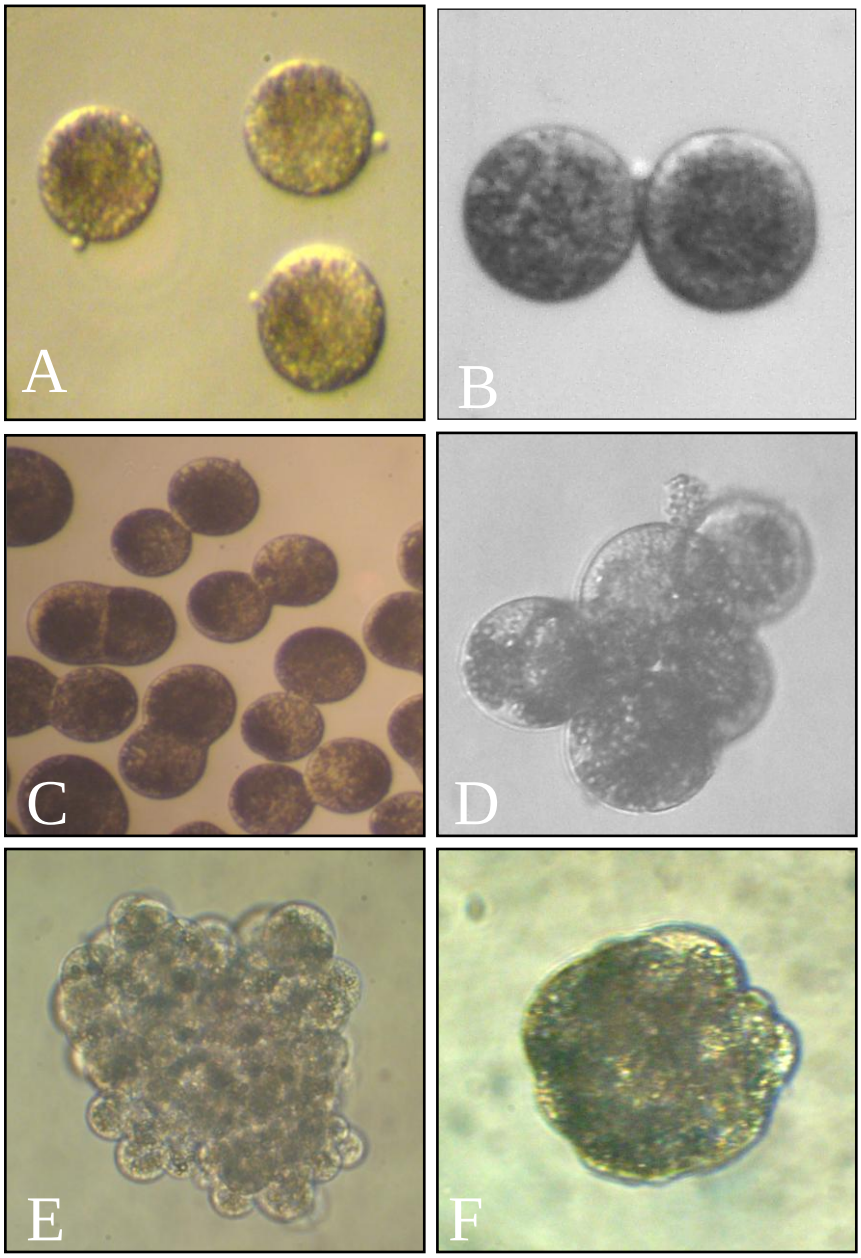

Fig.3

A) Couplet showing somatic cells attached to demi-cytoplasts
B) Triplet showing somatic cell between two demi-cytoplasts
C) Fused reconstructed embryos
D) 8 cell stage embryo
E) 32 cell stage embryo
F) Compacted morula

Aphidicoline at 2,4,6,μM dose. In all above experiments, cells were cultured under same conditions (5% CO_2 in air and high humidity at 37 ^{0}C) and upto same passaged level (4-5 passages). The flow cytometric analysis of cell cycle showed that cells confluency and combination of confluency and serum starvation is efficient as chemical synchronizer roscovitine to synchronize most cells in G0-G1 stage of cell cycle. In case of aphidicoline treatment all experimental groups cells died, so during the study, this chemical synchronizer was excluded out from the experiment.

Table 4.1 Percentage ± S.E.M. of buffalo fibroblast cells existing in various phases of cell cycle after various treatments

Treatment Groups	Cell cycle stages		
	G0-G1	S	G2-M
Cyclic fibroblast (60-70 % Confluency)	70.67±1.28	18.90±1.96	10.76±1.12
Full confluency	85.99±1.46	9.33±0.76	2.43±0.42
Serum Starvation	76.75±3.57	15.85±1.34	16.26±2.2
Full confluency with serum starvation	93.89±1.38	4.1±0.95	1.46±0.46
10 μM Roscovitine	88.90±1.5	6.08±1.2	5.6±0.71
20 μM Roscovitine	94.78±1.4	2.79±0.82	4.2±0.89
30 μM Roscovitine	96.26±1.74	1.95±0.45	1.98±0.30

To evaluate the effect of various treatments on the cell cycle, DNA content by FACS analysis was measured, and the relative percentage of the proportions of cells in the G0/G1 (2C DNA content), S (2C–4C) and G2/M (4C DNA content) stages were calculated (Table 4.1).

The table 4.1 shows that full confluency, combination of full confluency and serum starvation, 10μM ROS, 20μM ROS, 30μM ROS improved cell cycle synchrony compared to that of cyclic fibroblast and serum starved cells for 24 hours. As was evident from the greater

relative percentage of proportions of G0/G1 (Table 4.1) or greater DNA peaks (Figs. 4a & 4b), the vast majority of the cells at any given time, were in G0/G1 stage.

At concentration of 10μM roscovitine, synchronized cells in G0/G1 phase were similar as full confluent and 20μM, 30μM and combination of serum starvation synchronized most cells in G1/G0 phase above 90 percentage but most cells in 30μM roscovitine group underwent apoptosis and cell death (Fig.5D). Removal of roscovitine, followed by culturing washed cells in medium containing 10% FBS for 24 hours restored progression in the cell cycle comparable with cycling cells. During experiment, all treatment groups of aphidicoline cells died (Fig.6) after 24 hours of incubation, hence these cells were not processed for further analysis.

4.3 Effect of reprogramming time on developmental competence of intra-species Handmade cloned buffalo embryos

For successful nuclear transfer, the donor nucleus must be properly reprogrammed. During nuclear reprogramming, epigenetic marks were completely removed from genome of donor nuclei, resulting in erasure of tissue-specific gene expression pattern and programmed genome into totipotent state. Moreover, reprogramming of the genome is a highly complex process involving the timely activation and deactivation of the genes leading to a predetermined proteome expression. From experiments, it became clear that maternally inherited factors present within the oocytes also have the extraordinary property to restore the totipotency to a differentiated somatic nucleus when transplanted into it. This transformation of differentiated cell to a totipotent state is most widely understood meaning of reprogramming. Our study indicated that the timing between fusion and activation has impact on genomic reprogramming. The cleavage of embryos from holding time between 6-8 hours (84.4-85.37 %) was significantly higher ($P<0.05$) than that of embryos were holding time between 0-4 hours (61.24-62.78 %) in table 4.2. The rate of blastocyst development of

