1. INTRODUCTION

After the creation of Dolly in 1996, by Ian Wilmut, the cloning research entered into a ‘Renaissance Era’ with exponentially growing input which paved way for creation of several cloned animals of different mammalian species. The work and achievements in cloning research after Dolly is much greater than those obtained during the previous fifty years. The importance of somatic cell nuclear transfer (SCNT) is, without any doubt, beyond replicating superior animal genotypes. It has now developed into an invaluable experimental tool to address fundamental scientific issues such as nuclear potency, cell de-differentiation, chromatin structure and function, epigenetics, genome manipulation along with its most important applications in biomedical research.

Considering the limitations placed on working with mammalian oocytes, the current success in somatic cell nuclear transfer in mammals is truly amazing. Offspring have been obtained from an ever-growing list of species including cattle, buffalo, sheep, goat, domestic cat, gaur, mouse, pig etc. (Keefer, 2008). Cloning research has helped in conservation of species and presently efforts are ongoing for bringing back the extinct Tasmanian devil and the Wooly mammoth. Very little is known about specific factors or mechanisms involved with the ooplasm reprogramming a somatic nucleus. This incredible complexity and lack of understanding has been the basic reason for low success rate initially and continues to be reflected in the overall inefficiency of the cloning procedures. Dolly was just one cloned offspring that resulted after 277 attempts (0.3% efficiency) (Edwards et al., 2003).

Since the birth of Dolly, several mammalian species have been cloned from different somatic cell types, among which the fibroblasts are the most commonly used ones. A clear consensus has not been reached yet on superiority of somatic cell type for nuclear transfer, although it has been reported that cumulus cells has the highest cloning efficiency (Tian et al., 2003).
In spite of its enormous scientific significance, it is still in state of low success level and there seems to be substantial problems with the health aspects of the cloned animals. Till date no normal cloned animals has been reported which is basically due to the incomplete reprogramming of the imprinted genes of somatic cells used for nuclear transfer (Shi et al., 2003; Kang et al., 2003). Many laboratories in the world are greatly involved in erasing these epigenetic signals in order to produce a normal healthy cloned animal.

An insight into mammalian oogenesis shows that the developing oocytes accumulate proteins, mRNA, and molecular precursors in their cytoplasm. These resources contain messages needed for fertilization, early embryonic development and transcription factors which cause embryonic genome activation (Barnes and Eyestone, 1990). Thus any loss in ooplasm should result in the loss of these precursors and subsequent embryonic development.

Most of the studies on nuclear transfer have relied on mechanical enucleation by aspiration or squashing/compression of the matured oocytes. Depending on the efficiency of the micromanipulator, a loss of ooplasm between 5 to 50 per cent along with the first polar body and MII chromosomes has been reported (Westhusin et al., 1992, 1996). This loss of ooplasm adversely affects the quality of embryos and their developmental potency. Vast majority of data available in the literature show that more the amount of ooplasm lost during enucleation, fewer the cells would be obtained from embryos at morula or blastocyst stage, resulting into development of poor quality of embryos (Peura et al., 1998).

Hua et al., (2011) studied the development of SCNT embryos by removing different amounts of ooplasm and the effect of nucleus to cytoplasmic ratio (N/C) on quality on embryos and reported that the loss of more than 30 per cent ooplasm had an adverse effect on the chromosome constitution, morphological features and developmental potential of SCNT embryos. Hence, it would be interesting to assess the quality of embryo and its development potential by replenishment of about 10-20 per cent of ooplasm from exogenous in vitro matured enucleated oocytes.
Apoptosis study using molecular markers has been suggested as an additional criterion for morphological evaluation of embryos to assess embryo quality and effectively predict embryo viability (Pomar et al., 2005). There are many pro-apoptotic marker genes like Caspase-3, Bax, Bcl-2, Fas, P53 and anti-apoptotic marker genes like Mcl-1, Bcl-w, whose expression are considered as an important criteria to predict the subsequent embryo development (Melka et al., 2010).

The hypothesis behind this study was to test whether an addition of 10-20 per cent ooplasm from enucleated exogenous oocytes could improve the quality of embryos in terms of number of cells observed in morula. Replenishment was done in those oocytes having lost more than 30 per cent ooplasm during enucleation process (Lower the amount of ooplasm lost, lower the perivitelline space which created difficulties in replenishment of ooplasm) Hence oocytes which has lost more than 30 per cent of ooplasm during enucleation were selected for replenishment.

Keeping the above in view, the present experiment was undertaken to test the effect of replenishment of ooplasm in enucleated oocytes having lost more than 30 per cent of ooplasm during enucleation process. The embryos thus produced will be tested for its developmental qualities which will be assessed using morphological parameters and apoptotic molecular markers. Thus, this curricular project has been designed with following objectives:

1. To standardize the technique of production of SCNT embryos with replenished ooplasm and its comparison with normal SCNT embryos.
2. Comparison of conventional and replenished SCNT embryos by Hoeschst fluorescence stain.
3. To study the apoptosis gene expression in SCNT embryos using molecular markers.
2. REVIEW OF LITERATURE

2.1 Collection and Transportation of ovaries and oocyte recovery

The ovaries obtained from slaughter house can be brought to the laboratory in a saline solution or Dulbecco’s phosphate buffer solution at 30-35°C, without any detrimental effect on oocyte maturation (Pugh et al., 1991). Oocytes are recovered within 1-2 hours of slaughter of animal and ovaries stored at 30°C (First and Parrish, 1987). The temperature changes and time intervals mandated by handling and transport of ovaries from the slaughterhouse to the laboratory adversely affect the rate of oocyte recovery and their quality (Guignot et al., 1999).

Various techniques have been used for oocyte recovery in animals like aspiration of follicles, dissecting the intact follicles and slicing and puncturing of follicles (Das et al., 1996). Chauhan and Anand (1991) observed that goat oocytes isolated from 3-5 mm diameter follicles with compact cumulus mass, matured and in vitro fertilized better than other groups. Shukla (2003) reported average number of follicles in left and right ovary to be 9.06 and 9.11 respectively, while average recovery of oocytes from each ovary was 2.28.

Wang et al. (2011) concluded that a storage temperature of 15°C for a 3–4 hour period had a significant beneficial effect on the quality and developmental competence of oocytes used for somatic cell nuclear transfer (SCNT) due to the alleviation of stress on the oocytes compared with those subjected to storage temperatures of 25°C or 35°C.

2.2 Selection of oocytes for IVM and their in vitro maturation

Selection of bovine oocytes for in vitro maturation (IVM) and subsequent in vitro fertilization (IVF) as the basis of visual assessment of morphological feature was first examined by Leibfried and First (1979). Wani et al. (1999, 2000) classified oocytes as good, fair and poor on the basis of the cumulus cell layer and cytoplasmic characteristics in the laboratory, prior to maturation. Chauhan and Anand (1991) observed that goat oocytes
isolated from 3-5 mm diameter follicles with compact cumulus mass, matured and \textit{in vitro} fertilized better than other groups.

According to the Chauhan \textit{et al.} (1998), Cumulus Oocyte Complexes (COCs) are classified as Grade-I (compact COCs with unexpanded cumulus mass having $\geq 5$ layers of cumulus cells and a homogeneous cytoplasm), Grade-II (COCs with $\leq 4$ layers of cumulus cells and with a homogeneous cytoplasm), or Grade-III (oocytes without cumulus cell and uneven cytoplasm). This criterion has been reported to be reliable due to rate of maturation and cleavage, as well as the proportion of cleaved embryos that develop to morula and blastocyst stage, decrease linearly from Grade-I to Grade-III.

Quality of oocyte also depends upon size of follicles from which they are aspirated. Shukla \textit{et al.} (2003) recorded 90 per cent of Grade-I and 83 per cent of Grade-II oocytes from medium sized follicles whose diameter ranged from 3 to 5 mm and 5 to 7 mm respectively. Blondin and Sirard (1995) reported that very few or no embryos crossed the 8 cell stage using oocytes from the follicles with diameter of 2 mm or less, thus were developmentally less competent.

Maturation of oocyte involves both nuclear as well as cytoplasmic activation besides the associated change in the organization of plasma membrane. Cumulus-oocyte interaction is a critical requirement for normal follicular growth, acquisition of the oocyte developmental competence and cytoplasmic maturation to support male pronucleus formation (Chian \textit{et al.}, 1994). Singh (2004) reported that \textit{in vitro} maturation rates in Grade I oocytes varied from 40 to 75 percent in 8 experimental trials. The Grade II oocyte showed poor IVM rates and the overall average IVM rates in both A and B grade oocyte was 26.16 per cent.

Various medias like TCM-199 (Moore and Trounson, 1977; Suzuki \textit{et al.}, 1991; Madan \textit{et al.}, 1994), Ham’s F-12 (Fukushima and Fukui, 1985), Ham’s F-10 (Totey \textit{et al.}, 1993), minimum essential medium (MEM) and Weymouth medium (Ravindranath \textit{et al.}, 2001) have been widely used for
in vitro maturation of oocytes. Madan et al. (1994) have observed 79.89 per cent of maturation using TCM-199 supplemented with bovine estrous serum.

Hormone supplementations play a significant role in in vitro maturation (IVM) of oocytes. Gonadotropin (Lenz et al., 1983), follicle-stimulating hormone (Shukla et al., 2003), luteinizing hormone (LH) and estradiol 17-β (Totey et al., 1993) has been used successfully. Chauhan et al. (1997) reported that addition of FSH along with FBS in maturation medium increased the maturation up to 78 per cent. It has also been reported that supplementation of EGF in maturation medium increased the maturation rate up to 83 - 85 per cent (Gupta et al., 2002). Nema (2005) reported that the addition of epidermal growth factor increased the cumulus expansion rate of goat oocyte than control maturation media, however, the formation of polar bodies did not differ between the control and EGF group. Totey et al. (1993) reported that gonadotropin (FSH and LH) and oestradiol (E2) supplementation has resulted in significant improvement in the maturation rate of buffalo oocytes in vitro.

Keskintepe et al. (1994) studied the effects of medium supplementation with oestrous goat serum and glycoprotein hormones on caprine oocyte maturation in vitro (IVM) and reported that the oocyte maturation took place during 27 h in TCM-199 supplemented with 20 per cent estrous goat serum, oestradiol-17-β (1.0 µg /ml), and different combinations of FSH, LH, hCG and TSH.

You et al. (2012) reported that L-carnitine treatment did not improve the nuclear maturation of oocytes but significantly increased intracellular glutathione (GSH) levels, which led to a reduction of reactive oxygen species levels in IVM oocytes. Oocytes treated with L-carnitine showed higher rates of blastocyst formation after parthenogenetic activation and SCNT compared with untreated oocytes.

Catalá et al. (2012) observed that oocytes with higher content of Adenosine triphosphate (ATP) developed into good quality IVF blastocysts and the ATP content did not significantly affect the production of ICSI blastocysts.
Picco et al. (2012) observed that adding copper during oocyte maturation significantly increased both intracellular GSH content and DNA integrity of cumulus cells and conclusively inferred that for optimal embryo development to the blastocyst stage, oocytes were partially dependent on the presence of adequate copper concentrations during IVM.

2.3 Micromanipulation tools

During SCNT, for enucleation of matured oocytes and nuclear transfer, special instruments such as micromanipulators are required. The handling of these micromanipulators as well as the preparation of micropipettes used during micromanipulation are difficult tasks and require a specially trained and highly skilled workforce along with considerable investment (Gabor and Mickey, 2006). Some of the micro tools, which are used for oocyte/embryo manipulation, are as follows.

2.3.1 Holding pipette:

Holding pipette is used for holding oocyte and embryos during micromanipulation. An optimal holding pipette is found to be one whose lumen at the tip is about 1/3rd - 1/5th the diameter of the zona pellucida and the outer diameter is the same as the diameter of the zona pellucida (Wolfe and Kraemer, 1992). For enucleation, the denuded oocytes with the first polar body are held by a holding pipette having outer diameter approximately 110–120 µm (Daniel, 2006).

2.3.2 Enucleation needle:

Enucleation needle is used to make a rent in the zona pellucida prior to matured removal of first polar body. Therefore it should have a sharp tip and strong shank. Preferably the shank should be about 0.5 cm in length while the tip should be within 0.5 mm (Singla, 1995). If the shank is too narrow, it will bend with pressure on the holding pipette and will not cut the zona pellucida easily. It is made prepared using micropipette puller and then bent at the shank prior to micromanipulation (Wolfe and Kraemer, 1992).
2.3.3 Transfer pipette:

Transfer pipette is used for transferring somatic cell in to the enucleated oocytes. An ideal pipette should have a lumen, which is small enough to permit the aspiration of only one cell at a time, but also sufficient so as not to disturb the shape of the cells aspirated. It is made by pulling the capillary tubing with a pipette puller and cutting the tip with a microforge to get a final diameter of 15-20 µm (Daniel, 2006).

2.4 Denudation of matured oocytes

Cumulus oocyte complexes with expanded cumulus were treated with hyaluronidase (0.5 mg/ml) in HEPES modified M-199 (Shah et al., 2008) to remove the cumulus.

2.5 Preparation of Ooplasm

2.5.1 Dissolution of zona-pellucidae

The enzyme pronase dissolves the zona-pellucida, the outer layer of oocytes. Kragh et al. (2005) used 8 mg/ml pronase in HEPES-buffered TCM-199 supplemented with 20 per cent cattle serum for 10 seconds for zona pellucidae removal of porcine oocytes. Shah et al. (2008) incubated the buffalo oocytes with pronase (2.0 mg/ml in TCM-199 containing 10% FBS) for 8 minutes at 38.5ºC to digest the zona pellucidae.

