CELL CYCLE IN EARLY MOUSE EMBRYOS: IMPLICATIONS FOR NUCLEAR TRANSFER

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DECLARATION

I declare this thesis is my own composition and has not been submitted for application to any degree previously. The work and ideas contained herein are my own, unless otherwise stated. I acknowledge all help and assistance provided to me during the course of this study.

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ABSTRACT

The aim of the project was to optimize cell cycle co-ordination in murine embryos reconstructed by nuclear transfer. The project also involved studies of oocyte activation, cell cycle length and synchronization, evaluation of MPF, and DNA replication in early embryos.

Embryos were successfully arrested at mitosis by culturing them up to 16 hours with 2.5 mM 6-dimethylaminopurine (DMAP). However, the reversibility and the development after release was affected when exposed for more than 9 hours. A comparative study of different concentrations of 6-DMAP and 10 μM nocodazole for holding the embryos at mitosis for 12 hours showed a greater effectiveness of nocodazole. Treatment of embryos for synchronization with different concentrations of Taxol for different time periods was not successful because of its toxicity affects. Nocodazole was used as a method of synchronization in subsequent studies.

In most of the experiments 25 mM strontium chloride was used as the method for the artificial activation of cytoplasts and reconstituted embryos. Later on, a new method for parthenogenetic activation of metaphase II oocytes or recipient cytoplasts was established. The later method involved the exposure of metaphase II oocytes or recipient cytoplast to 25 mM strontium chloride for 60 minutes with subsequent exposure to 2.0 mM 6-DMAP for 3 hours. The proportion of parthenogenetically activated metaphase II oocytes that reached blastocyst stage was greater (P< 0.001) than using strontium chloride alone. The new protocol was better than the protocol using 5 μM Ionomycin for 5 minutes with subsequent exposure to 2.0 mM 6-DMAP. Moreover, when embryos were produced by transfer of 4-cell donor nuclei into recipient cytoplasts which were pre-activated with strontium chloride and 6-DMAP (25 mM strontium chloride (1 hour) + 2.0 mM 6-DMAP (3 hours)), an enhanced development to morula/blastocyst was achieved compared to the use of strontium alone.

The effect of meiosis/mitosis/maturation promotion factor (MPF) on the development of embryos reconstructed at different times in relation to activation was investigated and its level was studied by looking at nuclear envelope breakdown (NEBD) and nuclear envelope breakdown and premature chromatin condensation (NEBD&PCC). The first cell cycle length of the enucleated and non-enucleated parthenotes was also studied. In the absence of nuclear material, the cell

cycle in parthenotes was reduced by 5 hours. The lower development of the embryos reconstituted at different time in relation to activation by using 4-cell donor nuclei at G1 or G1/S to blastocyst suggested the need to re-investigate the duration of G1-phase because it was suggested that cell cycle stage was incorrectly classified.

After determining the timing of G1 duration and S-phase initiation of 3rd and 4th cell cycle, nuclear transfer experiments were performed to investigate the effect of nuclear donor cell cycle stage and its interaction with recipient cell cycle stage. Two methods for determining the duration of G1 and start of DNA replication in 3rd and 4th cell cycle were established. The start of DNA replication was detected by the technique of pulse labeling for proliferating cell nuclear antigen PCNA or by incorporating 5-bromodeoxyuridine (BrdU) by rapid pulsing. Embryos were then reconstructed either by using 2-cell donor nuclei at G2/M phase or 4-cell donor nuclei at G1, S and G2/M stage of cell cycle with MII cytoplasts or pre-activated cytoplasts. Further more, the embryos were reconstructed by transferring 2-cell donor nuclei at G2/M stage and 4-cell donor nuclei at G1/S, S and G2/M phases of cell cycle into metaphase II cytoplasts and then delaying activation by 4 hours. Delayed activation in embryos reconstructed using G2/M stage of cell cycle improved development to morula/blastocyst stage to the same proportion achieved with G1 donor nuclei transferred to MII cytoplasts.

The studies confirm the importance of cell cycle co-ordination in the development of reconstructed embryos. By appropriate co-ordination of cell cycle stages it has been possible for the first time to obtain similar development with 4-cell blastomeres with donor cell nuclei at any stage of the cell cycle . Moreover, the use of strontium chloride + 6-DMAP activated cytoplasts with 4-cell donor nuclei from different cell cycle can produce embryos that will develop with a higher frequency to morula/blastocyst than strontium chloride alone.

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ABBREVIATIONS

AI	Artificial Insemination
AI/NM	Artificial Insemination/Natural Mating
BPL	ß-propiolactone.
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
CB	cytochalasin B
6-DMAP	6-Dimethylaminopurine
DMSO	dimethyl sulphoxide
eCG	pregnant mare's serum gonadotrophin
ET	Embryo Transfer
FITC	fluorescein isothyocyanate
gM16	modified M16 culture media
GV	Germinal vesicle
HAU	haemagglutinating unit
HBSS	Hank's Balanced Salt solution
hCG	human chorionic gonadotrophin
hpa	hours post activation
hpf	hours post fusion
ICM	inner cell mass
IU	international unit
MPF	maturation / mitosis / meiosis promoting factor
NEBD	Nuclear envelope break down
PB	polar body
PCC	premature chromatin condensation
PCNA	proliferating cell nuclear antigen
pM16	modified M16 culture media
PN	pronucleus
sM16	Modified M16 culture media
UV	ultraviolet
-ive	Negative
+ive	Positive

CHAPTER 1

INTRODUCTION

Historically, animal scientists have been quick in taking up the advantages of new biotechnological techniques for the propagation of economically superior animals. In the past few decades, the spread of superior germ plasm of carefully selected proven bulls by artificial insemination has played a vital role in rapid genetic improvement. This technique allows each bull to have its genetic contribution to thousands of offspring but each cow can only produce 5 or 6 calves in her lifetime. In later years, the use of multiple ovulation embryo transfer (MOET) and cloning by embryo splitting have been developed which partially compensated this imbalance.

During recent years, the establishment of nuclear transfer procedures has provided new opportunities, at least in principal, to multiply large number of genetically superior animals. Previous experience with artificial insemination and multiple ovulation/embryo transfer suggest that it may be at least 10-20 years before this could become a routine procedure in livestock production. The techniques of nuclear transfer can be used in cattle and pigs because it is probably only in these species that the benefits are likely to justify the cost. Non surgical procedures of transferring embryos would need to be developed to keep the procedure simple on the farm and success rate has to efficiently improved.

In recent years since the introduction of techniques for cloning in domestic animal (Willadsen, 1986; Prather et al., 1987), embryo cloning has moved from experimental tool to its commercial application. Nicholas and Smith (1983) evaluated the genetic value from breeding, selection, and large scale commercial use of cloned dairy cattle and demonstrated its considerable potential to increase genetic gain. The cloned animals are expected to be more uniform than the random-bred animals and provide greater opportunity for investigating genotype-treatment interactions in research. For traits such as milk production, one monozygotic twin could replace more than 20 random-bred animals in the experiment. However, the study of Wilson et al., (1995), on the comparison of birth weight and growth characteristics of bovine calves produced by nuclear transfer (cloning), embryo transfer and natural mating suggested

that the variability in the birth weight for CLONE calves was four to 12-fold greater than for ET or AI/NM calves. The cause of this variability is not known.

In animal breeding, genetic progress is dependent on exploiting genetic variation and cloning has it's limited use within breeding programms. Some traits of economical importance are greatly influenced by environment and it is often difficult to assess the genetic merit of an individual animal. In cases like mastitis in dairy cattle , it might be beneficial to clone several copies of elite animal to accurately assess her susceptibility to these kinds of conditions. Cloning could also be integrated into breeding strategies aimed at multiplying endangered indigenous breeds of livestock, in order to conserve the genetic diversity of animals adapted to specific environments. The ability to produce large numbers of genetically identical animals would also have important benefits in experimental design. Genetic variations between individuals often alter the responses to test the diet and drugs. In those experiments, which require the slaughtering of animals to assess the carcass traits, the multiple number of genetically identical animals would be helpful. In addition to decreasing the number of animals required for an experiment, it is possible to investigate the interactions between genotype and various treatments for the first time (Biggers, 1986). Individuals reconstructed with identical nuclear genomes, but with different cytoplasmic environments also provide greater opportunities for evaluating the cytoplasmic inheritance and nuclear-cytoplasmic communication.

The ultimate aim for most of the commercial embryo cloning projects is the production of a large number of genetically identical animals from a superior embryo. One way to achieve this goal might be the implementation of multiple generational cloning. This procedure assumes that the success rate for each generation of cloning remains constant. However, very little information has been reported on the success rate of cloning in mammals. Stice and Keefer, (1993) reported the production of 54 genetically identical embryos that developed to morula stage from one parent embryo by recloning. Another way to get large number of genetically superior identical animals is the use of different cell lines as source of nuclei for transfer. In the mouse, it is possible to establish cell lines by the culture of embryonic cells in such a way that they divide but do not differentiate (Evans and Kaufman, 1981).

The production of live lamb "Megan and Morag" by nuclear transfer from cells of early embryos that had been cultured for several generations laid down a mile stone in history of nuclear transfer (Campbell *et al.,* 1995). This major breakthrough came because of the induction of quiescence in the donor cells. Later on the birth of Dolly

from an adult cell (Wilmut *et al.*, 1997) has opened a new era in developmental biology. The ability of nuclear transfer procedures to derive live animals from cultured cells provides an alternate way of producing transgenic farm animals. Moreover, the ability to manipulate millions of cells at once opens up the possibility of much more specific genetic modifications, including the deletion or substitution of specific genes or the introduction of the single letter changes in the whole genetic code that are typical of many human genetic diseases. At present transgenic sheep, goat and cattle are already being used to produce human proteins in milk. The technique of nuclear transfer will provide a more reliable way of producing transgenic animals, reducing the number of animals needed to establish each transgenic line. By using donor cells from established cell cultures with specific genetic modifications, this technique provides its future applications in the successful production of xenotransplants and nutrichemicals. At present, mice in which specific mutations have been deliberately introduced have often provided very useful model for studying human genetic diseases such as cystic fibrosis and obesity. The differences between mice and humans mean the effects of introduced mutation are not the same as in the human genetic disease. Nuclear transfer will extend the range of species in which gene targeting will be possible and thereby provide better models to test treatments for human diseases. In diseases like Parkinson's and leukemia, intact cells are used to treat the patients. These cells have to be obtained from the close relatives to avoid the problems to immune rejection. As the research will advance in the understanding of the process of reprogramming of the differentiated cells into all the cell types, the patient's own cells would be removed , converted into the desirable cell type in the laboratory and reintroduced into the patient for treatments. The technique of cloning using cultured cell also provides tools for future research in the fields of ageing & cancer.

During recent years significant progress has been made in understanding the interaction of cell cycle stages of donor nuclei and recipient cytoplasm in reconstructed embryos by nuclear transfer. This understanding has led to an increase in the frequency of development and also to development from later developmental stages. In reconstituted embryos the transplanted nucleus must fulfill a number of roles;

* firstly the nucleus must be able to respond to the new cytoplasmic environment in such a way that its genetic material remains undamaged,

* secondly the genetic information present must be able to redirect development of the reconstituted embryo.

The present project was thus planned to optimize cell cycle co-ordination in the development of murine embryos reconstructed by nuclear transfer. In the start of this project (1994), the hypothesis was that there is cytoplasmic state in MII cytoplasts after activation which could support development of embryos reconstructed with donor nuclei from any stage of cell cycle. The hypothesis of higher development after nuclear transfer from G1 donor was also tested. The efficiency of lower development in embryos reconstructed using different cytoplasmic environments of cytoplasts, suggested the need to re-investigate the length of the cell cycle and to map the cytoplasmic activity of MPF(Chapter 6).

To conduct experiments for evaluating the cell cycle co-ordination in the development of reconstructed embryos, there was need to synchronize the donor cell cycle. The experiments were also conducted to establish proper method for the synchronization of cell cycle (Chapter 4). The experiments in chapter 7 were conducted to test the hypothesis of a window of opportunity in the donor cell cycle; the DNA synthesis initiation timings were investigated. In chapter 8, the experiments were conducted using three nuclear transfer protocols to study their effect on reprogramming of donor nuclei. In the end of this project it was felt that activation stimuli used in previous experiments was not enough to create a cytoplast as "universal recipient". The experiment described in chapter 5 were conducted to establish proper activation protocol.

CHAPTER 2

REVIEW OF LITERATURE

The idea of nuclear transfer as a tool to investigate the potential of nuclei from differentiated cells was first proposed by Spemann (1938). He proposed nuclear transfer experiments by transferring nuclei from embryos more advanced in development into cytoplasts to study their ability to direct development. Briggs and King (1952) adopted this idea of performing nuclear transfer experiments after Spemann's death in 1941. They performed nuclear transfer experiments in amphibians in which donor nuclei were injected into cytoplasts of enucleated parthenogenones of *Rana pipiens*. It was demonstrated that the embryonic nuclei could replace the nuclei of fertilized ova and result in development. The cloning procedure as described by Briggs and King (1952) is as follows:

--- activation of the recipient oocyte, the process of initiating in the absence

   of fertilization

--- enucleation of the recipient ovum ; and

--- transfer of a nucleus intact, lysed blastomere to the enucleated ovum.

The detailed procedure has been described by Elsdale *et al.,* (1960) and Gurdon and Laskey (1970). By using slightly different methods of nuclear transplantation in *Xenopus,* Gurdon and his colleagues have obtained results suggesting that the nuclei of some differentiated cells may retain totipotency which progressively loss during increased development. Using the procedure of serial transplantation (which involved placing an intestinal nucleus into an egg and, when the egg had become a blastula, transferring the nuclei of the blastula cells into several more eggs), increased rate of development (7%) to the feeding tadpole stage was demonstrated (Gurdon, 1962). Moreover, seven of these tadpoles metamorphosed into fertile adult frogs (Gurdon and Uehlinger, 1966); these nuclei were totipotent. Kings and his colleagues, however, criticized these experiments, pointing out that (1) not enough precautions were taken to make certain that primordial germ cells-which

migrate through and often stay in the gut - were not used as source of nuclei, and (2) the intestinal epithelial cell of such a young tadpole may not qualify as a truly differentiated cell type. Such cells of tadpole still contain yolk platelets (DiBerardino and king, 1967; Mckinnell, 1978; Briggs, 1979). To answer these criticisms, Gurdon and his colleague cultured epithelial cells from adult frog foot webbing. When nuclei from these cells were transferred into activated, enucleated *Xenopus* oocytes, none of the first generation transfers progressed further than the formation of the neural tube shortly after gastrulation. By serial nuclear transplantation, however, numerous tadpole were generated (Gurdon *et al.*, 1975). Although these tadpole died prior to feeding, a single differentiated cell still retained incredible potencies. A single nucleus derived from an adult frog red blood cell (which neither replicate nor synthesizes mRNA) can undergo over 100 divisions after being transplanted into an activated oocyte and still retain the ability to generate swimming tadpoles (Orr *et al.*, 1986; DiBerardino, 1989). Although DiBerardino (1987) has observed that " to date, no nucleus of a documented specialized cell nor of an adult cell has been shown to be totipotent," such a nucleus can still instruct the formation of all the organs of a swimming tadpole.

In principle the procedure of Briggs and King (1952) is similar to that used today in mammals. The method of non-invasive ovum enucleation and use of fusogenic agent for blastomere fusion by McGrath and Solter (1983a) has created the revolutionary opportunities in animal breeding and research. The nuclear transfer procedure involves pre-treatment of recipient ova with Cytochalasin. This compound causes the recipient plasma membrane to become more elastic, making the recipient ovum more conducive to manipulation without lysis. The zona pellucida can be circumvented, either by making a cut with a glass knife to allow the insertion of a blunt enucleation pipette (Willadsen, 1986); or a sharpened bevelled pipette can be used to puncture it and subsequently enucleate the ovum (McGrath and Solter, 1983a).

2.1-PROCEDURES OF NUCLEAR TRANSFER IN MAMMALIAN SPECIES

In mammalian species there are two predominant methods of nuclear transfer which varies upon the point at which the recipient cell is enucleated. Both metaphase II (MII) oocytes and one cell zygote have been considered as recipients for nuclear transfer. Metaphase II (MII) oocytes which are to be enucleated are cultured in medium containing the microfilament inhibitor Cytochalasin D or B and

specific fluorochrome Hoechst 33342. The metaphase plate is removed by aspirating a small amount of cytoplasm from directly beneath the 1st polar body. The enucleation is confirmed by exposing the aspirated portion of cytoplasm to UV light for the presence of both polar body and the metaphase plate. Similarly, zygotes are cultured in medium containing Cytochalasin D or B with the addition of microtubule inhibitor colchicine. In mouse the pronuclei are visible under DIC optics and can be removed by aspiration. However, in ungulate species the zygotes have to be centrifuged to visualized the pronuclei. After enucleation the recipient cell is then ready to receive the donor cell (karyoplast). Donor cells (karyoplasts) can be derived from a variety of embryonic cell stages ranging from the 2-cell stage to the blastocyst stage (McGrath and Solter, 1983b; Robl *et al.*, 1986; Robl *et al.*, 1987; Prather *et al.*, 1987; Willadsen, 1986; Tsunoda *et al.*, 1988; Smith and Wilmut, 1989; Stice and Robl, 1989; Prather *et al.*, 1989; Bondioli *et al.*, 1990). Now a donor cell (karyoplast) is aspirated into the enucleation pipette, the pipette is inserted through the hole previously created in the zona pellucida and the karyoplast expelled into the perivitelline space. The karyoplast is then placed in close contact with the recipient cell or cytoplast. The karyoplast (donor cell) and recipient cell or cytoplast are ready for fusion. Fusion may be performed in two ways. Sendai virus ranging from 1000 to 9000 hemagglutination units (HAU) successfully fuses blastomeres and enucleated ova in sheep and mice (Graham, 1969; McGrath and Solter, 1983a; Robl *et al.*, 1986; Willadsen, 1986). Fusion may be induced by electroporation in cattle, pigs and rabbits as well as in sheep and mice. This is performed in a non ionic solution such as 0.3 M mannitol in distilled water and a fusion chamber consisting of two electrodes, ranging from 200 μm to 1 mm apart (Kubiak *et al.*, 1985; Robl *et al.*, 1987; Willadsen, 1986; Smith and Wilmut, 1989). The use of electric pulse for fusion also induces activation of MII oocytes (cytoplast) in most of the situations.

The establishment of these procedures have played a very important role in successful production of embryo clones in cattle, sheep, rabbit, pigs and mice (Prather *et al.*, 1987; Willadsen, 1986; Stice and Robl, 1989 ; Prather, 1989 ; Tsunoda *et al.*, 1987). Similar procedures of nuclear transfer have been used during that period to investigate the regulation of early development in mice (e.g. Surani *et al.*, 1987) and in livestock species for fundamental research (for review see Barnes *et al.*, 1990).

Amphibian eggs and embryos have , for a long time , been favoured material for analysis of problems in embryology primarily because of their large size

makes manipulation seem easier, and external fertilization and development makes observation straightforward. Wilson (1896) studied the early cell lineage's and proposed that nucleo-cytoplasmic interactions were important in inducing gradual changes in cell function. The most obvious change in cell activity between oocyte and early embryo is the resumption of the cell cycle.

2.2 THE CELL CYCLE

The cell cycle is the period during which events required for successful reproduction are completed. Two major events considered to be common to all cell cycles are S-phase (Laskey *et al.*, 1989), when chromosomes are replicated (DNA synthesis) and M-phase (McIntosh and Koonce, 1989), when the replicated chromosomes are segregated into two daughter cells (Mitosis). These two easily detectable components of nuclear activity are divided by a post-mitotic gap (G_1) and a second gap (G_2) between DNA synthesis and mitosis. Prescott (1976), and Smith and Martin (1973) studied the duration of these phases and reported that though all phases vary in length, but post embryonic cells show greatest variation in the length of G_1, both between cell type and within one population. In amphibians, the first embryonic cell cycle lasts 75 minutes and is followed by 11 synchronous cell cycles, each about 30 minutes. The early embryonic cell cycle of amphibians during cleavage is dramatically different in many ways.

Firstly, during early synchronous cleavage, G_1 and G_2 are largely suppressed (Graham and Morgan, 1966) which reflects the absence of growth in early embryos.

Secondly, the cell cycle is extremely rapid with cleavage recurring every 35 minutes (Newport and Kirschner, 1982).

Somatic cells are born small and must import nutrients so that they can grow and duplicate all the contents of the cell. On the other hand , eggs, however, are large and inherit with them a stock of nutrients from their mother, all the structural components of cells, and almost all the enzymes that catalysis the processes of DNA synthesis and mitosis (e.g. histones, Woodland and Adamson, 1977; DNA polymerase, Benbow *et al.*, 1975; tublin, Pestell 1975).

Thirdly, the recurrence of surface contraction wave in each cell cycle (Hera *et al.*, 1980). These waves have been interpreted as the autonomous cytoplasmic oscillator which directs the orderly repetition of DNA synthesis and mitosis. The

same type of cytoplasmic oscillators are also found in adult cell cycle (Klevecz, 1976).

2.2.1- REGULATION OF CELL CYCLE

Xenopus and starfish have been the main species whose oocytes were widely used to study the regulation of cell cycles (Maller, 1990; Meijer and Guerrier, 1984). These immature oocytes undergo meiotic maturation following the stimulation of harmones. Newport and Kirschner, (1982) have reported that the cell cycles of these embryos after fertilization were composed of S and M phases without any detectable G_1 and G_2 phases. However, the regulation of the cell cycle in mammalian oocytes seems to be different from that in Xenopus and starfish oocyte. The mitotic cell cycle of mammalian oocytes after fertilization is composed of all the four phases i.e. G_1, S, G_2 and M (Abramzuk and Sawichi, 1975). The progression of cell cycle in these phases depends on the "checkpoint" control (Hartwell and Weinert, 1989) operating at the two principal decision points of the cell cycle: $G_1 \Rightarrow S$ (DNA synthesis initiation) and $G_2 \Rightarrow M$ (mitotic induction). Van't Hof (1985) illustrated the design features of cell cycle control system to describe the progression and points of control in the mitotic cell cycle (Fig. 2.1). These transitions are regulated by a combination of transcriptional, post translational, and quaternary protein association mechanisms. The transcriptional controls are more prominent in the $G_1 \Rightarrow S$ than in the $G_2 \Rightarrow M$ transition.

During last 20 years, extensive biochemical and genetic studies have provided a better understanding of the regulation and control of the normal cell cycle in eukaryotes. The frog oocyte and eggs have been widely used in these studies to identify the key regulator of the cell cycle. The fully grown frog oocyte is arrested at GV, preceding the first meiotic division. It is induced by the release of progesterone from the follicle cells to proceed rapidly through meiosis 1 and prophase of meiosis II before its arrest at metaphase of meiosis II. Fertilization relieves the metaphase arrest and initiates a series of rapid, nearly synchronous cell cycles that proceed without any detectable G1 and G2 phases until there is an abrupt change to a more complex and asynchronous cell cycle after 12 division (Newport and Kirschner 1982).

Although the initial studies of meiotic maturation were focused on extra cellular signals like progesterone, a major innovation was the use of cytoplasmic transfer by microinjection to identify the intracellular regulators of meiosis. Studies

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of Masui and Markert (1971) and Reynhout and Smith (1974) revealed the presence of cytoplasmic activity (not progesterone) that could induce the meiotic maturation in immature oocytes. They named this activity maturation promotion factor or MPF.

Fig: 2.1 DESIGN FEATURES OF CELL CYCLE CONTROL SYSTEM
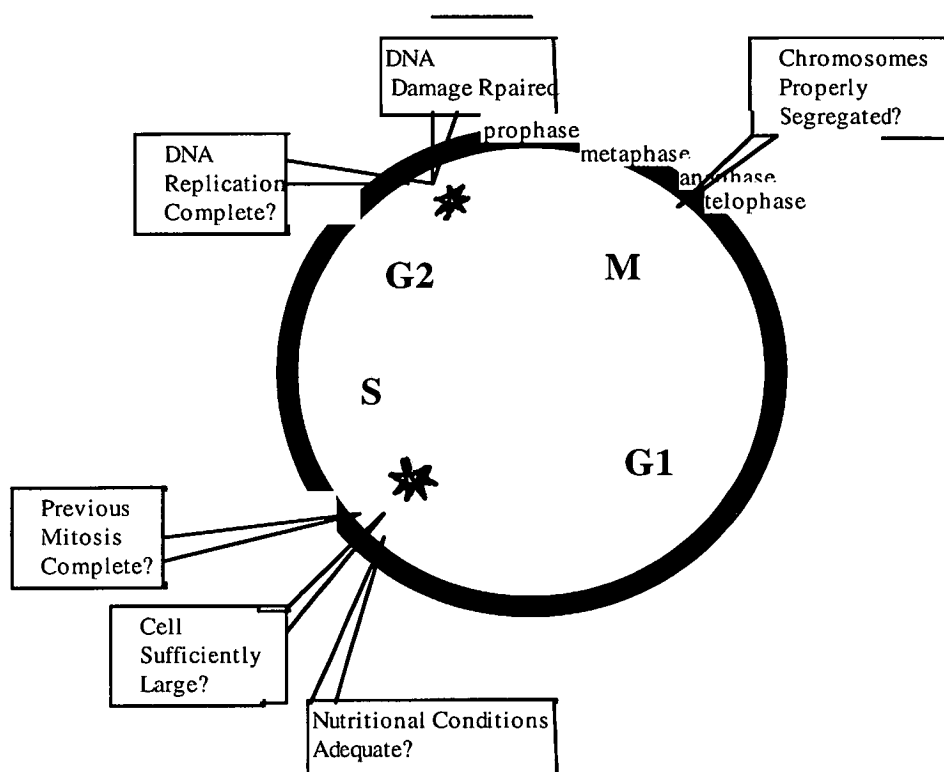
Fig. 2.1 Points of control in the mitotic cell cycle. Cells monitor essential conditions, primarily in late G1 and G2, but also elsewhere in the cell cycle before entering the next phase of the cycle. Labelled check marks indicate six points at which control systems operate to prevent cell cycle progress in the absence of a positive response to the questions posed in the boxes. Large asterisks within the circle indicate principal control points of the plant cell cycle(Van't Hof, 1985).

MPF also stand for mitosis and meiosis promoting factor, a name that more generally describes its widespread role in cell cycle. Oocytes can be induced to mature by injecting cytoplasm from mitotically arrested mammalian cells, suggests MPF existence in wide range of cell types. After meiosis 1 MPF activity

disappeared, but reappeared and was maintained at high levels as the oocyte entered and was stably arrested at, second meiotic metaphase in the unfertilized egg (Gerhart and Kirschner, 1984), suggesting that MPF is associated with the metaphase state. The discovery that MPF activity rises and falls in the meiotic and mitotic cell cycles of frog eggs strengthened the suggestion that MPF plays a key role in regulating the cell cycle. Further, experiments with enucleated oocytes proved that both the appearance of MPF and its disappearance during activation were purely cytoplasmic events independent of GV.

Maturation Promotion Factor (MPF) is central to cell cycle regulation. During meiosis the activity of MPF increases sharply before germinal vesicle breakdown (GVBD), remains high throughout first metaphase, declines slightly during anaphase and telophase and increases again at metaphase II. After activation of the egg by the fertilizing sperm MPF activity falls to basal levels (Fulka et al., 1992). High levels of MPF, seem to be needed around the time of sperm penetration in order to remove the sperm nuclear membrane (Peter et al ., 1990) and the breakdown of sperm nuclear envelope is required for the formation of male pronucleus. Recently Naito et al. (1992) reported that a high H1 kinase (MPF) activity of pig oocyte at the end of in vitro maturation was followed by a high incidence of male pronuclear formation (73.5%) after in vitro fertilization as compared with that (10.6%) of oocytes with low-level activity. So the elevated level of MPF at the time of sperm entry plays an important role in the formation of the pronuclei while the subsequent decline of MPF allows the progression of the zygote into the G1-phase of the first mitotic cell cycle .

MPF has been identified as a complex of two sets of proteins, cyclins (Swenson et al., 1986; Labbe et al., 1989) and p34^{cdc2} (Dunphy et al., 1988; Labbe et al., 1989). p34^{cdc2} is a protein kinase whose activity is measured by using exogenous histone H1 as a substrate. p34^{cdc2} is essential for the transition from G1 to S and G2 to M phase in the cell cycle. Activation of the p34^{cdc2} kinase requires cyclin (Murray et al., 1989), and the kinase activity of p34^{cdc2} cyclin complexes is regulated by the phosphorylation state of both components (reviewed by Clarke and Karsenti, 1991).

There are now known to be a number of proteins whose activity varies according to the phase of cell cycle. The first to be identified is cyclin. Cyclin is a prominent protein of 56 kD, which accumulates until the first mitotic division and then disappear at the end of mitosis. This protein accumulates in the next interphase

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and again disappear at mitosis. Because of its cyclic appearance, this protein was named as cyclin. In the late interphase, $p34^{cdc2}$ makes complex with cyclin and at G2/M transition, dephosphorylation of the complex provides $p34^{cdc2}$ with histone H1 kinase activity, generating active MPF. Similarly, $p34^{cdc2}$ is kept in the dephosphorylated active form during metaphase II arrest in oocytes. The kinase activity of MPF is believed to trigger a cascade of reactions leading to nuclear envelope breakdown, chromosome condensation, spindle formation (reviewed by Lewin, 1990), and, thereby, entry into and maintenance of second meiotic arrest.

In mouse as in most of other vertebrates, oocytes are arrested at the second meiotic metaphase by cytostatic factors (CSF) (Masui and Markert, 1971). CSF prevents ubiquitin-dependent degradation of mitotic cyclins and thus inactivation of MPF (Glotzer *et al.,* 1991; Murray *et al.,* 1989). Two components of CSF have been identified so far: Mos, the product of c-mos proto oncogene (Sagata *et al.,* 1989), and cyclin dependent kinase 2 (Cdk2) (Gabrielli *et al.,* 1993). The product of the oncogene *mos* is expressed early in oocyte maturation and disappears immediately after fertilization (Sagata *et al.,* 1989; Watanabe *et al.,* 1989). The Mos protein has the same effect as CSF in arresting mitosis at metaphase with high $p34^{cdc2}$ activity. It is believed that CSF is, in part or entirely, Mos and the second meiotic arrest is due to the transcription of *mos* as the oocyte matures. Mos will also trigger frog oocyte maturation (Sagata *et al.,* 1988). These observations suggest that it is Mos that need to be synthesized to take the oocyte through the G2/M transition (Minshull, 1993). However, in mouse oocytes that lack Mos, GVBD occurs normally (B. Maro, unpublished). Mos may be activating MAP kinase (Nebreda and Hunt, 1993). MAP kinase itself has CSF activity. MAP kinase is a serine / threonine kinase activated early in mitogenesis in response to growth factors (Thomas, 1992, Ruderman, 1993, Marshall, 1995). Originally called MAP kinase because it phosphorylated a MAP (microtubule-associated protein), it now represents mitogen-activated protein kinase. The study of Whitaker, (1996) indicates that MAP kinase is activated during oocyte maturation and that it regulate meiosis. In mouse oocytes it is activated at first meiosis, its activity remains high throughout oocyte maturation and during the second meiotic arrest at metaphase II (Kubiak *et al.,* 1993). Its activity falls after fertilization during the first mitotic cycle as the pronucleus forms. In mouse oocytes, It has been suggested that MAP kinase is responsible for maintaining condensed chromatin and suppressing S phase during the second meiotic division, taking over from $p34^{cdc2}$, the content of which decreases in the interval between meiosis I and meiosis II (Verlhac *et al.,* 1994).

In general, the calcium signalling system is responsible for breaking meiotic arrest, although there are some exceptions. The fertilization calcium signal is sufficient to trigger release from the metaphase II block in mature oocytes. Mollusc and ascidian oocytes arrest in metaphase I and frog and mammalian oocytes pause in metaphase II. In all these oocytes, fertilization sets off a calcium transient that is the trigger that releases meiotic arrest (Igusa and Miyazaki, 1983; Busa and Nuccitelli, 1985; Speksnijder, 1992; Galione et al., 1993; Whitaker and Swann, 1993). The Ca^{2+} transients stimulated at fertilization continue until the formation of pronuclei in the one-cell mouse embryo (Jones et al., 1995). The fertilization calcium transient activates calmodulin, which activates CaMKinase II, which activates cyclin destruction and degradation of Mos (CSF). There are three points to note, however. First, no link has been established between CaMKinase and cyclin destruction machinery. Second, cyclin content falls before Mos content declines (Watanabe et al., 1991; Lorca et al., 1993). Third, it is still unclear how the fertilizing sperm starts the Ca^{2+} transient.