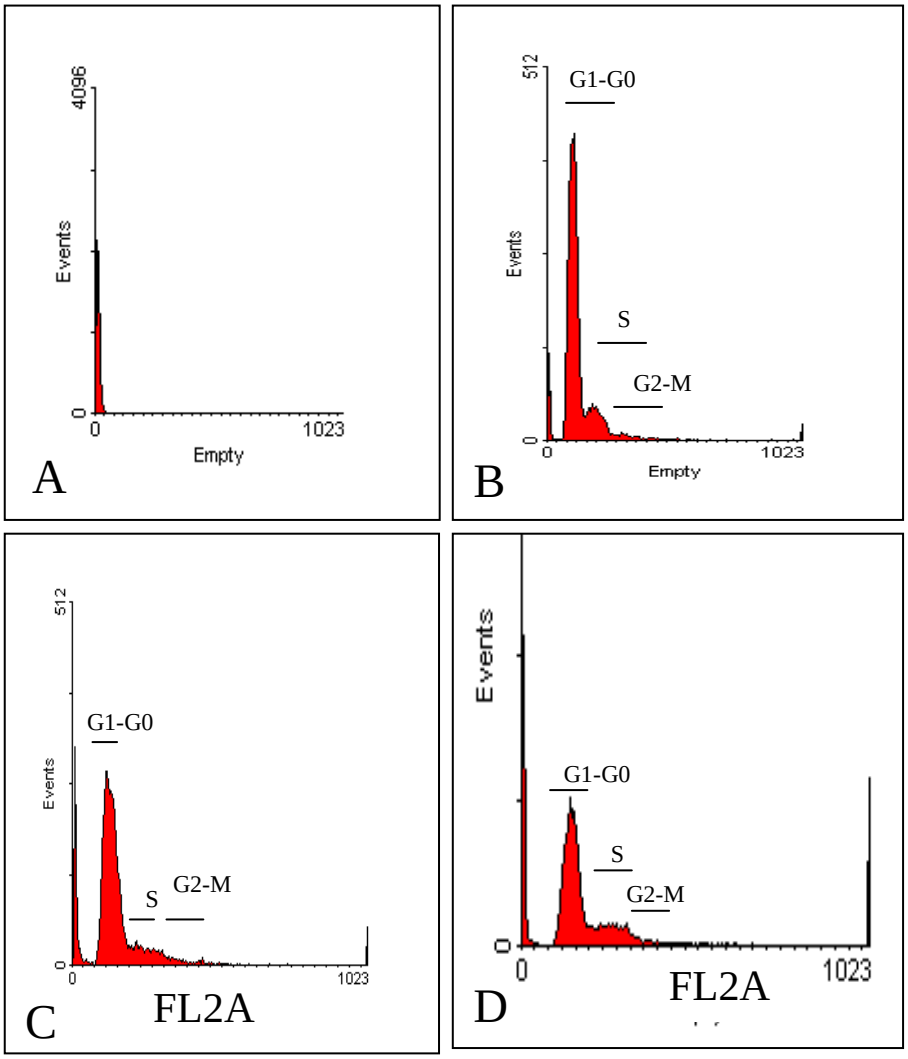

Fig.4a

A) Unstained population of cells (with out histogram)
B) DNA histogram of full confluent cell
C) DNA histogram of cyclic cells
D) DNA histogram of serum starved cells

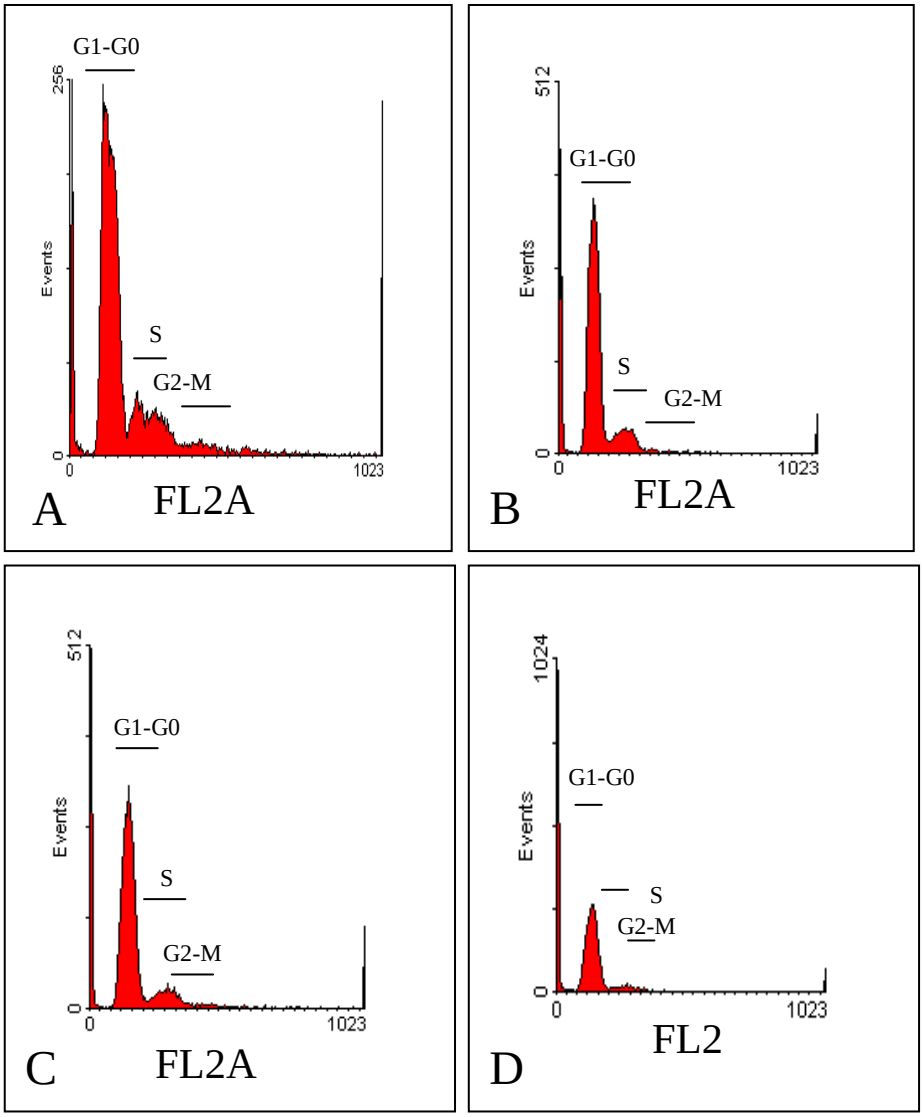

Fig.4b

A) DNA histogram of confluency with serum starvation cells
B) DNA histogram of 10μM ROS treated cells
C) DNA histogram of 20μM ROS treated cells
D) DNA histogram of 30μM ROS treated cells