2.5.2 Bisection/ Squashing and compression of oocytes for ooplasm

Bisection is done to remove the half part of oocyte containing polar body. Du et al. (2002) bisected the zona free oocytes with a microblade (viz., AB Technology, Pullman, WA, USA) into two parts and smaller part containing polar body is discarded. Bisecting an oocyte for handmade cloning is a crucial step because cytoplasmic volume is a limiting factor in the development of nuclear transfer embryos (Kumar, 2009; Bhat, 2010).

The developmental potential of embryo can be increased by increasing the amount of cytoplasm in the reconstituted embryo (Peura et al., 1998). Both Griesing et al. (1994) and Zakhartchenko et al. (1997) reported a decreased blastocyst development rate in nuclear transfer embryos when the cytoplasmic volume of the recipient oocytes was significantly reduced.
The maternal genetic factors and the complex nuclear-cytoplasmic interaction would affect the blastomere fragmentation, blastocyst formation embryonic gene expression and adult phenotype (Reik et al., 2001) which signifies the presence of vital factors in ooplasm.

Hua et al. (2011) reported that the rates of cleavage and blastocyst formation were significantly higher in embryos with low nucleus to cytoplasmic volume ratio (N/C). The number of blastomeres were significantly higher in low N/C embryos compared to medium and high N/C embryos. Also the number of copies of mtDNA was significantly higher in low N/C and IVF embryos when compared to medium and high N/C SCNT embryos stating the importance of ooplasmic part in embryo development.

2.6 Enucleation of oocytes

Enucleation of the recipient oocyte and transfer of somatic cells are the core steps in SCNT. Efficiency of enucleation procedure prior to nuclear transfer is of crucial importance to avoid aneuploidy, abnormalities with its detrimental effects on later development, to eliminate any genetic contribution of the recipient cytoplasm, for excluding the possibility of parthenogenetic activation and embryo development without the participation of the newly introduced nucleus (Dominko et al., 2000).

2.6.1 Optimum time for enucleation

At MII stage, the chromosomes are condensed in the form of the metaphasic plate (mitotic spindle) and in most of the cases spindle is located at the edge of the oocyte, in close proximity to the extruded polar body. When approximately 35 per cent of the cytoplasm around the polar body was removed blindly, a 65 per cent enucleation rate was obtained. In most of livestock species, the metaphase chromosomes are not visible by light microscopy due to presence of cytoplasm lipid. Therefore, the intercalating dye, Hoechst–33342 (bis–benzimide) is used to confirm that the chromosomes were removed from the oocyte (Westgusen et al., 1992). The presence of DNA binding fluorescent dye used to locate chromosome prior to enucleation is not recommended because excitable fluorochromes and ultraviolet (UV) light may damage the maternal cytoplasm and perhaps
reducing the developmental competence also (Bradshaw et al., 1995; Li et al., 2004).

For cloning by somatic cell nuclear transfer (SCNT) in goat, there are conflicting reports about the proper time of enucleation after IVM of oocytes, which varied from 20 to 27 hours (Das et al., 2003; Keefer et al., 2002; Daniel et al., 2008).

Ajithkumar et al. (2008) studied that with increase in maturation time an increase in perivitelline space occurred which caused displacement of the MII chromosomes reducing the success of proper enucleation and standardized that the optimum enucleation time for conventional SCNT is G1(20-23 hrs) and G2(23-26 hrs) in goat.

Jeon et al. (2011) reported that the number of oocytes showing the metaphase II (MII) located just underneath the first polar body was significantly higher in the pre-denuded oocytes at 12 hours of maturation and cultured for 6 hours in maturation media than those matured for 18 hours.

2.7 Development of skin fibroblast cell lines

The fibroblast cells are most common source for donor cells. These cells are easily harvested from either sex and cultured using standard tissue culture conditions. The plastic dishes/tissue culture flask has been employed by scratching through the explants to attach the tissue to the flask (Elliget and Lechner, 1992). Plasma clots have also been used to enhance attachment by placing a drop of plasma on plastic surface and embedding the explant in it. Alternatively, anchoring materials such as purified fibrinogen and thrombin have also been used as attachment matrix on the surface of culture vessels (Nicosia and Ottinetti, 1990).

In most of the current studies, the sterilized plastic tissue culture (TC) dishes or TC flasks with a coating of fibrinogen were employed for both primary culture as well as sub-culturing (Kubota et al., 2000). For developing skin fibroblast cell lines, ear tissue samples collected by biopsy have been utilized by different workers (Boquest et al., 1999; Kubota et al., 2000; Dinnye’s et al., 2001).
For skin fibroblast primary cultures, Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with antibiotics, L-glutamine and 15 per cent fetal bovine serum has been used by many workers (Boquest et al., 1999; Kubota et al., 2000). Gupta et al. (2002) reported that DMEM + Ham’s F-12 nutrient mixture supplemented with 15 per cent fetal bovine serum for transportation of skin samples and for development of primary cultures, resulted in migration of fibroblast cells from tissue explants within 4-5 days of seeding in TC flasks.

The dermal fibroblast from ear tissue of adult animals has been used for the production of cloned embryos in species, namely, porcine (Clark et al., 2003); rabbits, goats (Baguisi et al., 1999; Yu et al., 2003); Gaur (Lanza et al., 2001), cats (Shin et al., 2002) and bovine (Kashinathan et al., 2000; Knott et al., 2000; Tian et al., 2000; Pace et al., 2001). Six cloned calves were produced by using ear skin fibroblasts from a 17 year old bull by Kubota et al. (2000).

2.7.1 Sub-culturing and multiple passaging

According to Hayflick (1961), once the primary culture is sub-cultured (passaged or transferred) it becomes a cell line and the first subculture is referred as secondary culture. Various normal finite cell lines have been obtained from passaging human lung fibroblast (Nichols et al., 1977).

Sub-culturing or passaging is done by usual trypsinization at 70-80 per cent confluence and re-seeding in fresh culture media. According to Waymouth (1974) crude trypsin has been reported to be the most common enzyme used in tissue desegregation, as it is tolerated quite well by most of the cells. However, Cole and Paul (1966) reported that it is important to minimize the exposure of cells to active trypsin in order to preserve maximum viability. Desegregation in trypsin can be damaging to some epithelial cells but the fibrous connective tissue cultures are not affected. Other enzymes like collagenase have also been used for different fibrous tissues (Freshney, 1972). Bacterial proteases such as pronase (Glavin et al., 1996) and dispase (Compton et al., 1998) have also been used with varying degree of success.
Kubota et al. (2000) reported that primary cultures of skin fibroblasts are generally contaminated with epithelial cells and these may be removed by normal trypsinization and re-seeding by 5th passage. He also reported that the cloning competence of donor skin fibroblast cells improves with every passing and cells from 7-10 passages were found better for producing viable clones.

Melican et al. (2005) suggested that low-serum culture of transfected goat fetal cells and harvest by partial trypsinization may be more efficient for generating transgenic goats by somatic cell nuclear transfer.

2.7.2 Cell cycle stage and reprogramming of donor cells

Presently the low efficiency of SCNT is mainly attributed to faulty or incomplete epigenetic reprogramming of the donor genome (Rideout et al., 2001; Xue et al., 2002). Reprogramming has to erase epigenetic constraints imposed on the somatic donor genome during differentiation and convert the cell back in to an embryonic state. Another important aspect of the epigenetic status of the nuclear donor is its stage in the cell cycle. Since the landmark creation of Dolly by serum starvation method (Wilmut et al., 1997), some cell cycle stages may be more effective than others for nuclear cloning.

As mammalian cells progress through the cell cycle after cytokinesis, they require mitogens (growth factors) to progress through the G1 phase (Zetterberg et al., 1995). Once past the checkpoint late in G1, they can complete the rest of the cycle without further mitogenic stimuli (Connell-Crowley et al., 1998). Removing the mitotic signals by serum-deprivation will cause cells that are in G1 to exit the cell cycle and arrest in a nondividing state of low metabolic activity, called G0. As a result, G0 nuclei may be more easily modified by some “factor” in the oocyte cytoplasm, or become more amenable to respond to cytoplasmic signals, thus leading to complete nuclear reprogramming (Campbell, 1999). It has been suggested that the ability of a transferred nucleus to direct embryonic development to term depends on the “reprogramming of gene expression” by these cytoplasmic factors (Szollosi et al., 1988), and G0 nuclei may be more receptive to this action (Campbell, 1999).
Both quiescent G0 and proliferating G1 phase somatic donors have been successfully used to produce cloned offspring. For G0/ G1-phase donor nuclei, the donor chromatin has not yet replicated and chromosomes are not ready to segregate (Wells et al., 2003). The original claim of G0 cells as the major factor has been questioned by successful studies with non-serum-starved culture (Vignon et al., 1999) which started the debate, whether inducing quiescence is required for successful nuclear transfer or not.

To facilitate the remodeling and reprogramming of somatic cell nuclei, nonactivated metaphase II oocytes with high levels of maturation promoting factor are generally employed as recipient cytoplasts (Tani et al., 2001). Thus donor cells are usually required to be arrested at G0 or G1 stage of the cell cycle to avoid chromosomal damage and abnormal ploidy in resulting embryos (Lee and Campbell, 2006). It also helps in perfect reprogramming of the donor nuclei.

Serum starvation is commonly used method for arresting cells at G0 stage of cell cycle (Campbell et al., 1996; Wilmut et al., 1997). Kasinathan et al. (2000) evaluated methods for generating G0 and G1 cell populations and compared their development after cloning. They found that a high degree of confluence in culture was more effective than serum starvation for arresting cells in G0. They also concluded that in nuclear transfer, donor cell cycle stage is important, particularly affecting late fetal development. But both methods of synchronization often cause reduced cell survival and increased DNA fragmentation.

Sul et al. (2012) discovered that efficient cellular reprogramming can be done using RNA. Transcriptome Induced Phenotypic Remodelling (TIPER), the ability of RNAs to produce direct cell to cell differentiation, effects faster reprogramming skipping the pluripotent stages.

In donor cell cycle synchronization studies, the apoptosis rate of transgenic fibroblasts was not different form normal fibroblasts after 3 days of serum starvation or 2 days of contact inhibition and there was no effect on developmental capacity of reconstructed embryos; however, the kidding rate
of recipients in the serum starvation group was higher than that in the contact inhibition group (Wan et al., 2012).

All the scientists are equivocal about the fact that characterizing the underlying changes in chromatin organization will be important because these forms the molecular basis for epigenetic variation in the donor cell genome at different cell cycle stages which may ultimately influence the cloning efficiencies (Wells et al., 2003). Scientists are now adopting chemical reprogramming methods using aphidicolin (Shi et al., 2007), aphidicolin or butyrolactone (Kues et al., 2000), aphidicolin or staurosporine (Wells et al., 2003) which are reversible cell cycle inhibitors and are giving better results in SCNT.

2.8 Cloning competence of different somatic cell types

The ability to produce live offspring from a cultured cell line was reported in 1996 (Campbell et al., 1996). An adult-derived somatic cell was used to produce Dolly, the first animal cloned from an adult differentiated cell (Wilmut et al., 1997). Shortly after that report, reports of similar success with development to term in other species followed including goat (Keefer et al., 2002). In addition, many more successes using fetal cells were subsequently reported, most of them due to inducing quiescence in the donor cells prior to nuclear transfer: sheep (Wells et al., 1997; McCreath et al., 2000), cow (Cibelli et al., 1998; Zakhartchenko et al., 2001), mouse (Wakayama and Yanagimachi, 2001), goat (Baguisi et al., 1999; Keefer et al., 2001; Chen et al., 2002) and pig (Li et al., 2000; Onishi et al., 2000).

Many somatic cell types, including mammary epithelial cells (Campbell et al., 1996; Wilmut et al., 1997; Zakhartchenko et al., 1999b), ovarian cumulus cells (Keefer et al., 1994; Schuetz et al., 1996; Wells et al., 1997, 1998; Kato et al., 1998, 1999; Wakayama et al., 1998, 1999; Polejaeva et al., 2000), fetal fibroblast cells from skin and internal organs (Cibelli et al., 1998; Baguisi et al., 1999; Lanza et al., 2000b; Hill et al., 2000; Onishi et al., 2000; Knott et al., 2000; Du et al., 2002), sertoli cells (Campbell et al., 1996; Wakayama et al., 1998; Baguisi et al., 1999), macrophage and blood leukocytes have been successfully utilized for nuclear transfer.
Six cloned calves were produced by using ear skin fibroblasts from 17 year old bull by Kubota et al. (2000). Bovine skin fibroblasts have been used for the production of nuclear transfer derived clones (Zakhartchenko et al., 1999c; Kashinathan et al., 2000; Knott et al., 2000; Kato et al., 2000; Tian et al., 2000; Pace et al., 2001). Dermal fibroblast from ear tissue of adult animals has been used for the production of cloned embryos in goats (Baguisi et al., 1999; Yu et al., 2003).

Fibroblast cells are the most common source for donor cells. These cells are easily harvested from either sex and cultured using standard tissue culture conditions. Following nuclear transfer of either actively dividing or quiescent bovine cell cultures, rates of blastocyst development range from 21 to 60 per cent (Zakhartchenko et al., 1999b; Kubota et al., 2000; Hill et al., 2000). Given the slower growth rates of adult fibroblast in culture, the majority of cells are in G0/G1 phase of the cell cycle at any given time. Fibroblasts of aged animals remain competent for cloning and prolonged culture does not affect the cloning competence of adult somatic donor cells.