Cyclin are destroyed by controlled proteolysis. They are tagged with ubiquitin and presented to the proteasome, a large multifunctional protease. Cyclins have their own ubiquitin-conjugating enzymes in oocytes (Hershko et al., 1994; King et al., 1995) and so the most regulatory pathway would involve activating these enzymes. However, the proteasome itself is also regulated and peaks of activity coincide spatially and temporally with mitosis in the ascidian oocyte (Kawahara et al., 1992). So the proteasome may also be a direct or indirect target of CaMKinase II (Whitaker, 1993).

In Xenopus, cyclin has disappeared long before Mos, although the loss of both seems to be due to activation of CaMKinase (Lorca et al., 1993). Cyclin falls before Mos in mouse, too (Kubiak et al., 1993). The calcium transient seem to override the effect of Mos. Despite the fact that calcium increases explosively in the oocytes at fertilization, its effect are subtle and depend on the integrity of the mitotic spindle. This behaviour may be related to the fact that calmodulin and CaMKinase II are localized to mitotic (and presumably, meiotic) spindle.

The calcium-calmodulin signalling pathway is a candidate for the role of controlling anaphase onset and cyclin destruction in both oocyte and somatic cells (Whitaker and Patel, 1990; Lorca et al., 1993; Lu and Means, 1993). One possible effect of Mos/MAP kinase may be to suppress the endogenous mechanism that generates Ca^{2+} transient (Ciapa et al., 1994), forcing the oocyte to wait for large

Ca^{2+} transient triggered by the sperm. It is also hypothesized that p34^{cdc2} activates the cyclin proteolysis pathway directly (Felix et al., 1990).

Repeated calcium pulses are thought to be essential for normal development in mammalian oocytes (Ozil and Swann, 1995). There is correlation between p34^{cdc2} activity and Ca^{2+} oscillations in mouse oocytes. InsP$_3$ induces repetitive Ca^{2+} pulses in metaphase oocytes but only a single Ca^{2+} transient in oocytes when injected between meiosis I and II (Jones et al., 1995). The correlation between p34^{cdc2} and Ca^{2+} oscillations may be explained by the observations of Kono et al., (1995), that transplanting an interphase nucleus from the zygote generates Ca^{2+} oscillations in unfertilized mature oocytes. The Ca^{2+} transients begin when the nuclear envelope of the transplanted nucleus breaks down under the influence of the high p34^{cdc2} activity in the unfertilized oocytes. This may be due to the an oscillogen which is sequestered into the nucleus during interphase and is released when the nuclear envelope dissolves during mitosis.

Oocytes arrested at both first and second meiotic metaphase all show a calcium wave at fertilization (Whitaker, 1996). The wave is carried across the egg by calcium-induced calcium release (CICR) mediated by InsP$_3$ (Galione et al., 1993; Miyazaki et al., 1993) and ryanodine (Swann, 1992; Galione et al., 1993; Ayabe et al., 1995) receptors.

Upon fertilization, a diffusible, activating messenger may pass from sperm to egg after sperm-egg fusion. It has not been identified and could be any, or a mixture, of InsP$_3$, calcium, cGMP and cADP ribose (Whitaker and Swann, 1993). Or it could be a protein. Swann, (1993) has identified a cytoplasmic protein found in the equatorial segment of the sperm head that will trigger Ca^{2+} transients when microinjected into mature mouse and hamster oocytes.

From the above review, it is concluded that oocytes need p34^{cdc2} activation to go into meiotic metaphase and p34^{cdc2} inactivation to procede through anaphase into the next cycle. They need Mos/MAP kinase activation to induce second meiotic arrest. But the connection at molecular level between Mos/MAP kinase and p34^{cdc2} is unclear. In mature oocytes, the evidence for calcium signal that breaks the second meiotic arrest is strong. Fertilization or artificial activation releases the oocyte from meiotic arrest by inactivation of MPF (Newport and Kirschner, 1984; Murray and Kirschner, 1989; Murray et al., 1989). Studies by Jaffe, (1983) and Berridge, (1993) shows that at fertilization, the sperm triggers a transient increase in the

cytoplasmic free Ca^{++} which inactivates both MPF and CSF in a reaction mediated by calmodulin-dependent protein kinase II (Lorca *et al.,* 1993). This reaction first overcome the ability of CSF to prevent ubiquitin-dependent degradation of cyclin and thus inactivates MPF. This is then followed by a slower disappearance of CSF (Lorca *et al.,* 1991; Lorca *et al.,* 1993).

The purpose of this review is to gather informations regarding the role of cell cycle co-ordination in the development of embryos after nuclear transfer, particularly with reference to studies for DNA synthesis, chromatin structure and development after transferring of donor nuclei from different cell cycle stages of mouse preimplantation embryos including nuclei from some cultured cell lines.

2.3-NUCLEAR-CYTOPLASMIC EFFECTS ON TRANSFERRED NUCLEUS

Oocytes of amphibians and mammals represents particularly interesting experimental system for studying the behaviour of transfer nuclei. Following ovulation , mammalian oocytes remain blocked at the metaphase of second maturation division (metaphase II). Fertilization resumes the second meiotic division and the second polar body is extruded. This is followed by the decondensation of male and female chromatin and two pronuclei are formed. Shortly after the formation of pronuclei, DNA replication is initiated.

The level of MPF activity during oocyte maturation is maximal at metaphase of both the first and second meiotic division. MPF level remained high during metaphase II arrest and then its activity declines rapidly upon fertilization or artificial activation . This indicates the different states of cytoplasmic environments when using MII oocytes or pronuclear zygotes as cytoplast. Nuclei introduced into nonactivated (metaphase II) oocytes with high levels of MPF undergo premature chromosome condensation (PCC) and nuclear envelop breakdown (NEBD). The transplantation of interphase nucleus into an enucleated, metaphase II (MII) oocyte also results in premature chromosome condensation (PCC) in mouse (Czolowska *et al.,* 1984, 1986; Balakier and Masui, 1986, Szollosi *et al.,* 1988). In addition, PCC results in chromosome damage, the degree of which depends on the cell cycle stage of donor cell (Johnson and Rao, 1970; Collas *et al.,* 1992a). It was reported by Schwartz *et al.,* (1971) that PCC in G1 and G2 phases results in elongated chromosomes with single and double-stranded chromatids, respectively. S-phase chromatin, however, condenses less completely and displays extensive

fragmentation, a phenomenon also describes as "pulverization". The extent of development of nuclear transfer embryos is also influenced by the position of donor nucleus in the cell cycle. Collas *et al.,* (1992b) studied the influence of cell cycle stage of the donor nucleus on development of nuclear transfer rabbit embryos and reported that the transfer of nuclei in G1 into MII cytoplasm yields high rates of development to blastocysts, whereas development is dramatically impaired with S-phase nuclei. Therefore, the progress of donor nuclei in cell cycle reduces the development after transplantation into MII cytoplasm, likely due to the deleterious effect of PCC.

2.3.1-DNA SYNTHESIS IN RECONSTRUCTED

EMBRYOS

Donor and recipient cell cycle stage synchrony affects not only chromosome structure, but also the regulation of DNA synthesis in cell hybrids (Johnson and Rao, 1970; Rao and Johnson, 1970). During a single cell cycle all chromosomal DNA must be replicated once and only once. Experiments in somatic cell hybrids (Roeper *et al.,* 1977) and the Xenopus cell free system (Blow and Laskey, 1988) have shown that intact G2 nuclei are not induced to re-replicate when transferred to an S-phase cytoplasm . However, when the nuclear envelope is permeabilized, replicated nuclei undergo a second round of replication. The results of study by Campbell *et al.,* (1993) showed that all nuclei, regardless of cell cycle stage, undergo DNA replication when transplanted into metaphase II (MII) cytoplasts in which MPF activity was high. However, if the nuclei are transferred after the decline of MPF activity, when no NEBD occurred, then the replication was dependent upon the cell cycle stage of the transferred nucleus. Nuclei which were in G1 and S-phase initiate or continued replication respectively, whilst those which were in G2 were not induced to re-replicate previously replicated DNA (Campbell *et al.,* 1993). The results of this study suggests that when using MII oocytes as cytoplasts, then only nuclei at G1 phase of the cell cycle should be transferred. On the other hand when the nuclei are transferred after the decline of MPF activity then the chromosomal damage induced by PCC is avoided and all nuclei regardless of their cell cycle stage undergo co-ordinated DNA replication.

2.3.2- STAGE OF DEVELOPMENT OF DONOR CELL (KARYOPLAST)

The development of embryos after nuclear transplantation depends upon the cleavage stage of development of the donor embryo, and difference in stages within the cell cycle of karyoplasts and cytoplasts. Smith *et al.,* (1988) reported that the high incidence of development is observed when nuclei are transferred between embryos at exactly the same stage of development. However, when embryo reconstruction involves nuclear transfer between different stages of the same cell cycle or different cell cycles, a lower development of subsequent developmental success is achieved (Smith *et al.,* 1988). Mouse one celled cytoplasts have an extremely limited capacity to support development when reconstituted with nuclei from embryos in the second or later cleavage cycles (Howlett *et al.,* 1987). These studies suggest the need to regulate the cell cycle stage of both the donor and recipient and also the development stage of the donor.

Across species comparison suggests that: 1) the developmental potential of reconstituted embryos is influenced by the stage of development of donor embryo and II) the latest stage of donor embryo that is consistent with development of the reconstituted oocytes of different species to term may coincide with the stage at which the transcription of the embryonic genome begins (Table: 2.1). During their growth phase, mammalian oocytes synthesize and accumulate protein and mRNA (Schultz, 1986). Following fertilization, there is a period of time in which transcription from the embryonic genome is essentially absent or very low and these proteins and mRNAs, which constitute the maternal contribution to early development, direct early development at least up to the time when the embryonic genome is activated; this time corresponds to the maternal to zygotic transition. Zygotic gene activation (ZGA) is involved in replacing maternal transcripts that are common to both the oocyte and embryo, as well as generating new transcripts that are unique to the developing embryos. The destruction of these maternal transcripts, which frequently initiates during oocyte maturation and continues up to the time of ZGA, can result in the loss of greater than 90% of many mRNAs (Schultz, 1993). The first cell cycle following fertilization appears to be exclusively regulated by the information provided in the maternal cytoplasm (Flach *et al.,* 1982). Fertilization induces the synthesis of new proteins from the pool of maternal RNAs (Latham *et al.,* 1991) together with post-translational modifications of existing maternal proteins (Braude *et al.,* 1979; Howlett and Bolton, 1985; Bellier *et al.,* 1997), and these events may be involved in the acquisition of transcriptional permissive state at

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the G2 phase of the 1-cell embryo and during the minor phase of ZGA (Latham *et al.*, 1992). However, no direct molecular has yet been found between maternal information and the ZGA (discussed in Forlani and Nicolas, 1996).

The potential of donor nuclei to retain the ability to support development is longest in amphibians. In *Xenopus,* the transcription of zygotic genes is delayed until the mid-blastula transition at the 4,000-cell stage, whereas the potential is lost most rapidly in the mouse, a species in which acquisition of transcriptionally competent state occurs during the late 1-cell stage (Vernet *et al.*, 1992; Latham *et al.*, 1992; Ram and Schultz, 1993) and late 1-cell embryos are capable of synthesizing a paternally derived mRNA (Matsumoto *et al.*, 1994). This is the stage at which the embryo first begins producing its own RNA, whereas previously the embryo relied on maternally produced transcripts (Newport and Kirschner, 1982b). The major transcription- dependent expression of endogenous genes begins in 2-cell embryos about 2 to 4 hours after completion of the first mitosis (concurrent with DNA replication) and then increases significantly 8 to 10 hours later during G2-phase, concurrent with degradation of maternal RNA (see DePamphilis *et al.*, 1988). Expression of zygotic genes is controlled by a time-dependent mechanism (the "zygotic clock") that delays transcription-dependent gene expression until about 20 hours after fertilization, regardless of whether the 1-cell embryo has completed S-phase, entered mitosis, and cleaved into 2 cells (Conover *et al.*, 1991; Manejwala *et al.*, 1991; Wiekowski *et al.*, 1991).

Not all mammalian blastomeres are the same, however, and mammalian species differ greatly in the time of gene activation (ZGA) or time of transcription (Table: 2.1); which occurs early in mammalian development. The early start of transcription in mammalian species was thought to be a big barrier to clone mammalian species from the later developmental stages of the embryo and it was thought that cloning in mammalian species would not be successful despite some suggestions that it would work in *Xenopus.*

In frog (*Rana pipiens*), the tale bud-stage is the latest developmental stage from which a tadpole has been produced by nuclear transfer of a somatic nucleus, whereas 40% of germ cell nuclei of this stage are still totipotent (Smith, 1965). LeSimple *et al.*, (1987) obtained normal adults by nuclear transfer of germ cells isolated from larva of salamander (*Pleurodeles walt*). In amphibians, germ cells seem to retain totipotency longer than the somatic differentiated cells (DiBerardino, 1987). Later experiments with different mammalian species suggests that the loss of

developmental potential (totipotency) of donor nuclei is not associated absolutely with the initiation of transcription, for normal development has occurred following nuclear transfer from cells that have progressed through the stage of activation of the embryonic genome, including inner cell mass (ICM) cells in ruminants and primordial germ cells in salamander (Smith and Wilmut, 1989 and LeSimple *et al.*, 1987). The transfer of nucleus from a donor cell that has initiated transcription results in the need to reprogram gene expression such that transcription ceases and is then re-initiated in a stage specific manner. Any change in the gene expression of a nucleus transferred into a different cytoplasmic environment is defined as reprogramming of its gene expression. Reprogramming may be complete or incomplete. Reprogramming of gene expression of the donor nuclei of early cleavage stages is effective in some species. The use of present nuclear transfer procedures seems to be less effective in inducing the reprogramming of gene expression of donor nuclei more advanced in differentiation and also a lower rate of development to term is observed.

Table 2.1

Species	Mouse	Rabbit	Cow	Sheep	Xenopus
Start of transcription	Two-cell	Four-cell	8- 16 cell	8- 16 cell	4000
Nuclear totipotency -	Two-cell	32-cell	32-cell	64-cell	Tadpole intestinal epithelium

A comparison between the stage of development at which transcription from the embryonic genome begins and the most advanced stage of development from which nuclei transferred to enucleated oocytes have been able to support development to adulthood. *Data on nuclear transfer from Kono et al., 1991b; Collas and Robl, 1991; Bondioli et al., 1990; Smith and Wilmut, 1989; Gurdon, 1986. Data on transcription from Bolton et al., 1984; Van Blerkom and Manes, 1974; King et al., 1985, 1988; Camous et al., 1986; Calarco and McClaren, 1976; Newport and Kirschner, 1982.*

The transferred nuclei may be able to support normal development only if the nuclei are in an undifferentiated state or the donor cells are differentiated but the nuclei are amenable to reprogramming. So it is possible to produce totipotent nuclei

from many tissues. However, the pluripotential characteristics of embryonic stem (ES) cell have made them suitable candidates for future investigation. Cells are said to be totipotent when they are able to contribute to all the tissues of the conceptus including the placenta, whereas they are said to be pluripotent if they are able to contribute to all tissues of the fetus. Pluripotent embryonic stem (ES) cells or ES-like cells have been derived from preimplantation embryos of several species, including mice (Evan and Kaufman, 1981; Martin, 1981), hamsters (Doetschman *et al.*, 1988), cattle (Saito *et al.*, 1992; Strelchenko and Stice, 1994). ES cells also provide a large population of totipotent nuclei potentially useful for production of clonal offspring by nuclear transfer (First and Prather, 1991). The encouraging results with nuclear transfer of blastomeres and briefly cultured ICM (Inner cell mass) cells in species other than mouse (Keefer *et al.*, 1994; Sims and First, 1994; Stice *et al.*, 1994) opens up the possibility of producing viable animals through nuclear transfer of ES cells that retains totipotential properties.

2.3.3 - The recipient cell (cytoplast)

The state of cytoplasmic differentiation at transplantation is a major determinant governing the integration of a transplanted nucleus into a host cytoplast. In nuclear transfer experiments variety of recipients cells (cytoplasts) of different stages of development have been used from oocytes at metaphase I through metaphase II to pronuclear zygotes and 2-cell embryos. Each recipient cell (cytoplast) has advantages and disadvantages. The use of pronuclear zygote ensures the activation while on the other hand parthenogenetic activation is needed for the oocytes. The use of cleavage stage recipient cells is favoured in some species to the extent that reprogramming of gene expression is required. The 2-cell recipients are mainly used in mouse because transcription starts in this species at two cell stage (Tsunoda *et al.*, 1987). Studies using nuclear transfer in mouse embryos have shown that the ability of a blastomere nucleus to support development to the blastocyst stage *in vitro* and to term depends on the stage of the recipient cytoplasm to which it is fused. Recipient cytoplasts from one and two-cell embryos were used and it was shown that, while nuclei from four and eight cell blastomeres could support development to the blastocyst stage (Robl *et al.*, 1986) and on some occasions, to term (Tsunoda *et al.*, 1987) when transferred to two cell stage cytoplasm, no development could be obtained after transplantation to the cytoplasm of one-cell enucleated zygotes (McGrath and Solter, 1984; Surani *et al.*, 1987; Smith *et al.*, 1988). In livestock species the use of MII oocytes have become

the method of choice because of technical difficulties and lack of development while using the pronuclear zygotes.

After choosing the suitable recipient cell the enucleation of the chromosome material is done. Willadsen (1986) described the procedure of enucleation by splitting the oocyte into two halves, one of which was likely to be enucleated. One of the major disadvantage was the reduction of the cytoplasmic volume of the oocyte which is believed to accelerate the pattern of differentiation of the new embryo (Evsikov *et al.*, 1990). Smith and Wilmut, (1989) used different procedure to remove the chromosomes with minimum of cytoplasm. Aspiration of the Ist polar body and neighbouring cytoplasm removes the metaphase chromosomes in 67% of sheep oocyte. Now it has been proved that the use of DNA specific fluorochrome (Hoechst 33342) guaranteed the enucleation with minimum removal of cytoplasm (Tsunoda *et al.*, 1988). Although these MII cytoplasts have the proven ability, after activation, to induce nuclear remodelling and full subsequent development of nuclei obtained even as late as seventh mitotic cycle from ovine and bovine blastocysts, nevertheless , it is possible that cytoplasts from early meiotic stages may be superior to those from matured eggs (DiBerardino *et al.*, 1984). An alternate approach of non- invasive enucleation of the oocytes was used by some workers in mammals, whereas in amphibians, irradiation with ultraviolet light is used as a routine procedure (Gurdon, 1960).

2.3.4 - Effects of cell cycle co-ordination on the development of reconstructed embryos

The information gathered above about the effect of cell cycle co-ordination on development illustrates that the development after reconstruction is dependent on the cytoplasmic state of recipient cytoplasts. The transfer of nuclei at G1 stage of development into MII oocyte cytoplasts at the time of activation, when the MPF level is high, gives maximum development compared to the donor nuclei in S or G2 phase. All nuclei transferred at the time of activation when MPF levels are high, undergo nuclear envelope breakdown (NEBD) which is followed by premature chromosome condensation (PCC). The nuclear envelope is then reformed and DNA synthesis is observed in all nuclei. However, the results of different studies shows that in sheep the maximum development (61.3%) is observed (Campbell *et al.*, 1994) when donor nuclei in S- phase are transplanted after the decline of MPF i.e. in early S-phase cytoplasts. It reduces to 45.7% when the donor nuclei were transferred in mid S-phase of the recipient cytoplasts. In cow the transfer of nuclei

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at S-phase in MII oocyte cytoplasts at the time of activation at high levels of MPF, only 1.25% embryos developed. Studies on development in rabbits by Collas *et al.,* (1992) showed that maximum development (71%) occurs when the donor nuclei in G1 stage of cell cycle are transferred in MII cytoplasts at the time of activation. The results of the studies in mouse depicts the same trend of maximum development (77.8%) when the nuclei are transferred at G1 stage of development but when the donor nuclei are transferred at G2 stage of development in MII cytoplast showed lower rate (20.8%) of development (Cheong *et al.,* 1993). From the above results it may be predicted that the only nuclei which are in G1 would promote development when using MII cytoplasts and therefore, the percentage of development reflects the percentage of blastomeres in the G1 phase. This discrepancy in results also leads to another prediction that the recipient cytoplasm were not at MII at the time of embryo reconstruction.

2.3.5 - EVENTS AFTER NUCLEAR TRANSFER

After the transfer of a donor nuclei to an enucleated, activated oocyte various changes occur to the transferred nucleus. These changes are characterized by a remodelling of the nucleus and can be observed as a swelling of the transferred nucleus (Gurdon, 1964). This remodelling presumably results in a reprogramming of the transferred nucleus such that it behaves developmentally as if it was a one celled zygote.

2.3.5.1 - Nuclear Remodelling

Nuclear remodelling is a process of changes that occur to a nucleus after transfer to an oocyte. These morphological and biochemical changes in the nuclear structure are thought to be a result cytoplasmic components from the oocyte acting upon the transferred nucleus that result in a reprogramming of genomic expression.

2.3.5.1.1- Nuclear Remodelling in Amphibians and Mammals

The morphological indication of nuclear remodelling is the disappearance of the nucleoli after transfer to an oocyte, as nucleoli are present in *Xenopus* larval stage nuclei, but not in early cleavage stages (Gurdon and Brown, 1965). Another indicator is the swelling of the transferred nucleus in amphibians (Gurdon and Brown, 1965) as well as in mammals (mouse: Czolowska *et al.,* 1984; rabbit: Stice

and Robl, 1988; pig: Prather *et al.*, 1988) and is dependent upon the time of transfer in relation to the time of activation. Swelling does not occur if the recipient cell is an enucleated zygote, enucleated 2-cell stage blastomere (Barnes *et al.*, 1987), or an enucleated germinal vesicle oocyte (Dettlaff *et al.*, 1964).

Nuclear remodelling may be the result of exchange of proteins between donor nuclei and cytoplasmic compartment. The exchange of protein between the nucleus and cytoplasm does not appear to be limited by the nuclear envelope, but by selective binding sites within the nucleus (Feldher and Pomerantz, 1978). Some proteins appear to migrate out of or into the nucleus at different rates than other. Simultaneously, cytoplasmic proteins, both basic and acidic, are acquired by the nucleus (Merriam, 1969; Gurdon, 1986). This protein intake appears to be an inducer of the nuclear swelling and is not a consequence of nuclear swelling (Merriam, 1969).

In amphibians and mammals changes in the distribution of nuclear antigen have been observed during early cleavage (Dreyer, 1987; Stricker *et al.*, 1989). Changes are also observed in nuclear lamins. The A/C type of nuclear lamin becomes undetectable after the transition to zygotic control of development in the mouse, pig and cow (Schatten *et al.*, 1985; Prather *et al.*, 1989b).

2.3.5.1.2 - Chromosomal Abnormalities

Some of the chromosomal abnormalities which limit development are related to the regulation of DNA synthesis (DiBerardino, 1979) that appears to occur in the first cell cycle after nuclear transfer (DiBerardino and Hoffner, !970). Serial nuclear transfer reveals that these chromosomal abnormalities show stable developmental restrictions (Briggs *et al.*, 1964; DiBerardino and King, 1965). One of the other changes observed is the reprogramming of the RNA synthesis. There are two approaches to illustrate this. One is by evaluating the localization of snRNA protein within the nucleus and the other is evaluating the nucleolar morphology.

2.3.5.2 - DEVELOPMENT AFTER NUCLEAR TRANSFER

Nuclear transplantation studies with mice and rat have shown that enucleated zygotes which receive nuclei from either late 2-cell or more advanced embryos exhibited limited preimplantation development (McGrath and Solter, 1984; Robl *et al.*, 1986; Tsunoda *et al.*, 1987 and Kono and Tsunoda, 1988). Transplantation of

single nuclei from a 4-cell mouse embryo into one of the enucleated blastomeres of a 2-cell resulted in the successful production of identical triplet mice (Kono *et al.,* 1991a), but it is difficult to produce live young by such nuclear transplantation between highly asynchronous embryos. Development to term after nuclear transfer was achieved in sheep (Willadsen, 1986), cattle (Prather *et al.,* 1987) and rabbits (Stice and Robl, 1988). Although the mouse was the mammal species in which most of the early work was conducted, it appeared to be rather difficult to clone by nuclear transfer. Tsunoda *et al.,* (1987) reported the development of fertile mice after transplantation of 8-cell stage nuclei into enucleated 2-cell blastomere. The development to term have also been reported when a enucleated oocyte in metaphase II stage was used as recipient from late two cell stage nuclei (Kono *et al.,* 1991b) and early 8-cell nuclei (Cheong *et al.,* 1993). The production of identical sextuplet mice by transferring metaphase nuclei from four-cell embryos have also been reported by Kwon and Kono, (1996) by serial nuclear transfer.

Review of literature depicts very little information about the most advanced embryonic stage from which development after nuclear transfer could occur in mammals. Development to term has been reported from inner cell mass (ICM) nuclei in sheep (Smith and Wilmut, 1989) rabbit (Collas and Robl, 1991) and cow (Collas and Barnes, 1994). There are a few reports of development to blastocysts by using the various types of cells as donor nuclei, but no report of development to term has been observed until recently.

Moreover, development to blastocyst stage after nuclear transfer has been reported from thymocyte nuclei (Kono *et al.,* 1991c) and embryonic stem cells (Tsunoda and Kono, 1993) in mouse; cumulus granulosa cells and primary foetal fibroblast in rabbit (cited by Collas and Barnes, 1994); and cumulus granulosa cells in cow (Collas and Barnes, 1994). Mouse primordial germ cells lead to implantation sites although no development to term (Tsunoda *et al.,* 1989). However, the recent study of Wakayama *et al.,* (1998) has demonstrated the full term development of mice from enucleated oocytes injected with cumulus cell nuclei.

The results of the nuclear transfer experiment suggests progressively restricted development after nuclear transfer for nuclei more and more developmentally advanced cells. The decline in the development to term may be related with the transcriptional activity at different stage of development of the different species. Surani *et al.,* (1987) reported that the transcriptional activity of reconstituted embryos could affect the development of reconstituted embryo. In the

mouse the transcriptional activity of the embryonic genome occurs at the mid 2-cell stage (Piko and Clegg, 1982; and Bensaude *et al.,* 1983) but the later reports have shown that it starts at the late 1-cell stage (Latham et al., 1992; Ram and Schultz, 1993). Transferred nuclei from early 2-cell stage embryos have a greater developmental potential than nuclei derived from the late 2-cell stage embryos (Howlett *et al.,* 1987). However, Smith *et al.,* (1988) have reported better development following the transfer of late 2-cell nuclei after activation of embryonic genome. These conflicting results suggests that the transcriptional activity is not solely responsible for the development of reconstructed embryos. It is now believed that the cell cycle stage of the recipient cytoplasm and donor nuclei are critical for optimal development of the reconstituted embryos (Smith *et al.,* 1988).

Early development is controlled by product of maternal genome. These products, in the form of mRNA and protein are synthesized and stored within the oocyte during growth. The point during early development when genes unique to the embryos are first expressed is the maternal/zygotic transition (MZT; reviewed by Davidson, 1986; First and Barnes, 1989; Barnes and Eyestone, 1990).

In mammals the effects of donor nuclei obtained beyond the time of the MZT on embryo cloning are mixed. Experiments with mouse, where enucleated pronuclear eggs were used as recipients demonstrated that only nuclei obtained around the time of the MZT (2 cell stage) resulted in significant development (McGrath and Solter, 1984; Robl *et al.,* 1986). In addition to this , it has been postulated that development resulting from embryo cloning in mice is due to the overlap of developmental periods, (i.e. from the MZT to compaction). The transition of which may mark the irreversible points of commitment (Johnson, 1981; Robl *et al.,* 1986; Barnes *et al.,* 1987; Howlett *et al.,* 1987). Studies on embryo cloning of cattle and sheep suggest that the MZT does not represent an irreversible barrier of commitment. In sheep and cattle the MZT occurs at the 8- to 16-cell stage and 4- to 8-cell stage, respectively (Crosby *et al.,* 1988; Camous *et al.,* 1986; King *et al.,* 1989; Barnes and Eyestone, 1990). Nuclei from inner cell mass (ICM) cells of sheep, when transferred to meiosis II cytoplasm, result in substantial development to the blastocyst stage (Smith and Wilmut, 1989). In cattle the nuclei from 16- to 64-cell stage donor embryo result in blastocyst formation and are capable of resulting in offspring (Prather *et al.,* 1987; Bondioli *et al.,* 1990).The results of the above experiments demonstrate that the development to the blastocyst stage is not totally dependent on reprogramming.

There are two ways in which it may be possible to produced larger numbers of genetically identical calves : to repeat the transfer of nuclei from embryos derived by nuclear transfer or to use various cultured cell lines as donor cells. Serial transfer of nuclei which were themselves derived following nuclear transfer is progressively less efficient with each passage (Stice, 1992). The development of totipotent embryonic cell cultures has great value in producing genetically identical animals. One of the first differentiation events in mammalian embryonic development is the formation of two cell types, the trophectoderm (TE) and the inner cell mass (ICM). Historically, murine embryonic stem (ES) cell lines have been established from the inner cell mass (ICM) of blastocyst stage embryos by culture on feeder layers of embryonic fibroblast or buffalo rat liver cells. The ability to isolate embryonic cells directly from preimplantation mouse embryos and to maintain them *in vitro* has provided a powerful research tool.

The production of cloned offspring in mammalian species by the transfer of nuclei from embryonic stem (ES) cells could provide a useful method for the production of large numbers of identical offspring. These cells have the ability to differentiate into a wide variety of cell types . They are able to produce all tissues of new individual (Nagy *et al.,* 1993). Once isolated, ES cells may be grown *in vitro* for many generations. They produces an unlimited number of identical cells capable of developing into fully formed adult chimaeras (Evans and Kaufman, 1981; Martin, 1981; Bardley *et al.,* 1984; Robertson, 1987). Now researchers are trying to prove the possibilities of embryonic stem (ES) cells as a source of donor nuclei for nuclear transfer. Kono *et al.,* (1991) reported that the mouse embryonic stem (ES) cells have the potential to support development to term with present procedures, as the cells of the inner cell mass (ICM) do not do so. At the present time, the isolation of pluripotent embryonic stem (ES) cells or ES-like cells have been derived from preimplantation embryos of several mammals, including mice (Evans and Kaufman, 1981; Martin, 1981), hamsters (Doetschman *et al.,* 1988), pigs (Notarianni *et al.,* 1990), cattle (Saito *et al.,* 1992; Strelchenko and Stice, 1994), mink (Sukoyan *et al.,* 1992), rat (Iannaccone *et al.,* 1994) and rabbits (Graves and Moreadith, 1993). However, neither production of chimeric animals nor development to term following nuclear transfer has been shown except for mice.

Primordial germ cells are another stem cell population that has attracted particular interest as nuclear donor. Although primordial germ cells in mammals acquire pluripotency or totipotency after gametogenesis or fertilization, it is still

unknown whether the cells have these characteristic before gametogenesis begins. Tsunoda *et al.,* (1989) reported development from the enucleated unfertilized mouse oocytes receiving a single male germ cell into blastocysts, but live foetuses were not obtained after the transfer of reconstituted eggs into recipients. Observations on development after nuclear transfer suggest that primordial germ cells may be a suitable source of nuclei. In the mouse, 17% of manipulated eggs developed to blastocyst stage following transfer of nuclei to enucleated oocytes, and this proportion was increased to 62% by serial transfer from a two-cell egg to normal two-cell embryos (Tsunoda *et al.,* 1989). As the methods of nuclear transfer are improved, it may be possible to increase these proportions. There is also the probability that in other mammals, primordial germ cells will prove to have a greater developmental potential, perhaps in species in which genome activation occurs later in development. Of the different cell types primary foetal fibroblast cells are another candidates which might be used as donor nuclei.

2.3.5.2.1 - QUIESCENCE USE OF CULTURED CELLS

In normal cell cycle, a new-born cell while starting out a normal prereplicative program begins activating genes and accumulating things which commit it to a round of chromosome replication when they reach critical levels. If the medium lacks essential factors or contains the transcription inhibitor actinomycin D or a protein synthesis inhibitor such as cycloheximide at the time of cell birth, the cell cannot accumulate enough components to reach this critical commitment point, the so-called restriction point. If the cell is caught before the restriction point, it will stop advancing toward the S phase and, bearing marks of its aborted G1 transit, it will "park" itself indefinitely in a so-called G_0 state. This parking state in cell cycle is also said to be the G_0 state of quiescence. This state could occur in cultured cell from G1 phase into a quiescent G_0 state by lack of serum or a critical nutrient.

The knowledge about different cell lines suggests that most of the cells have very short G1 and G2 with long S phase. The reduced development or no development may be due to the reason that they have already initiated replication when transferred into cytoplast. This is confirmed by the results that the donor nuclei in G1 phase gives maximum development after nuclear transfer. In the same way the study with different cell type i.e. pluripotent embryonic stem (ES) cell have showed that the development to blastocysts is possible from ES or ES-like cells in different species of livestock but the development to term is still restricted. The restricted development by the use of the cells of different cell lines may due to their

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long S phase. As discussed earlier that the cells in G1 can be aborted from the normal cell cycle and go to G_0 state of quiescence, could be used as donor nuclei and may support development to term.