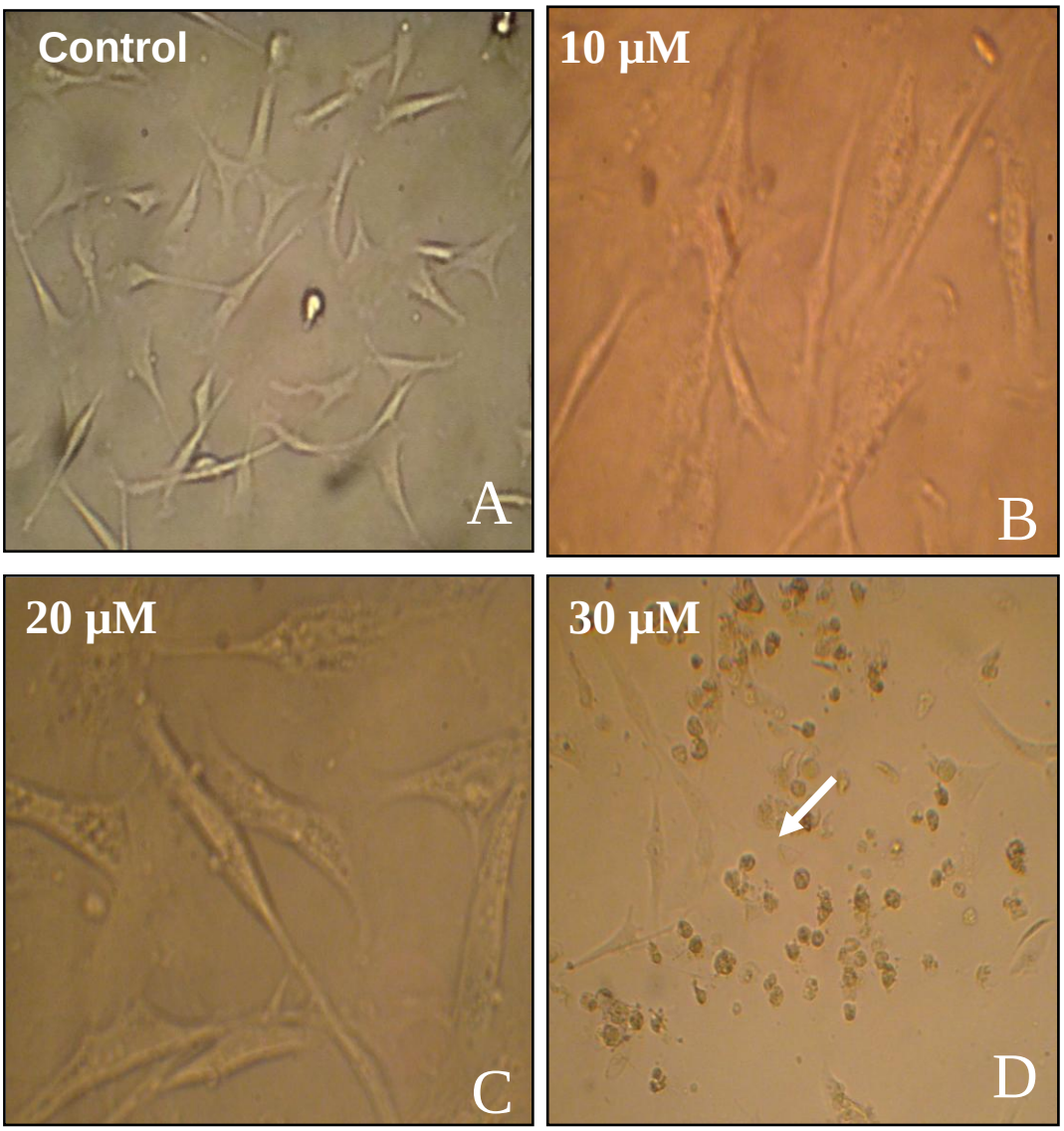

Fig.5

A) Cyclic fibroblast (60-70%) confluency
B) 10 µM treated roscovitine cells
C) 20 µM treated roscovitine cells
D) 30 µM treated roscovitine cells

Arrow indicates dead cells

embryos with holding time between 0-2 hours (16.04-22.44 %) was significantly lower than that of embryos activated at 4 and 8 hours after fusion (22.44-30.02 %) are shown in table 4.2 and Fig. 5b. The higher blastocysts percentage (52.0 %) was obtained when holding time between fusion and activation was 6 hours as compare to other holding time considered during study.

Table 4.2 Effect of reprogramming time on developmental competence of hand made cloned embryos

Reprogramming Time	Reconstructed Embryos	Cleaved	Blastocysts
0	226	61.24± 7.1[a] (128)	16.04± 6.3[a] (32)
2	92	62.78±7.2[a] (58)	18.36±1.4[a] (18)
4	208	61.66±5.0[a] (130)	22.44±3.7[b] (48)
6	142	85.37±1.0[b] (122)	52.0±3.4 [c] (74)
8	144	84.84±0.9[b] (122)	30.02±4.6[b] (44)

Figures quoted as mean percentages±s.e.m

Values in same columns with different superscripts differ significantly.

Aston K I *et al.,* 2006 study reported that, 2 hours time in hold between fusion and activation gives better result on embryonic development as compared to 1,1.5,2.5,3,3.5,4 hours of hold time, after 2 hours holding time the proportion of embryos possessing elongated or scatter chromosomes tend to increase with increasing time between fusion and activation. In our study we concluded that that 6 hours holding time between fusion and activation was best for reprogramming

of unidifferentiated genes of somatic cells into totipotent genes of embryos.

4.4 Efficacy of buffalo oocytes to support *in vitro* development of inter-species handmade cloned embryos.

The population of wild animals is decreasing day by day due to human activity such as habitat destruction, hunting competition with introduced herbivores (Holt and Pickard, 1999). Intensive selection, cross breeding and market demand also decrease the population of domesticated species. According to species survival commission survey in 2006, 1528 animal species are critically threatened, including 162 species of mammals. The major aim of animal conservation is to maintain ecological diversity. The habitat maintenance is one of best way to conserve biodiversity (Loi et al., 2001), Due to small population size, *In situ* conservation strategies are not able to maintain live population of animal in their adaptive environment. Hence *ex situ* conservation strategies have been developed for establishing viable population through cryopreservation of animal genetic resources like gametes, embryos, cells/tissue samples and DNA (Andrabi S.M.H. *et al.,* 2007). Assisted reproductive techniques (ART) including artificial insemination (AI), embryos transfer (ETT), *in vitro* embryos production (IVF), cryobiology, semen/embryos sexing, reproductive cloning, genome resource banking are valuable tools for *in situ* and *ex situ* conservation programs for endangered mammalian species. Major obstacle for applying ART is low availability of oocytes and lack of knowledge of reproductive physiology. Somatic cell nuclear transfer has been suggested as potential technique to become an integral part of wild life conservation programs, specifically interspecies cloning in which oocytes of domestic animal could combine with somatic cell from close related wild counterpart.

The objective of this study was to examine the competence of buffalo oocyte cytoplasm to reprogram adult somatic cell nuclei of different species and initiate embryonic development. Matured buffalo oocytes were enucleated between 21-24 hours after the start of maturation as recipient cytoplast.