Some researchers also reported that, apart from the type of somatic cell used, use of somatic cells from donors of various ages (Kato et al., 2000), passages and culture conditions (Kubota et al., 2000) and use of original stem cells with low levels of epigenetic markers (Toda et al., 2002) also influence cloning competency. It appears that cells from fetuses, as well as aged adults, can lead to comparable 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 (Tian et al., 2003).

A clear consensus has not yet been reached as to the superior somatic cell type for nuclear transfer. This is due in part to the fact that different laboratories employ diverse procedures and cell culture, nuclear transfer, and micromanipulation all which require critical technical skills. However, among the somatic cell types tested, the consensus from numerous
laboratories is that cumulus cells give the highest cloning efficiency and result in the least number of abnormalities in cloned animals (Tian et al., 2003).

Tang et al. (2012) showed that fibroblasts transfected with lentivirus vector carrying PSL1 and PSL 3 constructs can be used to produce transgenic animals with silenced myostatin gene. However, reduced expression of the exogenously introduced gene in sheep embryos may be attributed to the epigenetically modifying methylations which occur during reprogramming of genome of the reconstructed embryo.

2.9 Transfer of donor cells and ooplasm into enucleated oocytes

The most common way of inserting somatic cells is by injecting them under the zona pellucida and then using an electric impulse to induce cell membrane fusion between the enucleated oocyte and the somatic cell (Wilmut et al., 1997; Kato et al., 1998; Kubota et al., 2000; Du et al., 2002). Alternative ways include injection of the cytoplasm-free donor nucleus (Wakayama et al., 1998, Onishi et al., 2000) or the whole somatic cell into the cytoplasm (Lee et al., 2003). Earlier attempts to induce fusion with chemical and viral agents have been less successful and would now be rarely used (Vajta and Gjerris, 2006).

At the time of nuclear transfer, the preferred phase of the cell cycle for the donor nucleus is G1 if a metaphase II oocyte is used as a recipient because chromosome abnormalities and DNA replication may occur when S phase chromatin is forced to condense into metaphase chromosomes. Overall, a high proportion of cells with a G1 DNA content can be obtained by growing cultures to confluence (Kasinathan et al., 2000), serum deprivation (Campbell et al., 1996; Wilmut et al., 1997) or by use of chemicals like aphidicolin (Shi et al., 2007).

Donor cells at various levels of passage have been used by different scientists each having its own merits and demerits. Kasinathan et al. (2001) reported that cloning efficiency would decrease more quickly in cells from adults than in cells from calves or fetuses. The donor cells are usually obtained from the cell lines by trypsinising with 0.25 per cent trypsin–EDTA (Liu et al., 2007) or 0.05 per cent trypsin–EDTA (Du et al., 2006). After
enucleation a single cell was inserted into the perivitelline space of the enucleated oocyte by using a 15-20 µm (internal diameter) glass pipette (Cibelli et al., 1998) to facilitate close membrane contact for subsequent fusion. Small (<20 µm) donor cells with smooth plasma membrane (Keefer et al., 2002) and brightest cells (Arat et al., 2003) are preferred for transfer. Jang et al. (2004) evaluated the effect of age of donor cells (passages) on nuclear transferred embryos.

In somatic cell transfer, one of the main obstacles is the fusion efficiency between the donor cell and the oocyte cytoplasmic membrane. Phytohaemagglutinins have been widely used in cell biology for enhancing cell agglutination and fusion in various types of mammalian cells (Wu et al., 1994; Nishimura et al., 2004). Du et al. (2006) found that an optimized protocol of somatic cloning using phytohaemagglutinins (150 µg/ml, 20 min.) significantly improved fusion efficiency.

Re-appearance and up-regulation of telomerase activity has been used as an index of reprogramming efficiency and developmental potential (Jang et al., 2004).

2.10 Electrical activation of reconstructed oocytes

Karyoplast–cytoplast couplet, as an important link in nuclear transfer (NT), may partly influence the success rate of SCNT. Compared to other fusion methods, electrofusion is most extensively used, because of lower toxicity, good repeatability and high efficiency. Electrofusion could also be performed by chamber fusion (Kubota et al., 2000; Shiga et al., 1999; Wakayama and Yanagimachi, 1999; Choi et al., 2002), or by micro-electrode fusion (Takeuchi et al., 1999; Kishi et al., 2000; Du et al., 2006; Shen et al., 2006). In electrofusion the orientation of the cell must be such that the fusion membranes are perpendicular to the electro-magnetic fields. This is necessary as this is the point where the voltage membrane breakdown will first be reached (Zimmermann and Vienken, 1982). A low fusion rate may occur when the orientation of the donor cell and enucleated oocyte to the electrode is incorrect, or when there is little or no cell-to-cell contact (Miyoshi et al., 2001).
Electrical stimuli probably induce pores within the oocytes’ internal and external membranes. This may allow calcium ions to leak into the cytoplasm of the nuclear transplant embryos, resulting in activation. Activation of the reconstructed oocytes is initiated by a rise of the intracellular free calcium (Ca\(^{2+}\)) concentration after an electrical pulse (direct current). The transient increase of intracellular Ca\(^{2+}\) is immediately triggered by an influx of extra cellular Ca\(^{2+}\) from the fusion medium after electro stimulation (Sun et al., 1992).

Using micro-electrode method, Du et al. (2006) recorded a 33.0 ± 3.9 per cent fusion rate for couplets reconstructed with skin fibroblasts and enucleated oocytes and 59.0 ± 5.6 per cent fusion rate for couplets reconstructed using cumulus cells in cattle nuclear transfer (NT). Kishi et al. (2000) recorded a 56.0 ± 7.0 per cent fusion rate for couplets reconstructed using mammary gland epithelial cells and a 75.0 ± 4.0 per cent fusion rate for couplets reconstructed using fibroblast cells and enucleated oocytes in cattle NT.

Daniel et al. (2008) reported that the optimum DC electric field strength required for efficient cell fusion using goat cumulus and adult fibroblast cell was 2.0-2.5 kV/cm. Liu et al. (2007) compared chamber fusion (CF) and micro-electrode fusion in goat SCNT. They used a 15 µm tip-end, 100 µm frustum-end and 200 µm parallel micro-electrodes to perform micro-electrofusion with the aid of a micromanipulator and found that, the highest fusion and lowest degeneration rate were obtained in the 15 µm tip-end group. Compared with chamber fusion, the fusion rate was increased from 72.2 to 89.0 per cent for the granulosa cells, 77.1 to 94.6 per cent for fetal fibroblast cells and 51.2 to 78.0 per cent for mammary gland epithelial cells in goat SCNT.

When nuclear transplant embryos were given multiple electrical pulses there was an increase in the activation rate, as well as an increase in the rate of development to the blastocyst stage (Collas and Robl, 1990). Zimmermann cell fusion media is the commonly used media for karyoplast–cytoplasm couplet electrofusion.
The interval between fusion and activation is an important factor affecting the developmental competence of cloned embryos (Campbell, 1999) and prolonged exposure of the donor nucleus to oocyte cytoplasm before activation might promote developmental capacity of nuclear transferred embryos in both cattle (Wells et al., 1999) and mice (Wakayama et al., 1998). On the contrary prolonged intervals between fusion and activation might impair embryonic development (Choi et al., 2002; Aston et al., 2006; You et al., 2010). Melican et al. (2005) observed that re-fusion of non-fused couplet or an additional activation step was successful for producing live offspring.

2.11 Chemical activation

Activation of mammalian oocytes arrested in metaphase of the second meiotic division (MII) involves exit from meiosis and re-entry into the mitotic cell cycle, resulting in pronuclear formation and subsequent cleavage and development (Wassarman and Albertini, 1994). In normal fertilization, the fusion of a sperm cell with the oocyte is sufficient for activation, causing a transient intracellular rise in calcium ions (Sun et al., 1994; Lawrence et al., 1997). These calcium oscillations are responsible for the cascade of events that finally results in escape from MII arrest (Whitaker and Patel, 1990).

Protocols developed to artificially activate mammalian oocytes have attempted to mimic the biochemical and physiological events that normally occur during sperm-oocyte interaction. In somatic cell nuclear transfer, to activate the reconstructed oocytes different chemical stimuli are applied alone or in combination with some protein phosphorylation inhibitor like 6- Dimethyl aminopurine (6-DMAP) or protein synthesis inhibitor, cycloheximide (CHX) (Koo et al., 2000; Im et al., 2001). Oocytes activated with the protein phosphorylation inhibitor 6-DMAP may display an abnormal pattern of karyokinesis, indicative of altered DNA content during the first cell cycle (De La Fuente and King, 1998), while the protein synthesis inhibitor CHX may prevent translation of cytoplasmic proteins responsible for controlling DNA replication (Soloy et al., 1997)

There was a significant increase in the development of cloned embryos when the reconstructed embryos were cultured in cytochalasin-B for
5 hours after electrofusion, followed by 5 μM ionomycin for 5 minutes and a combination of 2 μM 6-DMAP and 5 μg/ml cytochalasin-B for 5 hours (Zou et al., 2002). 5 μM ionomycin and 2 mM 6-DMAP were used for chemical activation (5 minutes and 2.5-4 hours respectively) after post fusion treatment with 7.5 μg/ml cytochalasin-B for 1 hour for the production of cloned goats (Keefer et al., 2002) and only 5 μM ionomycin and 2 mM 6-DMAP were found effective for chemical activation in SCNT in which telophase enucleation was practiced in cloning of Asian yellow goat (Chen et al., 2007).

A delayed activation with ionomycin and 6-DMAP 3 hours after fusion was practiced in buffaloes (Shi et al., 2007). For chemical activation, reconstructed oocytes were cultured in 2.5 μg/ml cytochalasin-B and 10 μg/ml Cycloheximide for 1 hour, and then for an additional 4 hours in 10 μg/ml Cycloheximide in cattle (Du et al., 2006) and in goat (Daniel et al., 2008).

In case of zona-free parthenogenesis, there was a significant increase in cleavage rate and blastocyst yield when oocytes were activated by electrical pulse than when Ca-ionophore was used for activation and culturing of zona-free parthenogenetic embryos, flat surface was proved to be the best system (De et al., 2012).

2.12 Embryo culture

After SCNT the reconstructed oocytes were cultured further to observe cleavage and development of embryo. Optimal culture system is the crucial requirement to support embryonic development.

Use of oxygen and glucose together in culture medium had shown adverse effect as oxygen forms free radicals, which oxidizes glucose and affects development of embryo (Rieger, 1992). Totey et al. (1996) used glucose free Charles Rosenkrans-I medium (CR1aa) with low oxygen concentration for development of buffalo zygotes with better efficiency. Glucose supplementation in ovine embryo culture medium is not required until approximately day 3 or 4 of development, after which glucose improves development (Thompson, 2000).

Bovine serum albumin was originally used by Whitten (1957) and has since remained the most commonly used macromolecule, for the
culture of embryos in cattle (Wrenzycki et al., 2001) in sheep (Tervit et al., 1972) and in mouse (Whittingham, 1971). Kumar et al. (2007) suggested higher yields of day-8 morulae and blastosysts or day-9 blastocysts were obtained with mCR2aa and mSOFaa, even without serum supplementation and co-culture with somatic cells than those obtained when a complex medium with somatic cell support and serum supplementation. On day 8, the proportion of cleaved oocytes that developed to the morula + blastocyst stages was significantly higher for mSOFaa and mSOFaa + FBS (62.3% and 61.5% respectively) which in turn, was significantly higher when compared with TCM-199 + FBS (48.6%).

Additionally, even co-culture with cumulus cells or with non-reproductive tract cells such as BRL or Vero cells shows positive effects on embryonic development in vitro (Goto et al., 1994; Pegoraro et al., 1998). This may be due to the fact that not one or only few soluble factors are responsible for successful development but an ensemble of different signals and factors act cooperatively (Killian, 2004).

Jena et al. (2012) observed that handmade cloned embryos and parthenogenetic goat embryonic development was higher in the RVCL medium than modified synthetic oviductal fluid (mSOF).

2.13 Apoptosis gene expression studies of SCNT embryos

Apoptotic gene expression has been demonstrated by Melka et al. (2010) which has been found to affect the developmental potential of various pre-implantation embryos. During pre-implantation development, apoptosis is regulated by activity of pro-apoptotic and anti-apoptotic genes (Bergeron et al., 1998).

Pomar et al. (2005) suggested that incidence of apoptosis is an additional criterion to morphological evaluation of embryos to assess the embryo quality and effectively predict embryo viability.

Various genes have shown to cause apoptosis in pre-implantation embryos when expressed. The pro-apoptotic Bax is expressed in all bovine oocytes and the highest expression found in denuded oocytes (Yang and Rajamahendran, 2002), Caspase 3 associated with DNA
fragmentation (Walters et al., 2005; Spanos et al., 2002) and inhibition of Caspase-3 partially prevented DNA fragmentation, which indicated a caspase-dependent apoptosis pathway (Feugang et al., 2003).

Anti-apoptotic Mcl-1 has been found to increase the survival of cells and over expression confers resistance to apoptosis and targeting Mcl-1 by RNA interference induces apoptosis (Chohessy et al., 2006), anti-apoptotic Bcl-w mRNA found lower in good quality four cell embryos (Melka et al., 2010).