From the information about the transition of G1 phase of the cell into G_0 state of quiescence, it is hypothesized that the ES or ES-like cells can enter into G_0 state of quiescence and that can stay there without differentiation. The birth of sheep (Dolly) from the quiescent population of cultured cell make it possible to produce large number of cloned animals (Campbell *et al.*, 1996). This technique of quiescence could provide greater opportunity for the use of cultured cell population as donor nuclei for embryo reconstruction by nuclear transplantation in mice.

CHAPTER 3

GENERAL MATERIALS AND METHODS

All the experimental work described in this thesis have been conducted in murine preimplantation embryos and oocytes. The general facilities, animals, and methodology commonly used in most of the experiment during the project are described in this chapter. More details and specific methodology have been provided in each of the experimental chapters. The composition of different media, their modifications and the procedures followed in their preparation are described in appendix 1(section 1.1 to 1.6). Moreover, the details of stock solutions of chemical compounds and antibodies used in this project have been shown in the appendix 1(section 1.7.1 to 1.7.10).

3.1-GENERAL MANIPULATION FACILITIES

All the experimental studies on oocytes or embryo manipulation were conducted at the Roslin Institute. The basic equipment in this laboratory included: a humidified, 5 % CO_2 in air incubator (Flow Laboratories, Rickmansworth, Hertfordshire, UK) set a 37 °C; a binocular, dissecting microscope with transmitted light illumination system (magnification: X12.5-X80; M3Z, Wild) fitted with homemade heated stage for embryo handling and an embryo micromanipulator system (see section 3.4.3).

The sterile disposable plastic ware was mostly used as culturing material. The 60 mm (Costar corporation, Cambridge USA) petri dishes were utilized through out these experiments. The Gilson Pipetman digital pipettes (Scotlab Ltd. Strathclyde, UK) with the adjustable volume, with the capacity of 10 μl , 20 μl, 200 μl and 1000 μl and universal fit tips (Laser, Laboratory systems Ltd., Southampton UK) were used for microvolume measurements of the stock solutions and media. Also 30 ml universal tubes (Sterilin, Bibby Sterilin Ltd., Stone, Staffs, UK) and 1.5 ml Eppendorfs tubes (Sterilin, Bibby Sterilin Ltd., Stone, Staffs, UK) were used for storing the stock solutions, media and drugs. The embryological watch glass dishes were used. They were thoroughly cleaned and rinsed with water, dried and kept in dust free conditions.

3.2 *IN VITRO* CULTURE OF MOUSE EMBRYOS.

3.2.1 - *In Vitro* Culture Of Embryos.

The *in vitro* culture system consisted of a 5% CO_2 - 95% air atmosphere incubator and a microdrop culture system was set up with a modified M16 medium (Whittingham, 1971) in 60 mm disposable plastic dishes. The microdrops of the medium (35-40 μl) were dispensed on the bottom of the Petri dish and then covered with paraffin oil (Merck Ltd. Poole, Dorset, UK). The culture dishes were incubated 2-3 hours prior to the transfer of embryos into the drops to equilibrate the temperature and CO_2.

In the start of this project, M16 was used for the culture of embryos. But later on a modified M16 medium i.e. pM16 was used (PJ Otaegui, unpublished data, Ph.D. thesis). The pM16 medium was further modified by including L-glutamine and essential and non-essential amino acids and designated as sM16 (see appendix 1 section 1.1). The embryos cultured in this medium showed no cell block at 2-cell stage and reaches to blastocyst stage earlier (S, Ali, data not presented) The osmolarity of the modified medium used was around 260 mOsms. The sM16 medium was used in most of the experiments reported in this thesis.

A stock solution of the specific compound was made (see appendix section) and supplemented with the medium (sM16) 2-3 hours prior to the start of the experiment. This supplemented medium was then used to set up the microdrop culture and equilibrated for temperature and CO_2.

The stock solutions of all the chemicals required for the preparation of media (sM16) were prepared and stored at 4°C. It was convenient to prepare concentrated stock of the component of sM16 (see appendix 1 section 1.3). The bovine serum albumin (BSA) was dissolved at the rate of 4% in the end while preparing the sM16 media from the concentrated stocks. The media were sterilized by pushing it through Acrodisc syringe filters of 0.2 μm pore size (Product No. 4652 Gelman Science, Ann Arbor, Michigan, USA), aliquoted and stored at 4°C. The first 5 to 10 ml of sterilized media were discharged to avoid toxicity effects from the filter (Harrison *et al.,* 1990). Each aliquot was used once to minimize the chances of contamination. The media were prepared every week from the concentrated stocks and the stock solution were refreshed according to the requirements of their shelflife.

3.2.2 - Handling of the Embryos

The M2, an HEPES buffered medium M2 (Whittingham and Wales, 1969) was used during the recovery of embryos and manipulations procedures. The M2 medium was prepared freshly every week from the concentrated stock solutions (see appendix 1 section 1.4). It was sterilized through filtration by using the syringe filters and stored in 30 ml universals at $4^{\circ}C$. The media was equilibrated for room temperature before use. The embryos were washed 2-3 times with M2 medium into an embryological watch glass dishes at room temperature. The microdrops of M2 media was also used to store the embryos at $4^{\circ}C$ in the refrigerator.

3.3-PRODUCTION OF OOCYTES AND EMBRYOS

3.3.1 - Animal Management.

The animals were housed in a small animal unit (S.A.U.) where the computerized system had been installed to control the internal environments. The F1 females (C57BL/6 x CBA/Ca) were used to recover the oocytes but the embryos were recovered from F1 females (C57BL/6 x CBA/Ca) mated to F1 (C57BL/6 x CBA/Ca) males.

The photoperiod of 12 hours (08:00 hr to 20:00 hr) light and 12 hours dark (20:00 hr to 08:00) was practiced under this project to recover the oocytes in the morning and to perform the experiments during day time.

There were 20 air changes per hour. The temperature in the small animal unit was maintained at $21\,^{\circ}C \pm 2^{\circ}C$ and air humidity was kept at $50\,\% \pm 5\%$. There were 20 air changes per hour. The health status of the colony of the animals was being specific pathogen free (SPF). They were Staphylococcus aureus positive (an human comensal bacteria). The animals had "ad libitum" access to food and water.

3.3.2 - Superovulation Regime for the production of oocyte and embryos.

To superovulate the mice, two types of hormones were used: the pregnant mare's serum gonadotrophin hormone (eCG, Folligon; Intervet) to increase ovulatory follicle number and human chorionic gonadotrophin (hCG, Chorulon; Intervet) to induce the ovulation. The lyophilized powders of pregnant mare's serum gonadotrophin hormone (eCG) and human chorionic gonadotrophin (hCG) were

dissolved in sterile molecular water and a concentration of 50 IU/ml was achieved. The solutions were then aliquoted, stored at - 20 °C and used within two months.

Four to seven weeks old F1 females were superovulated and used as oocyte and embryo donor. Superovulation was induced by intraperitoneal injection of five IU of eCG at 20.00 hours, followed by 48 hours later (20.00 hours) five IU of hCG injection . When embryos were required, F1 females were mated with F1 males of the same strain immediately after hCG injection and copulation plugs were checked on the following morning.

3.3.3 - Harvesting of embryos and oocytes.

The females were killed by cervical dislocation a few minutes prior to embryo recovery. The females were laid on their backs on absorbent tissues and soaked thoroughly with 70 % ethanol, to reduce the risk of contamination during dissection from mouse hair. By holding the abdominal skin firmly upward with the forceps, a small cut was made in the midline. By grasping firmly, the both sides of the abdominal skin was pulled toward the head and tail until the abdomen was completely exposed and the fur was well out of the way. The body walls were then cut by using forceps and fine scissors. The coils of the intestines were pushed aside to locate the horns of the uterus, the oviducts and the ovaries.

Ovulated oocytes and early preimplantation embryos were recovered from the oviducts. The uterine horns were gently lifted with small forceps away from the abdominal cavity and a hole was made in the mesometrium with the tip of a fine scissors. The mesometrium was dissected away from the uterine horn, oviduct, ovary and fat pad and the oviducts were recovered by applying cut between ovary and uterine horn.

The oviducts were placed in a embryological watch glass containing embryo M2 medium at room temperature and transferred to the dissecting microscope under low magnification. The embryos were recovered by tearing the near by walls of the oviduct where the embryos were located and by gently squeezing the oviduct.

Fig 3.1 A diagrammatic representation of a holding pipette.

1 cm

2 cm
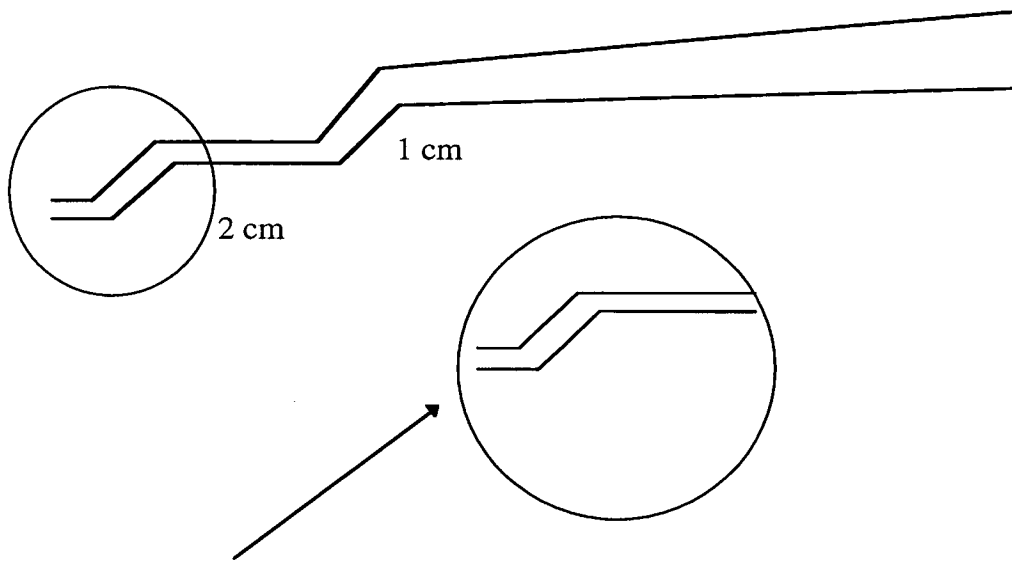
Tip of pipette closed from 150 to approx 20 μm.

Fig 3.2 A diagrammatic representation of an enucleation pipette

Detail of tip area
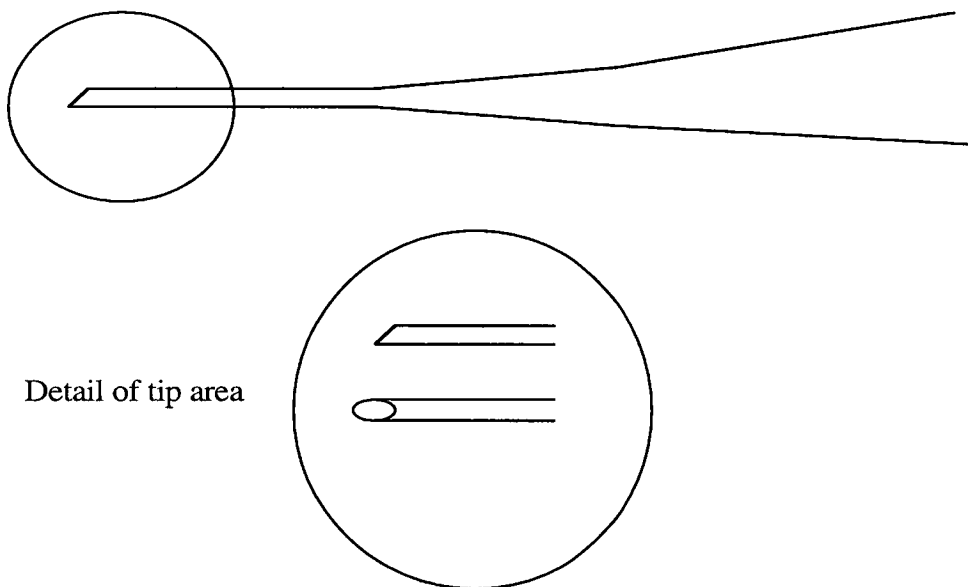
3.4-MICROMANIPULATION OF EMBRYOS AND OOCYTES.

3.4.1 - Preparation of Micro-Tools

3.4.1.1 - Holding pipettes

The holding pipettes were prepared by rolling a 10 mm long capillary tube (external diameter 1.0 mm; GC100-10, Clark Electromedical Instruments, Reading, UK) uniformly over a microburner flame and pulled by hand over a length of 5 to 8 cm with an external diameter of about 100 μm. The pulled capillary was mounted onto a microforge (MF1-microforge, Research Instruments Ltd. UK) and the first bend was made close to the start of the pulled part of the pipette at an angle of 45° (see fig 3.2) . The second bend was made approximately 1 cm from the first bend, making it parallel to the rest of the capillary. The third bend was created 2 cm away from the second bend and fourth very near to the third bend. All these bends were made by placing the capillary near the hot glass drop bead without touching it, in such a way that the heat would melt only the nearest side of the tube wall causing it to bend 45° downwards. The tip of the pipette was then cut flat by using the diamond pencil to mark the glass 2 mm from the final bend. The flat tip was then heat-polished, leaving an opening of approximately 20 μm diameter.

3.4.1.2 - Enucleation pipettes.

The enucleation pipettes were prepared by pulling thin-walled, 1.0 mm diameter glass capillary tubes (GC100T-15; Clark Electromedical Instruments, Reading, UK) using the moving coil microelectrode horizontal pipette puller (753 Micropipette Puller, Campden Instruments Ltd., UK). The settings of Microelectrode pipette Puller were adjusted to give an initial taper, which reduces the diameter of the capillary to slightly greater than the diameter required, with the second taper being almost parallel. The capillary was mounted on the microforge and the tip of the needle was then broken at right angles to an outside diameter of 15 to 20 μm, according to the intended use. A particular point of the needle with required diameter (determined by a graticular eyepiece) was placed near to the glass bead on the platinum wire heating filament. The capillary needle was brought into contact with slightly heated glass bead of the microforge resulting into fusion together. The filament of the microforge was switched off simultaneously and the spring-loaded connecting rod, holding the filament, was depressed causing the capillary to break at the point of contact with the glass bead.

Fig 3.3 Diagrammatic representation of micromanipulation chamber
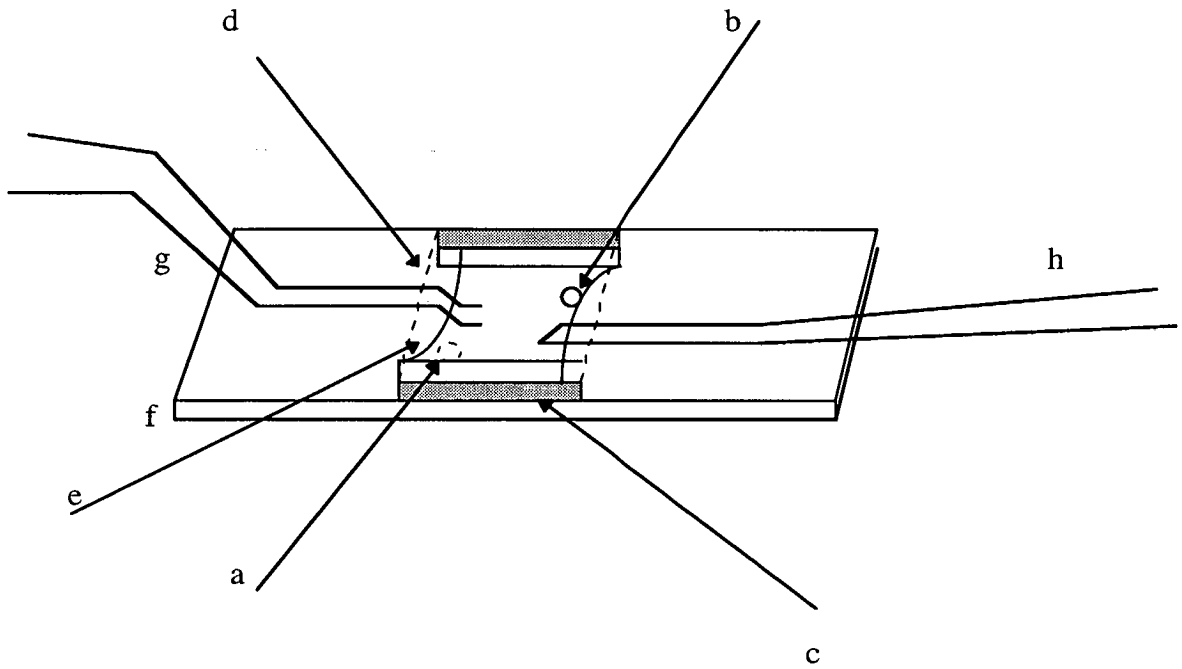
a) positioning of oocytes/embryos to be enucleated b) position of the enucleated oocytes or reconstructed embryos in the chamber c) glass support d) coverslip e) paraffin oil f) sliconised glass slide g) holding pipette h) enucleation pipette.

The tip of the pipette was then beveled to a 45° angle by positioning it on the beveller, built in the workshop of Roslin Institute with 1 micron Aluminum Oxide grading surface by passing a continuous flow of air through it, using a 50 ml syringe and a length of tubing (fig 3.2). The continuous air flow was applied to prevent dirt from entering into the pipette as it was ground. After bevelling, the tip was thinned and polished by rinsing with dilute 20%, v/v) hydrofluoric acid (Aldrich, USA) for 20-25 seconds by blowing continuos flow of air through it. The tip of the capillary needle was rinsed again with distilled water and then with 95% (v/v) ethanol. The bevelled capillary was then placed on to the microforge and a small point was pulled on the leading edge of the bevel. This was done by touching the tip of the pipette to the glass bead which was slightly heated to fuse the bead with the tip of the pipette and then drawing the pipette away to from a spike. If the tip was too long after being drawn out, the excess length was broken off to leave the shorter tip, suitable to pierce the zona pellucida without damaging the cytoplasmic membrane.

The enucleation pipettes were siliconised to prevent cellular contents from adhering to the pipette tip during manipulation. The pipette was connected to an empty 60 ml syringe and the tip of the pipette was dipped into a silicone solution (Sigmacote Sigma cat no. SL-2) to drawn it up and down 2-3 times. The pipettes after siliconization were placed overnight in an oven at 80 C° for drying.

3.4.2 - Micromanipulation Chamber.

The good quality glass slides were washed thoroughly with Decon detergent, rinsed at least five times in distilled water and dried overnight in an hot air oven. They were sliconised by dipping in "Sigmacote" (Sigma) and drying in oven. The micromanipulation chamber was made from a siliconised slide to which two rectangular glass supports (20 x 3 x 2 mm) were fixed by applying a line of a mixture of 5% sterile wax in Vaseline approximately 2.5 cm in length along both edges of the upper surface. The wax mixture was also applied on the top of each glass support. The micromanipulation medium was pipetted in the center of the slide (300 μl) in such a way that it was touching both glass supports and a cleaned coverslip was placed onto the mixture. The coverslip was pushed down to make a water-tight seal. In the end the both sides of the chamber were filled with paraffin oil to seal the chamber medium (see fig 3.3).

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3.4.3 - Micromanipulator Assembly.

The micromanipulator system consist of an inverted microscope (magnification up to x400; Diaphot-TMD; Nikon UK Ltd., UK) with condenser and objectives for differential interference contrast (Normarski optics); two micromanipulators (Micromanipulators M; E. Leitz Ltd., UK) with coaxial coarse and fine adjustment, tilting top and control for transverse and sagital movement and hydraulic system.

The micromanipulators were raised from the base to the level of the microscope stage on either side of the microscope. Each micromanipulator had double or single unit instrument holders which held micro tools-holders for the micro pipettes. The hydraulic system was controlled by 3x Narishige (IM-188) microinjectors that are modified to accept gas-tight micrometer syringes (Agla Micrometer Syringe Kit, Welcome Reagents Ltd., UK) which were connected through flexible polythene tubing (mod. 21.852-0062; Bel-art Products, UK) to the instrument tubes. The whole of the hydraulic system was filled with an inert mineral oil (Fluorinert 77, Sigma, cat no. F 4758 UK).

The holding pipettes were held in the left-hand manipulator, with its corresponding micrometer syringes situated on the right-hand side. The reverse arrangement was made for the enucleation pipette and its micrometer syringe. Before fixing the pipettes into the respective microtool-holders, the tubing was purged of air bubbles using a 20 ml syringe filled with Fluorinert, which was connected into the hydraulic line via a three-way tap. The pipettes were locked into the tool holders and using the reservoir syringes, each pipette was filled with Fluorinert. The pipettes were aligned parallel to each other and their tips introduced into the manipulation chamber. Medium from the chamber was then drawn a short distance into the pipettes using the microsyringes.

3.4.4 - Micromanipulation Procedures.

3.4.4.1 - Preparation and enucleation of oocytes.

The oocytes after their recovery from the oviduct were denuded from the cumulus cells prior to the start of manipulation procedures. The cumulus cells of the oocytes were removed by exposing the oocytes to M2 media containing hyaluronidase (300 μg/ml) for about 5 min. at room temperature with continuous pipetting. The oocytes were then separated from the cumulus cells and washed with M2 medium 2-3 times in embryological watch glass dishes. The oocytes were kept

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Fig: 3.4 - Micromanipulation Procedures

a) - holding the oocyte using holding pipette

b) - recognition of metaphase plate using UV light

c) - gently inserting the enucleation pipette into the oocyte

d) - suction of metaphase chromosomes

e) - removal of cytoplasm containing metaphase chromosomes from the
enucleation pipette

f) - reconstructed embryo by placing donor nuclei inside the perivitelline space
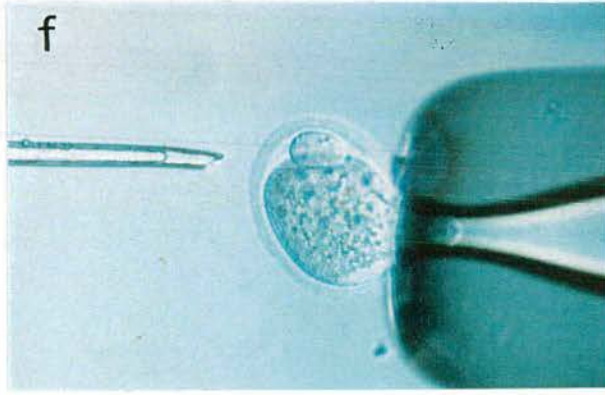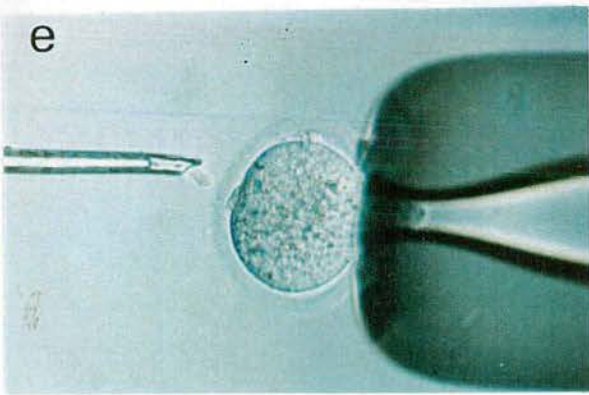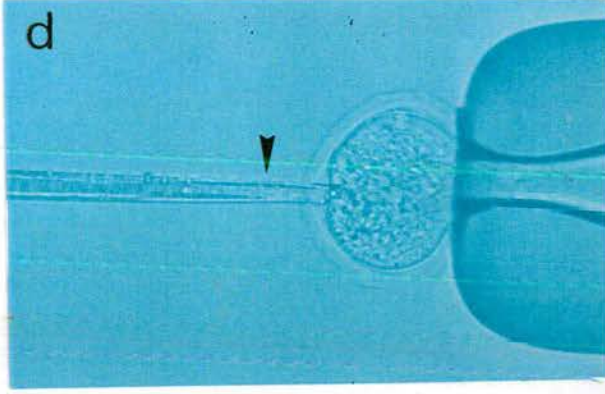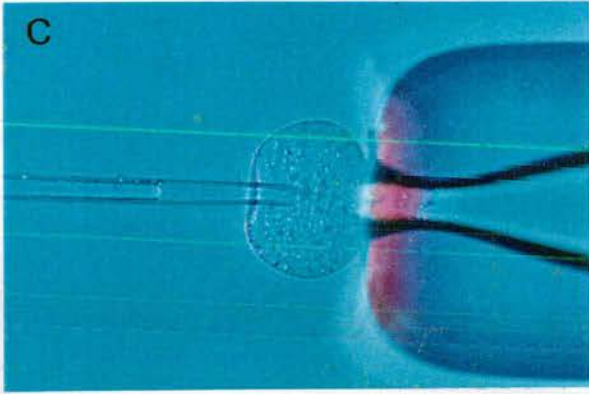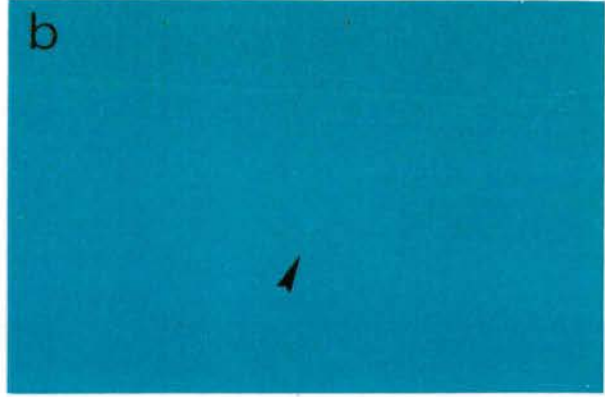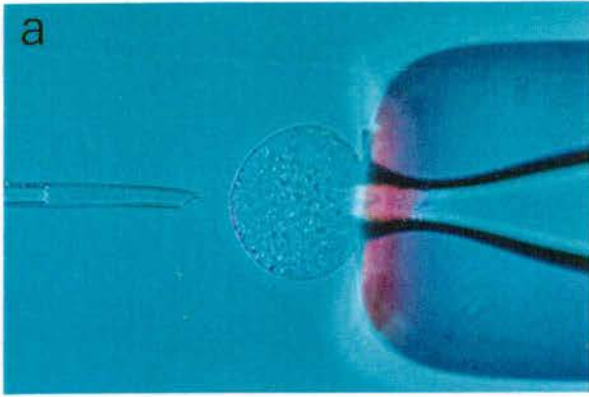
under paraffin oil at room temperature till their use for micromanipulation or some other treatments.

Before the start of enucleation procedure, the oocytes were incubated with Hoechst 33342 (0.8 µg/ml) in M2 for 5-6 minutes at room temperature. The oocytes were then washed with medium M2 and placed inside the micromanipulation chamber. The oocytes were placed in groups (10-15)before enucleation and the enucleation procedure was performed in the center of the chamber. The oocytes after enucleation were placed in the upper right corner of the chamber. The chamber medium was consisted of M2 medium supplemented with 10 µg/ml of Cytochalasin B, a microfilament inhibitor, which gave elasticity to the cytoskeleton to facilitate micromanipulation. Moreover, it also includes 10% FCS to minimize stickiness inside the enucleation pipette.

The oocyte picked up with the holding pipette, was positioned in the center of the chamber by moving the chamber. After recognizing the position of metaphase complex inside the oocyte by flushing the UV light at x 400 magnification, the enucleation pipette was introduced through the zona pellucida and the metaphase complex was aspirated into the enucleation pipette. It was possible to identify a clearer, non granular zone inside the cytoplasm that would reveal a circular structure when pushed gently with the enucleation pipette and enucleation could be performed without using the UV light. As the pipette was withdrawn from the oocyte, there was a formation of bridge extending between the aspirated cytoplast within the pipette and the enucleated oocytes within the zona pellucida. This bridge would stretch to a fine thread, pinch off and reseal. After enucleation, the enucleation pipette was exposed to the ultraviolet (UV) light for one or two seconds to confirm the aspiration of metaphase II complex of the oocyte.(see Micromanipulation procedures Fig. 3.4). The study of Tsunoda et al., (1988) demonstrate that the cytoplasm of mouse zygote is more resistant to UV irradiation after Hoechst staining. Eggs at the second metaphase, from which chromosomes have been removed under a fluorescence microscope, can be used as cytoplasm recipients for nuclear transplantation of inner cell mass nuclei. In bovine nuclear transfer embryos, no detrimental effects of short-term UV exposure and Hoechst dye on the development was observed (Betthauser et al., 1993).

3.4.4.2 - Nuclear Transfer

All nuclear transfer procedures were performed in the micromanipulation chamber. The M2 medium supplemented with inhibitors of both microfilament

(Cytochalasin B 10 μg/ml) and microtubule (nocodazole 10μM) polymerization and 10% FCS was used as chamber medium. The nuclear donor embryos were placed in the chamber and nuclear transfer was started 5-10 minutes later after achieving the elasticity of the cytoskeleton due to presence of drugs in the chamber.

The nuclear donor embryos were picked up by holding pipette and were positioned tightly by increasing the negative pressure. The enucleation pipette was then introduced through the zona pellucida and positioned adjacent to the nucleus to be removed. The karyoplast with small amount of cytoplasm surrounded by the cellular membrane was then gently sucked inside the enucleation pipette by increasing the negative pressure. By moving the pipette outside the zona a complete separation of the karyoplast was achieved through the formation, break and reseal of a cytoplasmic bridge.

In Sendai virus mediated fusion, the enucleation pipette was then moved to a 5 μl drop of the viral solution near to the main drop of the chamber medium. After picking up the small amount of viral suspension in the enucleation pipette, the enucleation pipette was moved back into the chamber.

The slide was then moved to focus the previously enucleated oocytes. The cytoplast was then oriented to the previous hole by repeated pushing and picking up moves with the holding pipette. This pipette was then introduced inside the perivitelline space of the cytoplast and a karyoplast was carefully placed. The reconstituted embryos were then washed twice with M2 and shifted into the microdrops of M16 at 37°C in the incubator.

3.4.4.3 - Fusion Procedure

3.4.4.3.1- Sendai Mediated Fusion

In most of the experiments unless otherwise mentioned in this thesis, The fusion between the transferred karyoplast and the recipient cytoplast was Sendai virus mediated. The karyoplast with small amount of inactivated Sendai virus suspension was expelled inside the perivitelline space of the zona pellucida of the recipient cytoplast for fusion which occurred within 30 minutes at 37°C.

3.4.4.3.2- Electrofusion of mouse embryos

Experiment were also performed using Electrofusion technique of fusing karyoplast and recipient cytoplast. This was achieved by homemade pulse generator. The parameters were set according to information provided by Kubiak

and Tarkowski (1985) and J-P. Renard of the Pasteur Institute, Paris, France. The pulse generator was capable of producing an AC pulse up to 5 V for 20 sec followed by also DC pulse up to 4 pulses of 10-200 μsec duration's and range of 0.25 to 2.0 kV/cm when it was used, fusion chamber consisting of two electrodes of 200 μm apart. A homemade fusion chamber was used in fusing the couplets. The fusion chamber consisted of two platinum wire electrodes of 100 μm diameter fixed parallel and 200 μm apart with glue to the bottom of a 4.5-cm glass pertri dish. The couplets were fused by first washing them with 500 μl of 0.3 M mannitol solution. About 200 μl mannitol solution was placed in the fusion chamber, spanning the electrodes and the couplets were placed in the fusion medium at one side. Each couplet was then placed between the electrodes and the plane of contact between the cytoplast and the karyoplast was oriented parallel to the electrodes with the help of the pipette. A 3V of 5 sec duration AC current was also applied to help in alignment of the couplet and the fusion was achieved by the following a single pulse of 1.5 kv/cm DC current of 100 μsec duration.

3.4.4.4 - Activation.
3.4.4.4.1- Strontium Activation Media

In most of the experiments Ca^{+2} and Mg free strontium activation media was used. The recipient cytoplasts and fused couplets were activated by using the 25 mM strontium chloride in Ca^{+2} and Mg free M16 (Otaegui *et al.*, 1994). The strontium chloride stock solution was made by dissolving 0.452 g/10ml of strontium chloride in distilled, deionised water. The final concentration of 25 mM was achieved by adding 3.67 ml of stock solution to the calcium magnesium free M16. Different stock solutions were mixed in a manner that stock B was added last to avoid any precipitation of salts and the final calibration of 25 ml was achieved by adding cold molecular water at 4°C. (see Appendix 1 table: 1.4).

In later experiments discussed in chapter 5, different activation protocols were used for activating the recipient cytoplasts. The protocol involved the use of Ionomycin and 6-Dimethylaminopurine.

3.4.4.4.2- Ionomycin Activation Media

The recipient oocytes were artificially activated by exposing them to 5.0 μM concentration of Ionomycin in M2 medium for 5-6 min. The stock solution of Ionomycin calcium salt (Sigma) was prepared by dissolving 1mg of Ionomycin

calcium salt with DMSO to the concentration of 1.0 mM (see Appendix 1 section 1.7.8).