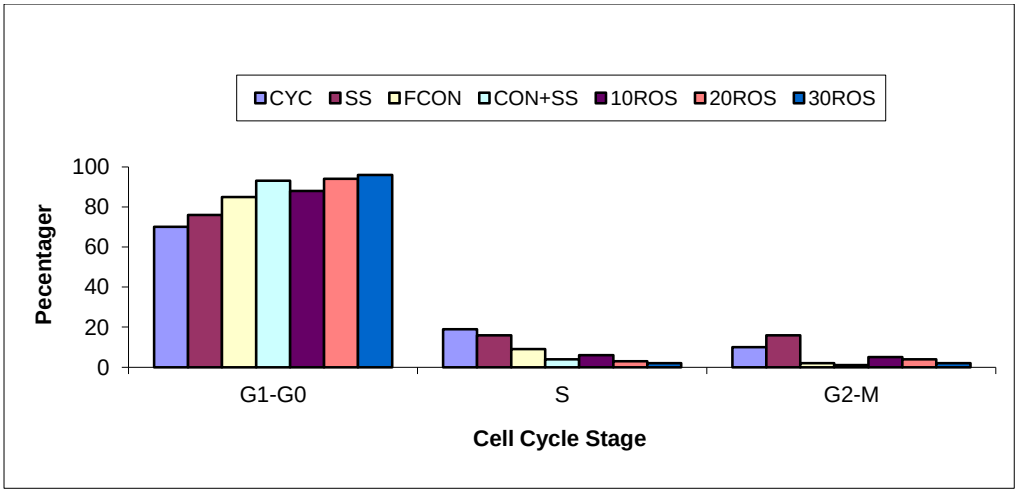

Fig.5a Cell cycle synchronization of culture buffalo fibroblast

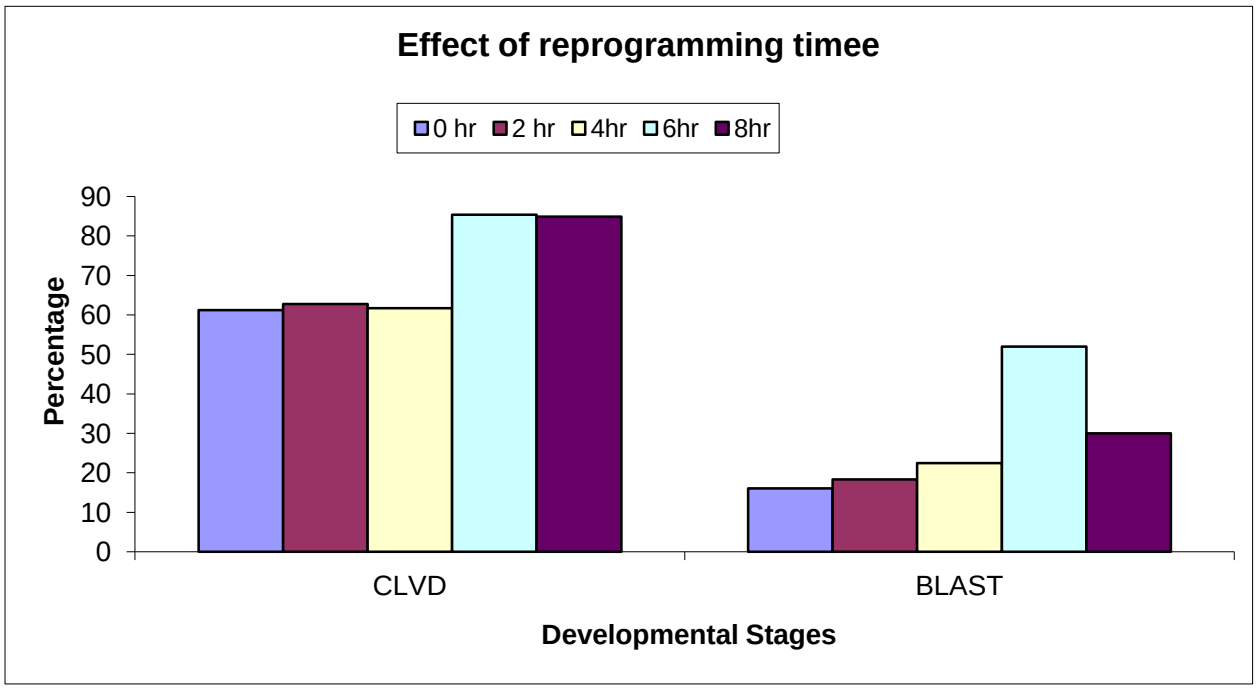

Fig.5b Effect of reprogramming time on developmental competence of intraspecies hand made cloned embryos