Fear and Hansen (2011) reported that the anti-apoptotic genes Bcl-2, Bcl -2L1, HSPA1A and DFFA showed a reduction in concentration as the embryo progressed above 16 cell stage and among the pro-apoptotic genes Bax, Bad and DFFB showed a distinct increase in expression above the 16 cell stage in vitro fertilized embryos. The genes HistH2A and DFFB did not vary between stages and hence used as housekeeping genes for comparison.

The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling reaction (TUNEL) (Gavrieli et al., 1992) enables in situ detection of apoptotic cells by labeling of extensive oligonucleosomal DNA fragmentation generated by endogenous DNase activity during the apoptotic process.

Park et al. (2007) studied the development of nuclear transferred embryos using somatic cells treated with apoptosis inhibitors. cDNA microarray gene expression analysis studies of Abdoon et al. (2012) concluded that the retarded development of parthenogenetic buffalo embryos could be due to down regulation of genes related to translation, nucleic acid synthesis, cell adhesion, and placental development.

2.14 Statistical analysis

The data reported in this manuscript are in expressed in percentage. All the data were analyzed using unpaired t test to compare the fusion, cleavage and further embryonic developmental rates of restructured embryos obtained by using two different methods of SCNT, i.e. Normally produces SCNT embryos and replenished SCNT embryos. Completely randomized design was
employed to compare the fusion rates of both the embryos and the developmental rates achieved in two culture systems.
3. MATERIALS AND METHODS

Experimental animals for the present study comprised of adult Black Bengal goat maintained at Animal Biotechnology Centre, MPPCVV, Adhartal, Jabalpur (M.P.). The ovaries for in vitro maturation and micromanipulation were obtained from the small animal slaughterhouse, Jabalpur (M.P.).

The protocol for somatic cell nuclear transfer was performed in the following steps:

- Oocyte (COC) aspiration.
- In vitro maturation of oocytes.
- Denudation of mature oocytes.
- Enucleation of oocytes and their confirmation.
- Preparation of Ooplasm
  a. (i) Removal of zona-pellucidae of enucleated oocytes
  (ii) Dissecting the ooplasm into 4-8 pieces using microblade
  b. Squashing and compression of enucleated oocyte to extrude ooplasm.
- Development of skin fibroblast cell lines.
- Preparation of donor cells for nuclear transfer after reprogramming of cell lines by serum starvation method.
- Transfer of donor cell into enucleated oocytes by conventional SCNT.
- Transfer of donor cell and ooplasm into enucleated oocytes by Replenished SCNT
- Phytohaemagglutinin treatment.
- Electrofusion of reconstructed oocytes
- Chemical activation of reconstructed oocytes and their in vitro culture using ionomycin, Cycloheximide and 6-DMAP.
- Embryo culture in RVCL media and Blastocyst media
- Embryo Staining
- Pooling of embryos in Trizol gradewise.
- RNA extraction and cDNA preparation.
- Real time expression analysis.
3.1. *In vitro* maturation of goat oocytes

3.1.1 Collection of goat ovaries

Goat ovaries were collected from small animal slaughterhouse, Jabalpur (M.P.). The ovaries were washed with sterile warm normal saline (35 – 38°C) and transported to the laboratory in warm Dulbecco’s phosphate buffered saline (DPBS) at ambient temperature. Before oocyte collection, the ovaries were washed 3 times with normal saline and 2 times with DPBS. (Both normal saline and DPBS contained gentamicin as antibiotic).

3.1.2 Oocyte collection

Oocytes were aspirated from all the visible medium sized follicles on the surface of ovaries with syringe (with 20 gauge needle) in aspiration medium (DPBS). Aspirated material was transferred in searching dish. Oocytes were searched under stereo-zoom microscope and were placed in 35 mm culture petri dish containing 50 µl DPBS drops and washed 4 times in it and transferred to washing medium for further washing (50 µl). The cumulus oocyte complexes (COCs) were then transferred to maturation medium.

3.1.3 *In vitro* culture medium solution

**Aspiration medium** - The oocytes were aspirated in DPBS (Dulbecco’s modified phosphate buffer saline) - polyvinyl alcohol (PVA). The composition of DPBS - PVA is shown in Appendix-I.

**Oocyte washing medium (1 ml)**

<table>
<thead>
<tr>
<th>COMPONENTS</th>
<th>QUANTITY (MICROLITER)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCM – 199 (Hyclone)</td>
<td>925</td>
</tr>
<tr>
<td>Fetal Bovine Serum</td>
<td>75</td>
</tr>
<tr>
<td>Gentamicin sulphate</td>
<td>1</td>
</tr>
</tbody>
</table>
Maturation Medium - (1 ml)

<table>
<thead>
<tr>
<th>COMPONENTS</th>
<th>QUANTITY (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCM - 199</td>
<td>900</td>
</tr>
<tr>
<td>Foetal Bovine Serum (FBS)</td>
<td>75.0</td>
</tr>
<tr>
<td>Follicle-stimulating hormone (FSH)</td>
<td>10.0</td>
</tr>
<tr>
<td>Luteinizing hormone (LH)</td>
<td>10.0</td>
</tr>
<tr>
<td>β-Estradiol</td>
<td>1.0</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>1.0</td>
</tr>
<tr>
<td>Gentamicin sulphate</td>
<td>1.0</td>
</tr>
</tbody>
</table>

**β-Estradiol (Sigma E8875)** : Dissolved in absolute alcohol @ 1 mg/ml and made aliquots of 5 µl and stored at -20°C.

**FSH (Sigma F2293)** : 2.5 unit (1 mg)/ml normal saline

Made aliquots of 15 µl and stored at -20°C

**LH (Sigma L5269)** : 2.5 unit (1 mg)/ml normal saline

Made aliquots of 15 µl and stored at -20°C.

**Sodium pyruvate (Sigma P4562):** 250 mM solution of sodium pyruvate was prepared in ultrapure deionized water. Aliquots of 5 µl were prepared and stored at -20°C.

**Gentamicin sulphate (Sigma G1264):** 50 mg of gentamicin was dissolved in 1 ml of deionized water. Aliquots of 5 µl were prepared and stored at -20°C.

### 3.1.4 Oocyte grading

**Grade-I (good quality):** Oocytes having compact cumulus oocyte complex (COC) with more than 4-5 layers of complete ring cumulus cells and homogeneous granular gray cytoplasm.
Grade-II (fair quality): Oocytes having not much complexes of cumulus oocyte complex with 2-3 layers of cumulus cells surrounding the zona pellucida and evenly granular ooplasm.

Grade-III (average quality): Oocytes with 1-2 layers of cumulus cells, which may be broken at several places/partially denuded with irregular dark ooplasm.

Grade-IV (poor quality): Oocytes without cumulus cells and having irregular dark ooplasm and oocytes with much expanded and scattered cumulus cells.

After grading of oocytes, grade I and II quality oocytes were used for *in vitro* maturation. Oocytes were washed thrice in medium (3 ml) and were transferred from one petridish / droplets to another.

3.1.5 Culture of oocytes for *in vitro* maturation

The 50 μl droplets of maturation medium were prepared in petri dish (35 mm) and oocytes were transferred in groups of 10-15 oocytes per droplet. The droplets were then covered with sterile pre-equilibrated (38.5°C) mineral oil (Sigma) and incubated in CO₂ incubator with >95 per cent humidity, 5 per cent CO₂ at 38.5°C for 24 hours.

3.1.6 Assessment of oocytes for *in vitro* maturation

Following methods were carried out for assessment of IVM oocytes:

i. Cumulus cell expansion

ii. Identification of polar body

(i) Cumulus cell expansion

Maturation of oocyte was classified on the visual assessment of the degree of cumulus cells expansion according to the procedure described by Hunter and Moor (1987). The assessment of matured oocytes was done in the following way:

Grade-A: Full cumulus cell expansion was characterized by adhering nature of cumulus cells and enlargement of the cumulus mass to at least 300 μm away from zona pellucida.
**Grade-B**: Moderate cumulus cell expansion was observed at the most 150-200 µm away from zona pellucida.

**Grade-C**: Slight expansion of cumulus cells was characterized by cumulus cells being tightly adherent to the zona pellucida.

(ii) **Identification of polar body**

Matured oocytes were collected in eppendorff tube and vortexed in DPBS (without Ca\(^{2+}\) and Mg\(^{2+}\) ions): PVA with 0.1 per cent hyaluronidase. The denuded oocytes were kept in 35 mm petri dish and observed under inverted microscope (Nikon). Locating one extruded small body between perivitelline membrane and zona-pellucida known as polar body and were selected for micromanipulation.

**Hyaluronidase (Sigma H4272)**: 30 mg/ 3 ml DPBS without Ca\(^{2+}\) and Mg\(^{2+}\).

Aliquots of 50 µl were stored at -20°C and before use 450 µl of DPBS without Ca\(^{2+}\) and Mg\(^{2+}\) was added and incubated in CO\(_2\) incubator with >95 per cent humidity, 5 per cent CO\(_2\) at 38.5°C for 1.5 hours.

3.2 **Preparation of micro tools for micromanipulation (Holding pipette, Enucleation needle, Transfer pipette and Microblade)**

(i) **Holding pipettes:**

Holding pipettes were prepared having an outer diameter of 100 µm and an internal diameter of about 40 µm (Wolf and Kraemer, 1992). These micropipettes were prepared from thick walled borosilicate glass tube with filament having an outer diameter 1 mm, inner diameter 0.58 mm and length of 150 mm (Sutter Instrument Co., Item No: BF100-58-15, California, USA). The pipettes were pulled using horizontal micro electro pipette puller (Narishige group model PN-30, Japan).

A piece of tubing was mounted in the pipette puller and melted in centre at 100.2°C for 2-3 seconds and pulled with sub magnet of 30.2 and main magnetic force of 60.2 respectively, giving two tapered ends. One of the pulled capillary was mounted in the microforge (Narishige, model MF-900, Japan) and observed through the stereo zoom microscope using the eye-piece article point at which the pipette diameter was determined. With the help
of glass cutter a scratch mark is made and sudden force is given to break with
even edges of the capillary tube. The glass capillary of desirable diameter
was mounted on microforge and the glass bead covering the filament was
then brought near the tip of the capillary. The power was turn on and as the
temperature increased, the pipette was evenly moved towards the glass bead
and fine polished until the edges melted, leaving only a narrow internal lumen
(15-20 µm). The first angle of 20-25° was given placing the capillary tube at
temperature of 75-80°C.

(ii) Enucleation needle:

These micropipettes were prepared from thin walled borosilicate
glass tube with filament having an outer diameter 1 mm, inner diameter 0.78
mm and length of 150 mm (Sutter Instrument Co., Item No: BF100-78-15,
California, USA). The pipettes were pulled using horizontal micro electro
pipette puller (Narishige group model PN-30, Japan).

The capillaries were pulled in centre at temperature 100.2°C for
2-3 seconds in same sub and main magnetic strength as of holding pipette.
The tip was tapered uniformly and sealed by gentle stretching after placed it
into glass bead. The first angle was given at temperature 55-60°C. These
sharp pipettes were used to cut zona pellucida of matured oocytes.

(iii) Transfer pipette:

The transfer pipettes were prepared with the same procedure as
the preparation of enucleation pipette except that the tip diameter was of 10-
15 µm.

(iv) Microblade:

Microblades were prepared for fine cutting of ooplasm into
smaller pieces under micromanipulator. The blades were prepared by
attaching a very fine piece of metal blade’s cutting edge with the glass
capillary having the diameter of 1 mm and length of 100 mm.
3.3  Enucleation of oocytes

3.3.1  Denudation

Matured oocytes were denuded by keeping the oocytes in 0.1 per cent hyaluronidase supplemented Ca\(^{2+}\) and Mg\(^{2+}\) free DPBS for 1 minute and vortexing for 2 minutes, and centrifuged at 1000 rpm for 5 minutes. Denuded oocytes were washed in enucleation medium (DPBS + 20% FBS).

3.3.2  Enucleation process

The denuded oocytes with the first polar body were selected for enucleation and were placed in 50 µl droplets of the enucleation medium and held by a holding pipette (which creates a negative pressure) in such way that the first polar body was positioned at 12 O’clock. The zona pellucida was pierced near the first polar body followed by squashing and compressing out the first polar body and its surrounding cytoplasm with an enucleation pipette; approximately 15 to 20 percent of the oocytes total ooplasmic volume was extruded through the slit made in the zona.

3.3.3  Testing of enucleated oocytes

For confirmation of successful enucleation the following procedure was followed:

- The oocytes were separately transferred into a drop of enucleation medium after the enucleation was over.
- The droplets of 20 µl of enucleation medium were made in line to smaller droplets of bis Benzimide (Hoechst 33342, Sigma).
- The extruded material was removed with fine pasteur pipette and was kept parallel to their respective drops of Hoechst 33342 stain for 10-15 minutes and observed under fluorescent microscope.
- Successful enucleation was confirmed if white fluorescence of MII chromosome and polar bodies were visible under epifluorescence UV light.
**Bis benzimide H 33342 trihydrochloride (Sigma B2261):** Stock solution is prepared as 1 mg/ml DPBS + 20 per cent glycerol. From this 15 µl was taken and added in 985 µl DPBS+ 20 per cent glycerol and made aliquots of 50 µl and stored at -20°C (kept away from direct light sources).