3.4.4.4.3 - 6-Dimethylaminopurine (DMAP) treatment.

The oocytes were subsequently exposed to 6-Dimethylaminopurine (DMAP), the protein kinase inhibitor. A stock solution of 6-dimethylaminopurine (Sigma) was prepared by dissolving with the distilled, deionised water and 20 μl aliquots were made containing 2.5 mM DMAP (see Appendix section 1.7.5). This solution was diluted with M16 or strontium activation media depending upon the type of treatment and the final concentration of 2.5mM/ml or 2.0 mM/ml was achieved.

3.5 - PREPARATION OF THE INACTIVATED SENDAI VIRUS SOLUTION.

The viral stock which produce growth in the embryonated chicken egg was a generous gift from Dr. Bill Christie (MRC, Edinburgh). The results obtained are described below together with methodology, which was based on Giles and Ruddle (1976) with slight modifications.

Seven day embryonated pathogen-free chicken eggs were candled and the top of the air space was marked with an arrow indicating the position of the embryo. A horizontal line approximately 5 mm long was marked at the level of inner shell membrane, which delimits the bottom of the air space, on the side of the egg opposite the embryo. The second mark was placed in a position to avoid injecting virus into the yolk sac, if the yolk sac rises close to the level of inner shell membrane. The eggs which appeared to be infertile during candling were discarded and the fertilized ones were grouped into batches of 5 for inoculation into the allantoic cavity opposite to the embryo. The eggs were surface-sterilized by two washes with 70% ethanol after complete drying of each wash. Using a sterile dissecting needle as a punch and scalpel handle as a hammer, two small holes were made, one at the point of arrow over the air space (vent hole) and the other at a point 2 to 3 mm above the horizontal line (site of injection). Six groups were injected with 0.2 ml of a 10^{-3} dilution of the infected allantoic fluid in Hank's Balanced Salt Solution (HBSS). Two groups were used as the control, one injected with HBSS alone and the other without any injection. The site of injection and the vent hole were sealed with small pieces of selotape. The injected eggs, eggs injected with

HBSS only and uninjected control were incubated at 35.5^{0}C in the moist chamber for 72 hours and turned three times a day.

At the end of incubation period all the eggs were candled again and any egg have dead embryo or any other abnormality was discarded. The eggs were placed in the cold room at 4^{0}C overnight to reduce the bleeding at harvesting procedure and also to kill the embryos. The surface of the eggs were sterilized and the inner shell membrane was exposed by cutting the shell above the air space with sharp scissors. Allantoic fluid (5 to 10 ml per egg) was aspirated by inserting a sterile syringe with 1.5 inch 18-gauge needle through the inner shell membrane into the allantois. At this point all necessary precautions were adapted to avoid getting virus into the air.

The allantoic fluid from the controls and the infected eggs was collected separately in sterile bottles and the titer or the haemagglutination activity was determined by an assay based on the Salk pattern method (see Cassals, 1967). A packed cell volume of 0.5 ml of chick red blood cells was washed twice in 10 ml aliquots of HBSS and resuspended in 100 ml of HBSS which was used throughout as the diluent. Serially increasing 2-fold dilution's were made in a tray (96 V shape wells; Sterilin, Bibby Sterilin Ltd., Stone, Staffs, UK) from an initial 1:10 dilution of allantoic fluid. To each 0.1 ml dilution, 0.1 ml of red blood cells suspension was added and mixed in each well. The tray was covered with Saran wrap and incubated for 2 hours at 4^{0}C. A negative agglutination reaction was indicated by a "button pattern", a diffuse or continuous sheath of red blood cells covering most of the wells. The virus titer was determined by highest dilution giving complete agglutination. The dilution was said to contain 1 haemagglutination unit (HAU) in the volume used (Cassals, 1967). The harvested allantoic fluid produced a titer of 12800 HAU/ml while the control had no titer.

The allantoic fluid from the injected eggs was centrifuged at 2000 X g in a refrigerated centrifuge for 20 min. The supernatant was aspirated and saved and the pellet discarded. The supernatant was centrifuged in an ultracentrifuge at 16000 X g with zero deceleration for 1 hours. The supernatant was completely removed and discarded. The pellets from all the centrifuged tubes were broken up with a glass rod having a rounded fire-polished end, and the material was worked into a paste-like consistency without lumps by adding small amounts of 1% bovine serum albumin (BSA) in HBSS without glucose (HBSS-G). The paste was resuspended in 1% BSA in HBSS-G to the volume one tenth of the starting volume, without atomizing the virus. This was achieved by expelling the suspension 8 to 10 times through the 25-gauge needle. BSA at this point was added to reduce the loss of

HAU which may occur during inactivation (Klebe *et al*, 1970) or low temperature storage (Neff and Enders, 1967). The virus suspension was centrifuged at 2000 X g for 20 minutes and transferred to a sterile bottle. At this stage the titer of the suspension was 102400 HAU/ml.

A chemical method of inactivation was chosen, using ß-propiolactone (BPL; Sigma, UK) which destroys the viral RNA. The procedure was carried out at 4^0C with all solution kept on ice and pipettes equilibrated to the working temperature. A 0.5% solution of BPL was diluted in a saline bicarbonate solution (1.68 g $NaHCO_3$ + 0.85 g NaCl + 0.2 ml of 0.5% phenol red + 100 ml of double distilled water). One ml of 0.5% BPL in saline bicarbonate was added to each 9 ml of Sendai virus suspension and agitated for 10 min. The solution was incubated at 37^0C for 2 hours with agitation and statically at 4^0C overnight to inactivate the remaining BPL. A sample was removed for HAU assay and infectivity determination. At this stage the titer had fallen to 12800 HAU/ml. The infectivity of the inactivated Sendai virus was determined by inoculating 7-day embryonated eggs with 0.2 ml of 1:4 and 1:20 dilution of the inactivated material for 72 hours for the first egg passage. Samples of 0.2 ml of undiluted allantoic fluid harvested for each series (1:4 and 1:20) of the first egg passage were used to initiate a second 72 hours egg passage. In the first egg passage 1:4 dilution gave 400 HAU/ml haemagglutination activity but no HAU was observed in a 1:20 dilution and in any of the following passages. It was considered that haemagglutination activity in the first passage of 1:4 dilution was the result of higher HAU after inactivation of virus with BPL. However, no HAU was observed in any of the further passages indicating that the virus had been completely inactivated. The virus suspension was stored in 1 ml aliquots at -70^0C till the completion of fusion experiments.

3.6 DNA labeling techniques

Two techniques of DNA labeling were employed to investigate the duration of G1 period and start of DNA replication in 3rd and 4th cell cycle of preimplatation mouse embryos: the pulse labeling for proliferating cell nuclear antigen (PCNA) and the rapid pulsing technique of incorporating 5-bromodeoxyuridine (BrdU). The detailed procedures have been discussed in chapter 6.

3.7-STATISTICAL ANALYSIS OF DATA.

Details of the methods used for the statistical analysis of the experimental data are given in each experimental chapters. Those analysis were conducted with the help and advice of Mr. Dave Waddington (Roslin Institute).

CHAPTER 4

SYNCHRONIZATION OF DONOR NUCLEI

4.1 INTRODUCTION

Synchronization of the donor nuclei plays an important role in evaluating the effect of cell cycle co-ordination in the development of reconstituted embryos. Asynchronous cleavage is the characteristic of early embryonic processes in mammals which progressively increases among blastomeres throughout preimplantation development. Synchronization of the blastomeres within an embryo is more difficult as the embryo approaches the late cleavage stages. In nuclear transfer experiments, it is believed that blastomere asynchrony causes inconsistent nucleo-cytoplasmic interactions in the embryos reconstructed by nuclear transfer. The lower success rate in nuclear transfer experiments has been reported due to the negative effects of the absence of cell cycle synchrony between nuclear donor and recipient cytoplasm (Smith *et al.*, 1990; Campbell *et al.*, 1994 and Otaegui *et al.*, 1994).

The study of Otaegui *et al.*, (1994) in this laboratory demonstrated that nocodazole, an inhibitor of tubulin polymerization could successfully be used to synchronize donor blastomeres and to hold the donor embryos at mitosis stage for several hours. The 2-cell embryos exposed to nocodazole (3.0 µg/ml) for 12.5 -14.5 hours showed no toxic effects on development of embryos after release and 98% reached to blastocyst stage as demonstrated by Kato and Tsunoda (1992). However, prolonged exposure to nocodazole showed negative effect on the division rate of blastomeres in bovine embryos (Tanaka *et al.*, 1995). The longer exposure to nocodazole also leads to chromatin dispersion in mouse embryos which could result in chromosomes abnormalities (Samake and Smith 1996a, b). The previous reports emphasized the need for a reliable method for synchronization to arrest the cell cycle of mouse embryos for longer periods of time, enabling the blastomeres to reach synchrony together and be able to used for reconstruction of embryos by nuclear transfer with convenience. Several cell cycle arrest drugs are available with specific mechanism of action in cell cultures for the inhibition of cell cycle progression.

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An inhibitor of maturation promotion factor (MPF), 6-dimethyl aminopurine (6-DMAP) has been used to prevent germinal vesicle breakdown (GVBD) during invertebrate (Guerrier and Doree, 1975; Doree *et al.*, 1983) and vertebrate oocyte maturation (Rime *et al.*, 1989). The dependency of mouse oocyte maturation on protein synthesis and protein phosphorylation has been previously reported by many workers. These experiments shows that the germinal vesicle breakdown (GVBD) in mouse oocyte maturation does not require protein synthesis (Schultz and Wassarman, 1977; Hashimoto and Kishimoto, 1988) but needs protein phosphorylation (Rime *et al.*, 1989). The critical period after which protein synthesis is required seems to be linked to the transition from prophase I to metaphase I (Hashimoto and Kishimoto, 1988) and metaphase I requires protein synthesis to maintain chromosomes in a condensed form (Clarke and Masui, 1983; Hashimoto and Kishimoto, 1988) but Rime *et al.*, (1989) have postulated a need for protein phosphorylation rather than protein synthesis. It has also been reported that, the transition from metaphase I to the end of the first meiotic division is MPF independent (Hashimoto and Kishimoto, 1988), and formation of metaphase II and its stability until fertilization of the oocyte depends on protein synthesis (Siracusa *et al.*, 1978; Clarke and Masui, 1983; Hashimoto and Kishimoto, 1988; Rime *et al.*, 1989).

Phosphorylation has been shown to play an important role in the G2/M transition of mitosis (Adlakha and Rao, 1987; Cross *et al.*, 1989) and meiosis (Lohka and Maller, 1987; Maller *et al.*, 1989). As meiosis I proceeds towards GVBD, a large increase in phosphorylation of cellular protein occurs in conjunction with the appearance of maturation-promotion factor (MPF) activity (Lohka and Maller, 1987; Ozon *et al.*, 1987). This large burst of phosphorylation may involve elements necessary for nuclear dissolution (e.g., lamins, histones, nucleoplasm, nucleolar proteins, intermediate filaments, etc.; reviewed by Adlakha and Rao, 1987) The 6-DMAP has been shown to inhibit the GVBD in starfish and sea urchin (Neant *et al.*, 1989; Neant and Guerrier, 1988a), and mouse (Rime *et al.*, 1989).

On the other hand, Taxol is an antimitotic agent and is an important new drug for the treatment of certain cancers (Rowinsky *et al.*, 1990). Amongst the many microtubule-directed drugs, the Taxol family are unique in that they stabilize cytoskeletal microtubules against depolymerization. Taxol promotes the formation of discrete bundles of stable microtubules that result from the reorganization of the microtubule cytoskeleton (Schiff PB and Horwitz SB, 1980). The novel characteristic of Taxol is its ability to polymerize tubulin *in vitro* in the absence of

guanosine $5'$-triphosphate (GTP), which is normally required for tubulin assembly (Schiff et al., 1979). It is only drug known to bind at a specific site on the microtubule polymer (Parness and Horwitz, 1981), and study with a Taxol photoaffinity analogue have identified the amino-terminal 31 amino acids of β-tubulin as the main site of photoincorporation (Rao et al., 1994). The other indicates a 2.5-fold higher labelling of β- tubulin than α- tubulin (Combeau et al., 1994). The recent study of Isabelle and Richard by using guanyl-(α&β)-methylene-diphosphonate (a non-hydrolyzable GTP analogue), showed that microtubule stabilization with Taxol is accompanied by a conformational change in the microtubule lattice and, implicitly, in the tubulin dimer. Taxol blocks the cell cycle in its G1 and M phases by stabilizing the microtubule cytoskeleton against depolymerization--the basis of its clinical use in cancer therapy. In vitro , it decreases the critical concentration of tubulin assembly, increases the polymerization rate and stabilizes microtubules against the effects of cold, the presence of calcium, dilution or the action of other drugs. Because of its microtubule stabilization property, it is also used in experiments where stable microtubule assemble is required.

In recent years 6-dimethylaminopurine (6-DMAP), the inhibitor of protein phosphorylation has been successfully used to arrest cleavage in 4-cell mouse embryos (Samake and Smith, 1996a). So it was decided to evaluate the effect of 6-DMAP for the synchronization of cell division and on the other hand the effect of Taxol, the microtubule stabilizer drug on the cell cycle arrest was also studied.

4.2 EMBRYOS

The F1 female mice (C57BL/6 x CBA/ Ca) of 4-7 weeks old were superovulated by using superovulation regime (see section 3.3.2) and mated with the same F1 males immediately post hCG injections. The mice were killed by cervical dislocation 44-48 hours post hCG injections and mating. The 2-cell embryos were recovered from the oviducts in M2 medium and washed 2-3 times with M2. The embryos were stored at low temperature (4°C) in a glass dish containing M2 covered with mineral oil until required till their exposure to different synchronization treatments

4.3 - STATISTICAL ANALYSIS.

The data was analyzed using Generalized Linear Mixed Model (Breslow and Clayton, 1993) looking at the effect of treatment whilst allowing for differences

between days. The treatment effects were tested using a Wald statistics (distributed as chi-squared) and, if significant, differences between means were assessed by an approximate t-test. Means declared to differ, differ at the 5% level or better.

4.4- EXPOSURE TO 6-DIMETHYLAMINOPURINE (6-DMAP)

The aim of the present study was ; To determine the reliability of 6-dimethylaminopurine (6-DMAP), an inhibitor of maturation promotion factor, to arrest and to synchronize blastomere division in cleavage stage murine embryos and to assess its toxicity in vitro.

The experiments were planned to establish the minimum dose concentration of 6-DMAP required to hold and maintain the cell cycle at mitosis. The second objective of this study was to determine the maximum period of time in the particular concentration, compatible with normal development to blastocyst after release. In most of the experiment performed under this Ph.D. project, 4-cell donor were used as nuclear donor for development studies. There was a need to establish a more reliable method for the synchronization of 2-cell mouse embryos.

4.4.1 EXPERIMENTAL DESIGN

The 2-cell mouse embryos were recovered 48 hours post hCG injections and exposed to 6-DMAP to evaluate the effect of different concentrations and time of exposure on the proportion of embryos arrested at mitosis and development after release. The embryos were washed 2-3 times with M2 and were exposed to different concentrations of 6-DMAP in sM16 medium. For this purpose 60 mm petri dishes containing 30 µl microdrops of 5.0, 2.5 and 1.25 mM concentrations of 6-DMAP in sM16 were prepared at least 2 hours prior to the start of each treatment and were equilibrated at 37°C in 5% CO_2 in air in the humidified incubator .

After the end of each treatment, the ability of the different concentrations of 6-DMAP to arrest the cell cycle and to hold the embryos at mitosis was determined. The number of cells in each embryo were counted to determine the proportion of cells divided after exposure to the treatment. They were also treated either with Hoechst 33342 (1.0 µg/ml) in M2 for 10 minutes or they were stained with aceto-orcene to determine the proportion of nuclei at mitosis.

The embryos were washed several times with M2 and sM16 to remove the affect of 6-DMAP from the embryos and cultured in the microdrops of sM16 under liquid paraffin at 37°C in an atmosphere of 5% CO_2 in air and the developmental potential of the embryos was determined.

4.4.2- RESULTS

The effect of different concentrations of 6-DMAP and length of exposure on the embryos arrested at mitosis is presented in Table 4.1. All embryos exposed to 5.0 mM and 2.5 mM DMAP treatments for 9, 12 and 16 hours showed 100% cell cycle arrest at interphase (G2-M) (Figure 4.1), it was not possible to conduct formal statistical analysis with large number of treatments having a proportion of one.

When the embryos were exposed to 1.25 mM concentration of 6-DMAP for 16 hours, 66% showed cell cycle arrest compared to control group where 96% of the embryos had shown cleavage to 4-cells. The exposure of the treated embryos at the end of treatment to Hoechst staining (1.0 µg/ml) revealed that all those embryos exposed to the minimum concentration of 2.5 mM 6-DMAP and above for 12 hours were with intact nuclear envelope (Fig. 4.2). The prolonged exposure of embryos to 5.0 mM and 2.5 mM concentrations of 6-DMAP for 24 hours showed some negative effect on the embryos as 19% and 5% of the embryos had fragmented or appeared to be fluid filled respectively. In contrast, 100% of the embryos in the control group had divided.

4.4.2.1- Development After Release

The effect of toxicity of different concentrations of 6-DMAP treatment for 9, 12 and 16 hours on the development of 2-cell embryos is presented in tables 4.2 to 4.4 respectively. The development of treated embryos to 4-cell was shown to be greatly effected (p< 0.001) with varying concentrations of DMAP and higher proportions of development with decreasing concentration of DMAP was observed. However, different concentrations of DMAP when compared for 9 and 12 hours, provided no detectable difference in the development of embryos to 4-cell. All the embryos exposed to different concentrations of 6-DMAP for 16 hours when released from the drug exposure and further cultured in sM16 medium for 4 days, showed no development as compared to the control group where 89% development to morula stage was observed (Table: 4.4). When embryos were exposed to different concentrations of 6-DMAP for 9 hours, maximum development (77%) to morula and

Fig. 4.1 Two-cell mouse synchronized with 6-DMAP

a) Two-cell mouse embryos synchronized with 5.0 mM 6-DMAP after 24 hours

b) Two-cell mouse embryos synchronized with 2.5 mM 6-DMAP after 24 hours

c) Two-cell mouse embryos synchronized with 1.25 mM 6-DMAP after 24 hours

Note: 1.25 mM concentration is unable to hold the cells at mitosis and the embryos are shown to be fragmented.
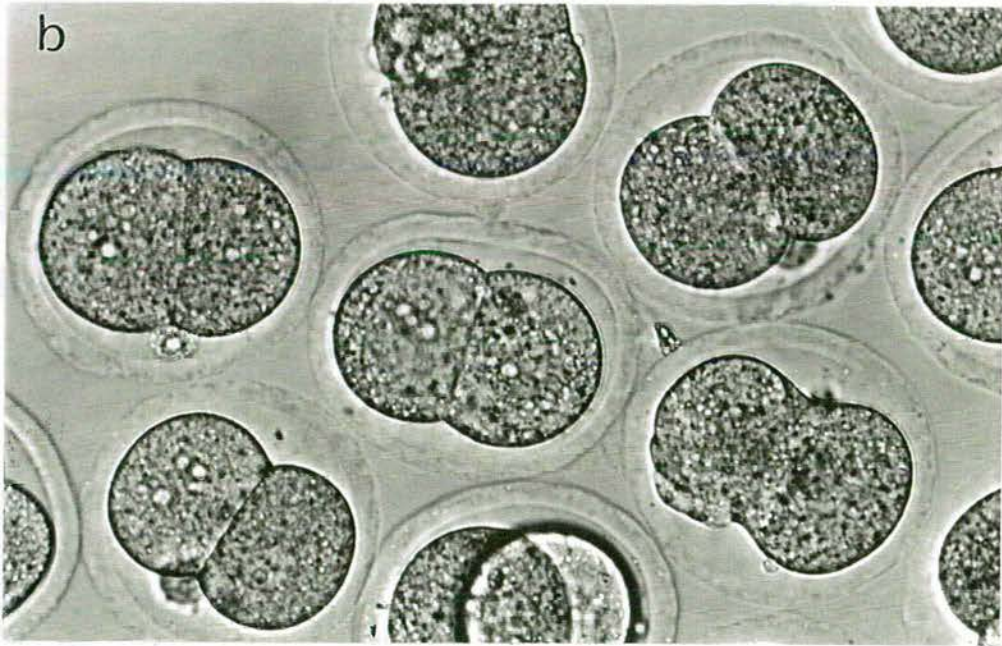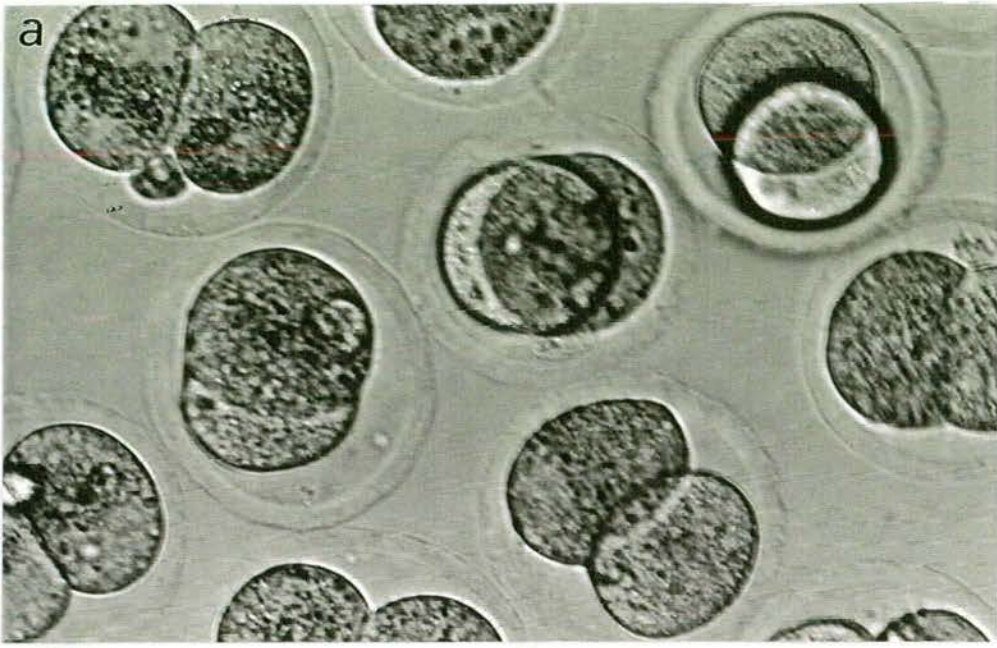
Fig. 4.2 Hoechst staining of two-cell mouse embryos synchronized with 6-DMAP

a) Hoechst staining of 2-cell embryos after 12 hours of synchronization with 5.0 mM concentration of 6-DMAP (Condensed nuclei)

b) Hoechst staining of 2-cell embryos after 12 hours of synchronization with 5.0 mM concentration of 6-DMAP (normal nuclei)

c) Hoechst staining of 2-cell embryos after 12 hours of synchronization with 2.5 mM concentration of 6-DMAP (looks normal)

d) Hoechst staining of 2-cell embryos after 12 hours of synchronization with 2.5 mM concentration of 6-DMAP (disturbed chromatin)
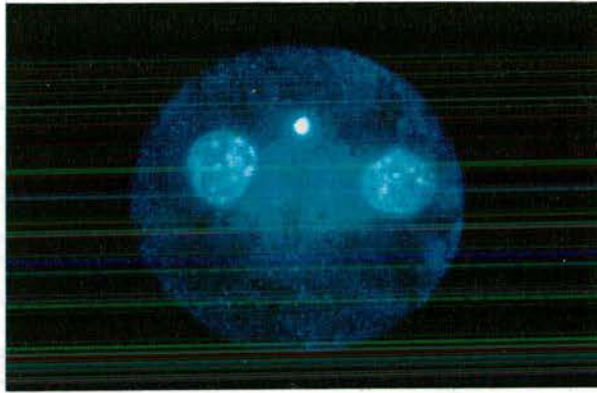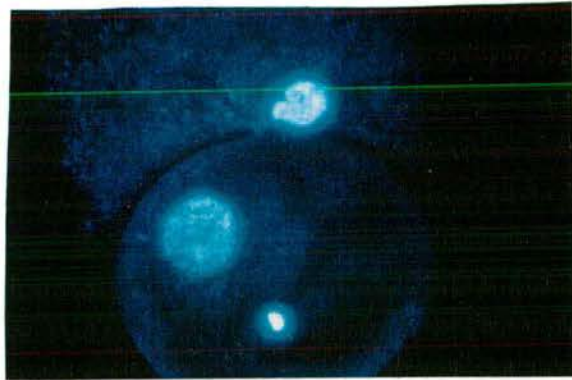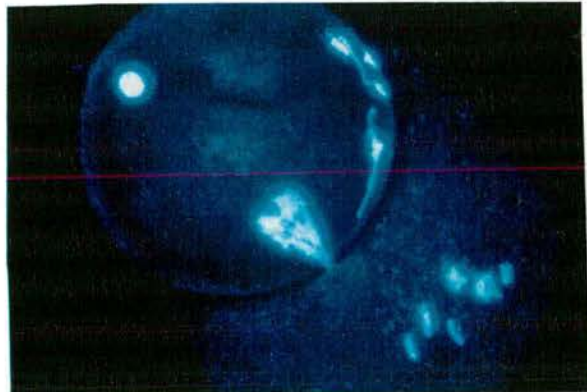
Fig. 4.3 Development after release of two-cell mouse embryos from synchronization with 6-DMAP for 9 hours

a) Development to blastocyst of embryos exposed to 5.0 mM 6-DMAP for 9 hours after 3 days of culture.

b) Development to blastocyst of embryos exposed to 2.5 mM 6-DMAP for 9 hours after 3 days of culture.

c) Development to blastocyst of embryos exposed to 1.25 mM 6-DMAP for 9 hours after 3 days of culture.

d) Development to blastocyst stage of the control group.
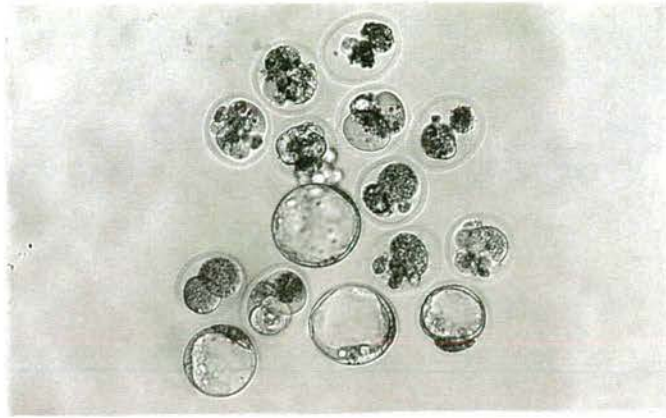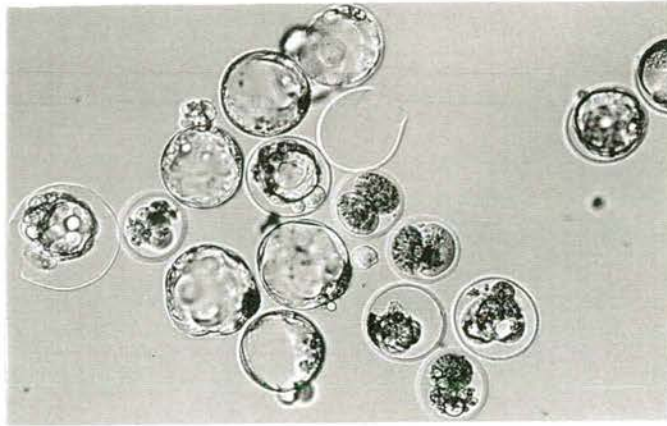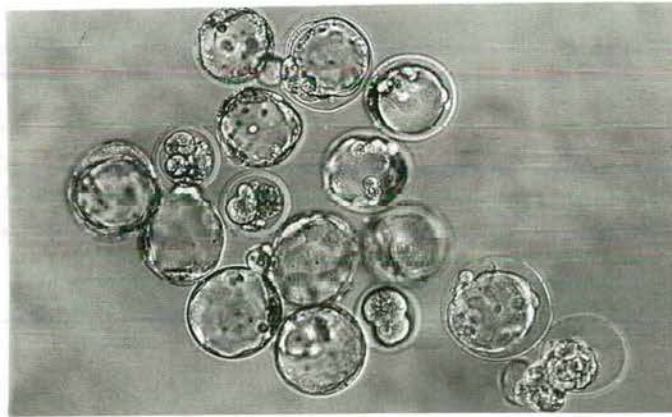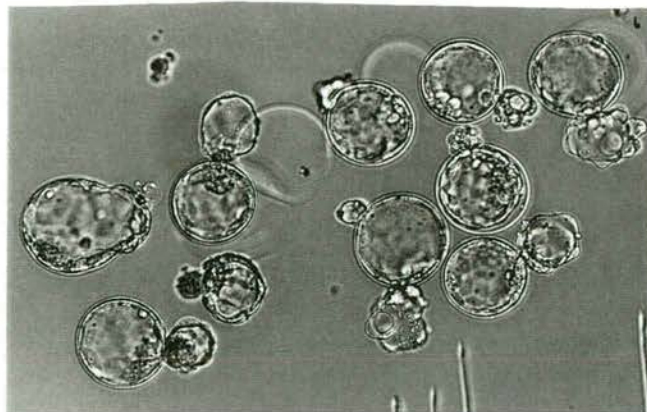
Table: 4.1 Effect of different concentrations of 6-DMAP and length of exposure on the proportion of 2-cell embryos arrested at mitosis.(3 Rep.)

Concentrations	9 Hours	12 Hours	16 Hours	24 Hours
5.0 mM	55/55(100)[a]	78/78(100)	60/60(100)	39/48(81)
2.5 mM	43/43(100)	110/110(100)	80/80(100)	57/60(95)
1.25 mM	51/60(85)	33/80(41)	38/58(66)	3/60(5)
Control	9/45(20)	8/45(18)	2/45(4)	0/45(0)

a Number (%) of embryos arrested at 2-cell at the end of each treatment.

Table: 4.2 Development after release of 2-cell embryos synchronized for 9 hours with different concentrations of 6-DMAP.

Conc.	No Change (2-cell)	4-cells	8-cells	Morula	Blastocysts
5.0 mM	24/40(60)[a]	16/40(40)	14/40(35)	11/40(28)	11/40(28)
2.5 mM	14/47(30)	33/47(70)	31/47(66)	31/47(66)	31/47(66)
1.25 mM	11/47(23)	36/47(77)	36/47(77)	36/47(77)	36/47(77)
Control	3/37(8)	34/37(92)	33/37(89)	33/37(89)	30/37(81)

a Proportion (%) of development after release from synchronization for 9 hours.

Table: 4.3 Development after release of 2-cell embryos synchronized for 12 hours with different concentrations of 6-DMAP.

Conc.	No Change (2-cell)	4-cells	8-cells	Morula	Blastocysts
5.0 mM	21/36(58)[a]	14/36(39)	14/36(39)	13/36(36)	13/36(36)
2.5 mM	23/57(40)	34/57(60)	20/57(35)	20/57(35)	20/57(35)
1.25 mM	3/35(9)	32/35(91)	32/35(91)	32/35(91)	32/35(91)
Control	3/37(8)	34/37(92)	33/37(89)	33/37(89)	30/37(81)

a Proportion (%) of development after release from synchronization for 12 hours.

Table: 4.4 Development after release of 2-cell embryos synchronized for 16 hours with different concentrations of 6-DMAP.

Conc.	No Change (2-cell)	Dead	4-cells	8-cells	Morula	Blastocysts
5.0 mM	48/48(100)[a]	0/48(0)	0/48(0)	0/48(0)	0/48(0)	0/48(0)
2.5 mM	27/47(57)	18/47(38)	0/47(0)	0/47(0)	0/47(0)	0/47(0)
1.25 mM	40/40(100)	0/40(0)	0/40(0)	0/40(0)	0/40(0)	0/40(0)
Control	3/37(8)	0/37(0)	34/37(92)	33/37(89)	33/37(89)	30/37(81)

a Proportion (%) of development after release from synchronization for 16 hours.

blastocyst stage was revealed with the lowest concentration of 6-DMAP (1.25 mM) used in this experiment (Table: 4.2) which was comparable to the control group (89%) (Fig. 4.3).

The exposure to the 2.5 mM concentration for 9 hours showed slightly less development to morula and blastocyst i.e. 66%. In contrast, when the embryos were exposed to different concentrations of 6-DMAP for 12 hours, the developmental competence to morula and blastocyst was shown to be much higher (91%) in the embryos treated with 1.25 mM concentration of 6-DMAP as compared to the 35% development to morula/ blastocyst stage in the group treated with 2.5 mM concentration. For 5.0 mM concentration development to blastocyst was similar. These patterns resulted in an interaction between DMAP concentrations and exposure time ($p < 0.01$). Overall, blastocyst development enhanced by lower concentrations of DMAP ($p < 0.001$).