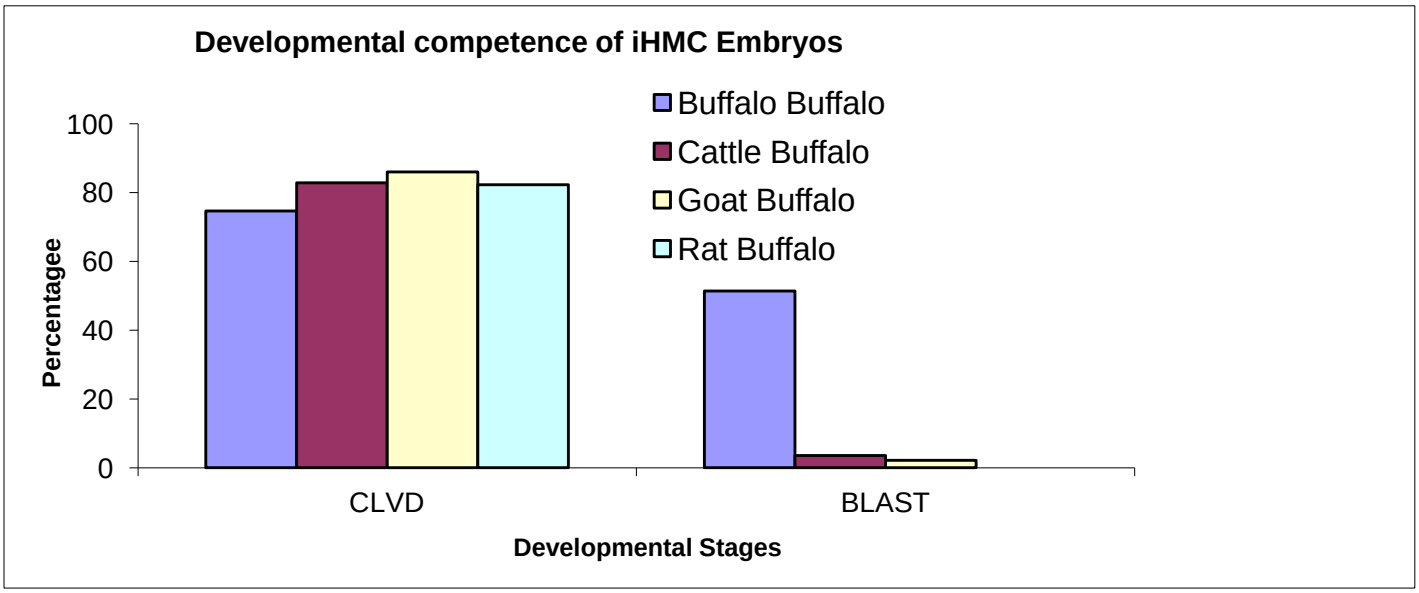

Fig.5c Developmental competence of interspecies cloned embryos

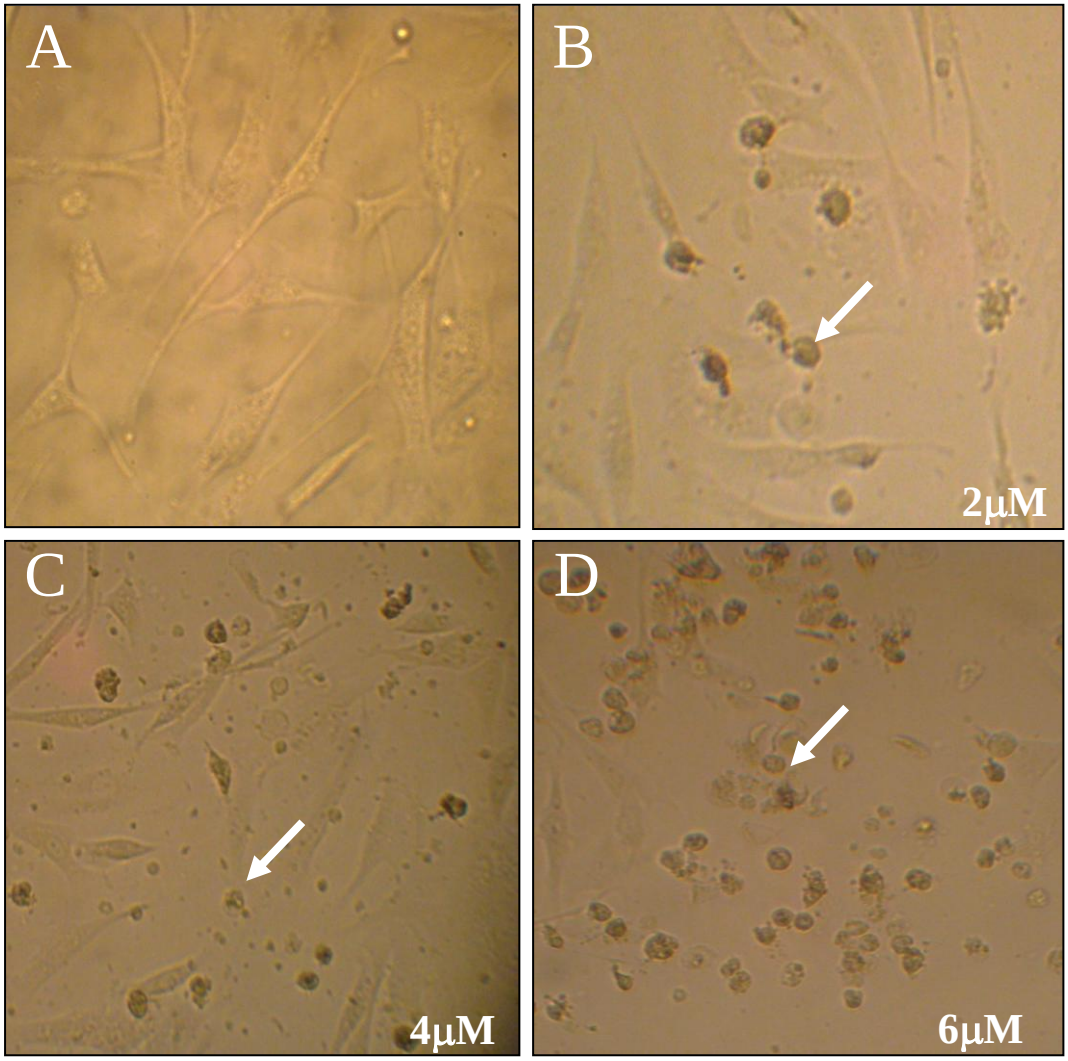

Fig.6

A) Control cyclic fibroblasts
B) 2 µM treated aphidicoline cells
C) 4 µM treated aphidicoline cells
D) 6 µM treated aphidicoline cells

Arrow indicates dead cells

TABLE 4.3 *In vitro* development of intra- and interspecies handmade cloned embryos produced in buffalo oocyte recipient cytoplast.

Donor nuclei	Recipient oocyte	Reconstructed Embryos	Cleaved	Blastocysts
Buffalo (MU) *	Buffalo	280	74.63±3.8[a] (208)	51.4±2.6 [a] (145)
Cattle (SH) *	Buffalo	256	82.79±5.3 [a] (211)	3.5±1.0[b] (9)
Goat (AB) *	Buffalo	180	85.99±4.9 [a] (154)	2.2±0.9 [b] (4)
Rat (WR) *	Buffalo	184	82.27±3.6 [a] (152)	0±0[c]

Figures quoted as mean percentages±s.e.m

Experiment carried out from 30/11/2008 to 11/04/09

Values in same columns with different superscripts differ significantly.

Data from 5 trails.

* MU, SH, AB and WR represent Murrah, Sahiwal, Alpine × Beetal breeds of animal and Wister strain of rat respectively.

Adult fibroblasts were isolated from skin samples of buffalo, cow, goat and rat used as donor nuclei. Cell cycle of the donor cells was synchronized in G0 or G1, stage by culturing cells to full confluent stage for 4-6 days prior to nuclear transfer. Mature zona free oocytes were enucleated by manual bisection of cytoplast on basis of protrusion cone and stained by Hoechst 33342 DNA fluorochrome for checking enucleation efficiency. The single somatic cell attached demicytoplast were fused with other demicytoplast. After activation with ionomycin (5 µM for 5 min) and 6-DMAP (2 mM, 6 h), all NT embryos were cultured on flat surface. NT units were examined 3 and 6 day of culture for cleavage and blastocyst stages of development. Most of the NT units cleaved 24 hours after activation. Cleavage rates were 74.63%, 82.79%, 85.99% and 82.27% for buffalo, cow, goat, and rat

NT embryos respectively. (Table 4.3) and blastocyst rates were 51.4%, 3.5%, 2.2%, and 0% for buffalo, cow, goat, and rat NT embryos (table 4.3 and Fig.7 & 5c) Dominko *et al.,* 1999 study reported that bovine oocytes had ability to reprogramme somatic cells from porcine, ovine and primate upto blastocysts stage but no pregnancy was established in any interspecies cloned embryo transfer animals, and all interspecies rat embryos were arrested at 2 cells stage. During our study, it was shown that cleavage rate in rat embryos did not significantly differ ($p<0.05$) to that of other species but none of rat embryos developed upto the blastocyst stage (Fig.7D and table 4.3)

4.5 Intra and interspecies embryo transfer in synchronized recipients

Various workers reported the variable pregnancy rates upon transfer of cloned blastocysts to recipient buffaloes. Saikhun *et al*. (2004) reported that, 50% of animals were pregnant at 30 days after transfer of cloned embryos to swamp buffaloes, which was reduced to 25% at day 60 and none of the pregnancies could continue beyond 90 days. Shi *et al*. (2007) reported transfer of 42 cloned blastocysts to 21 recipients, out of which four (19% of recipients) were found pregnant and only a single (4.7% of recipients) surviving calf has resulted. Shah *et al.,* 2009 reported the transfer of 69 hand made cloned blastocysts to 23 recipients, three were pregnant out of 23 (13.04 %). They also showed that out of the group of recipient buffaloes to which 12 newborn fibroblast reconstructed blastocysts were transferred, the pregnancy rate was found to be 25% at Day 40. The pregnancy rate was 20% in the group of recipient to which 14 fetal fibroblast reconstructed blastocysts were transferred. The transfer of embryos reconstructed using adult skin fibroblasts or cumulus cells 16 did not result in any pregnancy, and reported world first buffalo calf born through hand made cloning technique.