### 3.4 Development of skin fibroblast cell lines

#### 3.4.1 Preparation of medium (plain medium)

Powdered Dulbecco’s Modified Eagle’s Medium (DMEM) + Hams’ F-12 nutrient mixture with 2.5 mM glutamine and 15 mM HEPES (Hyclone SH30004.02) was dissolved in ultra-pure deionized water. To this 1.2 g of sodium bicarbonate was added. Finally, 1 litre of medium was prepared and filtered through 0.22 µm filter (Nalgene) in sterilized glass bottles and stored at 4°C.

**Fetal bovine serum (FBS, Hyclone SH30070.03)**

Frozen 500 ml fetal bovine serum bottle stored at -20°C, was slowly thawed at 4°C and was aliquoted (1 ml) in sterile polypropylene tubes and stored at -20°C for future use.

**Gentamicin (1000X) stock solution**

Gentamicin sulfate : 50 mg
Deionised water : 1 ml

Gentamicin was dissolved in 1 ml deionised water, filtered through 0.22 µm filter (Nalgene), and aliquoted as 50 µl in sterilized eppendorf tubes and stored at -20°C for future use.

#### 3.4.2 Preparation of different types of complete medium (100 ml)

For tissue collection and regular passaging, plain medium was finally reconstituted with fetal bovine serum (FBS), L-glutamine and antibiotic (gentamicin) to prepare working medium to be used for tissue culture. Stock solutions used in working medium were prepared as below:
### Components

<table>
<thead>
<tr>
<th>Components</th>
<th>For collection of tissue pieces and setting of primary culture</th>
<th>For washing and cleaning of tissue pieces in laboratory</th>
<th>For regular passaging/sub culturing</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM (Hyclone)</td>
<td>83.5</td>
<td>93.5</td>
<td>88.5</td>
</tr>
<tr>
<td>FBS (Hyclone)</td>
<td>15</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>L-Glutamine (Sigma)</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Gentamicin (Sigma)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>100 ml</strong></td>
<td><strong>100 ml</strong></td>
<td><strong>100 ml</strong></td>
</tr>
</tbody>
</table>

All the above ingredients were mixed and filtered through 0.22 µm (Nalgene) filter. Medium was kept in incubator at 38.5°C with 5 per cent CO₂ and was checked for contamination if any, one day prior to use.

**Phosphate buffered saline (10X)**

(Quantity in g.)

<table>
<thead>
<tr>
<th>Components</th>
<th>PBS – A</th>
<th>PBS – B</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>80.0</td>
<td>80.0</td>
</tr>
<tr>
<td>KCl</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>11.5</td>
<td>11.5</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>MgCl₂.6H₂O</td>
<td>--</td>
<td>1.0</td>
</tr>
<tr>
<td>CaCl₂.2H₂O</td>
<td>--</td>
<td>13.3</td>
</tr>
</tbody>
</table>

The above constituents were dissolved in ultra-pure de-ionized water to make 1000 ml.
For PBS-B all the constituents except CaCl$_2$.2H$_2$O were dissolved in de-ionized H$_2$O and CaCl$_2$.2H$_2$O was dissolved separately in water and added drop by drop with slow and gentle agitation in above solution to prevent precipitate formation. Final volume of 1 litre buffer solution was stored at 4°C after filtering through 0.22 µm filters.

### 3.4.3 Preparation of trypsin-EDTA solution (for 100 ml)

<table>
<thead>
<tr>
<th>COMPONENTS</th>
<th>QUANTITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>0.030 g</td>
</tr>
<tr>
<td>Trypsin</td>
<td>0.250 g</td>
</tr>
<tr>
<td>PBS-A (1X)</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

EDTA was dissolved in 20 ml PBS-A solution at pH 8.0 (add 1N NaOH drop by drop). Trypsin was dissolved separately in 50 ml of PBS-A when both the ingredients were dissolved completely, mixed them and volume was made 100 ml by adding PBS-A. The TE solution was filtered through 0.22 µm filter and 1 ml aliquots were stored at -20°C.

### 3.4.4 Collection and transportation of skin tissue pieces

Skin (ear pinna) tissue pieces (0.5 x 0.5 cm) of Black Bengal goats (Age 2 years and 1.5 years) were collected from the Animal Biotechnology Centre, MPPCVV, Adhartal, Jabalpur (M.P.).

Following protocol was adopted for tissue biopsy cleaning and primary transplant:

- Ear site was shaved with a scalpel blade on both sides, and then scrubbed both sides with soap solution. The site was cleaned with 1 per cent iodine solution and sprayed with 70 per cent ethanol. The sample area was not touched after this.
- A notch-shaped ear sample of about 1 x 1 cm was cut with help of biopsy forceps and cut piece was gently rinsed with 70 per cent ethanol for few seconds and then placed in a sterile eppendorf tubes containing complete medium (15% FBS) with gentamicin and
antimycotic and transported to laboratory. Tubes were labeled properly with information of about animal number, date of collection etc.

- In laminar flow-hood, sample was transferred into a petri dish and cleaned with scalpel to remove hair, if any. Rinsed in 70 per cent ethanol for 1-2 seconds followed by three washings with PBS.
- Clean tissue was transferred into a petri dish containing 2 ml washing medium with antibiotic and antimycotic.
- Tissue sample was cut medially, exposing the basal epidermis on both sides. Any cartilage observed was removed and were chopped into small tissue pieces of 1 mm².
- Tissue blocks were transferred into a 25 cm² tissue culture flask (BD Falcon), in few drops medium and kept in CO₂ incubator at 38.5°C maintaining 5 per cent CO₂ with 98 per cent humidified environment for 4 hours.
- When tissue pieces adhered to the surface of flasks, 4 ml of complete medium (15% FBS and without antimycotic) was added slowly from the side and incubated further in CO₂ incubator for 24 hours, at 38.5°C maintaining 5 per cent CO₂ and 98 per cent humidity.
- Flasks were observed after 24 hours, under inverted microscope (Leica) to check the contamination if any, or floating tissue. Floating tissues were reseeded again in new tissue culture flasks.
- Flasks were observed after 3-4 days and media was replaced by primary culture medium, without antimycotic. Culture was allowed to grow until 20 to 30 per cent confluency was attained before reseeding for better growth.

3.4.5 Primary cell culture

Primary cell culture refers to the cells that grow in culture flask directly from tissue explants. Tissue pieces were removed when growing fibroblast cells attained 20-30 per cent confluence and were reseeded again in new culture flasks for obtaining more primary cells.
At 70-80 per cent confluence, skin fibroblast cells were removed enzymatically by trypsin: EDTA (0.125 g, 0.015 g in 50 ml of PBS-A) treatment. Cultures were checked for epithelial cell colonies. The fibroblast monolayers came out first, while layer of epithelial cells were left stuck on to tissue culture flasks.

3.4.6 Purification of fibroblast cells by partial TE treatment

In earlier passages, epithelial cells were also screened along with skin fibroblast cells in tissue culture flasks. These were harvested carefully by giving partial treatment of TE for 4-5 minutes. Upper layer of skin fibroblast cell removed faster than epithelial cells on action of TE. Cells were collected and seeded in new culture flask while epithelial cells remained stuck in TC flask.

3.4.7 Subculture / passaging of cell

Fibroblast cells are anchorage dependent cells and hence, they grow up to a limit, forming a monolayer on surface of tissue culture flask but stop to divide or come out of the cell division to non-availability of surface to form monolayer. Secondary culture/subculture provides a way in which the cells get a new surface, nutrient and other conditions for a better growth.

- Skin fibroblast cells growing in tissue culture flasks were harvested at late log phase when 70-80 per cent confluence was observed.
- Culture medium was removed and monolayer of fibroblast cells was washed with 2 ml of PBS-A in 25 cm² flask.
- To each tissue culture flask 1 ml Trypsin-EDTA (for 25 cm² flask) was added and a wetting layer over the monolayer was formed.
- Flasks were kept for 3-5 minutes at 38.5°C for trypsin reaction. After incubation, the flasks were observed under inverted microscope for rounding of fibroblast cells and coming out of their fibres.
- When all the cells became rounded up, 2 ml of complete medium (10% FBS) was added to stop the enzymatic activity of Trypsin-EDTA on cells to avoid cell wall thinning.
• Surface of flask was gently rinsed with medium and cells were collected into 15 ml centrifuge tube (Axygen, USA) and centrifuged for 10 minutes at 1000 rpm.
• After centrifugation, supernatant was removed carefully and the cell pellet was resuspended into complete medium to make 1 ml volume of cell suspension.
• From this cell suspension, 10 µl suspension was transferred to eppendorf tube and was stained with 0.4 per cent trypan blue (Gibco BRL) and cells were counted on Neubauer's haemocytometer.

Total cell count (T.C.C.) = Number of cells in 10 squares x dilution factor x 10³.

3.6 Reprogramming of cell lines by serum starvation

Donor cells of passage 6-10 derived from skin fibroblast cells were used for nuclear transfer. The cell lines were grown for about 80 per cent confluency in DMEM medium with 10 per cent FBS and then they were kept in media having 0.5 per cent FBS for 72 hours. These cell lines were used for nuclear transfer after trypsinisation.

3.7 Preparation of Ooplasm

3.7.1 Dissection method

(i) Removal of zona pellucidae

A few enucleated oocytes with were selected and incubated with proteinase K (2.0 mg/ml) in TCM containing 10 per cent FBS for 8 minutes at 38.5°C, 5 per cent CO₂ and 98 per cent humidity.

(ii) Dissection of ooplasm

Dissection of ooplasm was carried out under micromanipulator. The zona free enucleated oocytes were bisected by microblade into two halves and further bisected to give rise to smaller masses of ooplasm containing 15 to 20 per cent of total ooplasm volume. These ooplasm pieces were transferred to pre equiliberated TCM containing 20 per cent FBS and incubated for 20 minutes at 38.5°C for rounding up.
3.7.2 Squashing and Compression method

A few enucleated oocytes were selected as donors of ooplasm. Ooplasm for transfer were prepared by squashing and compression of the already enucleaed oocyte to extrude an approximately equal amount of ooplasm as the amount lost during enucleation. The ooplasmic mass was manipulated in such a way so as to be taken into a transfer pipette along with a somatic cell for transfer.

3.8.1 Protocol for preparation of cells for nuclear transfer

Following media/chemicals were used in preparation of cells for nuclear transfer:

1. Cell dissociation medium
   Trypsin-EDTA (TE)

2. Washing medium
   DPBS-A

3. Cell suspension medium
   DMEM + 10 per cent FBS

The following procedure is followed for the preparations of cells for SCNT:

- The culture medium was removed from culture petri dish
- The cells were washed twice with 1 ml of DPBS-A
- 0.5 ml of TE was added, immediately 300-400 µl was removed in which 100-200 µl of TE was left in culture petri dish
- TE treated cells were incubated for 2-3 minutes followed by addition of 1 ml of DMEM +10 per cent FBS
- The contents were taken in a centrifuge tube and centrifuge at 1000 rpm for 10 minutes
- The supernatant was discarded and the cells were collected in TCM containing 20 per cent FBS for use as nuclear transfer.
3.9 Transfer

3.9.1 Transfer of cells into enucleated oocytes

The enucleated oocytes and dispersed donor cells were manipulated in TCM-199 containing 7.5 per cent FBS. The oocyte was held by holding pipette in such a way that, slit in the zona pellucida made during enucleation was clearly visible. The individual donor cell, which is of 15-20 µm diameter, having smooth plasma membrane was picked up with transfer pipette and introduced into the perivitelline space through the same slit.

3.9.2 Transfer of Ooplasm for Replenishment

The ooplasm was initially picked up using a transfer pipette of 15-20 µm diameter followed by an individual donor cell and introduced into the perivitelline space through the same slit made during the enucleation process. Thus, the somatic cell was sandwitched between principal and transferred exogenous ooplasm.

3.9.3 Phytohaemagglutinin treatment to reconstructed oocytes

The reconstructed oocytes were washed 4 times in enucleation media (DPBS + 20% FBS) and transferred to TCM-199 + 7.5 per cent FBS supplemented with 150 µg/ml phytohaemagglutinin-L (PHA-L, L-4144), classified as a leucoagglutinin and derived from Phaseous vulgaris (red kidney bean) for 20 minutes.

3.10 Electro fusion of reconstructed oocytes

Reconstructed oocytes were incubated for 5 minutes in Zimmerman cell fusion medium.
Zimmerman cell fusion medium

<table>
<thead>
<tr>
<th>COMPONENTS</th>
<th>QUANTITY (g/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>9.5840</td>
</tr>
<tr>
<td>Magnesium acetate tetrahydrate</td>
<td>0.0107</td>
</tr>
<tr>
<td>Calcium acetate</td>
<td>0.0016</td>
</tr>
<tr>
<td>K$_2$HPO$_4$.3H$_2$O</td>
<td>0.0288</td>
</tr>
<tr>
<td>L-Glutathione</td>
<td>0.0031</td>
</tr>
<tr>
<td>BSA</td>
<td>0.0010</td>
</tr>
</tbody>
</table>

All the ingredients were dissolved in 100 ml ultra-pure de-ionized water adjusted to pH 7.0 and filtered through 0.22 μm filter (Nalgene) in sterilized glass bottle and stored at 4°C.

The cells were overlaid in Zimmerman's cell fusion medium and fusion was performed at room temperature with manually oriented fine electrical rods (diameter of 120 μm) where the contact surface between the cytoplast and donor cell were made parallel to the electrode. Cell fusion was induced by a double electrical pulse of 2.0-2.5 kV/cm for 10 microseconds (μs), delivered by a BTX Electro cell manipulator (BTX 2001, San Deigo, CA, USA). After electrical stimulation, oocyte donor cell complex were incubated for 15-30 minutes in DPBS +20 per cent FBS at 38.5°C before being subjected to further activation procedures. Fusion rates were checked 90 minutes after the electrical pulse by observing the notch formation for confirmation.