4.4.3 - CONCLUSIONS.

The minimum concentration of 2.5 mM could successfully be used to arrest the cell cycle up to 16 hours. However, the length of exposure to different concentration of 6-DMAP for more than 12 hours showed a negative effect on the development of all treated embryos as 0% development was observed in this experiment . The embryos exposed to 2.5 mM concentration of 6-DMAP for 9 hour could provide synchronized population of donor nuclei for nuclear transfer experiments. The lower concentration of 1.25 mM DMAP gave rise to better blastocyst production.

4.5 EXPOSURE TO TAXOL.

The aim of the present experiment was to assess the reliability of a microtubule stabilizing agent, Taxol for arresting and synchronizing blastomere division in cleavage stage murine embryos and to assess its reversibility and toxicity in vitro.

4.5.1 EXPERIMENTAL DESIGN.

To determine the reliability of a microtubule stabilizing agent, Taxol for arresting and synchronizing blastomere division in cleavage stage murine embryos and to assess its reversibility and toxicity in vitro, 2-cell embryos were recovered

from the F1 females 44-48 hours post hCG injection and mating with the same F1 males. The embryos were washed 2-3 times with M2 medium. The embryos were kept at low temperature (4°C) under paraffin oil in M2 medium till required. In this experiment, the 2-cell embryos were exposed to zero, 0.5 µM, 1.0 µM, 5.0 µM and 10.0 µM concentrations of Taxol in M16 medium. The 30 µl microdrops were made using 60 mm dishes and covered with paraffin oil. The dishes were prepared 2 hours prior to the start of each treatments and were equilibrated at 37°C in the atmosphere of 5% CO_2 in air.

To determine the effect of concentrations of Taxol and length of exposure on the proportion of mouse embryos arrested at metaphase, the embryos were exposed to different concentrations of Taxol for different periods of time. The ability of different concentration of Taxol to hold the cell cycle was assessed by counting the number of cells in individual embryos at 8, 12, 16, 20 and 36 hours after exposure to the different concentrations of Taxol. They were also exposed to fluorescence staining Hoechst 33342 (0.1 µg/ml) to observe the nuclei in the treated embryos.

To assess the reversibility and toxicity, 2-cell embryos were released from the treatments by washing them several times with M2 and sM16 to remove the affect of drug and cultured in sM16 medium. The observations on the developmental competence of the exposed embryos were recorded.

4.5.2- RESULTS.

At the end of each treatment, all embryos with 2 cells were considered arrested and those with more than 2 considered non-arrested (Fig.4.4a). The 2-cell embryos were exposed to different concentrations of Taxol for various length of time and the results are presented in Table: 4.5. The analysis of data showed no detectable interaction between Taxol concentration and exposure time in the proportion of 2-cell embryos arrested at mitosis, however, a highly significant effect of the presence of Taxol verses controls (p< 0.001) and exposure time (p< 0.001) was observed. The chromatin morphology were assessed by staining them with Hoechst 33342 and depicted in (Fig. 4.4 c&d). The results of the experiment based on the cell count of the treated embryos showed cell cycle arrest in 100% of 2-cell embryos treated with 5.0 µM concentration of Taxol for eight hours. The results in Table 4.5 also showed the ability of even the minimum (0.5 µM) concentration of Taxol to arrest the cell cycle in 95% of embryos exposed. No difference was observed in the proportion of the embryos treated with 0.5 and 1.0 µm of Taxol for eight hours. When the

Fig. 4.4 Two-cell embryos synchronized with Taxol

a) normal morphology of the two-cell mouse embryos arrested at mitosis with 1.0 μM for 13 hours.

b) most of the embryos in control groups are showing growth to 3-4 cells

c) normal morphology of the nucleus of the 2-cell embryos synchronized with 10 μm Taxol for 8 hours.

d) disturbed nuclear morphology of the 2-cell mouse embryo synchronized with 10 μm Taxol for 12 hours.
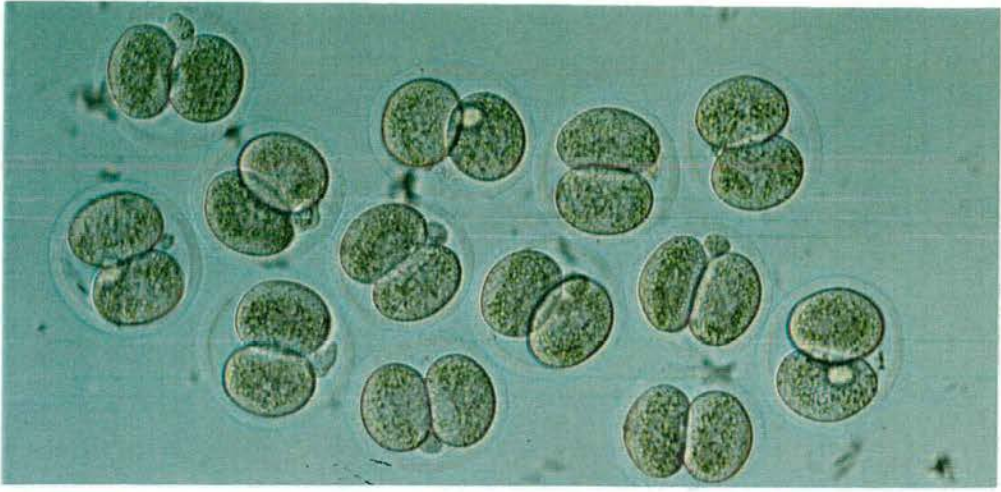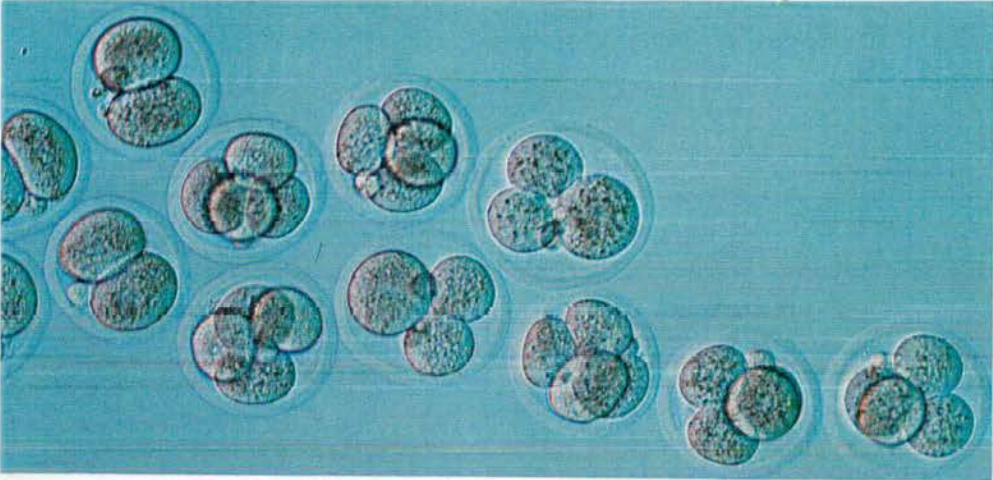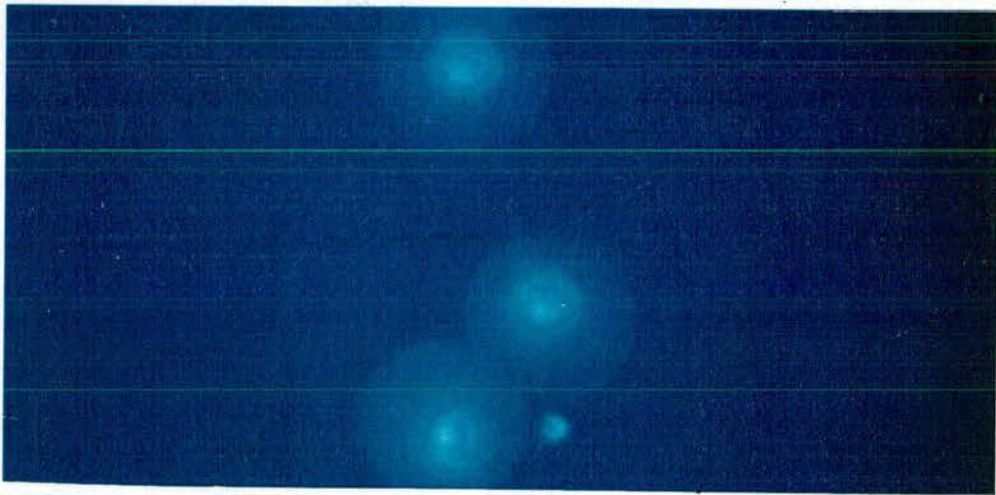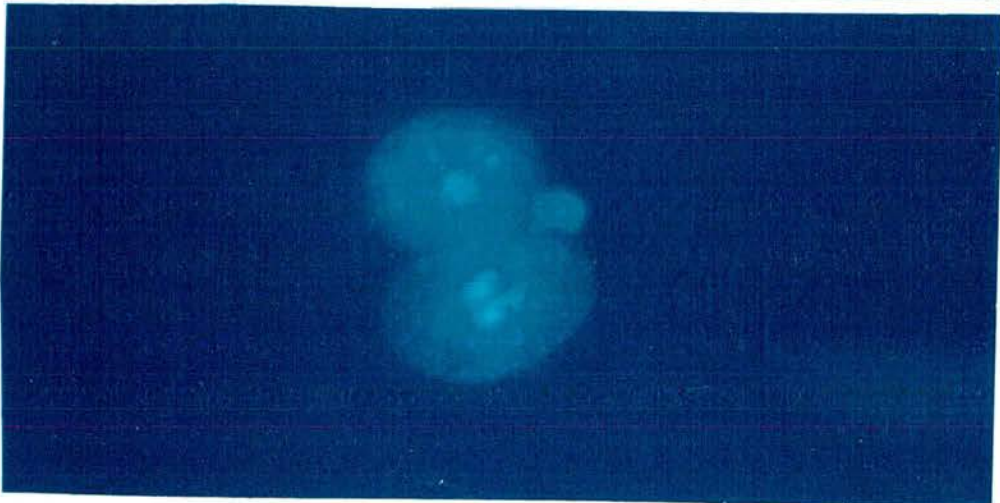
Table:4.5- Effect of concentration of Taxol and length of exposure on the proportion of mouse embryos arrested at metaphase(%).

Treatments	8 Hours	12 Hours	16 Hours	20 Hours	36 Hours
Control	7/21(33)[a]	7/59(12)	7/42(17)	2/40(5)	1/19(5)
0.5 μM	21/22(95)	33/58(57)	30/40(75)	22/42(52)	9/23(39)
1.0 μM	21/22(95)	35/60(58)	32/41(78)	25/43(58)	9/22(41)
5.0 μM	22/22(100)	33/59(56)	32/40(80)	25/40(63)	7/23(30)
10.0 μM	19/22(86)	35/61(57)	40/45(89)	34/45(76)	8/23(35)

a Number (%) of embryos arrested at the end of each treatment. Values are assessed by counting the number of cells in each embryos.
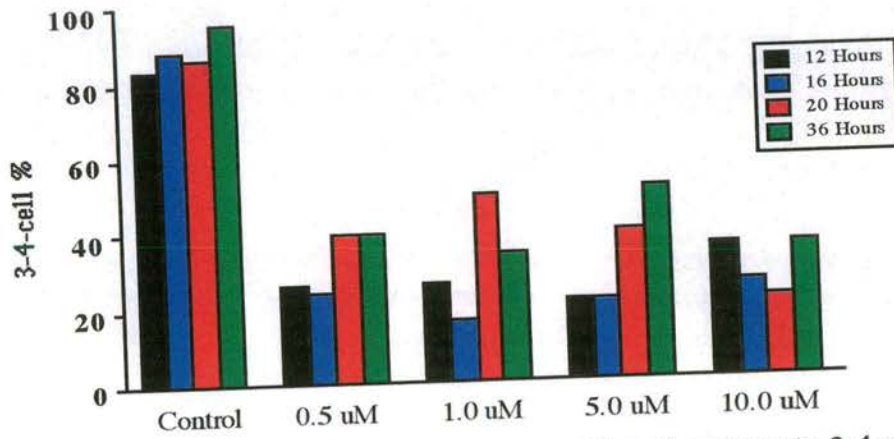
Fig: 4.5 Graph showing development of 2-cell embrys to 3-4-cell
release from synchronization treatmentof Taxol for various
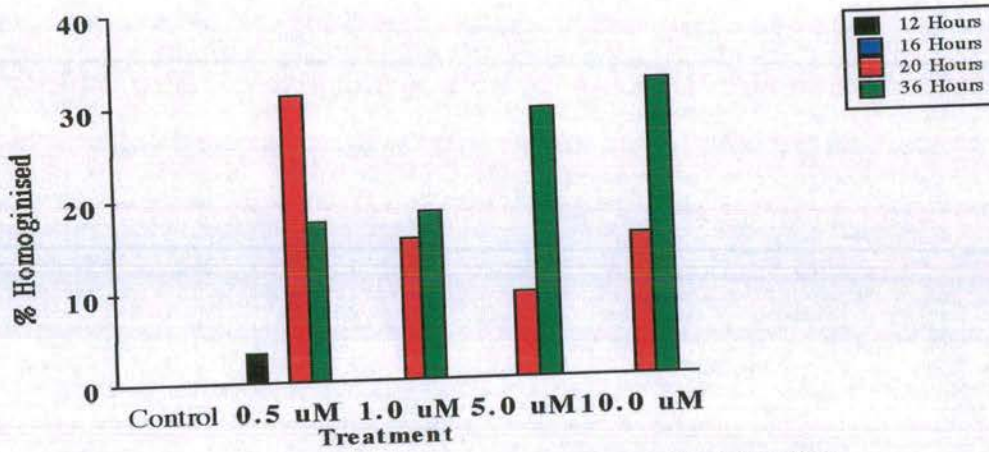
Fig: 4.6 Graph showing the precetage of embryos homoginised after their
release from synchronization with Taxol for various durations
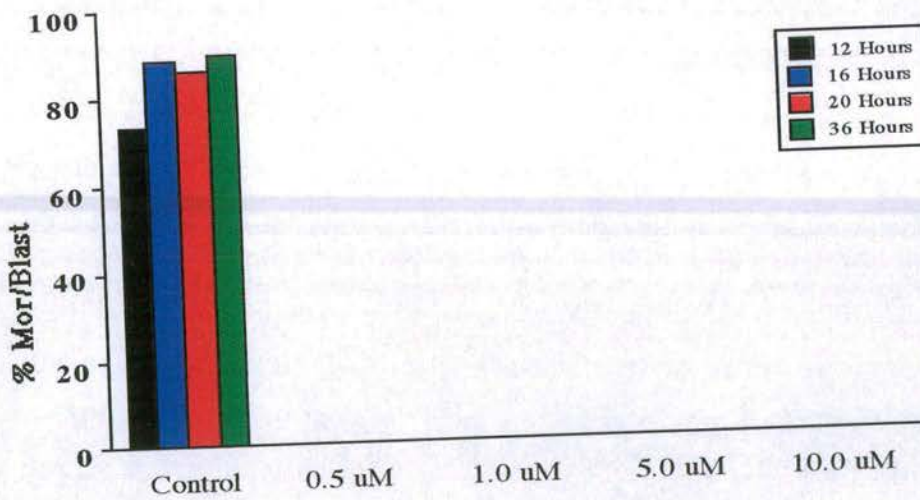
Fig:4.7 Graph showing 2-cell development to Mor/Blast. after their
release from synchronization with Taxol for various durations

embryos were exposed for 12 hours to different concentrations of Taxol, the cell cycle arrest in 2-cell embryos observed was 57, 58, 56 and 57% when exposed to 0.5, 1.0, 5.0 and 10.0 µM concentrations of Taxol as compared to the control (non-treated) groups (Fig. 4.4b), where 12% showed cell cycle arrest . The overall results showed in Table 4.5 demonstrated the positive effect (more than 30%) of all the concentrations of Taxol in arresting the cell cycle even up to 36 hours. The nuclear morphology of the embryos after different treatments showed disturbed metaphase assembly in the embryos exposed to 1.0 µM and 5.0 µM concentrations of Taxol for 12 hours. This conditions was observed in all the embryos exposed to more than 12 hours to even the minimum concentration of 0.5 µM of Taxol.

4.5.2.1 DEVELOPMENT AFTER RELEASE

The effect of toxicity of Taxol on the developmental competence of treated embryos for 12, 16, 20 and 36 hours to different concentrations of Taxol ranging from 0.5 µM to 10.0 µM was evaluated by culturing them after washing the treated embryos several times with M2 and M16. The results are presented in Fig: 4.5 to 4.7. The analysis of the data for development of embryos to 3-4-cells out of the total showed no detectable interaction between Taxol concentrations and exposure time, however, significant effect of Taxol verses control ($p<0.001$) and exposure time ($p<0.01$) was observed. The results indicated absolutely no growth beyond 3-4 cells in all the embryos exposed to different concentrations of Taxol. In fact, in most of the embryos, the homogenized appearance was observed. Hoechst staining of all those embryos which developed to 3-4 cell showed disturbed/ scattered chromatin.

4.5.3 CONCLUSIONS

Taxol could be used to hold the cell cycle arrest. Minimum concentration of 0.5 µM of Taxol is able to arrest the cell cycle in 95% of 2-cell embryos. The exposure of cells to Taxol is said to be having a favorable effect on stabilizing spindle for microtubule kinetic studies. However, after the use of Taxol in preimplantation embryos for holding the cell cycle arrest, no embryo developed to morula or blastocyst stage.

4.6- COMPARISON OF NOCODAZOLE AND DIFFERENT COMBINATIONS OF 6-DMAP.

In this laboratory, exposure of mouse blastomeres to 10 μM nocodazole was the preferred method of choice for synchronization. To evaluate the differences in the ability of different concentrations of 6-DMAP to hold the cell cycle at mitosis and to assess the difference in the developmental competence, it was planned to compare these differences with the previously preferred method for synchronization of mouse blastomere i.e. 10 μM nocodazole.

4.6.1 EXPERIMENTAL DESIGN.

The 2-cell embryos were exposed to 5.0 mM, 2.5 mM and 1.25 mM concentrations of 6-DMAP in sM16 medium. For comparison purposes the groups of 2-cell embryos were also exposed to 10 μM nocodazole. The control groups were also prepared to compare the reversibility of both groups of embryos after exposure to the drugs. After the end of each treatment, the 2-cell embryos were washed with M2 and several drops of sM16 and cultured in the microdrops of M16 in 60 mm dishes prepared at least 2 hours prior to their transfer. The observations about the developmental competence of the embryos exposed to different treatments were recorded.

4.6.2 RESULTS

To compare the effect of different concentrations of 6-DMAP with nocodazole, the embryos treated with 10 μM nocodazole and different concentrations of 6-DMAP ranging from 1.25 mM to 5.0 mM for 12 hours were washed and cultured in sM16 for 4 days and results are presented in Table 4.6. No difference in cell cycle arrest of the 2-cell mouse blastomeres was observed either exposed to 10 μM nocodazole or 2.5 mM and above concentration of 6-DMAP. Complete (100%) cell cycle arrest was observed in the embryos treated with 10 μm nocodazole, 2.5 mM and 5.0 mM concentrations of 6-DMAP as compared to the group of embryos exposed to 1.25 mM concentration of 6-DMAP where 75% showed cell cycle arrest. The embryos arrested out of the total by treatment with 10 μM Nocodazole, 5.0 mM and 2.5 mM DMAP showed proportions equal to one, hence it was not possible to compare the treatment effect through statistical method.

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Table: 4.6 Effect of nocodazole (10 μM) and different concentrations of 6-DMAP for 12 hours on the proportion of 2-cells arrested at mitosis and their development in vitro.

Treatments	Replications.	2-Cell Embryos	Arrested at Mitosis (%)	4-Cells (%)	Blastocyst (%)
Control	4	80	--	78/80(98)	77/80(96)
Nocodazole (10 μM)	4	125	125/125(100)	119/125(95)	119/125(95)
6-DMAP (5.0 mM)	3	65	65/65(100)	25/65(38)	22/65(34)
6-DMAP (2.5 mM)	3	60	60/60(100)	38/60(63)	20/60(33)
6-DMAP (1.25 mM)	3	60	45/60(75)	55/60(92)	54/60(90)

The results presented in table: 4.6 showed that the development to blastocysts/total after there release was greatly effected by different treatments (p< 0.001). The embryos exposed to 10 μM nocodazole and 1.25 mM DMAP for 12 hours showed better (p< 0.05 or above) development to blastocysts/total as compared to 5.0 mM and 2.5 mM DMAP treatment and were comparable to control group. No toxicity effect of 10 μM Nocodazole treatment on the developmental competence of the treated embryos was observed as 95% developed to blastocyst stage. On the other hand, although the 2.5 mM and 5.0 mM concentrations of 6-DMAP were quite good for holding the cell cycle but development after release to blastocyst stage was adversely affected and remained restricted to 33 and 34% respectively. The lower concentration (1.25 mM) of 6-DMAP had little effect on development to blastocyst and results are comparable to the control group and to the group treated with nocodazole.

4.6.3 CONCLUSIONS

There is no difference in development of embryos to blastocyst after their release form 10μM Nocodazole and 1.25 mM DMAP treatments for 12 hours and the results are comparable to control group. But, as depicted in Table 4.6, the inability of the 1.25 mM DMAP treatment to hold the cell cycle at mitosis for 12 hours suggests that nocodazole treatment is better and could successfully be used to arrest the cell cycle up to 12 hours with no affect on subsequent development to blastocyst stage. In contrast, exposure of embryos to 2.5 mM and 5.0 mM concentrations of 6-DMAP for 12 hours though good for holding the cell cycle, adversely affected the subsequent development to blastocyst stage. The lower concentration of 6-DMAP is unable to completely arrest the cycle but development is comparable to the control group and the group treated with 10 μM nocodazole.

4.7- DISCUSSION

Experiments were conducted to study the effect of interactions of donor nuclei and recipient cytoplasms on the development of nuclear transfer embryos. The synchronized population of donor nuclei was required to capture different stages of donor cell cycle for interaction studies. Since the cell cycle synchronization of donor nuclei in the embryos is more difficult in early pre-implantation embryos, there is a need for a reliable method to synchronize mouse embryos.

In the present study, the results of experiments showed that 2.5 mM/ml concentration (or above) of 6-DMAP can successfully be used to arrest the cell cycle of 2-cell mouse embryos up to 16 hours. The exposure of embryos for more than 12 hours showed negative effect on development after release from synchronization treatment. In this study, the exposure of embryos to 2.5 mM/ml concentration of 6-DMAP for 9 hours, indicated that the effect of 6-DMAP is reversible and good proportion (66%) of embryos reached to blastocyst stage after their release. However, higher dose concentration of 6-DMAP (3.0 mM/ml) was suggested in previous studies (Samake and Smith, 1996a, 1997) to successfully and reversible arrest the 4-cell and 8-cell mouse embryos.

An inhibitor of meiosis/mitosis/maturation promotion factor (MPF), 6-Dimethylaminopurine (6-DMAP) has been successfully used to prevent the germinal vesicle breakdown during invertebrate (Guerrier and Doree, 1975; Doree et al., 1983 and vertebrate (Rime et al., 1989) oocyte maturation. The kinase inhibitor 6-DMAP caused significant reduction in the proportion of oocytes with spindles and condensed chromatin, indicate the necessity for phosphorylation events in the resumption of meiosis (Henry et al., 1997). Since DMAP inhibits the normal phosphorylation of proteins and interferes with the activity of MPF, the cell division is prevented (Neant et al., 1988a). Studies in marine invertebrates showed that DNA synthesis regulation in mitotic cells relies on 6-DMAP insensitive event (Neant and Dube, 1996) and in mammalian cell, though the presence of 6-DMAP after activation enhances the efficiency of activation and the speed of pronuclear formation but no effect on DNA synthesis was observed (Ledda et al., 1996). Since 6-DMAP inhibits the normal phosphorylation of protein and has no effect on DNA synthesis, it is predicted that 6-DMAP treated embryos are arrested at late G2 stage of the cell cycle, just before the rise of MPF activity. In this study, most of the embryos stained with Hoechst after their exposure to 6-DMAP have shown intact nuclear envelope as evident from fig. 4.2 suggests the synchronization of embryonic cells at late G2 stage of cell cycle and the absence of MPF activity. The results of the present study to synchronization nuclear donor cells are in agreement with previous reports of Samake and Smith (1996a, 1997) indicating that 6-DMAP can be used to synchronize the donor cell at late G2-phase. The results concludes that the exposure of 2-cell embryos to minimum dose concentration of 2.5 mM of 6-DMAP can successfully arrest the cell cycle (100%) upto 16 hours and with higher reversibility and development to blastocyst (77%), when released after 9 hours of exposure. The failure in development of embryos to blastocysts after prolonged exposure of 16 hours to 6-DMAP is expected due to its toxicity causing chromosomal abnormalities.

The experiment for synchronization of donor embryos were also conducted with Taxol. The results of the experiments indicated that embryos can successfully be arrested from cleavage with even 0.5 µM/ml concentration for 8 hours. But showed drastic effect on development after release. The development remained restricted at 3-4 cells and no embryo progressed further. In stead, they become homogenized after 3 days of incubation. The negative affect on development after release may be due to its toxicity, even with the lowest concentration (0.5 µM) or due to the exposure time. However, it is suggested that further experiments using concentrations lower than 0.5 µM be conducted to find the possible beneficial role of Taxol in cell synchronization studies.

Taxol is a new drug for the treatment of certain cancers (Rowinsky et al., 1990) which blocks the cell cycle in its G1 and M phases by stabilizing the microtubule cytoskeleton against depolymerization. It was inferred that this was due to the stabilization of cytoplasmic microtubules. Indirect immunofluorescence was used to show that Taxol treated cells displayed bundles of microtubules radiating from a common site in addition to their cytoplasmic microtubules. Ultarstructural observations showed that the mitotic cells contained microtubule bundles but no normal spindle. It was concluded that the inability of the cells to form a mitotic spindle in the presence of Taxol could be due to the fact that the cells were unable to depolymerize their microtubule cytoskeletons (Schiff PB and Horwitz SB, 1980). The results of the present experiment have shown drastic effect on the reversibility of treated embryos treated with different concentrations (0.5-10.0 µM) of Taxol is expected due to its toxicity as disturbed and scattered chromatin in treated embryos was observed after staining with Hoechst 33342.

Taxol apparently induces the assembly of free microtubules in the cytoplasm, not attached to the centrsomes or kinetochores. In this experiment when the embryos were exposed to the higher concentrations of Taxol continuously for longer duration, the preexisting microtubules, attached to the organizing centers may not stabilized and disappeared and disturbed chromosomal configuration was observed (fig: 4.4). The study of Maro et al., (1985), demonstrated that even at very low doses of Taxol, only large bundles of microtubles in the spindle area of some cells were observed. Moreover, for higher doses (and/ or longer incubation time) cytoplasmic asters located near the cell surface were observed. In this study, the use of higher doses and longer duration could be the cause of disturbed chromatin and no development after release from Taxol.

Previous studies for synchronization of 2-cell mouse embryos with microtubule-disruptive drugs, colcemid and Nocodazole demonstrated that Nocodazole was more reversible than of colcemid in the majority of embryos treated with Nocodazole (Maro and Bornens, 1980; Johnson *et al.,* 1988; Tacke and Grunz, 1990, Otaegui *et al.,* 1994). In this study the results of the experiment for comparing the effect of Nocodazole with different concentrations of 6-DMAP showed Nocodazole effect comparable with 1.25 mM/ml of 6-DMAP when reversibility was considered but 1.25 mM/ml 6-DMAP was unable to completely hold the cell cycle so Nocodazole was preferred in further experiments.

The importance for synchronization between donor nucleus and recipient cytoplasts in nuclear transfer experiments is emphasized by previous studies in various mammalian species (Smith *et al.,* 1988, 1990; Collas *et al.,* 1992a; Campbell *et al.,* 1993, 1994; Cheong *et al.,* 1993; Otaegui *et al.,* 1994; Stice *et al.,* 1994). The present study concludes that 6-DMAP can successfully and reversibly be used to arrest the cell cycle at mitosis. In contrast, Taxol though is successful in arresting the cell cycle, but reversibility is greatly effected after release. Furthermore, Nocodazole proved better in successfully arresting the cell cycle for 12 hours with higher reversibility.

CHAPTER 5

ESTABLISHMENT OF PROTOCOL FOR THE ACTIVATION OF PARTHENOTES AND RECONSTRUCTED EMBRYOS

5.1 INTRODUCTION

Most amphibian and mammalian oocytes become developmentally arrested at the germinal vesicle stage in prophase of the first meiotic division (reviewed by Masui and Clarke, 1979). Upon appropriate stimulation, meiosis resumes, the germinal vesicle breaks down, the first division is completed and the oocyte then becomes arrested at metaphase of second meiosis. This arrest is maintained by the cytoplasmic activity i.e. the activity of maturation promotion factor (MPF), which is central to cell cycle regulation. During meiosis the activity of MPF increases sharply before germinal vesicle break down (GVBD), remains high throughout first metaphase, declines slightly during anaphase and telophase and increases again at metaphase II. MPF has been identified as a complex of two sets of proteins, cyclins (Swenson $et\ al.$, 1986; Labbe $et\ al.$, 1989) and $p34^{cdc2}$ (Dunphy $et\ al.$, 1988; Labbe $et\ al.$, 1989). MPF activity is partly a function of calcium (Ca^{2+}). Any imbalance of components of the multi-cellular complex which is required for the cell cycle arrest may make the oocyte sensitive to activation stimuli during aging. Activation events include cortical granule release, completion of meiosis with the extrusion of the second polar body (PB2), and pronuclear (PN) formation.

At fertilization the activation of the oocyte and inactivation of MPF is triggered by a series of intracellular Ca^{2+} oscillation (Cuthbertson and Cobbold, 1985; Collas $et\ al.$, 1993; Swann and Ozal, 1994). These Ca^{2+} oscillations are known to be important for resumption of the cell cycle (Kline and Kline, 1992). The studies in which Ca^{2+} was increased artificially showed a critical role of Ca^{2+} in activation. Direct injection of Ca^{2+}, or application of Ca^{2+} ionophore A23187, both trigger parthenogenetic egg activation and PN formation in mammalian oocytes

(Steinhardt *et al.*, 1974; Fulton and Whittingham, 1978; Kline and Kline, 1992; Vincent *et al.*, 1992). The findings that there are many other parthenogenetic agent cause a Ca^{2+} increase in mammalian oocytes suggests that Ca^{2+} is both necessary and sufficient trigger for activation (Whittingham, 1980; Swann and Ozal, 1994).

Parthenogenetic activation of the oocytes may be used instead of fertilization by sperm to prepare the oocytes for nuclear transfer experiments. Parthenogenesis is the production of embryonic cells, with or without eventual development into an adult, from a female gamete in the absence of any contribution from a male gamete (Kaufman, 1981). It is now established that the natural activation stimulation induced by sperm penetration causes a series of Ca^{2+} transients in the egg which persists for many minutes or even hours (Miyazaki *et al.*, 1993; Kline and Kline., 1992 and Carroll *et al.*, 1996). These transient spikes are propagated throughout the fertilized egg in the form of a wave and initiate both cortical granule exocytosis (Miyazaki., 1990) and escape from metaphase II arrest (Whitaker and Patel., 1990).

In mammals, the activation of metaphase II oocytes can be induced by a variety of chemical and physical stimuli whose effectiveness increases with increasing post-ovulatory aging of the oocyte (Kaufman, 1981). The effectiveness of these procedures varies for parthenogenetic activation and development in mouse. Mouse oocytes have been activated by exposure to Ca^{2+} -Mg^{2+} free medium containing Hyaluronidase (Graham, 1970), exposure to ethanol (Cuthbertson, 1983), strontium (Sr^{2+}) (Bos-Mikich *et al.*, 1995), Ca^{2+} ionophores or chelators (Steinhardt *et al.*, 1974; Kline and Kline, 1992), inhibitors of protein synthesis (Siracusa *et al.*, 1978, Bos-Mikich *et al.*, 1995) and electrical stimulation (Tarkowski *et al.*, 1970, Swann and Ozil, 1994). The parthenogenetic studies shows that the mammalian parthenogenetic agents such as cycloheximide (Siracusa *et al.*, 1978; Clarke and Masui, 1983) and puromycin (Balakier and Casper, 1993) mediate their effects by inhibiting protein synthesis, and it is not clear if this effect is relevant to fertilization or other parthenogenetic stimuli.