To evaluate the post-implantation development of intra and interspecies hand made cloned embryos, the blastocysts (at day 7 of

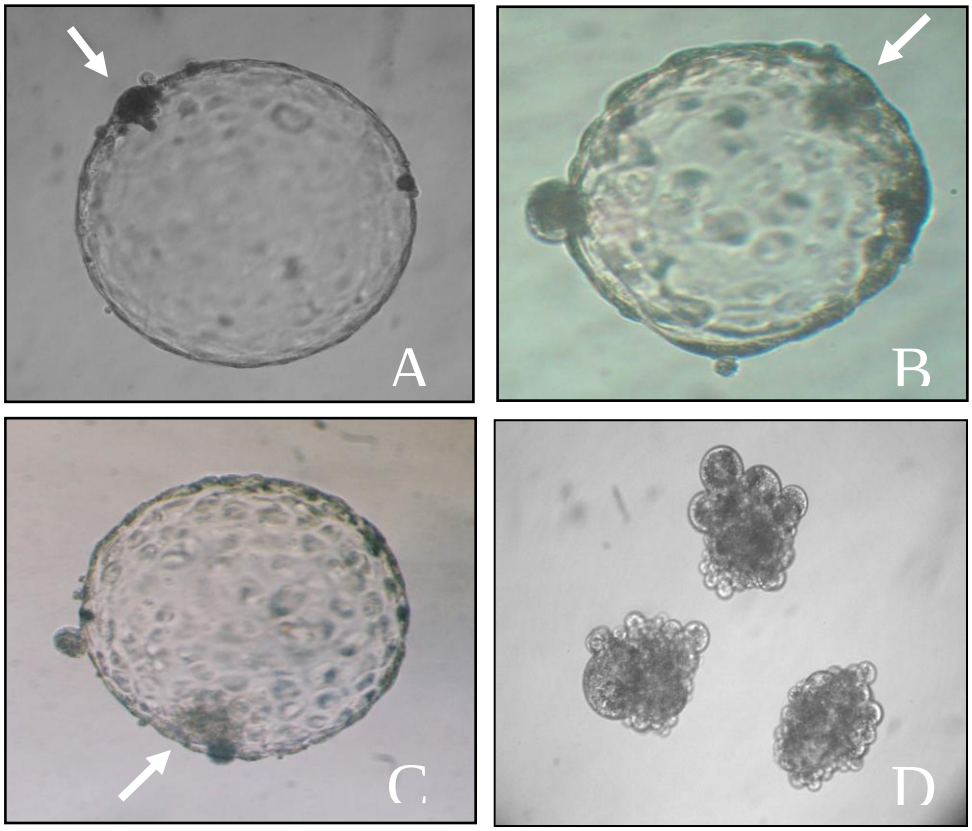

Fig.7

A) Intraspecies buffalo blastocyst
B) Interspecies goat blastocyst
C) Interspecies cattle blastocyst
D) Interspecies rat embryos arrested at 32 cell stage

Arrow indicated ICM

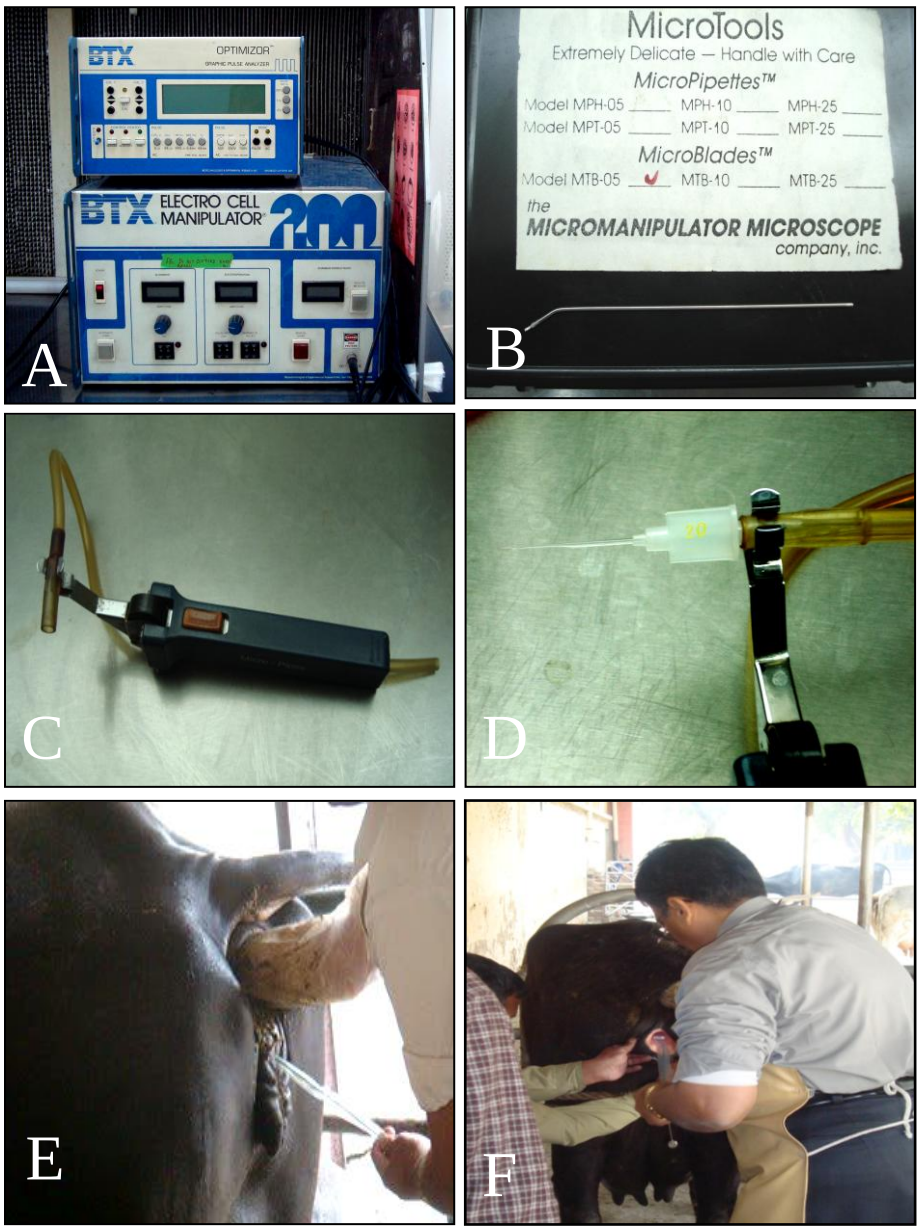

Fig.8

A) BTX electrofusion machine

B) Microblade

C) Unopette

D) Unopette with flame pulled micro capillary tip

E) Embryo transfer in buffalo

F) Embryo transfer in cattle of day 7th blastocyst.

IVC), having a good number of ICM and TE cells (Fig.7), were transferred to synchronized recipients (Figs.8 E & F) and the pregnancies diagnosed through ultrasonography. Intra and interspecies cloned embryos were transferred to 6 and 3 recipients, respectively. All six intraspecies and 3 interspecies embryo recipients were examined after 45 days of transfer and three (50.00%) intraspecies embryos recipients were found to be pregnant (Table 4.4). All the three pregnant recipients could not sustain the pregnancy beyond 80-90 days and aborted. We could not trace the fetus/ membrane etc, as the animals were kept in loose housing system. None of the interspecies embryo recipients were found to be pregnant, although the data pertaining to transfer is not enough to draw a conclusion. Further experimentation is required to comment upon the pregnancy outcomes.

Table 4.4 Intra and interspecies cloned embryos transfer to recipients and pregnancies established

Donor Cells	Recipient cytoplasts	Blastocysts transferred (n)	Recipients (n)	Examined (n)	Pregnant (n)	Pregnant %
Adult buffalo fibroblasts	**Buffalo**	22	6	6	3	50.00
Newborn cow Fibroblasts	**Buffalo**	5	3	3	0	0.0

In present study, the transfer of interspecies embryos to recipients was accomplished to evaluate the post developmental competence of the cloned blastocysts produced through interspecies cloning in buffalo using the handmade cloning technique.