3.11.1 Chemical activation of reconstructed oocytes

Following chemicals were prepared, aliquoted, stored at -20°C and thawed for use as and when required.

**Ca-ionophore A23187 (Sigma C7522)**

Ca-ionophore (Sigma) 500 μM solution was prepared in ethanol and aliquoted in sterilized eppendorf tubes (0.2 ml) containing 10 μl solution and stored at -20°C.
Cycloheximide (CHX): (Sigma C7698)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycloheximide</td>
<td>1 mg</td>
</tr>
<tr>
<td>Ethanol</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

Mixed well and aliquots of 10 µl were prepared and stored at -20°C.

6-DMAP (6- Dimethyl amino purine): (Sigma D2629): 250 mM

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-DMAP</td>
<td>40.8 mg</td>
</tr>
<tr>
<td>DPBS</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

Aliquots of 10 µl were stored at -20°C.

3.11.2 Chemical activation protocol

Following protocol was used for chemical activation of reconstructed oocytes:

- The reconstructed oocytes were activated by incubating in TCM supplemented with 20% FBS and 5 µM calcium ionophore for 5 min at 38.5°C.
- These oocytes were then washed thrice in 20 µl drops of TCM containing 20% FBS.
- Another 4 hr activation was done at TCM-199 + 20% FBS supplemented with 2 mM 6-DMAP in CO₂ incubator having 95% humidity with 5% CO₂ at 38.5°C.

3.12 Embryo culture

The fused reconstructed oocytes were washed six times and transferred in 50 µl pre-equilibrated RVCL media with 1 per cent fatty acid free bovine serum albumin (FAF-BSA) in 35 mm (BD falcon) petridish covered with mineral oil and incubated in CO₂ incubator at 38.5°C and 5 per cent CO₂ for 72 hours. After 72 hours the oocytes were checked for cleavage and development. Only those oocytes which showed cleavage and development were selected and put in the petridish having the 50 µl drop of Blastocyst media (Cook®) with 1 per cent BSA for further 5 days. Different stages of embryos were recorded during this period. The embryos were collected, washed with DPBS without calcium and magnesium and then transferred to 1
ml 0.01 per cent DEPC (Diethyl pyrocarbonate) treated eppendorff tubes containing TRI® reagent (Sigma, USA) for RNA extraction.

### 3.13 Embryo Staining

Preparation of bis Benzimide H 33342 trihydrochloride (Hoechst dye, Sigma B2261):

Stock solution - 1 mg/ml DPBS + 20% glycerol

From this, 15 µl was taken and added in 985 µl DPBS + 20 per cent glycerol and made aliquots of 50 µl, stored at -20°C (kept away from direct light sources).

**Procedure for Embryo Staining:**

- Embryos were removed from culture medium and washed two times in 50 µl drops of DPBS containing 1 mg/ml polyvinylpyrrolidone (PVP).
- They were transferred to a 50 µl drop of Hoechst 33342 stain.
- The embryos were kept in the dye for 10 minutes in a dark chamber.
- They were washed in 50 µl drops of DPBS-PVP.
- The embryos were then transferred to a clean slide with a minimal volume media and were allowed to dry for 15 minutes at room temperature in a dark compartment.
- Finally, covered with a clean cover slip and cell nuclei were counted under the fluorescent microscope with a UV filter.

### 3.14.1 Isolation of Total RNA from SCNT Embryos

All the material used for RNA isolation were first treated with 0.01 per cent DEPC for an overnight duration to make them RNase free and were then taken out from DEPC treated water, wearing hand gloves and dried at 60°C in hot air oven. After drying they were autoclaved at 15 lbs pressure for 20 minutes for inactivation of DEPC. Total RNA isolation was done by trizol method with some modifications (Sambrook and Russel, 2001).
Procedure for RNA Isolation

- 2 ml microcentrifuge tube having SCNT embryos in trizol reagent in -20°C (Sigma, U.S.A) were thawed and incubated at room temperature until melted.
- 200 µl of chloroform was added, mixed well by hand for 15 sec. and left at room temperature for 2-3 minutes.
- Centrifuged at 12,000 rpm for 15 minutes at 4°C.
- The colourless upper phase i.e. supernatant was taken out carefully and transferred to a DEPC treated microcentrifuge tube.
- 500 µl of isopropyl alcohol along with 20 µg of glycogen was added, mixed well by hand and incubated at -20°C at least for an overnight time period for RNA precipitation.
- The contents were centrifuged at 12,000 rpm for 10 minutes at 4°C. A small white gel like pellet of RNA was seen at the bottom of the tube after this step.
- The supernatant was slowly and completely discarded using a pipette.
- The RNA pellet was rinsed with 1 ml of ice-cold 75% ethanol (in DEPC treated water) to remove salt for 2-3 times.
- Centrifuged at 9500 rpm for 5 minutes at 4°C.
- Ethanol was discarded carefully by using pipettes.
- The pellet was allowed to air dry for 5 to 10 minutes to remove any remaining ethanol.
- Then air dried RNA pellet was dissolved in 10 µl DEPC treated water by incubating in water bath at 55-60°C for 10 minutes. Concentration of the total RNA was determined by Nanodrop ND-1000 spectrophotometer V3.5 (OD at 260). The extracted RNA was further used for synthesis of cDNA immediately or stored at -20°C until further use.

Glycogen (Invitrogen: 10814-010): 100 µl (20 µg/µl) stock contains 2 mg glycogen dissolved in 100 µl of nuclease free water and stored at -20°C until further use.
3.15 cDNA preparation and quantification

cDNA was prepared from 11 μl of RNA using RevertAid™ first strand cDNA Synthesis kit (Fermentas) as per manufacturer’s instructions. All the glasswares and plasticwares used for cDNA preparation were made nuclease free before use.

The following reagents were added into a sterile, nuclease-free tube on the ice as per the indicated order:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity(μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA template</td>
<td>11</td>
</tr>
<tr>
<td>Random hexamer primer</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>12</strong></td>
</tr>
</tbody>
</table>

The tube was mixed gently, centrifuged briefly and incubated at 65°C for 5 minutes. Then the following components were added in the indicated order:

<table>
<thead>
<tr>
<th>Components</th>
<th>Quantity(μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X reaction buffer</td>
<td>4</td>
</tr>
<tr>
<td>5X reaction buffer</td>
<td>4</td>
</tr>
<tr>
<td>RiboLock™ RNase inhibitor</td>
<td>1</td>
</tr>
<tr>
<td>10 mM dNTP mixture</td>
<td>2</td>
</tr>
<tr>
<td>RevertAid™ M-MuLV reverse transcriptase (200 unit/ μl)</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>20</strong></td>
</tr>
</tbody>
</table>

The above reaction mixture was mixed gently and centrifuged briefly. An RT-PCR reaction containing all components except reverse transcriptase was also prepared as a negative control.
3.15.1. Reaction protocol for cDNA synthesis

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>65°C</td>
<td>5 min.</td>
</tr>
<tr>
<td>II</td>
<td>25°C</td>
<td>5 min.</td>
</tr>
<tr>
<td>III</td>
<td>42°C</td>
<td>60 min.</td>
</tr>
<tr>
<td>IV</td>
<td>70°C</td>
<td>5 min</td>
</tr>
<tr>
<td>V</td>
<td>4°C</td>
<td>∞</td>
</tr>
</tbody>
</table>

The concentration of all the prepared cDNAs was determined by using the Nanodrop spectrophotometer (OD at 260 nm) to ensure that similar amounts of cDNA were being used for real time RT-PCR analysis.

3.16. Primers

Description of forward (F) and reverse (R) primers used to assess expression of apoptosis regulatory genes (Melka et al., 2010).

<table>
<thead>
<tr>
<th>No</th>
<th>Primer</th>
<th>Primer Sequence (5'-3')</th>
<th>Primer size (-mer)</th>
<th>Tm (°C)</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>β-actin(F)</td>
<td>CCAGATCATGTTCGAGACTTTCA</td>
<td>24</td>
<td>55.0ºC</td>
<td>132bp</td>
</tr>
<tr>
<td></td>
<td>β-actin(R)</td>
<td>TCCCCAGAGTCCATGACAAATG</td>
<td>21</td>
<td>56.7ºC</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Mcl-1(F)</td>
<td>AGGCCGCATCAGAAATGTG</td>
<td>19</td>
<td>55.9ºC</td>
<td>200bp</td>
</tr>
<tr>
<td></td>
<td>Mcl-1 (R)</td>
<td>TCCATCGCTAGGTGATGGC</td>
<td>19</td>
<td>57.0ºC</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Caspase-3(F)</td>
<td>CCGCACCCTTCTGTGATGA</td>
<td>20</td>
<td>62.4ºC</td>
<td>80bp</td>
</tr>
<tr>
<td></td>
<td>Caspase-3(R)</td>
<td>AGAAGTACGCGAGGTCCACT</td>
<td>21</td>
<td>60.9ºC</td>
<td></td>
</tr>
</tbody>
</table>

3.17. Real time PCR reaction protocol

Real time RT-PCR (ABI Prism™ 7300) was performed by SYBR green chemistry (ABI, USA) using cDNA as template. The real time PCR protocol included the following cycling conditions.
<table>
<thead>
<tr>
<th>Stages</th>
<th>Stage-I</th>
<th>Stage-II</th>
<th>Stage-III</th>
<th>Stage-IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cycles</td>
<td>1</td>
<td>1</td>
<td>45</td>
<td>1</td>
</tr>
<tr>
<td>Temperature</td>
<td>57 °C</td>
<td>95°C</td>
<td>95°C &amp; 60°C</td>
<td>95°C &amp; 60°C</td>
</tr>
<tr>
<td>Time</td>
<td>1 min.</td>
<td>10 min.</td>
<td>95°C-15 sec</td>
<td>56°C-1 min.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>95°C-15 sec</td>
<td>56°C-30 sec</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>95°C-15 sec</td>
<td>95°C-15 sec</td>
</tr>
</tbody>
</table>

The specificity of amplification during real-time RT-PCR was monitored by evaluating the melting/dissociation curve and running the products on 2 per cent agarose gel (Appendix II).

### 3.18 Statistical analysis

Unpaired t-test was performed to compare the fusion, cleavage and further embryonic developmental rates between normal and replenished reconstructed embryos. Completely randomized design (CRD) was used to compare the cleavage and embryonic developmental efficiency of replenished and conventional SCNT for RVCL BSA and RVCL Blast BSA culture groups in different experimental trials. CRD was also used to compare the number of blastomeres in Conventional and replenished embryos as well as in the RVCL BSA and RVCL Blast BSA culture groups.
4. RESULTS

4.1 In vitro maturation of slaughterhouse goat oocytes

Ovaries were collected from small animal slaughterhouse, Jabalpur (M.P.) and oocytes were aspirated and matured in vitro in TCM-199 fortified with FSH, LH and 17β-estradiol in 16 experimental trials. The data representing the number of oocyte aspirated, grading of oocytes, cumulus expansion and polar bodies observed are presented in Table 1. Out of 595 ovaries 1678 oocytes were aspirated indicating an average of 2.82 oocytes recovered per ovary. Among the aspirated oocytes 35.10 per cent of grade-I, 35.00 percent of grade-II and 29.90 per cent of grade-III & IV oocytes were observed (Fig.1a).

The in vitro maturation of oocytes were evaluated by cumulus expansion and polar body formation after incubation at 38.5°C in maturation media in a CO₂ incubator with 5 per cent CO₂ and 97 per cent relative humidity. Different grades of cumulus expansion of oocytes recorded have been shown in Fig. 1b. Out of 1176 oocytes set for maturation, 687 oocytes (58.4%) showed good (Grade A) cumulus expansion, followed by 309 oocytes (26.3%) Grade B and 180 oocytes (15.3%) of Grade C. Out of 996 in vitro matured oocytes, 702 oocytes (70.48%) showed the first polar body formation (Fig. 1c and Fig. 1d).

4.2 Enucleation

In vitro matured A and B grade oocytes were used for SCNT. Denuded oocytes showing first polar body were held as shown in Fig. 2a using a holding pipette. The zona was pierced just above the polar body thereby creating a slit (Fig. 2b). This was followed by squashing and compression with an enucleation needle to extrude the first polar body along with the surrounding ooplasm having MII chromosomes (Fig. 2c). Approximately 15-20 per cent of the ooplasm from oocytes were extruded through the slit. The extruded mass was stained using bis-benzimide H-33342 trihydrochloride stain and observed under a fluorescent microscope to confirm
enucleation (Fig. 2e and Fig. 2f). The successfully enucleated oocytes were selected for transfer and preparation of ooplasm.

### 4.3 Preparation of Ooplasm

A few successfully enucleated oocytes were zona dissolved and further dissected into smaller 8-10 pieces having 15-20 per cent of the total ooplasm volume using specially designed microblades as in Fig 3a. Alternately, zona intact enucleated oocytes were squashed and compressed further to extrude 15-20 percent ooplasm for replenishment of enucleated oocytes (Fig. 3b).

### 4.4 Nuclear Transfer for Conventional and Replenished oocytes

Skin fibroblasts of passage 6-10 were used for transfer after reprogramming by serum starvation for both conventional and replenished transfer. Transfer was performed by a transfer pipette of 10-15 µm in diameter so as to make it possible to enter the enucleated oocyte through the previously made slit to produce conventional reconstructed oocytes (Fig. 4b). This was followed by transfer of ooplasm through the same pipette along with the somatic cell to produce replenished reconstructed oocytes.