Fertilization and parthenogenetic development in mouse is also dependent on post ovulatory aging. Freshly ovulated oocytes are generally activated at low rates (Siracusa *et al.*, 1978; Vincent *et al.*, 1992; Swann and Ozil, 1994). It is also reported that in contrast to the oscillations at fertilization most parthenogenetic agents cause monotonic increase in Ca^{2+} (Cuthbertson *et al.*, 1981; Miyazaki and Igusa, 1981; Swann and Ozil, 1994). A clear exception to this rule is Sr^{2+} -containing medium, which induces Ca^{2+} oscillations (Kline and Kline, 1994). The study of Bos-Mikich *et al.*, (1995) has demonstrated the ability of the two parthenogenetic agents, strontium (Sr^{2+}) and cycloheximide, to activate mouse oocytes. The results of

their study shows that Sr^{2+} and cycloheximide act synergistically to promote parthenogenetic activation up to the pronuclear stage in oocytes collected immediately after ovulation. It is further demonstrated that parthenogenetic agents may have two important loci for activating mammalian oocytes and that the combined effect on Ca^{2+} release and protein synthesis is most effective.

5.2- 6-DIMETHYLAMINOPURINE (6-DMAP), A REVERSIBLE INHIBITOR OF THE TRANSITION TO METAPHASE.

The breakdown of the nuclear envelope, chromatin condensation, microtubule polymerization and the activity of microtubule organization centers (MTOCs) are the first indications of oocytes maturation (Szollosi *et al.* 1972a, b). These activities are regulated by a soluble cytoplasmic maturation factor (MPF) whose activity is temporally correlated with the M phase of the cell and is assumed to be controlled by a phosphorylation/ dephosphorylation cascade (reviewed by Maller, 1983; Ozon *et al.,* 1987). Protein synthesis inhibitors like cyclohexamide, neither affect the initial appearance of MPF nor GVBD, but the reappearance of MPF at the time of first polar body extrusion is inhibited (Hasimoto and Kishimoto, 1988) which suggests that protein synthesis is required for the formation of MPF during the second meiotic cycle.

It has been reported that phosphorylation plays an important role in the G_2/M transition of mitosis (Adlakha and Rao, 1987; Cross *et al.,* 1989) and meiosis (Lohka and Maller, 1987; Maller *et al.,* 1989). A large increase in the phosphorylation of cellular protein occurs in conjunction with the appearance of MPF activity as meiosis 1 resumes to GVBD (Lohka and Maller, 1987; Ozon *et al.,* 1987). The puromycin analog 6- dimethylaminopurine (6-DMAP) reversibly inhibits GVBD in starfish oocytes stimulated by the natural hormone 1-methyladenine (Neant and Guerrier, 1988b). In the metaphase 1 oocytes from the limpet *Pateellaa vulgata,* 6-DMAP similarly induces decondensation of the chromosomes and reformation of the nuclear envelope (Neant and Guerrier, 1988a). In both these species, 6-DMAP appears to be an inhibitor of MPF formation. These investigations show that 6-DMAP inhibits the burst of phosphorylation that normally occurs shortly before GVBD, without affecting the overall rate of protein synthesis. In nuclear transfer experiments, some protocols requires the use of the "Universal recipients". So there is need to establish a suitable activation protocol to activate mouse MII cytoplast to use them as "Universal recipients", with basal level of MPF.

The aims of the present series of experiments were: 1) to study the effect of 6-DMAP on the chromatin configuration and development after exposure of mouse oocytes in metaphase II stage 2) to establish a suitable method for parthenogenetically activating the metaphase II oocytes and to use them as "universal recipients" and 3) the extent of parthenogenetic development in mouse oocytes by combining the standard activation protocol with the techniques which inhibits protein kinase activity by reducing phosphorylation of cellular protein ultimately suppressing the MPF activity in the metaphase II oocytes.

The second objective of these experiment was to assess the rate of development of the embryos reconstructed by using the donor nuclei from different stages of 3rd cell cycle and pre-activated recipient oocytes (Universal recipients) with no MPF or with basal level of MPF.

5.3 EFFECT OF INCUBATION TIME IN 6-DIMETHYLAMINOPURINE, STRONTIUM CHLORIDE AND THEIR COMBINATION ON THE CHROMATIN MORPHOLOGY OF METAPHASE II OOCYTES.

This study was conducted to determine the effect incubation time with 6-DMAP (protein kinase inhibitor), strontium chloride, and their combinations treatments on the chromatin morphology of metaphase II oocytes.

5.3.1 EXPERIMENTAL DESIGN

Cumulus free oocytes of 14-15 hours post hCG were exposed to the following treatments: 1) one hour exposure to 2.5mM 6-DMAP in M16; 2) one hour exposure to 2.5mM 6-DMAP plus 25 mM strontium chloride in M16 (calcium magnesium free strontium activation media); 3) one hour exposure to 25 mM strontium chloride in M16 (strontium activation media) alone ; 4) five hours exposure to 2.5mM 6-DMAP in M16; 5) sequential treatment with 25 mM strontium chloride for one hour and 2.5 mM 6-DMAP for two hours; 6) sequential treatment with 25 mM strontium chloride plus 2.5mM 6-DMAP for one hour and 2.5 mM 6-DMAP for two hours; and 7) sequential treatment with 2.5mM 6-DMAP for one hour, 25 mM strontium chloride plus 2.5mM 6-DMAP for one hour and 2.5 mM 6-DMAP for one hour. Microdrop culture dishes for each treatment were made and equilibrated at 37°C in 5% CO_2 in air at least 2 hours prior to the initiation of each treatment. In Strontium chloride alone treatment group, the oocytes were exposed to 25 mM

Strontium chloride for 1 hours and then cultured after washing with M2 medium in the microdrops of sM16. After 3 hours from the start of each treatment incubation (except for 5 hours DMAP group), the oocytes were washed with M2 medium and were mounted on the slides. They were fixed in an acetic acid: methanol (1:3, v/v) for 24 hours and were stained with 2% aceto-orcene. The chromatin morphology of the oocytes were examined under phase contrast microscope and observations on the different form of chromatin configurations and pronuclei were recorded.

5.3.1.2- STATISTICAL ANALYSIS

In this experiment, because many observations were equal to zero, the data was analyzed differently from all subsequent analysis. The actual numbers in the table were subjected to an extension of the Fisher exact test for interaction between treatments and category of chromatin morphology (Program STATXACT, Cytel Software Corporation). The proportions were then analyzed by using Generalized Linear Mixed Model (Breslow and Clayton, 1993) looking at the effect of treatment whilst allowing for differences between days. The treatment effects were tested using a Wald statistics (distributed as chi-squared) and, if significant, differences between means were assessed by an approximate t-test. Means declared to differ, differ at the 5% level or better.

5.3.2 RESULTS

The effect of 6-DMAP, strontium chloride and a combination of these treatments on chromatin morphology is shown in Table: 5.1 (Fig. 5.1). The statistical analysis showed a significant interaction (p< 0.001), indicating that different treatments result in different patterns of chromatin configuration. All those oocytes (MII) who had left their metaphase II arrest following their exposure to any treatment were expected to be activated. The exposure of oocytes to combined treatment of strontium and 6-DMAP showed accelerated response to pronuclear formation. Exposure of metaphase II oocytes to 6-DMAP incubation for one hour induced activation in 8.6% of oocytes as they showed one pronucleus and one polar body configuration. On the other hand 80% of oocytes showed no affect of 6-DMAP incubation and were observed at metaphase II. The prolonged exposure of oocytes to DMAP for 5 hours showed abnormalities as 81.3% of ooctyes with disturbed metaphase were observed.

Fig. 5.1 Effect of 6-DMAP, strontium chloride and combined treatment on the chromatin morphology of metaphase II oocytes.

a) MII oocyte after the treatment of 6-DMAP for one hour

b) anaphase stage of the oocyte after the treatment of St+6-DMAP for one hour

c) anaphase/telophase stage of the oocyte after the treatment of St+6-DMAP for one hour

d) telophase stage of the oocyte after the treatment of St+6-DMAP for one hour

e) condensed chromosomes in some of the oocytes after the treatment of 6-DMAP for one hour

f) disturbed metaphase assembly after the treatment of St+6-DMAP for one hour in some of the oocytes

g) one pronucleus formation (PN) in some of the oocytes after the treatment of 6-DMAP one hour then St+6-DMAP for one hour and again 6-DMAP for one hour

h) 1PN+1PB condition in some of the oocytes treated with strontium chloride for one hour and then 6-DMAP for 3 hours

i) 2PN condition in some of the oocytes after the treatment of 6-DMAP one hour then St+6-DMAP for one hour and again 6-DMAP for one hour

j) more than 2 PN condition in some of the oocytes after the treatment of 6-DMAP one hour then St+6-DMAP for one hour and again 6-DMAP for one hour

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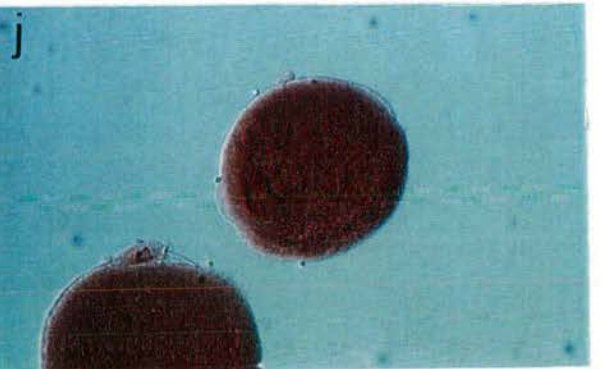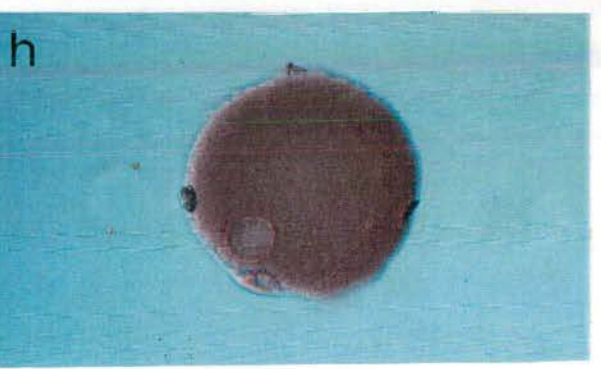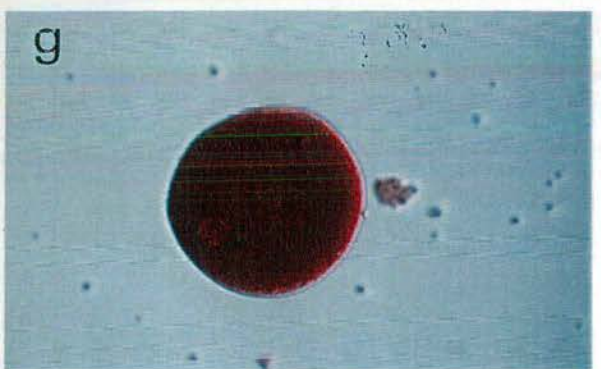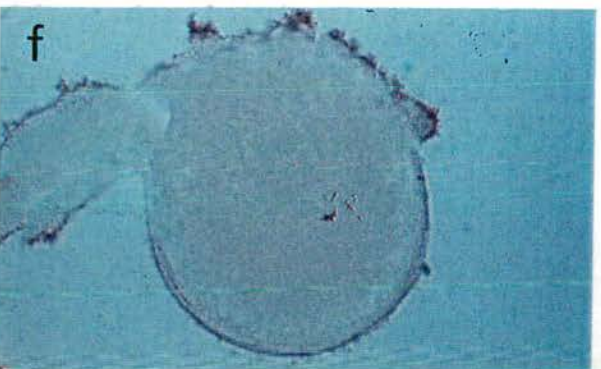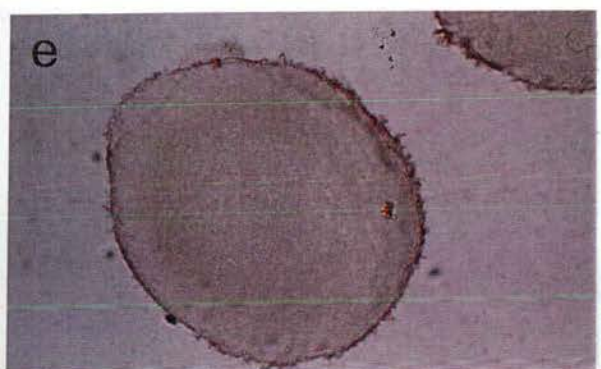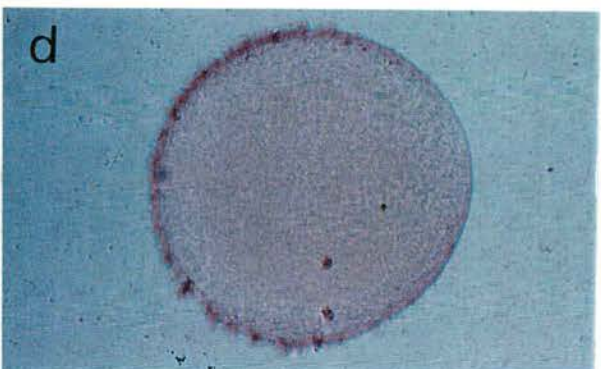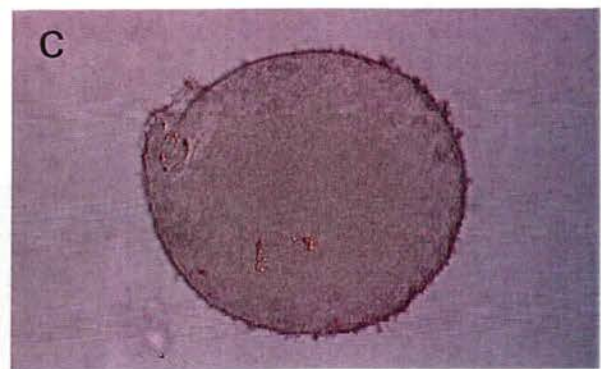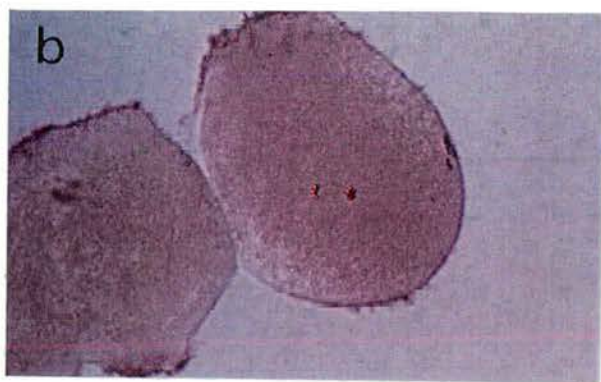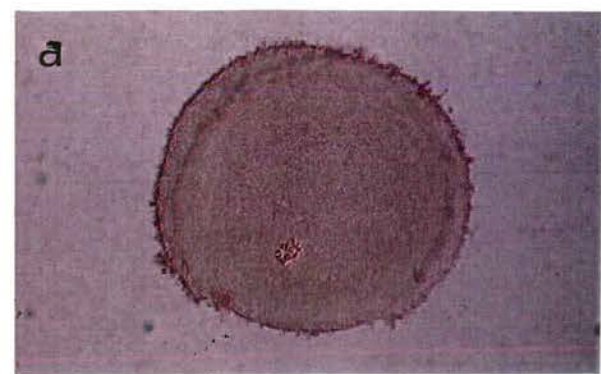
The combined treatment of 6-DMAP plus strontium chloride for one hour showed higher rate of activation as, 81.8% of oocytes showed exit from metaphase II arrest as compare to 38.9% which remained arrested in the treatment group which was exposed to strontium chloride alone for one hour. The chromatin configuration of oocytes exposed to 6-DMAP+Sr group, were observed to be as 30.3%, 30.3% and 21.2% in anaphase, telophase and anaphase-telophase stage respectively. Staining also revealed 16.7% of oocytes with disturbed MII when exposed to strontium chloride treatment for one hour. The effect of combined treatments is also shown in table 5. 1.

When examined after a 3 hours of activation , there were difference in the pattern of activation. The combined treatment of strontium chloride (1h) then 6-DMAP (2h), 6-DMAP+Sr.(1h) then 6-DMAP (2h) and 6-DMAP (1h) then DMAP+Sr.(1h) then 6-DMAP (1h) gave higher percentage of pronuclei between 85 and 94%. These percentages were shown to be similar, but for these oocytes there was a difference between treatments in the proportion with 2PN (p< 0.05). The combined treatment of strontium chloride (1h) then 6-DMAP (2h) and 6-DMAP+Sr.(1h) then 6-DMAP (2h) showed major effect on the formation of 2 pronuclei (PN). The results shows 48.4% and 45.4% formed 2PN when exposed the combined treatments of strontium chloride (1h) then 6-DMAP (2h) and 6-DMAP+Sr.(1h) then 6-DMAP (2h) respectively. The treatment with strontium chloride (1h) then 6-DMAP (2h) also induced 1PN+1PB configuration in 25.8% oocytes. On the other hand treatment with and 6-DMAP+Sr.(1h) then 6-DMAP (2h) did not induce the 1PN+1PB but 24.2% showed 1PN configuration. The data showed very less difference (16.1%v15.2%) between these two treatments as far as the percentage of more than 2PN are concerned. The data also revealed the mixed effect of combined treatment i.e. sequential treatment of 6-DMAP (1h) then DMAP+Sr.(1h) then 6-DMAP (1h) on the configuration of exposed oocytes as 21.2, 27.3, 18.2, 24.2, and 3% showed 1PN+1PB, 1PN, 2PN, >2PN and MII configurations.

5.3.3 CONCLUSION

Treatment of metaphase II oocytes with 6-DMAP regardless of the time of incubation suggests that 6-DMAP itself have negligible effect activation, but it potentiates the effect of strontium, in that it apparently increased the proportion of activated ones and accelerated the response.

Table: 5.1 Effect of 2.5 mM 6-DMAP and 25 mM Srontium chloride treatments and their combinition on the percentage of chromatin configuration of metaphase II oocytes.

	Tot.	MII	Dis. MII	C.C	Anaphase	Telophase	Ana-Tel	1PN+1PB	1PN	2PN	>2PN
*Stron.Chloride (1Hour)	36	38.9	16.7	0	44.4	0	0	0	0	0	0
*DMAP(1Hour)	35	80.0	0	5.7	0	0	0	8.6	0	0	5.7
DMAP(5 Hours)	64	15.6	81.3	0	0	0	3.1	0	0	0	0
*DMAP+Sr (1Hr)	33	0	0	18.2	30.3	30.3	21.2	0	0	0	0
*Sr. Chlride(1Hr) +DMAP(2Hours)	31	3.2	0	0	3.2	0	0	25.8	3.2	48.4	16.1
*DMAP+Sr (1 Hr) DMAP(2 Hours)	33	6.1	0	9.1	0	0	0	0	24.2	45.4	15.2
*DMAP(1 Hour) + DMAP+Sr(1 Hour) DMAP(1 Hour)	33	3.0	0	6.1	0	0	0	21.2	27.3	18.2	24.2

* The oocytes were fixed after 3 from the start of each treatment. All those oocytes which are at anaphase, telophase, anaphase-telophase, or showed pronuclear formation (1PN+1PB, 1PN, 2PN) are considered to be activated. The oocytes showing > 2PN are abnormal may be due to the effect of DMAP treatment.

5.4 EFFECT OF TIME OF INCUBATION IN 6-DIMETHYLAMINOPURINE, THE PROTEIN KINASE INHIBITOR ON THE PARTHENOGENETIC DEVELOPMENT OF METAPHASE II OOCYTES.

The study was conducted to determine the effect of protein kinase inhibitor 6-DMAP on activation and parthenogenetic development of metaphase II oocytes. The second objective of this study was to compare its effect with standard activation protocol i.e. one hour exposure to strontium chloride and its combined treatments with 6-DMAP on the activation and parthenogenetic development of metaphase II oocyte.

5.4.1 EXPERIMENTAL DESIGN

The oocytes recovered 14 hours post hCG were denuded using 300 i.u. of Hyaluronidase/ml in M2 medium. They were washed twice in M2 medium. The oocytes were randomly allotted to the following treatment groups : 1) control (-ive control); 2) one hour incubation in the microdrops of sM16 containing 2.5 mM 6-DMAP; 3) one hour exposure/incubation with 25 mM strontium chloride in calcium magnesium free M16; 4) combined treatment of 2.5 mM 6-DMAP plus 25 mM strontium chloride in calcium magnesium free M16 for one hour; 5) sequential treatments of 2.5 mM 6-DMAP in sM16 for five hours and one hour incubation in strontium activation media containing 25 mM strontium chloride; and 6) sequential treatment of 2.5 mM 6-DMAP in sM16 for five hours and one hour incubation in strontium activation media containing 25 mM strontium chloride plus 2.5 mM 6-DMAP. The microdrops for each treatment medium were prepared in 60 mm plastic dishes and were covered with liquid paraffin oil. These dishes were equilibrated at 37°C in an atmosphere of 5% CO_2 in air, well ahead before the start of experiment. The experiment was repeated twice. The observations with the developmental pathway were recorded. The blastocysts quality was assessed by staining them with Hoechst 33342 for 15 minutes and number of cell per blastocysts were counted.

5.4.1.2- STATISTICAL ANALYSIS

The data on the proportion of parthenogenetic development of oocytes treated with Strontium chloride, DMAP and their combinations were analyzed by using Generalized Linear Mixed Model (Breslow and Clayton, 1993) looking at the effect of treatment whilst allowing for differences between Days. The treatment effects

were tested using a Wald statistics (distributed as chi-squared) and, if significant, differences between means were assessed by an approximate t-test. Means declared to differ, differ at the 5% level or better.

5.4.2 RESULTS

The effect on the parthenogenetic development of metaphase II oocytes exposed to 6-DMAP , strontium chloride and combined treatments for different time and combinations is depicted in Table 5.2. The oocytes in negative control group and those exposed to 6-DMAP alone for one hour showed no change after 20 hours of incubation in M16. However, on the second day fragmentation occurred in 48% of the control group and 46% of the DMAP group. A significant difference ($p<0.01$) between different treatments was observed when compared for development to 2-cell/total. Strontium chloride (1 hour) treatment induced maximum rate of activation (81%) as compared to the combined treatment of DMAP+Sr (1 hour), which showed 41% development to 2-cell stage ($p< 0.01$). The development to 2-cells stage in treatment groups where two groups of oocytes were first exposed to DMAP for 5 hours and then each subsequently exposed to strontium chloride (1 hour) and DMAP+Strontium chloride (1 hour) showed 79% and 54 % respectively. The rate of development to morula stage was observed to 48% and 46% in the groups treated with strontium chloride (1 hour) and the combined treatment of DMAP (5 hour) with subsequent treatment of DMAP+Sr (1 hour). The rate of development by treating the oocytes with strontium chloride and other combined treatments was higher as compared to negative control and DMAP (1 hour) alone. The analysis of the data also showed a significant difference in different treatment when compared for development to blastocysts stage from treated oocytes.

5.4.3 CONCLUSIONS

The DMAP treatment is unable to induce any activation in metaphase II oocytes. The treatment of oocytes with strontium chloride provide good activation treatment and maximum number of parthenotes developed to 2-cells and stage. There is no difference in the development of parthenotes up to morula stage exposed to strontium alone and the parthenotes treated with DMAP (5 hours) then subsequent exposure to DMAP+Sr.(1 hour).

Table:5.2 Parthenogenetic development of oocytes treated with 2.5mM 6-DMAP and 25mM strontium chloride for different length of time and combinitions (%)

	Total	2PB	Frag.	No change	2-cell	4-8 cell	Morula	Blastocysts	Dead
Control	31	0/31(0)	15/31(48)	16/31(52)	0/31(0)	0/31(0)	0/31(0)	0/31(0)	0/31(0)
DMAP(1 Hour)	35	0/35(0)	16/35(46)	19/35(54)	0/35(0)	0/35(0)	0/35(0)	0/35(0)	0/35(0)
Strontium Chloride (1 Hour)	31	0/31(0)	4/31(13)	2/31(6)	25/31(81)	18/31(58)	15/31(48)	12/31(39)	0/31(0)
DMAP+Strontium Chloride (1 Hour)	32	0/32(0)	5/32(16)	13/32(41)	13/32(41)	7/32(22)	6/32(19)	6/32(19)	2/32(6)
DMAP(5Hours)→ Stron.Chlor.(1 Hour)	39	33/39(85)	8/39(21)	0/39(0)	31/39(79)	15/39(38)	8/39(21)	8/39(21)	0/39(0)
DMAP(5 Hours)→ DMAP+Sr^{++}(1 Hour)	37	0/37(0)	4/37(11)	0/37(0)	20/37(54)	17/37(46)	17/37(46)	17/37(46)	13/37(35)

observations after 3 days of culture

5.5- ACTIVATION AND PARTHENOGENETIC DEVELOPMENT OF METAPHASE II OOCYTES.

The aim of the present study was to find out the reliable and suitable method for oocytes activation and the subsequent effect of activation treatment on the parthenogenetic development of metaphase II oocytes. A comparison was made between strontium and Ionomycin and their co-activation protocols.

5.5.1 EXPERIMENTAL DESIGN

The cumulus free metaphase II oocytes (14-15 hours post hCG) were exposed to different treatments explained as follows: 1) control group (negative control); 2) one hour incubation in strontium activation media (25 mM strontium chloride); 3) 6 minutes in 5 mM Ionomycin (Sigma) in M2 medium; 4) one hour exposure of metaphase II oocytes to the strontium activation media containing 25 mM strontium chloride and then 3 hour incubation in M16 containing 2.0 mM 6-DMAP; and 5) 6 minutes exposure to 5 mM Ionomycin in M2 and subsequent exposure to 2.0 mM 6-DMAP in M16 for 3 hours. The control group (negative control) was made to assess any spontaneous parthenogenetic activation/ development and its comparison with the other treatments. The treatment groups 2 and 3 were used to evaluate the effect of two different chemical stimuli on activation and parthenogenetic development of parthenotes. The treatment (4&5, the co-activation protocols) were used to study the effect of protein kinase inhibitor (6-DMAP) on activation and parthenogenetic development of parthenotes. The oocytes after each treatment were washed at least 3 times with M2 to minimize any nasty affect of drugs. At the end of each treatment each treatment group was incubated at 37^0 C in the atmosphere of 5% CO_2 in air in the microdrops of separately made dishes for each treatment a well ahead of time. They were observed for pronuclear formation 8 hour after their incubation in sM16. The observations along their developmental pathway up to blastocyst stage were also recorded. The blastocysts developed after each treatments were stained with Hoechst 33342 (0.1µg/ml) for 15 minutes and the number of cells/blastocyst were recorded to evaluate the quality of blastocysts. The data obtained was analyzed by using the method described in section 5.4.2.

5.5.2 RESULTS

The effect on the extrusion of second polar body and pronuclear formation is shown in Table: 5.3. These observation were recorded 8 hours after beginning of activation treatment. No change was observed in negative control group. The pattern of activation varied with treatments. The higher percentage of 2PB were observed (30 and 37%) in strontium and Ionomycin groups as compared to negative control and co-activation groups. The 1PN+1PB also revealed higher percentage (47 and 33%) in the parthenotes treated with strontium chloride and co-activation protocol of strontium chloride and subsequent exposure to DMAP. The 2PN formation was significantly higher (p< 0.001) in the parthenotes that were also exposed to DMAP.

The developmental competence of parthenotes is shown in Table: 5.4. The analysis of data revealed highly significant difference (p< 0.001) between different treatments when compared for development to 2-cell from treated oocytes. Strontium Chloride is better (p< 0.001) than the Ionomycin treatment (51% Vs 18%) and with DMAP treatment (94 and 59%) showed better (p< 0.05 or more) than without DMAP treatment (51, 18%) and control group (5%) when the development from start to 2-cell was compared. In the absence of DMAP, more oocytes activated with strontium developed to morula and blastocyst stages (30% and 15%) than with ionomycin (3% and 0%). Activation followed by treatment with 6-DMAP increased these frequencies for both strontium (75% and 70%) and ionomycin (48% and 47%). The overall analysis of the data indicates that treatment with co-activation protocol of strontium chloride and DMAP is the best treatment when development from start to blastocyst stage was compared.

5.5.3 CONCLUSIONS

The development of the parthenotes with the co-activation protocol of strontium chloride and DMAP suggests the need for longer exposure of the parthenotes to activation stimuli with subsequent exposure to DMAP.

Though the 2PN formation is slightly higher in the parthenotes treated with Ionomycin co-activation protocol, the subsequent development from 2-cell to morula and blastocyst stage is much higher in the parthenotes treated with strontium co-activation protocol (Fig 5.2a) with higher cell number (Fig 5.2b). The results of experiments also favours the use of the protein kinase inhibitor 6-DMAP as an inhibitor of phosphorylation in activating the metaphase II oocytes.

Table 5.3: Effect of different treatment for activation on the extrusion of second polar body and pronuclear formation (%) after 8 hours post activation (hpa).

	-ive Control	25 mM Strontium Chloride(1 Hour)	5 μM Ionomycin (6 minutes)	25 mM strontium chloride(1 Hour) + 2 mM 6-DMAP (3 Hours)	5 μM Ionomycin (6 minutes) + 2mM 6-DMAP (3 Hours)
2 PB	0/92 (0)	28/92 (30)	35/95 (37)	2/91 (2)	4/94 (4)
1 PN + 2 PB	0/92 (0)	43/92 (47)	7/95 (7)	30/91 (33)	6/94 (6)
2 PN + 1 PB	0/92 (0)	4/92 (4)	0/95 (0)	59/91 (65)	66/94 (70)

hpa= hours post activation

Table : 5.4 Effect of different treatment for activation on the parthenogenetic development of activated oocytes .(%)

	-ive Control	25 mM Strontium Chloride(1 Hour)	5 μM Ionomycin (6 minutes)	25 mM strontium chloride(1 Hour) + 2 mM 6-DMAP (3 Hours)	5 μM Ionomycin (6 minutes) + 2mM 6-DMAP (3 Hours)
2-cells	5/92 (5)	47/92 (51)	17/95 (18)	86/91 (95)	55/94 (59)
4-cells	--	44/92 (48)	5/95 (5)	79/91 (87)	48/94 (51)
8-cells	--	29/92 (32)	3/95 (3)	76/91 (84)	46/94 (49)
Morula	--	28/92 (30)	3/95 (3)	68/91 (75)	46/94 (49)
Blastocyst	--	14/92 (15)	0/95 (0)	64/91 (70)	44/94 (47)

Fig. 5.2 Development after parthenogenetic activation of MII oocytes using the activation protocol of Strontium chloride one hour with subsequent exposure to 6-DMAP for 3 hours.

a) development to blastocysts after 5th day

b) Hoechst stained blastocyst showing higher number of blastomere nuclei on 5th day
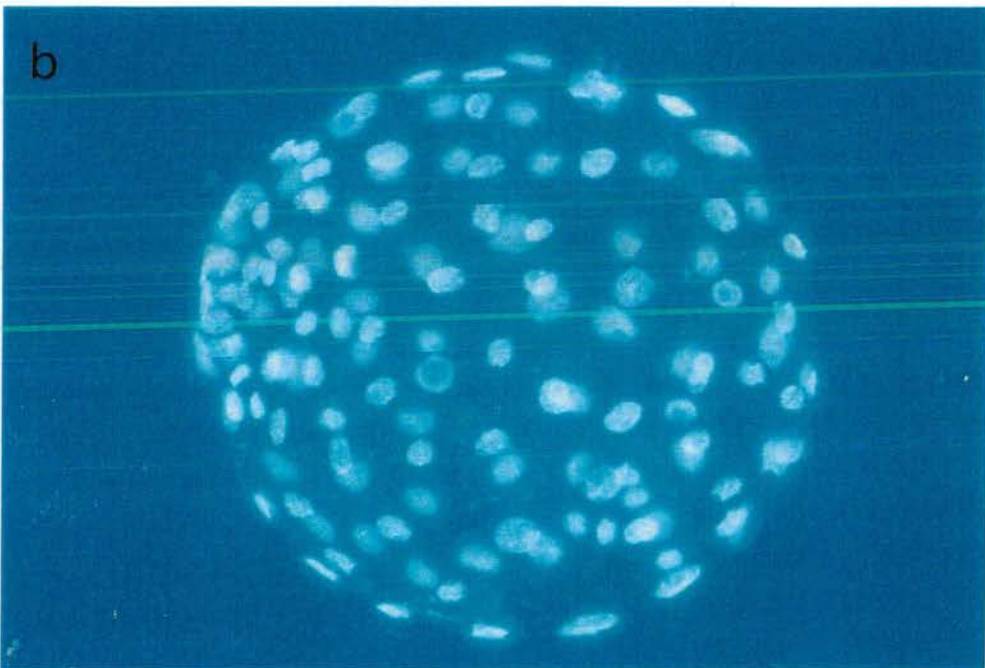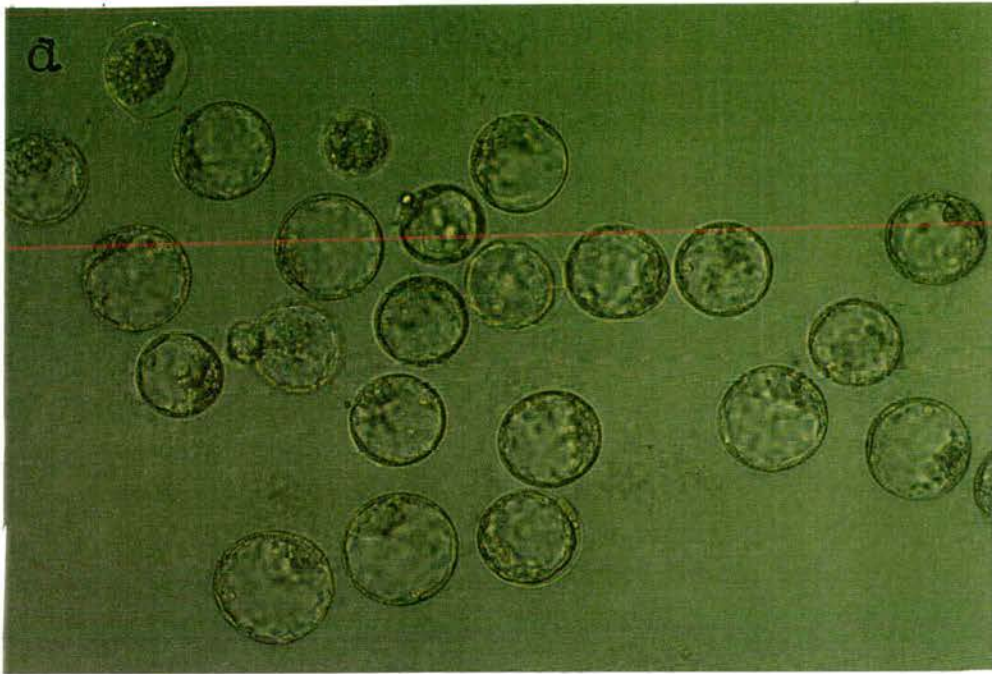
5.6 DEVELOPMENTAL POTENTIAL OF THE RECONSTRUCTED EMBRYOS USING MOST FAVOURED ACTIVATION PROTOCOL.