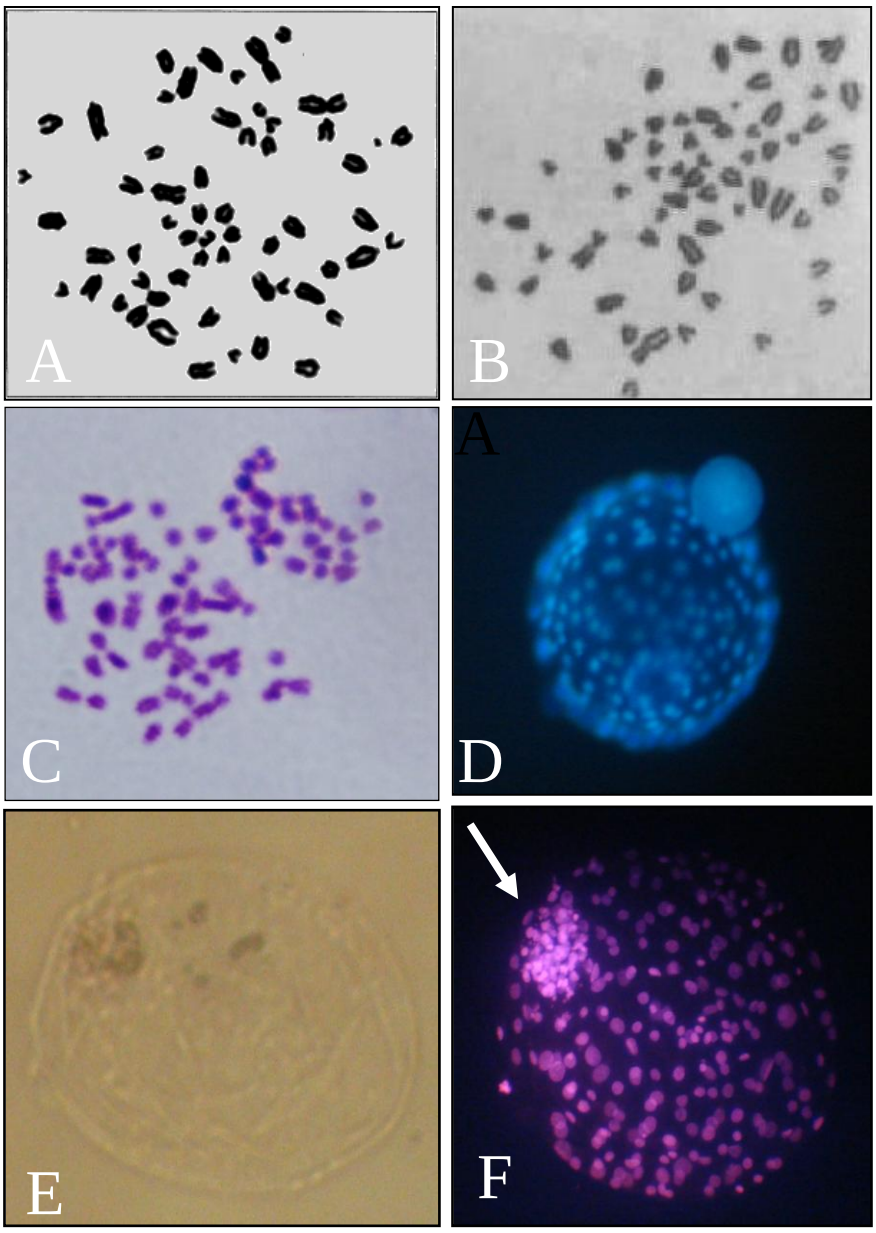

Fig.9

A) Chromosomes of cattle fibroblast at passage 5th showing diploid number (1000X); B) Chromosomes of goat fibroblast at passage 5th showing diploid number (1000X); C) Chromosomes of buffalo fibroblast at passage 5th showing diploid number (1000X); D) Blastocyst stain with H33342 for total cells count (UV) E&F) differential staining in bright and UV respectively, showing ICM (arrow) and TE cells

SUMMARY AND CONCLUSIONS

Nuclear transfer (NT) technology has potential applications in agriculture, animal husbandry and biomedicine, but the technology is hindered by low efficiency and extremely high costs involved. Nuclear transfer technology is supposed to play a tremendous role in future for faster multiplication of genetically elite animals including, high yielding female animals for milk production and elite sires for production of semen. The interesting and direct applications of NT to the human welfare include the production of transgenic animals (gene pharming) and patient-specific embryonic stem cells production without the fear of rejection by host's immune system.

India has the best and highest population of buffalo breeds adapted to low-input systems. Exploiting the animal cloning to propagate indigenous buffalo breeds could be of great concern, not only in view of its enhanced milk and meat production, but also for genome conservation (as some breeds of buffalo are facing the risk of extinction or being lost from the global gene pool). NT procedure is a powerful tool for faster multiplication of superior germ plasm, however the efficiency in terms of the numbers of live offspring born is very low (2 to 7%) because of inefficient reprogramming and higher incidences of fetal losses after transfer to the recipient animals. As the increasing demand for cloned embryos and offspring is likely to rise, the need is felt for the faster development of nuclear transfer procedures that are better in both efficiency and ease of operation. Micromanipulation has been used for nuclear transfer to produce cloned animals in most of the species but it requires, sophisticated equipment like micromanipulators, specialized equipments for making microtools and highly skilled and qualified personnel. To overcome this problem, micromanipulator- free "Handmade Cloning (HMC)" procedure can be used for nuclear transfer. Several laboratories have now adopted HMC technique to generate live animals. This procedure allows the manual bisection of zona-free oocytes, selection of individual enucleated ooplast by

Hoechst-33342 staining and UV illumination and fusion of a somatic cell and ooplast and their activation to produce a cloned embryo. This new technique is less demanding in terms of equipment, time and skill.

Modern reproductive techniques (ART) including artificial insemination (AI), embryos transfer (ETT), in vitro embryos production (IVF), cryobiology, semen/embryos sexing, reproductive cloning, genome resource banking are valuable tools for *in situ* and *ex situ* conservation programs for endangered mammalian species. Major obstacle for applying ART is lack of availability of oocytes and lack of knowledge of reproductive physiology. Somatic cell nuclear transfer has been suggested as potential technique to become an integral part of wild life conservation programs, specifically interspecies cloning in which oocytes of domesticated animals could combine with somatic cells from closely related wild counterpart. Keeping this in view, the present study was carried out with the following objectives:

1. To study the efficiency of different methods for synchronization of cell cycle in G1/G0 phase of cultured buffalo fibroblast.

2. To study the effect of reprogramming time on developmental competence of intra-species Handmade cloned buffalo embryos.

3. To study the efficacy of buffalo oocyte to support *in vitro* development of inter-species Handmade cloned embryos.

The methodology adopted for HMC in buffalo by shah. *et al.,* 2008 was used in this study after incorporating necessary modifications. Donor somatic cells were derived from the skin tissues of adult Murrah buffaloes, newborn Sahiwal cattle, adult goat (AB breed) and Wister rat available at the cattle yard, NDRI. The cells were maintained and passaged as well as cryopreserved for future replacement. Oocytes derived from slaughtered buffalo ovaries were matured *in vitro* for 21 hours (h). Matured oocytes were made free from surrounding cumulus and zona pellucida by enzymatic treatment. Zona-free oocytes were bisected manually under zoom

stereomicroscope using a microblade, and enucleated (without surface protrusion cone) half-ooplasts selected. Such enucleated demi-ooplasts were attached to a single donor cell (derived by trypsinization of over confluent monolayer fibroblast culture) and fused with demi-ooplasts having no somatic cells attached through electrofusion. Fused and rounded reconstructs were chemically activated (using calcium ionophore and 6-DMAP) and cultured in flat surface culture system. Cleavage and blastocyst rates were observed after culturing, undisturbed for 7 days in humidified CO_2 incubator at 38.5^{o}C. Healthy and expanded cloned blastocysts were transferred, non-surgically, into estrus synchronized mature recipient buffalo and cattle to establish pregnancies. Pregnant animals were diagnosed after 45 days of transfer through ultrasonography equipment.