### 4.5 Electrofusion

Trypsinized reprogrammed skin fibroblast cells (Fig. 3d) were used as nuclear donors to produce conventional and replenished reconstructed oocytes. Somatic cell alone was transferred to produce conventional reconstructed oocytes and somatic cell followed by ooplasm was transferred to produce replenished reconstructed oocytes. Following reconstruction the oocytes were kept for electrofusion where 2.0 - 2.5 kV/cm DC current with double pulse of 2 and 10 µs were applied through BTX Electro cell fusion machine.

### 4.6 Fusion Rate of Normal and Replenished Reconstructed Oocytes

The fusion rate of reconstructed oocytes after standard D.C. (direct current) electric pulse has been presented in Table 3. In two experimental groups, ten replications of normal and replenished reconstruction procedure has been shown. Out of 367 normally reconstructed
oocytes, 164 showed fusion which is 44.57 ± 4.92 per cent fusion efficiency. In the replenished reconstructed group, out of 306 oocytes, 184 fused showing a fusion rate of 59.50 ± 7.18 per cent. On comparing the results, a significant difference (P<0.05) in the fusion rates were observed between the two groups indicating a higher efficiency of the fusion process of replenished reconstructed oocytes.

4.7 Comparison of SCNT Embryos Development

4.7.1 Development of normal reconstructed embryos

The cleavage rates and developmental stages of normally reconstructed embryos observed are shown in Table 4.

In 10 experimental trials of normal reconstructed group, out of 164 electrofused normal reconstructed oocytes, 33 (19.84 ± 4.96%) were successfully activated showing cleavage after chemical activation and cultured for subsequent embryonic development. The different stages of embryos observed were 4 cell stage-31 (18.84 ± 4.16%), 8-16 cell stage-30 (18.21 ± 5.48%), 16-32 cell stage-25 (15.16 ± 4.20%), morula 21 (12.70 ± 3.70%) and no blastocyst was observed.

4.7.2 Development of replenished reconstructed embryos

In 10 experimental trials of replenished reconstructed group (Tab. 4), out of 184 electrofused replenished reconstructed oocytes, 48 (25.50 ± 4.23%) were successfully activated showing cleavage after chemical activation and cultured for subsequent embryonic development. The various sequential stages of embryos observed were 4 cell stage- 47 (24.91 ± 4.89%), 8-16 cell stage- 42 (22.46 ± 4.76%), 16-32 cell stage- 39 (21.62 ± 5.26%), morula 29 (15.09 ± 5.19%) and one early blastocyst (0.54%) was observed.

The cleavage and initial development rate of 4 cell stage up to 16-32 cell stage of replenished reconstructed embryo group were significantly higher (P<0.05) as compared to normal reconstructed group. However, the development of morula did not differ significantly (P<0.05) between the groups.
4.8 Developmental rates of SCNT embryo achieved in different culture systems

Embryo development in two culture systems, RVCL BSA and RVCL Blast BSA media, were recorded to assess the degree of support provided by the media for embryo growth. The comparative developmental potential (Tab. 3) of RVCL Blast BSA media with RVCL BSA, was as follows; cleavage (22.42 ± 5.13% vs. 23.42 ± 3.04%), 4 cell (21.17 ± 4.68% vs. 22.09 ± 2.29%), 8-16 cell (20.28 ± 3.73% vs. 19.84 ± 3.33%), 16-32 cell (19.26 ± 4.98% vs. 15.61 ± 2.56%), morula stage (16.06 ± 3.22% vs. 10.56 ± 2.97%) and blastocyst stage (0.48% vs. 0.00%).

The developmental rate of SCNT embryos irrespective of the method of reconstruction, the RVCL Blast BSA culture system did not differ significantly during the initial developmental stages with RVCL culture system. But during the advanced developmental stages, especially the embryonic developmental rate from 16-32 cell stage to morula, RVCL Blast BSA culture system proved to be more supportive significantly (P<0.05) than the RVCL culture system.

4.9 Morphological evaluation by assessing the blastomere nuclei number by Hoechst staining.

The number of blastomeres developed during embryo culture were assessed by staining the 4th day (96 hours) embryos with Hoechst 33342 stain. After staining, the embryos were subsequently observed under an epifluorescence microscope (Leica/Nikon) and number of blastomere nuclei were counted. On morphological comparison of conventional and replenished SCNT embryos, a higher number of blastomeres count was recorded in replenished SCNT group (27.00 ± 0.74) as compared to conventionally produced SCNT embryos (22.19 ± 0.91). The replenished reconstructed SCNT embryos were found to possess significantly more number of blastomeres (P<0.05) than the conventionally produced SCNT embryo group.

Comparative study of embryonic development of conventional and replenished embryonic groups were conducted with culturing in two types
of culture conditions *viz.* RVCL BSA and RVCL Blast BSA. The number of blastomeres observed in embryos developed in RVCL Blast BSA media (27.06 ± 0.68) was significantly higher (P<0.05) as compared to embryos developed in RVCL BSA group (23.13 ± 0.94).

### 4.10 Gene Expression Study

Pooled samples of morphologically good and poor quality SCNT embryos were used in the present study. β-actin is a housekeeping gene taken as endogenous control for the present expression analysis. Caspase-3 was selected as pro-apoptosis marker and Mcl-1 was selected as an anti-apoptosis marker.

The primer concentration for all the real time PCR reactions were optimized at 0.2 picomolar. Real Time PCR was carried out with ABI prism® 7300 SDS Instrument and the products were run on a 2 per cent agarose gel at a constant voltage gradient of 5-7 Volts/cm. For all the genes taken for study, faint bands were observed on the gel under a Gel Documentation system UV illuminator (Syngene). An amplification of the endogenous control, β-actin was observed in both the pools of morphologically good and poor quality SCNT embryos (Fig. 7a) suggesting their constitutive expression along with pro-apoptotic (Caspase 3) and an anti-apoptotic marker gene (Mcl-1). Caspase-3 amplification was observed in poor quality embryo group which was absent in good quality embryos. On the other hand, Mcl-1 expression was recorded in morphologically good quality SCNT embryo group and was absent in poor quality embryo group.
5. DISCUSSIONS

5.1 *In vitro* oocyte maturation

In the present study cumulus expansion and polar body formation were the criteria for evaluation of *in vitro* maturation of oocytes. The cumulus expansion for Grade-1 was better than other grade oocytes and also showed more percentage of polar body formation. These results indicated that the degree of cumulus expansion and formation of polar bodies largely depend on the quality of oocytes set for maturation.

Oocytes aspirated from mammalian slaughterhouse ovaries by aspiration or dissection of follicles have the capacity to develop into blastocysts (Martino *et al*., 1995; Pawshe *et al*., 1996). Keskinetpe *et al.* (1994) investigated the effects of medium supplemented with glycoprotein (LH, FSH, hCG, and TSH) on *in vitro* maturation (IVM) of goat oocytes and concluded that such glycoprotein hormones were required in maturation media to develop oocytes for successful *in vitro* fertilization (IVF). Supplementation of the IVM media with hormones, especially LH, FSH and estradiol, alone or in combination, has been considered by some authors to be essential for obtaining high maturation rates (Totey *et al*., 1996). Olson *et al.* (1991) reported the higher cleavage rate in media supplemented with oestradiol as compared to FSH and LH alone. In the present experiment a combination of FSH, LH and oestradiol were used and out of 996 oocytes *in vitro* matured, 702 oocytes (70.48%) showed the first polar body formation.

Jeon *et al.* (2011) reported higher number of matured oocytes showing first polar body when oocytes were denuded at 12 hours of maturation and subsequently cultured for another 6 hours. The percentage maturation was significantly higher than those matured for 18 hours without denudation.

5.2 Enucleation

Oocyte enucleation is the initial step to be performed in nuclear transfer procedure after *in vitro* maturation. The polar body and MII chromosome are located close to each other for a few hours during
maturation and removal of polar body by gentle pressure simultaneously removes the MII chromosomes also, thus resulting in complete enucleation.

In the present study the squashing and compressing technique was applied successfully that resulted in 70-75 per cent confirmed enucleation after Hoechst staining. Enucleation was done after 24 hours of maturation. It seems to be the ideal time of enucleation as the first polar body and MII chromosomes are located in close vicinity of each other as reported by Ajithkumar et al. (2008).

In an experiment to standardize the optimum time of enucleation Ajithkumar et al. (2008) reported that with increase in maturation time the perivitelline space also increases causing displacement of the MII chromosomes from the polar body, thus reducing the success of enucleation. The time 20-23 hours and 23-26 hours enucleation time for conventional SCNT is exhibited a significantly higher percentage of successful enucleation as compared to the other time groups i.e. 17-20 hours and 27-30 hours in goat.

Various methods of enucleation has been reported by various investigators which are laser assisted micro dissection of zona pellucida (Schmoll et al., 1999) aspiration of polar body and MII chromosome using enucleation pipette (Wilmut et al., 1997) squashing and compressing method (Das et al., 2003; Shen et al., 2006; Du et al., 2006) and chemically assisted method (Yin et al., 2005). The cloned animals have been reported to be produced from most of the above methods of enucleation like Dolly sheep by aspiration method (Wilmut et al., 1997), Amy cow by squashing and compressing method (Kubota et al., 2000).

5.3 Loss of ooplasm and its replenishment

The developmental potential of reconstructed oocytes largely depends on the volume of ooplasm removed along with nuclear material during enucleation. Gordon (2003) mentioned that that aspiration of 1/3rd of ooplasm may lead to altered metabolic activity in reconstructed embryo after nuclear transfer.
In the present study a new concept was developed for replenishment by substituting about 10-20 per cent of ooplasm from other in vitro matured enucleated oocytes. This was performed by transferring the ooplasm through the already made transfer pipette.

The developmental potential of reconstructed oocytes largely depends on the volume of ooplasm removed along with nuclear material lost during for enucleation (Peura et al., 1998). Depending on the efficiency of the micromanipulator, a loss of ooplasm between 5 to 50 per cent along with the first polar body and MII chromosomes has been reported (Westhusin et al., 1992, 1996). Both Griesing et al. (1994) and Zakhartchenko et al. (1997) observed a decreased blastocyst development rate in nuclear transfer embryos when the cytoplasmic volume of the recipient oocytes was significantly reduced. Thus the amount of ooplasm present was critically important for the development of good quality SCNT embryos.

Hua et al. (2011) studied the development of SCNT embryos by removing different amounts of ooplasm and the effect of nucleus to cytoplasmic ratio (N/C) on quality on embryos. The developmental rates of the three groups’ viz. high, medium and low N/C ratio embryos were similar and about 60 per cent of the ooplasm were sufficient for embryo development.

The authors further studied the mitochondrial DNA in above three groups and explained that the factors present in 60 per cent of cytoplasm were sufficient to regulate the timings of early cleavage division or the developmental process of SCNT embryos. Further, less than 30 per cent loss of ooplasm did not significantly affect the chromosome constitution in SCNT embryos, thus did not have detrimental effect as evident by morphological characteristics. However the highest rate of blastocyst formation was derived from those embryos with a low N/C ratio. The probable reason may be due to the fact that mitochondria of the ooplasm do not replicate from 2 cell to blastocyst stage. However, the expression of mitochondrial genes may increase with progress of embryonic development because expression of mitochondrial gene is vital for cellular function, especially for the generation of adenosine triphosphate (ATP). A small amount of ATP may be sufficient for early preimplantation embryo
development, but as the development progresses, the requirement of ATP would increase, therefore ATP deficiency may influence normal physiological function and embryonic quality (Van Blerkom et al., 1998).

No comparable results of research have been found in literature on developmental potential of such replenished reconstructed SCNT embryos. This seems to be the first attempt for development of SCNT embryos by replenishment technique.

5.4 Somatic cell for nuclear transfer

Many somatic cell types, including mammary epithelial cells, ovarian cumulus cells, fetal fibroblast cells from skin and internal organs (Cibelli et al., 1998; Du et al., 2002) have been successfully utilized for nuclear transfer. Fibroblast cells are the most common source for donor cells. These cells are easily harvested from either sex and cultured using standard tissue culture conditions. Following nuclear transfer of either actively dividing or quiescent bovine cell cultures, rates of blastocyst development range from 21 to 60 per cent (Zakhartchenko et al., 1999b; Kubota et al., 2000; Hill et al., 2000). In the present experiment serum starved fibroblast cells were used as nuclear donors.

5.5 Conventional and replenishment transfer

For conventional transfer the somatic cell was transferred individually into the perivitelline space of the oocytes through the same slit in zona pellucida made during enucleation using a 15-20 µm inner diameter transfer pipette was used as per the method described by Cibelli et al. (1998).

For replenishment method of SCNT the somatic cell along with ooplasm were transferred together into the perivitelline space of the oocytes through the same transfer pipette. In the present study, a 15-20 µm inner diameter transfer pipette was used as per the method described by Cibelli et al. (1998). It enabled to place the moderate sized viable cells into perivitelline space without difficulties. No comparable techniques for replenishment transfer have been found in literature. This seems to be the first attempt for such replenishment transfer of exogenous ooplasm for improving the developmental potential of SCNT embryos.
Various methods of somatic cell transfer has been reported by various workers like cell transfer into perivitelline space and subsequent fusion (Kubota et al., 2000; Shen et al., 2006; Du et al., 2006), direct intracytoplasmic injection of nucleus (Onishi et al., 2000), direct injection of somatic cell using piezo drill and activation by injection of sperm cytosolic extract in horse (Choi et al., 2002; Lee et al., 2003). The intracytoplasmic injection method has been used to a limited extent to produce embryo and live clone embryo and live calves (Goto, 1997). The commonly used method of nuclear transfer is cell transfer into perivitelline space and subsequent electrofusion. Many successful births of livestock have been reported in different species (Du et al., 2006; Kubota et al., 2000).