This study was planned to study the effect of most favourable activation protocol (one hour incubation in strontium activation media containing 25 mM strontium chloride and subsequent exposure to 2.0 mM 6-DMAP) for the parthenogenetic development of parthenotes to blastocyst stage, on the developmental competence of embryos reconstructed by using donor nuclei of different cell cycle stage of 3rd cell cycle and enucleated pre-activated oocytes with no MPF or with basal level of MPF.

5.6.1 EXPERIMENTAL DESIGN

The oocytes were recovered 14 hours post hCG injections. The cumulus were removed using 300 i.u. of Hyaluronidase/ml in M2 medium. The denuded oocytes were exposed to the Hoechst 33342 staining (0.8 mg/ml) for five minutes in M2 medium. They were first washed with M2 medium immediately after enucleation to wash out the effect of Nocodazole and Cytochlasin B drugs used during manipulation in the manipulation chamber. The enucleated oocyte were then exposed to the activation protocol as follows: one hour exposure to the strontium activation media containing 25 mM strontium chloride; washed with M2 and then exposed for 3 hours to 2.0 mM 6-DMAP in sM16. Donor embryos recovered at late 2-cell stage (44-48 post hCG) were synchronized using 10 mM Nocodazole (Otaegui, Ph.D. thesis) and were released according to the requirement of the experiment at different stages of 3rd cell cycle.

During an earlier experiment it was noted that donor nuclei at G1 stage of cell cycle contained more than 5 nucleoli. In this experiment and the next experiment, donor nuclei were selected having more than 6 nucleoli as a convenient and effective criterion. The donor nuclei at G1 stage of cell cycle were obtained 60-75 minutes after release from synchronization. Nuclear transfer was performed by using donor nuclei at G1, S and G2 (Otaegui, 1995 Ph.D. thesis) phases of 3rd cell cycle to the pre-activated (4 hpa) enucleated oocytes by using the most favoured activation protocol mentioned above. The couplets were fused by using 3 volts AC for 3.5 second followed by single DC pulse of 1.5 kv for 100 μ sec. in electrofusion chamber containing 0.3M manitol solution. The couplets were then washed and incubated in the microdrops of sM16 containing 10 μM Nocodazole and 10 μg/ml

Cytochalasin B. After one hour the fusion rate was assessed and the fused embryos were washed several times with M2 and drops of sM16 to remove the effect of drugs and cultured in sM16. Two control groups, one which got activation stimuli to verify the quality of oocytes and the other, not exposed to activation stimuli to detect any non-intended activation. The donor group was also included in this experiment to confirm the quality of karyoplasts. The observations were recorded along the developmental pathway to blastocyst stage. The quality of the blastocysts was assessed by counting the cell number in each blastocyst after staining with Hoechst 33342 for 15 minutes.

5.6.2 RESULTS

There were significant effect of donor cell cycle stage effect (p< 0.001) on development to morula and blastocyst after nuclear transfer (Table: 5.5). The embryos reconstructed with donor nuclei at G1/S stage and control group activated with co-activation protocol showed 97 % rate of development to 2-cell was significantly better (p< 0.001) as compared to the embryos reconstructed by using S-phase (81%) and G2-phase (84%) of cell cycle stage. The subsequent development to morula and blastocyst stage was significantly (p< 0.001) higher (85 and 79%) in the embryos reconstructed using donor nuclei at G1/S phase of cell cycle stage as compared to the embryos reconstructed by using S-phase (40 and 23%) and G2-phase (47 and 27%) of cell cycle stages. The analysis of data for development also indicated that embryos reconstructed by using G1 or G1/S donor nuclei are best donor and development was comparable with donor controls. Fig. 5.3 shows the example of blastocyst developed following nuclear transfer of G1 and S phase donor nuclei. There was difference in the morphology of the blastomeres nuclei as observed after Hoechst 33342 staining. The appearance of the blastomeres in some of the blastocysts produced following transfer of 4-cell S-phase donor nuclei was unhealthy (Fig. 5.3b) whilst the blastocysts from G1 donor appeared normal (Fig. 5.3a)

Table 5.5: Development after nuclear transfer of reconstructed embryos using donor nuclei from different stages of 3rd cell cycle and pre-activated oocytes (4 hpa) with strontium chloride (1 hour) + 2mM 6-DMAP (3 hours) protocol.(%)

Donor cell cycle stage	Replicates.	Total	Fused	2-cell/ Fused	4-cell/ Fused	Morula/ Fused	Blastocyst/ Fused
G_1 or G_1/S	3	57	39 (68)	38 (97)	36 (92)	33 (85)	31 (79)
S	3	79	47 (59)	38 (81)	21 (45)	19 (40)	11 (23)
G_2	3	79	49 (62)	41 (84)	36 (73)	23 (47)	13 (27)
+ive control	9	119	--	115 (97)	87 (73)	73 (61)	64 (54)
-ive control	9	127	--	7 (5)	--	--	--
Donor control	2	21	--	--	19 (90)	17 (81)	16 (76)

Fig. 5.3 Difference in nuclear morphology of the blastocyst's nuclei of embryos reconstructed with G1 and S-phase stages.

a) blastocyst nuclei of the embryos reconstructed by transferring donor nuclei from 4-cell at G1 stage of cell cycle into cytoplasts pre-activated using strontium and 6-DMAP protocol.

b) blastocyst nuclei of the embryos reconstructed by transferring donor nuclei from 4-cell at S-phase of cell cycle into cytoplasts pre-activated using strontium and 6-DMAP protocol.

a
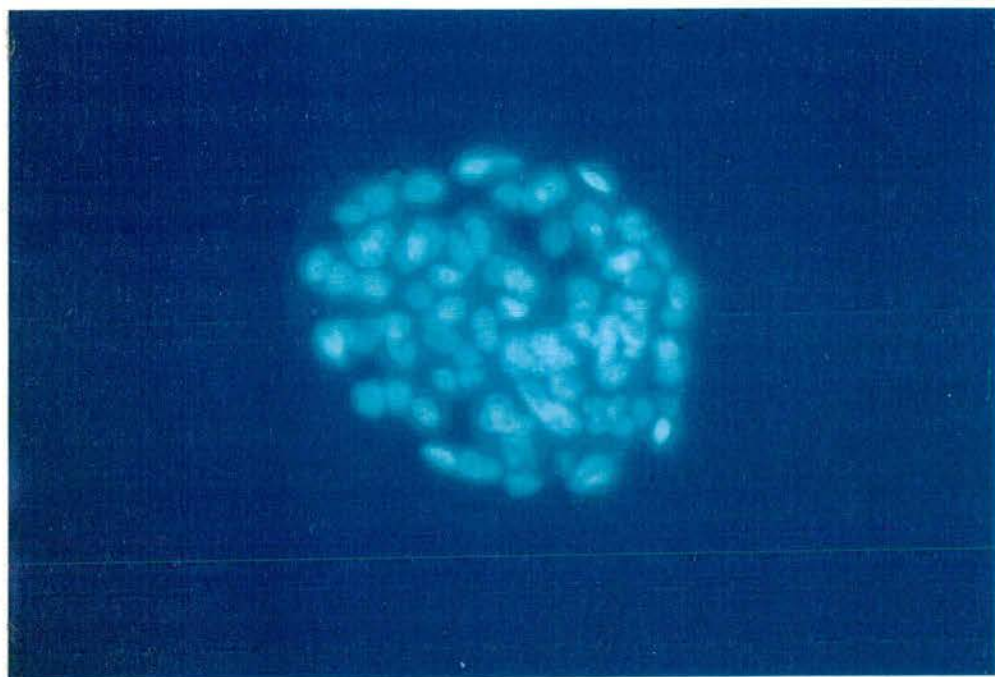
b
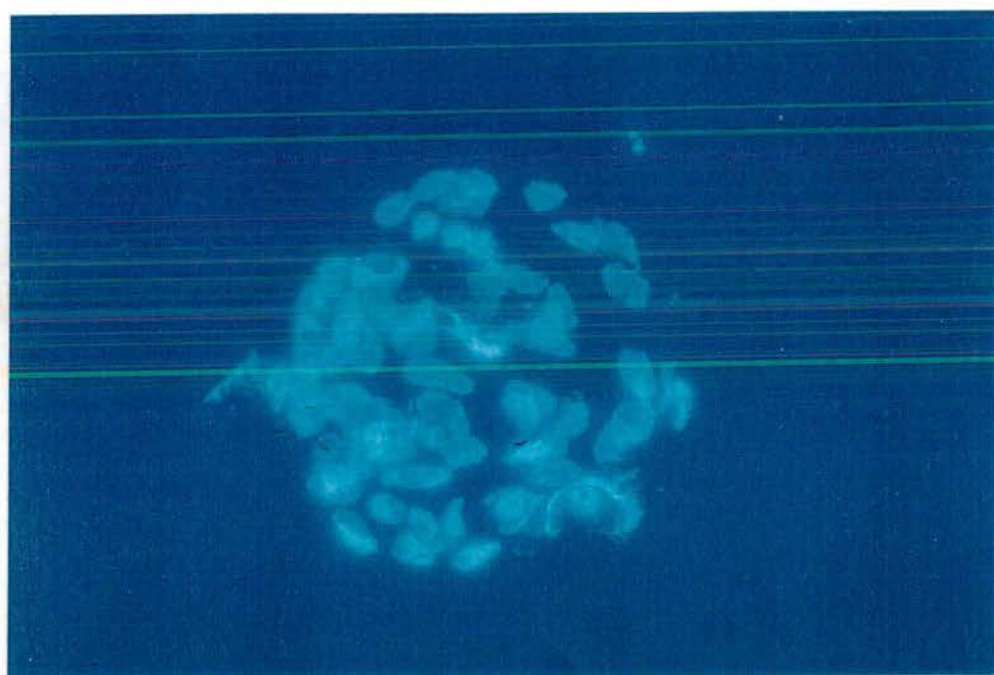
5.6.3 CONCLUSIONS

A number of reconstructed embryos were examined post fusion for evidence of NEBD (Table; 5.6). In one case the membrane was non distinct, but the chromosomes had not condensed. These observations confirms that the activation protocol did lower MPF activity as obvious from the intact nuclear envelope of the transferred nuclei (Fig 5.4).

Although all three nuclear transfer protocols were expected to maintain normal ploidy, there were significant differences in development. Development was more frequent when the donor cell was in G1 or G1/S phase.

The results of the present experiment suggests that though there is role of MPF in the development of reconstructed embryos but their could be other cytoplasmic factor which plays their role in remodelling and reprogramming of the transferred nuclei or cell cycle stage of the donor nuclei (the stage effect) could be the cause of lower development in embryos reconstructed by using S-phase and G2-phase stage of cell cycle. For G2 donor nuclei tetraploidy could be another cause of lower development.

5.7 COMPARISON OF THE EFFECTIVENESS OF TWO PROTOCOLS: ONE HOUR IN STRONTIUM ACTIVATION MEDIA VERSUS ONE HOUR EXPOSURE TO STRONTIUM ACTIVATION MEDIA (25 mM Sr) WITH SUBSEQUENT EXPOSURE OF THREE HOURS TO 2.0 mM 6-DMAP, THE PROTEIN KINASE INHIBITOR.

The results of the study (Table: 5.3) demonstrated maximum development (79%) by using the favourable activation protocol based on the parthenogenetic development of metaphase II oocytes, with the embryos reconstructed from the G1/ S phase of 3rd cell cycle by using enucleated pre-activated (4 hpa) oocytes by using favoured activation protocol i.e. one hour incubation in strontium activation media containing 25 mM strontium chloride and subsequently 3 hours exposure to 2.0 mM 6-DMAP in sM16. On the basis of these results it was decided to compare the effect of two different protocols on the development of the embryos reconstructed by using nuclear donors at G1/S phase of the 3rd cell cycle with the enucleated oocytes activated using above mentioned protocols.

Table: 5.6 Effect of activation protocol (25 mM Strontium chloride 1 hour + 2.0 mM 6-DMAP 3 hours) on the morphology of transferred nuclei after one hour of fusion.(%)

Donor cell stage	Total	Fused	1PN	Intact Nuclear/ observed	NEBD/ observed	NEBD+PCC/ observed
G1 or G1/S	14	12	--	11/11(100)	0/11(0)	0/11(0)
S-phase	12	11	--	10/11(91)	1/11(9)	0/11(0)
Control	25	--	--	24/25(96)	0/25(0)	0/25(0)

Fig. 5.4 Maintenance of nuclear envelope in embryos reconstructed from S-phase stage of donor nuclei using cytoplasts pre-activated with strontium and 6-DMAP protocol
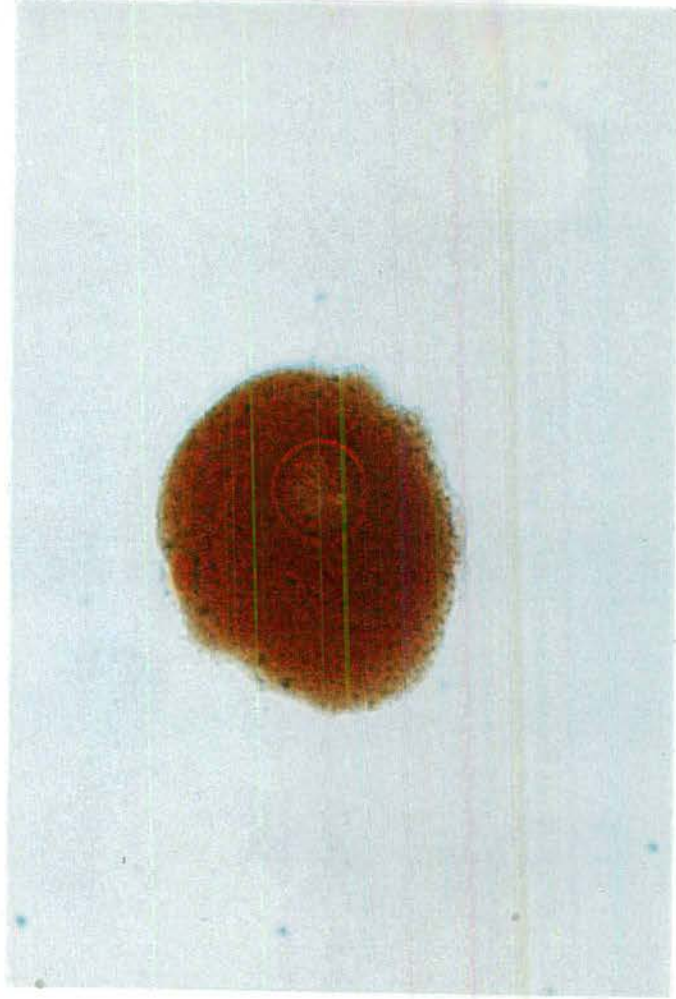
5.7.1 EXPERIMENTAL DESIGN

The oocytes were recovered 14 hours post hCG injections. They were denuded by using 300 i.u. of Hyaluronidaze .They were stained by using Hoechst 33342 (0.8 mg/ml). Enucleation of the oocytes was performed in the micromanipulation chamber containing 10 µM of Nocodazole and 10 µg Cytochalasin B in M2 medium. The enucleated oocytes were washed thoroughly with M2 medium to remove any affect of drugs used. They were activated by using calcium magnesium free strontium activation media containing 25 mM strontium chloride for one hour at $37^{o}C$ with 5%CO_2 in air. The activated oocytes were divided into two groups after washing with M2 with several changes. One group was incubated in the microdrop of sM16 medium for three hours and the other was exposed to 2.0 mM DMAP for three hours.

The donor nuclei at 2-cell stage were recovered 44-48 hours after hCG injections followed by mating with F1 males. They were synchronized for 9-12 hours using 10 µM of Nocodazole in M16 medium at 37°C. They were released in groups by washing them thoroughly with several changes of M2 to remove the effect of Nocodazole. The released embryos started dividing after 60 minutes. They were used for nuclear transfer 75 minutes after their release (Otaegui, 1995 Ph.D. thesis). The enucleated oocytes and the donor 4-cells were transferred into the micromanipulation chamber containing Nocodazole (microtubule inhibitor) and Cytochalasin B (cytoskeleton inhibitor). The stage of the cell cycle was recognized by evaluating the number of nucleoli. All those containing 6 or more than 6 nucleoli were considered in G1-phase. After reconstruction the couplets were fused by using 3 volts AC for 3.5 second followed by single DC pulse of 1.5 kv for 100 µ sec. in an electrofusion chamber containing 0.3M mannitol solution. The control groups were exposed with the same treatment but without enucleation to evaluate the ability of activation protocols and to compare them with the reconstructed groups. -ive control groups were used to identify any spontaneous activation in the oocytes with age or any other stimuli. The observations along the developmental pathways were recorded. The blastocyst's quality was evaluated by staining them with Hoechst 33342 for 15 minutes by exposing them to the UV light. The data was analyzed by using the method described in section 5.4.2.

5.7.2 RESULT

The fusion rate of the reconstructed couplets by using enucleated and pre-activated oocytes with strontium activation protocol was 68% as compare to the embryos reconstructed using enucleated and pre-activated oocytes with strontium co-activation protocol (80%) (Table: 5.7). Developmental competence of embryos to 2-cells stage also showed significant effect (p< 0.001) when two different activation protocols were compared. The subsequent development up to morula and blastocyst was higher in the embryos reconstructed by using pre-activated oocytes with strontium co-activation protocol as compared to those reconstructed by using strontium protocol , but statistical analysis showed non significant difference. Their was also a difference in the development of fused embryos to blastocyst (p< 0.05) within the group of strontium protocol between reconstructed embryos and non enucleated parthenotes which was not present within the strontium co-activation group. The overall analysis of the data on development from start to 2-cell indicated that strontium co-activation treatment did better than the strontium alone and the embryos reconstructed using G1 or G1/S donor nuclei developed better than the control. When development from 2-cell to 4-cell was compared, the Strontium co-activation protocol proved better than the Strontium alone protocol. However, the comparison of development from start to blastocyst showed Strontium co-activation treatments (+DMAP) not distinguishable from donor control and (-DMAP, G1 or G1/S) but better than (-DMAP, control). Moreover, donor control were better than the both Strontium alone treatments.

5.7.3 CONCLUSIONS

The results of the experiment proved the use of strontium co-activation protocol for the activation of enucleated oocytes, when pre-activated recipient with no MPF would be required.

Table 5.7: Development after nuclear transfer of reconstructed embryos using donor nuclei from G1 stage of 3rd cell cycle and pre-activated oocytes (4 hpa) with strontium chloride (1 hour) and strontium chloride (1 hour) + 2mM 6-DMAP (3 hours) protocols.(%)

Donor cell cycle stage	Activation Protocol	Replicates	Fused	2-cell/Fused	4-cell/Fused	Morula/Fused	Blastocyst/Fused
G1 or G1/S		3	36/53(68)	30/36(83)	21/36(58)	21/36(58)	21/36(58)
	Strontium Chloride						
+ Control*		3	--	16/32(50)	14/32(44)	10/32(31)	5/32(16)
G1 or G1/S		3	36/45(80)	45/45(100)	41/45(91)	41/45(91)	35/45(78)
	Strontium Chloride+						
+ Control*	DMAP	3	--	30/34(88)	27/34(79)	26/34(76)	24/34(71)
Donor control*	--	3	--	--	38/43(88)	38/43(88)	38/43(88)
-ive Control*	--	3	--	3/32(9)	2/32(6)	0/32(0)	0/32(0)

* The percentages are worked out of their total number.

5.8 - DISCUSSION

The results of the series of experiments presented in this chapter demonstrates the beneficial role of using 6-DMAP when used in combination with the activation stimuli. The results highlighted that 6-DMAP itself has negligible effect on activation but potentiates effect of Strontium, in that it apparently enhanced proportion activated and accelerated the response (table 5.1). There are different methods of activation available. In this study three methods of activation were tried to find out the optimum activation protocol for mouse oocytes activation. Studies on the parthenogenetic activation of mouse oocytes using divalent ions like Strontium, barium and manganese has suggested that these ions releases Ca^{+2} from intracellular stores (Whittingham and Siracusa, 1978; Fraser, 1987; O' Neill et al., 1991). The results shown in table 5.5 demonstrates that when the oocytes after activation were exposed subsequently with 6-DMAP, a higher activation proportion was achieved using strontium co-activation protocol.

6-DMAP, inhibits protein phosphorylation without inhibiting protein synthesis, in sea urchin, starfish, and mouse oocytes (Rebhun et al., 1973, Neant and Guerrier, 1988a,b, Neant et al., 1989, Rime et al., 1989, Szollosi et al., 1993). In mouse eggs, 6-DAMP inhibits protein phosphorylation by approximately 20% (Rime et al., 1989). Thus it is apparent not all protein kinases are inhibited by 6-DMAP. For example protein kinase C (PKC), which is activated by the phorbol ester, phobol 12-myristate 13 acetate (PMA) (Nishizuka, 1984), including parthenogenetic activation in mouse eggs (Cutterbertson and Cubbold, 1985), does not appear to be inhibited by 6-DMAP, instead 6-DMAP enhanced PMA-induced egg activation (Moses and Masui, 1994). The results presented in table 5.1 demonstrates that the combination treatments of strontium and 6-DMAP for 3 hours enhanced pronuclear formation (more than 85%) as compare to strontium alone (1 hour) when observed 3 hours after staining.

The data on the observation of polar body and pronuclear formation (8 hpa)(table 5.3) demonstrates the effectiveness of the combined protocol of strontium (1 hr) with subsequent exposure to 6-DMAP (3 hr) on oocytes activation, all of the oocytes exposed to this combined protocol showed polar body formation. In contrast, 81% and 44% of the exposed oocytes to strontium and ionomycin alone showed polar body formation. The exposure of oocytes to strontium and Ionomycin alone showed very little effect of Ionomycin exposure on parthenogenetic activation. But when the embryos were subsequently exposed to DMAP, enhanced response to

development was noted. Poor activation rate (44%) was observed in oocytes exposed to ionomycin alone but when the oocytes were exposed to the subsequent exposure of 6-DMAP, the activation rate was enhanced to 80%, comparable to strontium alone. The maximum development to morula/blastocyst was achieved using strontium co-activation protocol.

In mouse, the shorter exposure to Ionomycin may not be helpful in completely activating the oocytes. In contrast, the higher activation rate in strontium treatment group may due to longer exposure of oocytes (60 min.) to Strontium. The fact that strontium treatment produces Ca^{2+} transient comparable to sperm activation, the difference observed in parthenogenetic development may be due to the mode of intracellular calcium elevation. Ionomycin actually mobilizes the intracellular stores (Hoth and Penner, 1992) but induces only a single wave as compare to the repetitive series of calcium waves generated during fertilization. In contrast, prolong exposure to Strontium may be helpful in generating a series of calcium transients. Poor activation rates of freshly ovulated oocytes treated with some parthenogenetic agent such as A23187 has been suggested because they may generate only a single Ca^{2+} increase (Ozil, 1990; Swann and Ozil, 1994). These studies highlighted the function of Ca^{2+} oscillation in oocyte activation. This is supported by the observations that 100% of freshly ovulated mouse and rabbit eggs can be activated by repetitive electric pulse application to generate repetitive Ca^{2+} transient (Ozil, 1990; Swann and Ozil, 1994).

In most of the mammalian species, sperm entry into oocyte provides natural stimuli for egg activation. It is establishes that the natural activation stimuli induced by sperm penetration induces a series of Ca^{2+} transients in the egg which persists for many minutes or even hours (Miyazaki *et al.,* 1993; Kline and Kline, 1992; Carroll *et al.,* 1996). In mouse first sperm-induced Ca^{2+} transient is distinctive and unlike the later Ca^{2+} transient; it consists of small pacemaker-like rise in intracellular Ca^{2+}, followed by a rapid rise to a large, long lasting plateaus phase. Several fast Ca^{2+} spikes of large amplitude and short duration are often superimposed on the first transient (Kline and Kline, 1994). The release of Ca^{2+} from intracellular stores in oocytes is thought to be due to inositol 1,4,5-triphosphate receptors (insP$_3$ R) and ryanodine receptors (RyR); InsP$_3$ R channels being involved in propagation and maintaining the Ca^{2+} waves and RyR channels have been linked to the conversion of the zona glycoprotein (ZP$_2$) to its post fertilization form ZP$_2$ f (Yue *et al.,* 1995; Kenneth and Yue, 1996). The increase in intracellular calcium concentration that result from fertilization, inactivates MPF and CSF and releases the eggs from

metaphase arrest (Meyerhof and Masui, 1977; Murray and Kirschner, 1989b). So the efficiency of the parthenogenetic activation agent depends on its ability to generate repetitive Ca^{2+} transients.

When the oocytes were subsequently exposed to DMAP, the 2PN formation was observed in 65 to 70% of oocytes. The study of Maro *et al.,* (1986) shows that 6-DMAP inhibits spindle rotation and second polar body formation. These two events are controlled by interactions between the chromosomes and the oocyte cortex, resulting in the formation of a microfilament-rich domain overlying the spindle. Normal activation requires close association between one of the rotating spindle poles and the cortical actin layer. The lack of this contact in the presence of 6-DMAP suggests that protein phosphorylation is involved in the maintenance of spindle pole-cortex association and successful spindle rotation. Extrusion of first polar body was also often inhibited by 6-DMAP during mouse oocyte maturation (Szollosi *et al.,* 1991) as well as the formation of both polar bodies in maturing oocytes of Echinoderms (Neant *et al.,* 1989). The inhibition of second polar body in present study resulted in the formation of 2PN in oocytes exposed to 6-DMAP after activation with strontium or ionomycin. However, only 65% of the oocytes showed 2PN formation when treated with strontium co-activation protocol, may be due to the exposure time to strontium (60 min.) before its exposure to 6-DMAP. All those oocytes activated in the start of the exposure to strontium may have organized their spindle and spindle rotation which ultimately resulted in the formation of 1PN + 1PB. In contrast, the oocytes activated later with strontium was unable to form their spindle due to their exposure to 6-DMAP, resulted in the formation of 2PN. This is evident from the results of experiment (table 5.3) that oocytes exposed to strontium co-activation protocol showed 33% oocytes with 1PN + 1PB formation as compared to the ionomycin co-activation protocol, 6% showed 1PN + 1PB formation.

The observations of the present study suggests that 6-DMAP accelerates the formation of pronuclei in oocytes activated in presence or absence of 6-DMAP (table 5.1). These observations are in line with the previous studies of Szollosi *et al.,* 1993; Ledda *et al.,* 1996. However, formation of single pronucleus after DMAP exposure was also reported by Ledda *et al.,* 1996; Loi *et al.,* 1998 in bovine and ovine respectively. The exposure of oocytes to the co-activation protocol also enhanced the cell cycle progression, and higher number of cells in blastocysts on 4th day and 5th day was observed (data not shown) as compare to the strontium and Ionomycin alone. The morphology of the blastocyst from the strontium co-activation treatment group was observed to be better than the strontium or Ionomycin treated ones (Fig

5.2). The development of parthenotes to blastocyst stage (table 5.4) suggests difference in the effectiveness of different protocols used in this study. The formation of number of pronuclei and polar bodies is expected to alter the ploidy of the parthenotes. All those oocytes who showed 1PN + 1PB are expected to be haploid but those showing 2PN + 1PB represents two sets of haploid. However, the developmental potential of the parthenotes on this basis was not evaluated in these experiment. It is suggested that further experiments should be performed to evaluate the developmental potential of the haploid and diploid parthenotes. Previous study of Surani and Kaufman, (1977) using Ca^{2+} and Mg^{2+} free or Ca^{2+} alone medium resulting in suppression of second polar body extrusion demonstrated a proportion of cases with two pronuclear eggs or eggs that underwent immediate cleavage. However, the use of Cytochalasin D (CCD) or B could also be successfully used to block the polar body formation and the population of parthenogenetic oocytes with 2PN (haploid) could be achieved. In this study the observation of 2PN + 1PB shows the presence of two haploid pronuclei. However, it is not studied that either the oocytes divided before the synthesis of another set of chromosomes or they cleaved immediately. The cytogenetic studies of the parthenotes could provide solid informations in recognizing the population of haploids and diploids.

The observation on the nuclear morphology post fusion in embryos reconstructed by transferring G1 or G1/S phase and S-phase donor nuclei into pre-activated oocytes using Strontium co-activation protocol showed no NEBD and PCC. The results provides the evidence that strontium co-activation protocol did lower the CSF and MPF activity. In contrast when the embryos were activated using Strontium chloride alone, the basal level showed 41.7% ± 4.22 NEBD and 10.25% ± 3.7 NEBD & PCC in reconstructed embryos (Fig: 6.2) which is the evidence of the presence of some MPF activity. These observations suggests the need for longer exposure to strontium or the use of strontium co-activation protocol (strontium 1 hr then 6-DMAP 3 hr) to activate the recipient cytoplast to use them as "Universal recipient". The results of experiments also demonstrates that the cytoplasts activated using Strontium co-activation protocol support development with higher proportions of embryos reconstructed by using donor nuclei from any stage of 3rd cell cycle.

The results presented in table 5.6 that except one, none of the embryos reconstructed by nuclear transfer showed NEBD or NEBD & PCC. As evident from figure 5.4, no nuclear envelope breakdown and premature chromosome condensation occurred in the donor nuclei after fusion, suggesting the absence of MPF activity. Campbell *et al* ., (1993) suggested the use of pre-activated oocyte with

no MPF to reconstruct the embryos in nuclear transfer experiments . It was also suggested that if no NEBD and PCC occurs, all the donor nuclei will support development regardless of their cell cycle stage. These changes in donor nuclei are induced by the complex of p34cdc2 and cyclin B (Nurse, 1990). The maturation promotion factor is regulated by its phosphorylation state. The higher cytoplasmic activity of MPF causes NEBD and chromatin condensation. All embryos reconstructed by nuclear transfer into MII cytoplasts when MPF activity was high underwent NEBD & PCC (Fig: 6.2). The results of the experiments presented in table 5.5 are in agreement with Fulka *et al.,* 1996 and Campbell *et al.,* 1993) who reported development in nuclear transfer embryo reconstructed after lowering the level of MPF. The enhanced development of embryos reconstructed by using cytoplasts activated with Strontium co-activation protocol (+DMAP) is interpreted in the sense of the treatment of cytoplasts with DMAP, preserved the integrity of the nuclear envelope and normal synthesis of DNA. The use of DMAP treatment in these experiments helped in lowering the MPF activity and embryos reconstructed by nuclear transfer from donor nuclei of different stages in the absence of MPF undergo for development.

These experiments established that the sequential exposure to strontium and 6-DMAP provide a more effective means of activating represent oocytes than the use of strontium alone. However, the study of Wakayama *et al.,* (1998) demonstrated the use of lower concentration (10 mM) of strontium for longer duration to properly activate the reconstituted embryos. The study also concludes that treatment of cytoplasts with 6-DMAP after activation provides good opportunity to create "Universal recipients" and to enhance development by transferring donor nuclei from all stages of 3rd cell cycle. It further suggests that the integrity of the nuclear envelope could be maintained and normal ploidy is expected in nuclear transfer embryos by using strontium co-activation protocol and difference in development between treatments is expected due to cell cycle stage effect of donor nuclei .