Data pertaining to developmental rates (cleavage and total blastocyst percentages) was analyzed using SYSTAT 6.0 (SPSS Inc. Chicago, IL, USA) after arcsine transformation of percentage values. The differences between means were analyzed by one-way ANOVA followed by Fisher's LSD test. Significance was determined at $P<0.05$.

From cell cycle synchronizing study, we concluded that full confluency, combination of full confluency and serum starvation, 10μM Roscovitine (ROS), 20μM ROS, 30μM ROS improved cell cycle synchrony in G0-G1 phase as compared to that of cyclic fibroblasts and serum starved cells for 24 hours. At concentration of 10μM roscovitine synchronized cells in G0/G1 phase were as similar as full confluent, 20μM, 30μM ROS, combination of serum starvation with confluency were synchronized most cell (above 90%) in G1/G0 phase, but most cells in 30μM roscovitine group underwent apoptosis and cell death. Removal of roscovitine, followed by culturing washed cells in medium containing 10% FBS for 24 hours restored progression in the cell cycle comparable with cycling cells which indicated reversible affect of roscovitine on cells. During experiment all treatment groups of aphidicoline cells died after 24 hours of incubation, hence these groups were not processed for further analysis.

The reprogramming time had impact on embryonic development in consistent with our results where cleavage of embryos for holding time between 6-8 hours (84.4-85.37 %) was significantly higher ($P<0.05$) than that of embryos holding time between 0-4 hours (61.24-62.78 %). The higher blastocysts percentage (52.0 %) was obtained when holding time between fusion and activation was 6 hours. Thus we concluded that, 6 hours holding time was optimum reprogramming time for *Invitro* development of hand made cloned embryos of buffalo. There was no significant different between cleavage rate of all intra and interspecies a cloned embryo indicating that early embryonic development does not depend upon source of donor nuclei. Blastocyst rates were 51.4%, 3.5%, 2.2%, and 0% for buffalo, cow, goat, and rat NT embryos, revealing that buffalo oocyte has ability to reprogramme somatic cells of cow and goat upto blastocyst stage.

Intra species cloned blastocysts (day 7) derived from adult fibroblasts were transferred to 6 recipients, out of them, three were found to be pregnant after when examined at 45 days of transfer. All the three recipients could not sustain pregnancy beyond 90 days of gestation. Interspecies cloned blastocysts derived from newborn cells of sahiwal were transferred to 3 recipients, but none of them was found to be pregnant. Pregnancy rate of 50% in case of intraspecies buffalo cloning, the transfer of embryos to recipients was accomplished to evaluate the developmental competence of the cloned blastocysts produced from adult fibroblast and 6 hours of optimum reprogramming time in buffalo using the handmade cloning technique.

Various conclusions, drawn from the results of the present study are;

- 96 % cells in 30 μM ROS treatment were synchronized in G0-G1 phase, but cells viability was very low.
- Cell confluency and serum starvation with confluency is as efficient as chemical synchronizer, roscovitine for inducing arrest in the G0/G1 phase of the cell cycle in fibroblast cells of buffalo.

- Buffalo oocytes were able to reprogramme somatic cells from cow and goat upto blastocyst stage, where as most iHMC embryos arrested at 16-32 cells stage.
- All iHMC embryos of Rat could not developed beyond 32 cells stage.
- Cleavage rate did not depend upon source of donor nuclei.
- 6 hours was optimum reprogramming time for *in vitro* development of handmade cloned embryos in buffalo.
- Pregnancy was established through transfer of intra species cloned buffalo blastocysts produced, using adult fibroblast cells to synchronized recipients.

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ANNEXURE

ANNEXURE

Solutions for oocyte collection

Normal saline containing antibiotics

Composition	-	Volume (1000ml)
Sodium chloride	-	9.0 gm
Penicillin G	-	0.06 gm
Distilled water	-	1000 ml

Aspiration medium (for about 250-300 ovaries)

Composition	-	Volume (100 ml)
TCM-199 (Hepes modified)	-	100 ml
BSA	-	0.3 gm
Gentamycin	-	50μg/ml
L-Glutamine	-	0.68 mM

In vitro maturation media

Washing medium

Composition	-	Volume (40 ml)
TCM-199 (Hepes modified)	-	36 ml
FBS	-	4 ml
Sodium pyruvate	-	0.0036 gm
Gentamycin	-	50μg/ml
L-Glutamine	-	0.68 mM

Maturation medium

Composition	-	Volume (10 ml)
Washing medium	-	10 ml
Porcine FSH	-	5 μg/ml
Estradiol 17 β	-	1μg/ml
Follicular Fluid	-	500μl

Cell Culture Media

Culture Media

Composition	-	Volume (10 ml)
DMEM	-	9 mL
Gentamycin	-	50 μg/ml
L-Glutamine	-	2.0 mM
Fetal Bovine Serum	-	1 mL

Handmade Cloning Media

T0

Composition	**-**	**Volume (10 ml)**
HEPES (H-7006),	-	2.5 mM
NaHCO3 (S-4019),	-	5 mM
Sodium pyruvate (P-3662)	-	0.2mM
Gentamycine sulfate (G-1264)	-	50 µg/mL

Adjusted to pH 7.4, 280 mOsm.

T2 &T20

T0 supplemented with 2 and 20% FBS, respectively.

Hyaluronidase - 0.5 mg/mL solution in T2.

(Store in 500µL aliquots in 2 mL Eppendorf tubes)

Protease - 2 mg/mL solution in T10

(Sore in 500 µL. Centrifuge before use)

Cytochalasin B - 5 mg/mL stock dissolved in DMSO

(Store in 2µL aliquots)

Fusion medium

Composition	**-**	**Volume (10 ml)**
D-mannitol (M-9546)	-	0.3 M
$MgSO_4$ (M-2393)	-	0.1 mM
$CaCl_2$ (C-7902)	-	0.05 mM
Polyvinylalcohol (P-8136)	-	1 mg/mL

(**Store in 5mL aliquots)**

Phytohemagglutinin - 5 mg/mL stock solution in T0

(Store in 50µL aliquots)

Calcium ionophore II - 1 mg/mL stock in DMSO (D-2650).

(**Keep in a small dark bottle with tightly closed cap)**

Hoechst 33342 - Prepare 1mg/mL stock in distilled water.

(Store in 20µL aliquots)

Propidium Iodide - 100 µg/mL

Embryo culture Media

Research Vitro Cleave

RVCL	:	4 ml
BSA (FAF)@1%	:	40 mg

Embryo Transfer Media

Blastocyst holding medium

Composition	**-**	**Volume (10 ml)**
DPBS	-	10 ml
BSA (FAF)	-	40 mg

During the preparation of above all media, all the components were mixed well and incubated in a CO_2 incubator at 38.5°C, 5% CO_2 for 2 h for stabilization of pH and temperature before further use. The media were filtered through 0.22 µm filter just before use. The pH and the osmolarity of the media were checked, each time a fresh lot of the medium was prepared.