5.6 Electrofusion efficiency of normal and replenished SCNT.

Karyoplast–cytoplast couplet, as an important link in nuclear transfer (NT), may partly influence the success rate of SCNT. Compared to other fusion methods, electrofusion is most extensively used, because of lower toxicity, good repeatability and high efficiency.

In the present experiment double pulse and DC electric field strength of 2.0-2.5 kV/cm was used as described by Daniel et al. (2008) for efficient cell fusion. The consistent increased efficiency of fusion in the replenished reconstructed SCNT oocytes (59.50 ± 7.18) may be due to the complete and proper contact of the ooplasm with the somatic cell as seen in Fig. 4d than conventionally reconstructed oocytes (44.57 ± 4.92) as in Fig. 4c, where the contact with ooplasm might be incomplete giving reduced fusion efficiency. Here in replenished method of SCNT, the somatic cell which is being sandwiched between the principal and replenished ooplasm, may be getting maximum contact for efficient fusion.

5.7 Chemical activation and cleavage efficiency of conventional and replenished reconstructed oocyte

In the present study, chemical activation of reconstructed oocytes was done by culturing them in TCM-199 + 20 per cent FBS supplemented with 5 μM ionomycin (Calcium Ionophore) for 5 minutes
followed by an additional 4 hours activation in TCM-199 + 20 per cent FBS supplemented with 2 mM of 6-DMAP.

Ionomycin treatment is thought to mimic the intracellular calcium spike that occurs immediately in the oocytes following fertilization (Alberio et al., 2001). Incubation with a calcium ionophore such as ionomycin followed by exposure to phosphokinase inhibitor 6-DMAP has been successfully used in many species, including successful SCNT in both cattle (Cibelli et al., 1998) and goats (Keefer et al., 2001). Wani et al. (2009) treated the reconstructs 1 hour post-fusion with 5 µM ionomycin followed by exposure to 6-DMAP for 4 hours for the chemical activation of reconstructed oocytes in their SCNT experiment for the production of first cloned dromedary camel.

As per Albeiro et al. (2001), 6-DMAP is believed to induce the activation by preventing the phosphorylation of cdc25, which is normally responsible for activating maturation promoting factor (MPF). Drop in the levels of MPF induces the resumption of meiosis and chromatin condensation.

In the present study, it was seen that replenished reconstructed oocytes gave significantly higher (P<0.05) cleavage rates (25.50 ± 4.23%) than normally reconstructed oocytes (19.84 ± 4.96%).

5.8 Development of conventional and reconstructed SCNT embryos

In the present study, after chemical activation the reconstructed embryos showed varying rates of embryonic development which were classified according to the method of SCNT adopted.

The embryonic developmental rates observed in replenished reconstructed SCNT embryos were significantly higher (P< 0.05) as compared to conventional SCNT embryos for different stages viz. cleavage, 4 cell, 8-16 cell and 16-32 cell stage. A higher number of blastomeres were also recorded in the replenished reconstructed group when compared to the conventionally reconstructed group. This may be due to adequate or increased ooplasmic factors which play a role in the reprogramming of the nuclear donor and development. However, during the stages of embryonic development to the morula/blastocyst stages, although there was a higher rate of development, it
was not significantly different (P< 0.05) between the two groups of SCNT embryos.

5.9 Developmental rates of SCNT embryos achieved in different culture systems

A suitable culture system for supporting the embryonic development in vitro is critical in order to increase the IVEP efficiency. In the present study, two different culture systems were compared for embryonic development i.e. RVCL-BSA medium in comparison to RVCL Blast BSA medium. The embryo growth was and development up to morula/blastocyst observed in RVCL Blast BSA was found to be superior when compared to the RVCL BSA group (P< 0.05) and higher number of blastomeres were observed after Hoechst staining in the RVCL Blast BSA media when compared to RVCL media. The development of morula was 16.06 ± 3.22 per cent in RVCL Blast BSA group vs. 10.56 ± 2.97 per cent in RVCL BSA group. An early blastocyst was observed only in RVCL Blast BSA group (0.48%) with no blastocyst development in RVCL-BSA culture system.

In the present study, development of embryos to blastocyst stage recorded after chemical activation of reconstructed oocytes was poor. Failure to obtain good number of blastocysts in the present study using RVCL or RVCL Blast BSA as a culture media may be due to a variation in acceptability and compatibility of medium for different species. RVCL is a commercial media from Cook’s (Australia) which is basically used for cleavage; however, it has been successfully used for development up to blastocyst stage in buffalo as reported by Shah et al. (2008). The authors reported significantly higher rate of blastocyst development by using RVCL + 1 per cent BSA medium as compared to mCR2 and mSOF media.

From the above study, it seems that although RVCL Blast BSA can be used for blastocyst production in goat the protocol may to be improved by for better production of blastocysts.

5.10 Real time gene expression

Apoptotic gene expression has been demonstrated by Melka et al. (2010) which has been found to affect the developmental potential of
various pre-implantation embryos. During preimplantation development, apoptosis is regulated by activity of pro-apoptotic and anti-apoptotic genes (Bergeron et al., 1998).

β-actin, an endogenous housekeeping gene in the present study, encodes one of six different actin proteins. Actins are highly conserved proteins that are involved in cell motility, structure, and integrity. This actin is a major constituent of the contractile apparatus and one of the two non-muscle cytoskeletal actins. As they are associated with structure and integrity, they are being expresses at constant amounts even under different treatment conditions and thus, may be used as endogenous control for PCR, western blotting and protein degradation studies (Caradec et al., 2010). Therefore, the β-actin housekeeping gene was selected as a control for expression analysis as an endogenous control for both morphologically good and poor quality embryos. In the present experiment, amplification of β-actin was seen both in morphologically good and poor quality embryos.

Various genes like Bax, Fas, Caspase 3, p53, Bcl-xs, etc. have shown to cause apoptosis in pre-implantation embryos when expressed. Caspase 3 was detected in morphologically poor quality embryo group alone in the present experiment. The pro-apoptotic Caspase 3 is associated with DNA fragmentation (Spanos et al., 2002; Walters et al., 2005) and inhibition of Caspase-3 partially prevented DNA fragmentation, which indicated a caspase-dependent apoptosis pathway (Feugang et al., 2003). The Caspase 3 protein is a member of the cysteine-aspartic acid protease family. They are activated in the apoptotic cells both by extrinsic (death ligand) and intrinsic (mitochondrial) pathways. Caspase-3 has also been found responsible for chromatin condensation and DNA fragmentation (Ghavami et al., 2009). Thus it may be inferred that the unevenly developed embryos might have undergone a caspase dependent apoptosis pathway giving a lesser developmental potential.

Various genes like Bcl-w, Bcl-2L1, HSPA1A, ATPase 6, Mcl-1 etc. have shown to cause resistance to apoptosis in pre-implantation embryos when expressed. Mcl-1 expression was seen only in morphologically good quality embryos in the present experiment. Anti-apoptotic Mcl-1 has been
found to increase the survival of cells and over expression confers resistance to apoptosis and targeting Mcl-1 by RNA interference induces apoptosis (Chohessy et al., 2006). Mcl-1 inhibits apoptosis by partially inhibiting the pro-apoptotic protein Bak by binding and rendering it inactive and maintaining it as a monomer (Luo et al., 1998) and also by directly inhibiting Ca\(^{2+}\) signals within mitochondria. Therefore, it may be inferred that the morphologically good quality embryos expressing Mcl-1 may develop further to advanced embryonic stages.

Somatic cell nuclear transferred embryos are more prone to apoptotic changes due to the loss of mitochondria along with the ooplasm. More the loss of ooplasm during enucleation, more the deficiency of ATP occurs. ATP deficiency influences the normal physiological function as well as the embryonic quality (Van Blerkom et al., 1998). Hua et al. (2011) using expression studies on NP1, Cyt b and ATPase 6 genes reported that removal of less than 10 per cent of the cytoplasmic volume did not significantly affect the quality and development of SCNT embryos, whereas a higher degree of loss of ooplasm during enucleation would prove detrimental, thus inducing the apoptosis pathway.
6. SUMMARY, CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK

6.1 Summary and conclusions

Cloning by nuclear transfer from adult somatic cells is a remarkable demonstration of developmental plasticity. The protocol widely used for creation of somatic cell clone has several important steps like, creation and reprogramming of somatic cell line, enucleation of oocyte, transfer of somatic cell in enucleated oocyte, fusion of reconstructed oocyte and chemical activation for embryonic division along with their further development.

In the present study, to increase the quality of SCNT embryos, an attempt was made to produce replenished reconstructed embryos which contain exogenous ooplasm and their comparison with conventionally reconstructed oocytes. The \textit{in vitro} developed embryos were then assessed for quality by counting the number of blastomeres. Gene expression studies were also conducted in order to assess the developmental potential of the morphologically good and poor quality embryos.

The \textit{in vitro} maturation of oocytes was evaluated by cumulus expansion and polar body formation after incubation at $38.5^\circ \text{C}$ in maturation media in CO$_2$ incubator with 5 per cent CO$_2$ and 97 per cent relative humidity. Out of 1176 oocytes set for maturation, 687 oocytes (58.4\%) showed good (grade A) cumulus expansion, followed by 309 oocytes (26.3\%) grade B and 180 oocytes (15.3\%) of grade C. Out of 996 \textit{in vitro} matured oocytes, 702 oocytes (70.48\%) showed the first polar body formation.

In the squashing and compressing technique was applied successfully for enucleation of IVM oocytes that resulted in 70-75 per cent confirmed enucleation after Hoechst staining. These enucleated oocytes were used for both conventional and replenished transfer.

The somatic cell was transferred individually into the perivitelline space of the oocytes through the same slit in zona pellucida made during enucleation using a 15-20 µm inner diameter transfer pipette for conventional
transfer and the somatic cell along with ooplasm were transferred together into the perivitelline space of the oocytes through the same transfer pipette.

In the present study a new concept was developed for replenishment by replenishing about 10-20 per cent of ooplasm from other in vitro matured enucleated oocytes. This was performed by transferring the ooplasm through the already made transfer pipette.

The reconstructed oocytes were fused by applying standard D.C. double electrical pulse of 2-2.5 KV/cm for 10 μs under BTX electro cell manipulator. Out of 367 normally reconstructed oocytes 164 showed fusion which is 44.57 ± 4.92 per cent fusion efficiency. In the replenished reconstructed group, out of 306 replenished reconstructed oocytes 184 oocytes fused revealing a fusion rate of 59.50 ± 7.18 per cent. Thus a significant increase in the fusion rate was seen in the replenished SCNT group.

In this study, the chemical activation of reconstructed oocytes was done by incubating them in TCM-199 supplemented with 20 per cent FBS and 5 μM calcium ionophore for 5 minutes at 38.5ºC followed by another 4 hours activation in TCM-199 + 20 per cent FBS supplemented with 2 mM 6-DMAP in CO₂ incubator. The cleavage and initial development rate of 4 cell stage up to 16-32 cell stage of replenished reconstructed embryo group were significantly higher (P<0.05) when compared to normal reconstructed group. Even though there was an increase in the development of morula (15.09 ± 5.19% vs 12.70 ± 3.70%) it did not differ significantly (P<0.05) between the groups.

The developmental rate of conventional and replenished SCNT embryos was made under two different culture medias. During the advanced developmental stages, especially the embryonic developmental rate from 16-32 cell stage to morula, RVCL Blast BSA culture system proved to be more supportive significantly(P<0.05) than the RVCL culture system.

The number of blastomeres developed during embryo culture were assessed by staining the 4th day embryos. On observing under fluorescence microscope, a significantly higher number of blastomeres
(P<0.05) were recorded in replenished SCNT group (27.00 ± 0.74) as compared to conventionally produced SCNT embryos (22.19 ± 0.91). The number of blastomeres observed in embryos developed in RVCL Blast BSA media (27.06 ± 0.68) was significantly higher (P<0.05) as compared to embryos developed in RVCL BSA group (23.13 ± 0.94).

Real Time PCR carried out with ABI prism® 7300 SDS revealed an amplification of the endogenous control, β- actin in both the pools of morphologically good and poor quality SCNT embryos. For all the genes taken for study, faint bands were observed. An amplification of the endogenous control, β- actin was observed in both the pools of morphologically good and poor quality SCNT embryos. Caspase-3 amplification was observed in poor quality embryo group which was absent in good quality and Mcl-1 expression was recorded in morphologically good quality SCNT embryo group and was absent in poor quality embryo group suggestive of advancement of developmental potential.

6.2 SUGGESTIONS FOR FURTHER WORK

i. Development of transgenic cloned goat with improved meat quality.

ii. To study the expression of various genes to unravel the mechanism of growth, differentiation and identification of markers for developmental potential ranging from immature oocyte to blastocyst.

iii. Extensive investigation of specific molecular signatures that can be used to evaluate SCNT embryos.

iv. Development of transgenic cloned goat as a bioreactor for the production of important pharmaceutical drugs and nutraceuticals.