CHAPTER 6

THE EFFECT OF CYTOPLASMIC ENVIRONMENT

6.1- INTRODUCTION

Previous experiments on nuclear transfer in mammalian species have demonstrated the role of cytoplasmic environment on the morphological changes in the donor nuclei after transfer and fusion with the enucleated metaphase II oocytes. These include nuclear envelope breakdown (NEBD), premature chromosome condensation (PCC), dispersal of nucleoli, reformation of nuclear envelope and nuclear swelling (Collas *et al.*, 1992). In contrast, when pronuclear zygotes were used as recipient cytoplasm, no NEBD and PCC was observed. Reports of the previous studies have shown that NEBD and PCC are essential for the reprogramming of gene expression and these events increase the developmental potential of the reconstructed embryos (e.g., in the rabbit [Collas *et al.*, 1992a]). However, the extent and timing of these events vary between species and the stage of the donor cell cycle (Collas *et al.*, 1992b).

The development to blastocysts of reconstructed embryos have been reported to be greatly effected by the stage of the cell cycle of the donor nuclei, transfer of donor nuclei at G1 or early S-phases of cell cycle proved better as compared to those transferred at late S and G2 phases (Collas *et al.*, 1992a). The morphological examination of the embryos reconstructed by using donor nuclei at G1 and early S-phase had suggested very little effect of cytoplasmic environment on the chromosomal constitution of the donor nuclei. In contrast, large chromosomes and other chromosomal abnormalities have been observed in the embryos reconstructed from late S phase (Collas *et al.*, 1992b). Reports of previous studies suggests that this effect is induced by high level of maturation promoting factor (MPF) activity, present in MII oocytes and in mitotic cells (reviewed by Nurse, 1990 and Maller,

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1991). MPF was shown to be universal regulator of G2/M transition in eukaryotes (reviewed by Masui, 1992). MPF is the complex of two sets of proteins, cyclins (Swenson *et al.,* 1986; Labbe *et al.,* 1989) and p34^{cdc2} protein kinase (Dunphy *et al.,* 1988; Labbe *et al.,* 1989) whose activity is measured using exogenous histone H1 as a substrate. Activation of the p34^{cdc2} kinase requires cyclin (Murray *et al.,* 1989) and the kinase activity of p34^{cdc2} -cyclin complexes is regulated by the changes in the phosphorylation state of both components (reviewed by Clarke and Karsenti, 1991).

Mammalian oocytes remain arrested at metaphase of second meiotic division until they are fertilized or activated (spontaneously or artificially). This arrest is maintained by high levels of maturation promotion factor (MPF) (Kubiak *et al.,* 1993), which is stabilized by the action of another cytoplasmic component, the cytostatic factor (CSF) (Gerhart *et al.,* 1984 and Watanabe *et al.,* 1989). The cytostatic factor (CSF) is now known to be the *c-mos* proto-oncogene product (Mos) (Sagata *et al.,* 1989). At fertilization, a series of repetitive Ca^{2+} transients occur (Cuthbertson and Cobbold, 1985; Igusa and Miyazaki, 1986 and Taylor *et al.,* 1993) which are responsible for the destruction of MPF and re-entry into the cell cycle (Kline and Kline, 1992). The Ca^{2+} transients stimulated at fertilization continue until the formation of pronuclei in the one cell mouse embryo (Jones *et al.,* 1995). In nuclear transfer experiments, the Ca^{2+} releasing activity of karyoplasts from fertilized embryos has been demonstrated previously by their ability to activate MII-arrested oocytes (Kono *et al.,* 1995). The study of Kono *et al.,* (1996) demonstrates that fertilized embryos generate transients at nuclear envelope breakdown (NEBD) and during mitosis. In contrast, parthenogenetic embryos produced by 2-hr exposure to strontium containing medium do not generate detectable Ca^{2+} transients at NEBD or in mitosis. These studies suggests that the donor nuclei at all cell cycle stages except M-phase when transferred into MII-arrested cytoplasts generate Ca^{2+} transient at NEBD. The findings that local Ca^{2+} changes (Cheong *et al.,* 1993; Yao and Parker, 1994) and that NEBD releases Ca^{2+} supported the view that local Ca^{2+} transients in the nuclear region may induce NEBD and progression through mitosis.

CSF or Mos is also inactivated by the transient increase in the cytoplasmic free calcium (Meyerhof and Masui, 1977; Watanabe *et al.,* 1989) and it was demonstrated that CSF inactivation is the primary cause of MPF inactivation on meiotic release (Sagata *et al.,* 1989; Newport and Kirschner, 1984; Gerhart and Kirschner, 1984;). Degradation of the proto-oncogene product p39mos is not necessary for cyclin proteolysis and exit from meiotic metaphase (Lorca *et al.,* 1991). The study of Watanabe *et al.,* (1991) in *Xenopus* eggs demonstrates that at

molecular level, cyclin subunit of MPF are degraded before Mos is degraded and, at physiological level, that MPF activity is inactivated before CSF activity during activation. These results have provided the evidence that a calcium transient on fertilization induces a CSF-independent pathway for MPF inactivation, whereas CSF inactivation during meiotic release serves only to allow the fertilize egg to enter mitosis. So the activity of CSF after activation decreases slowly.

After reconstruction of embryos with the technique of nuclear transfer, nuclear envelope breakdown (NEBD) and premature chromosome condensation (PCC) occur in the donor nucleus (e.g. in mouse Czolowska *et al.,* 1984). The induction of NEBD and PCC is mediated by maturation promotion factor (MPF), (reviewed by Nurse 1990 and Maller, 1991). Studies of Campbell *et al.,* (1993) in bovine, suggests that all nuclei transferred into MII cytoplasts in which MPF activity is high undergo NEBD and PCC. In contrast when the embryos were reconstructed after the disappearance of MPF activity (10 hours post activation), no NEBD and no PCC were observed. However, the study showed the level of MPF after activation as measured by H1 kinase reached to its basal level of $20 \pm 6\%$ at 120 minutes. The study further suggested that if MII cytoplasts were used to reconstruct the embryos then only G1 should be transferred at the time of activation. In contrast, the use of pre-activated cytoplast (Universal Recipient) creates a cytoplasmic environment able to support development of embryos reconstructed using donor nuclei from any stage of cell cycle. In mouse, it may be difficult to establish "Universal recipient".

Thus the present series of experiments was planned to optimize the time for embryo reconstruction using G1 and G2 donor nuclei into different cytoplasmic environment after cytoplast activation and to find out the cytoplasmic environment representative of "the Universal recipient " in mouse.

The results of lower development in the above mentioned experiment suggested the need to re-ensure the length of cell cycle in enucleated and non-enucleated oocytes. So the experiments were also performed to evaluate the cell cycle length of enucleated and non-enucleated oocytes.

The third series of experiments were planned to map the level of MPF and to find out the basal level of MPF representative of the "Universal recipient".

6.2- DEVELOPMENT AFTER NUCLEAR TRANSFER

The study of Otaegui, 1995 (Ph.D. thesis), investigating the effect of interaction between donor nuclei and recipient cytoplast cell cycle phases demonstrated the use of cytoplasts at different stages of cell cycle. However, the proportion of development to blastocysts in embryos reconstructed by transferring S-phase and G2 phase remain restricted at low level. As the level of MPF activity varies after activation which give rise to different type of cytoplasmic environments, it was decided to reconstruct the embryos at various times post activation to optimize the time of G1 and G2 donor nuclei for reconstruction of embryos and to find out the cytoplasmic environment representative of "the Universal recipient " in mouse.

6.2.1- EXPERIMENTAL DESIGN

Oocytes recovered after 14 hours post hCG injection were denuded using 300 i.u. hyaluronidase/ml in M2 medium. The oocytes were enucleated by using micromanipulation procedures (see section 3.4.4). The enucleated oocytes (cytoplasts) were activated by using 25 mM strontium chloride in M16 medium for 1 hour. The donor 2-cell embryos were recovered at 44-48 hours post hCG injections after mating with the same F1 hybrid males. Until required they were placed at low temperature in M2 medium under paraffin oil. They were synchronized by culturing them in sM16 medium containing 10 μM Nocodazole for 8-12 hours. They were released in batches after washing them thoroughly with M2 medium several times to remove the effect of Nocodazole. They were cultured in sM16 and were used for reconstruction of embryos as soon as possible to capture the G1 stage of 3rd cell cycle (4-cell). The donor nuclei started dividing within 60 minutes and used for nuclear transfer 75 minutes after their release from synchronization. The donor nuclei at this time were considered at G1 or G1/S stage of cell cycle. The embryos were reconstructed at zero, 2-3, 4, and 6 hours post activation. On the other hand 2-cell donor nuclei were recovered at 40-44 hours post hCG injection and mating, to use them at G2 stage of 2-cell cycle and the embryos were reconstructed at 3, 4, and 6 hours post activation of cytoplasts. All couplets were fused by using sendai virus (8000 HAU). The observations on the development competence of the reconstructed embryos were recorded.

6.2.1.1- STATISTICAL ANALYSIS

The data was analyzed using Generalized Linear Mixed Model (Breslow and Clayton, 1993) looking at the effect of treatment whilst allowing for differences between days. The treatment effects were tested using a Wald statistics (distributed as chi-squared), and if significant differences between mean were assessed by an approximate t-test. Means declared to differ, differ at the 5% level or better.

6.2.2- RESULTS

The experiments were conducted to reconstruct the embryos at different times post activation of the cytoplasts and the results are presented in Table 6.1. The fusion rates are appreciably higher in the embryos reconstructed at 4 hours (96%) and 6 hours (87%) post activation by using 2-cell (G2) donor nuclei as compare to the embryos reconstructed by using 4-cells (G1 or G1/S) donor nuclei.

Using 4-cell (G1 or early S) donor nuclei at 0, 2-3, 4 and 6 hours post activation, the development to 2-cell was observed 34, 47, 50 and 29% respectively. A significant time effect (p< 0.01) was observed when the development to 2-cell from fused were compared. The development to 2-cell is shown to be good when embryos were reconstructed using cytoplasts at 2-3 or 4 hours post activation as compared to the embryos reconstructed 6 hours post activation. However, the analysis showed no distinguishable difference from all others when compared with the embryos reconstructed at 0 hours post activation.

The maximum development to morula/ blastocysts was observed to be 39% and 29% respectively, when 2-3 and 4 hours pre-activated oocytes were used at the time of G1 blastomere deposition. In contrast when 2-cell (G2) stage donor nuclei were used for reconstruction of embryos at 3, 4 and 6 hours post activation, 42, 46 and 26% respectively, development to 2-cell was observed and the analysis of the data showed a significant time effect (p< 0.01). The development to morula/blastocyst (Table: 6.2) stage is shown to be 11.1, 14 and 2% respectively. Slight difference in the development up to 2-cells stage of embryos reconstructed at 3,4, and 6 hours post activation using donor nuclei at 4-cell (G1 or G1/S) and 2-cells (G2) stages is observed, however, the analysis showed no donor effect.

Table. 6.1

Development after nuclear transfer of embryos reconstructe at various times post activation of enucleated oocytes by using 2-cell (G2) and 4-cell (G1 or early S) blastomeres

Nucl.Donor	Trt.	Rep.	Tot. Fused	% 2-cell/fu	% Mor/Bl.
4-cell (G1 or G1/S)	0 hr	3	64	34	8
	2-3 hpa	4	49	47	39
	4 hpa	4	34	50	29
	6 hpa	2	17	29	6
2-cell(G2)	3 hpa	4	36	42	11.1
	4 hpa	7	96	46	14
	6 hpa	5	87	26	2

hpa = hours post activation

6.2.3- CONCLUSIONS

Development to morula/blastocyst stage in the embryos reconstructed using donor nuclei from 4-cell embryos is shown to be maximum when 4-cell donor nuclei (G1 or early S) were fused at 3 and 4 hours post activation of oocytes .

No significant difference was observed in the development of the reconstructed embryos to 2-cell, by transferring donor blastomeres from 4-cell (G1 or G1/S) and 2-cell (G2) at 3, 4, and 6 hours post activation of cytoplasts, however, further development to morula/blastocyst is shown to be much higher in the embryos reconstructed at 2-3 and 4 hour post activation of cytoplast using 4-cell (G1 or G1/S) blastomeres.

The development using 4-cell donor nuclei at G1 stage of cell cycle at the time of activation (0 hpa) was unsuccessful. This could be explained due to improper use of donor cell cycle stage. The donor nuclei might not be at G1 stage of cell cycle because later experiments showed enhanced rate of development with G1 donor blastomeres. The lower rate of development by using 2-cell (G2) nuclei as donor blastomeres is expected due to partial exposure to MPF, unable to cause complete chromatin condensation or necessary reprogramming.

6.3- MAPPING OF FIRST CELL CYCLE LENGTH

The reconstruction of embryos by transferring donor nuclei at different stages of recipient cell cycle failed to reveal the stage of recipient cytoplast representative of the universal recipient and suggests that perhaps there is a small window in the cell cycle for basal level of MPF. So it was advisable to check the length of the first cell cycle. Thus the present experiment was planned to investigate the length of the 1st cell cycle of the enucleated and non enucleated oocytes.

6.3.1- EXPERIMENTAL DESIGN

To map the cell cycle length, the oocytes were recovered 14 hours post hCG injections and were treated with hyalauranidase (300 i.u./ml) to denude them from the cumulus cells. The oocytes were divided into two groups. One group of oocytes were enucleated by using micromanipulation procedures (see section 3.4.4). Both the groups (nucleated and non-enucleated) of oocytes were activated by using 25 mM of strontium chloride in Ca, Mg free M16 for one hour. The oocytes after activation

were washed with several changes of M2. They were incubated in the microdrops of sM16 at 37°C in 5% CO_2 in air, in 60 mm plastic petri dishes prepared at least 2 hours prior to incubation. Both the groups were observed every hour after activation until the time of first fragmentation / cell division and then observed after every 30 min. to record the observations.

6.3.2- RESULTS

The first cell cycle length, assessed by activating the non-enucleated and nucleated oocytes, is presented in Table 6.2. The length of the 1st cell cycle of the non-enucleated oocytes ranged from 18.7-22 hours (Table: 6.2) with an average of 20.41 hours. In contrast in the enucleated oocytes fragmentation (taken as attempted cleavage) was observed from 13.2-17.5 hours (Table 6.2) with an average of 14.5 hours.

6.3.3- CONCLUSIONS

In fact the cell cycle of enucleated oocytes is short. The length of 1st cell cycle in non enucleated parthenotes was observed to be 20.41 hours (Fig. 6.1). In contrast the length of the 1st cell cycle in enucleated parthenotes is shown 5.91 hours earlier i.e. 14.6 hours (Fig. 6.1).

MPF activity starts declining after activation and reaches its basal level around 4 hours post activation and then starts rising again after 6 hours post activation (Fig. 6.1), the rise in MPF in the absence of nucleus causes fragmentation.

6.4- MAPPING THE LEVEL OF MPF, ITS EFFECT ON DONOR NUCLEI AND DEVELOPMENT

The length of the 1st cell cycle in enucleated parthenotes is short which suggested the need for mapping the level of MPF and to investigate how long the MPF level stays at basal level. As the level of MPF declines with the passage of time after activation of the cytoplasts, the observations of the proportion of NEBD and NEBD&PCC of the transferred donor nuclei into the cytoplasm could provide good estimation of the level of MPF.

The study aimed to map the level of MPF by evaluating its effect on the morphology of transferred donor nuclei through NEBD and NEBD&PCC..

Fig: 6.1 Graph showing the timing of fragmentation or cleavage in enucleated and non-enucleated mouse parthenotes respectively
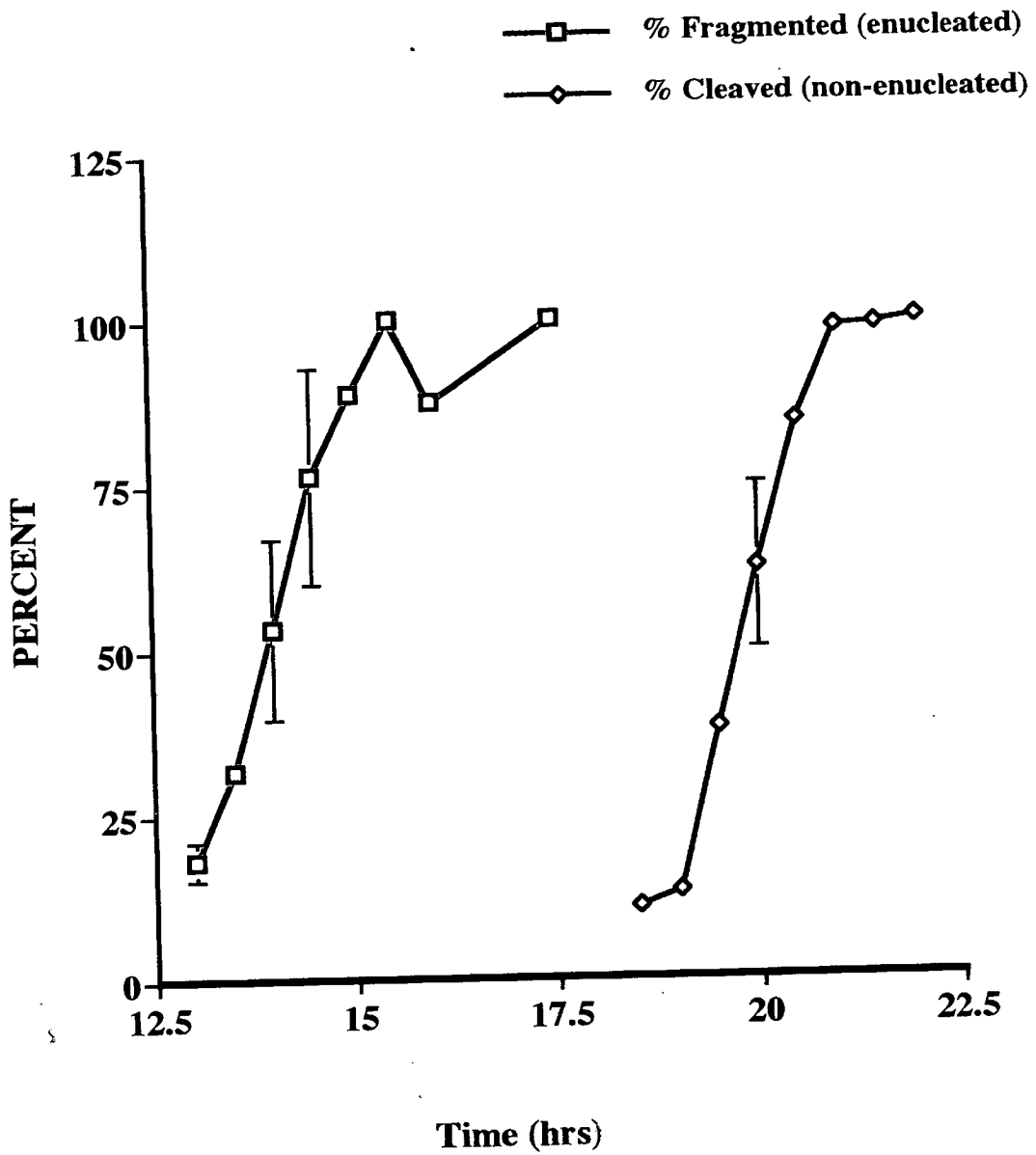
Table. 6.2
First cell cycle length after activation of non-enucleated and enucleated oocytes with 25mM Strontium chloride for 1 hours

Groups	Rep.	No.	Range	Av. Cl/Frag(Hrs)
Enucleated	4	34	13.2-17.5	14.5
Non-enucleated	3	94	18.7-22.0	20.41

6.4.1- EXPERIMENTAL DESIGN

4-6 weeks old F1 hybrid (C57 BL/6 X CBA) female mice were superovulated and the oocytes were recovered 14 hours post hCG injections. The oocytes were denuded by using 300 i.u. hyaluronidase/ml in M2 medium. Oocytes were treated with 1μg/ml Hoechst 33342 for 5 min. to examine the chromosomes and stage of maturation. The 2-cell donor nuclei were recovered 40-44 hours post hCG injections and mating.

The oocytes were enucleated within 3 hours of their recovery by using micromanipulation procedures (see section 3.4.4). The enucleated oocytes (cytoplasts) were activated by using 25 mM strontium chloride in Ca Mg free M16 medium for 1 hour. Late 2-cell (G2) blastomeres were used as nuclear donor (Karyoplasts). The embryos were reconstructed at zero, 2-4, 4-6, 6-8, 8-10, and 10-12 hours post activation. The zero hour of activation was considered, when they were first exposed to the activation medium (Strontium chloride). The donor nuclei were deposited into the perivitiline space of the zona pellucida of the cytoplast in a micromanipulation chamber containing 7.5 μg Cytochalasin B, 10 μM Nocodazole and 10% FCS in M2 by using holding and injection pipettes. The fusion was Sendai virus mediated. After reconstruction, the couplets were immediately washed with M2 medium with several changes and incubated in the previously prepared dishes containing microdrops of sM16 medium having 10 μM Nocodazole until fusion. One hour after fusion, the oocytes were washed with M2 medium and were mounted on to the slides. They were fixed in an acetic acid: methanol (1:3, v/v) for 24 hours and were stained with 2% aceto-orcene. The nuclei of reconstructed embryos were examined for NEBD and NEBD&PCC under phase contrast microscope, photomicrographed and observations were recorded.

6.4.2- RESULTS

The effect of MPF activity on the NEBD and NEBD&PCC is shown in figure: 6.2. The scanning of reconstituted orcene stained embryos for NEBD and PCC under high magnification revealed that all donor nuclei fused at 0 hours of activation had undergone nuclear envelope breakdown (Fig. 6.3 photomicrograph for NEBD) and 90% ± 3.22 showed NEBD and PCC (Fig. 6.3 photomicrograph for NEBD&PCC).

Fig: 6.2 Graph showing NEBD and NEBD+PCC activity in reconstructed embryos (Mean±S.E.M.)
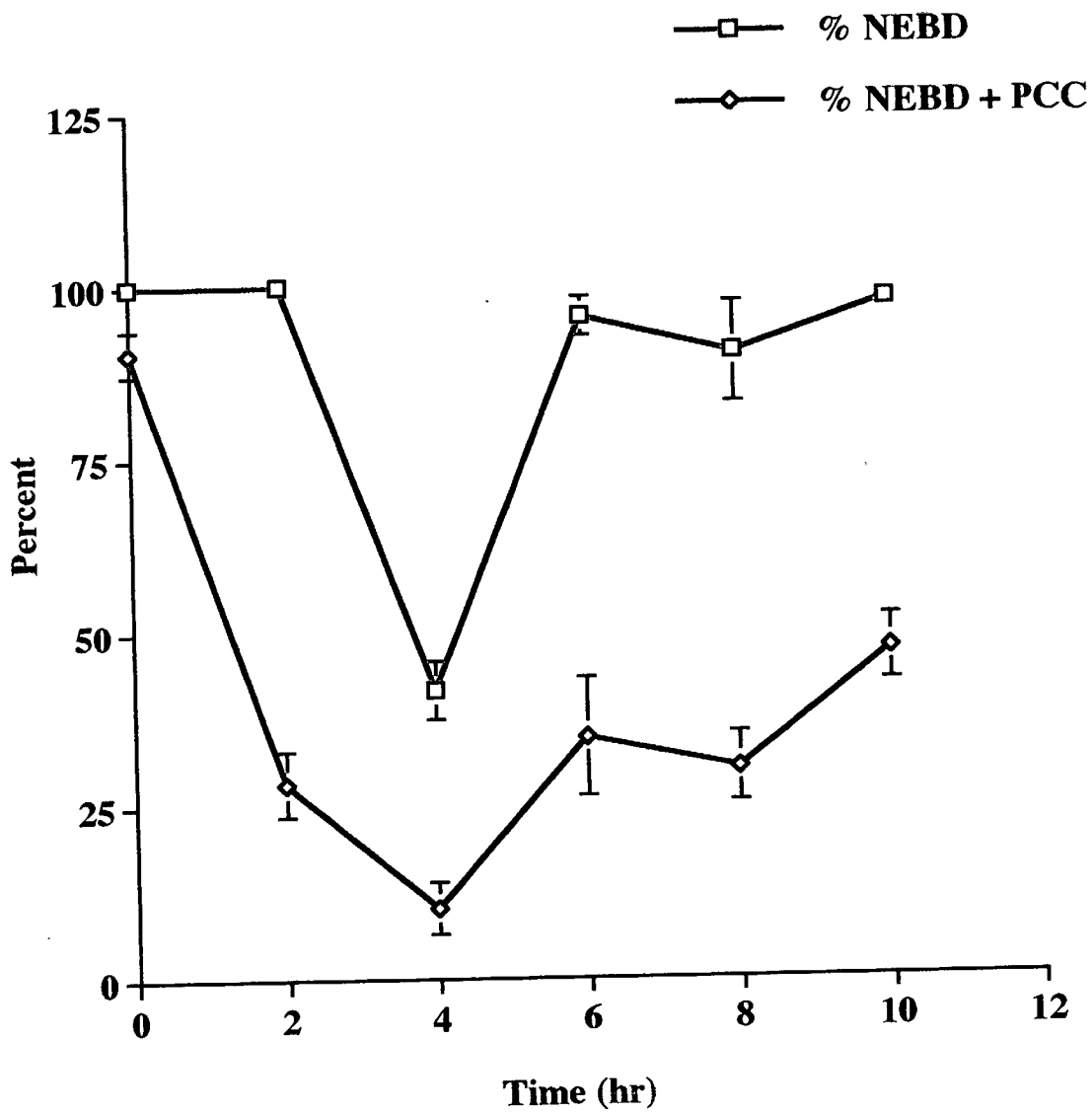
Fig. 6.3 Effect of MPF on the nuclear morphology of transferred nucleus.

a) NEBD and PCC in the embryos reconstructed at zero hour of activation

b) NEBD in the embryos reconstructed 2 hours post activation

c) embryo showing nuclear envelope when reconstructed 4 hours post activation
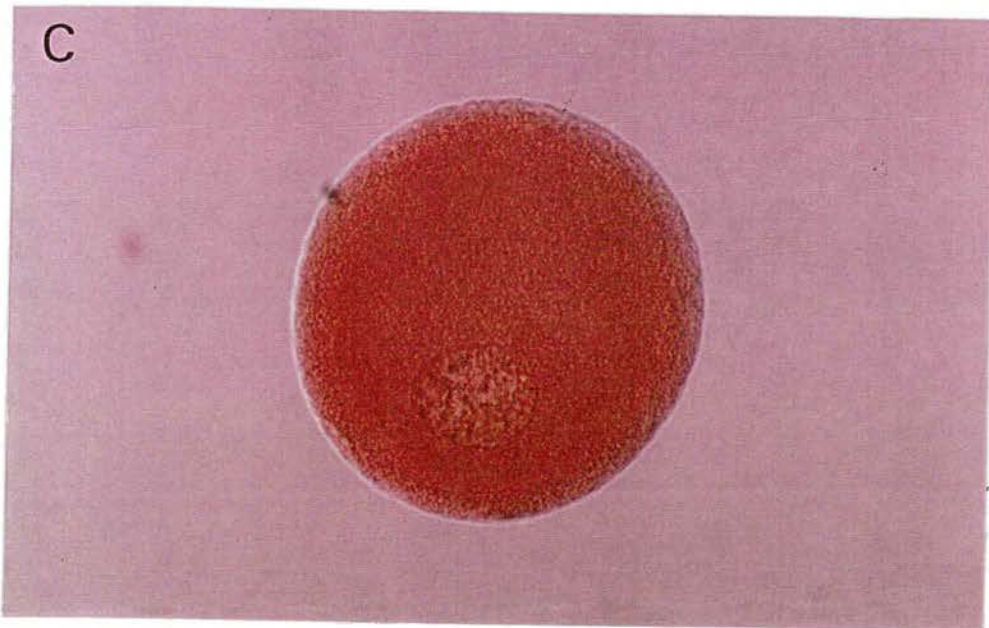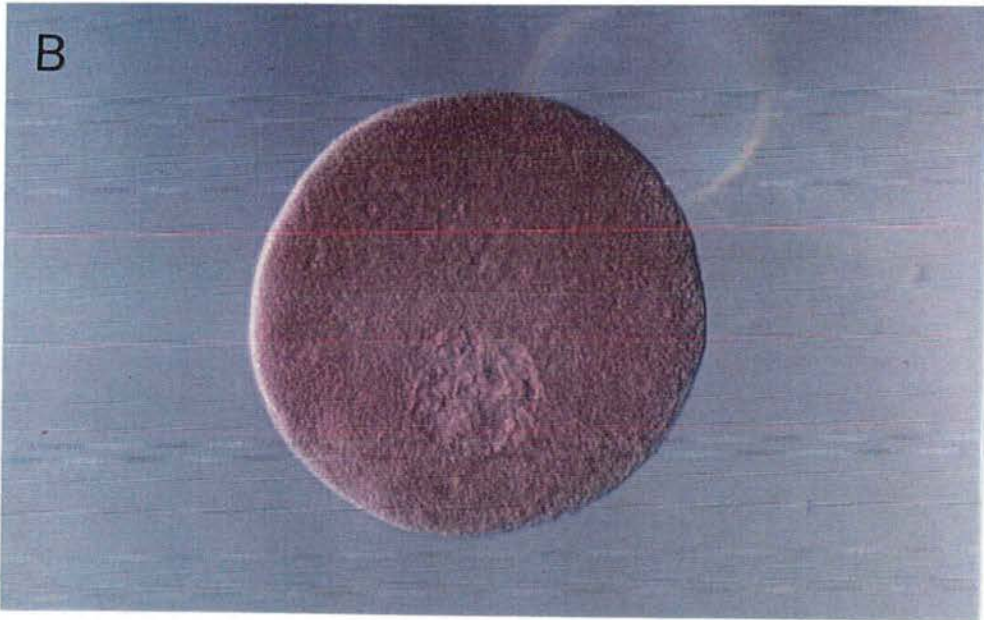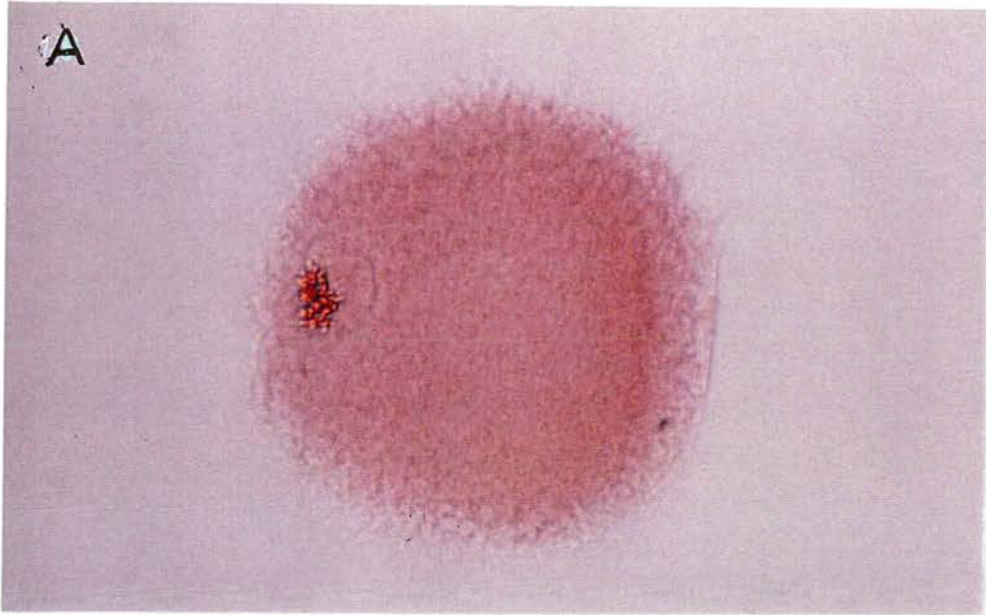
The frequency of NEBD+PCC declines rapidly after 2 hours post activation as the percentage of donor nuclei which undergo NEBD & PCC decreased to 28.24% ± 4.69. The basal level of MPF is observed 4 hours post activation where the percentage of NEBD and NEBD & PCC reaches to 41.7%±4.22 and 10.25% ±3.7 respectively (Fig. 6.4 showing the nuclear envelope). The level of MPF starts rising again after 6 hours post activation as the percentage of reconstituted embryos which undergo NEBD and NEBD & PCC increases to 95.5%±2.78 and 34.86% ± 8.5 respectively.

The level of MPF almost reached pre-activation level after decay was attained at 10 hours post activation as the percentage of NEBD and NEBD & PCC was observed 97.78% ± 2.2 and 47.42% ± 4.6 respectively.

6.4.3- CONCLUSION

MPF activity in murine oocytes starts declining rapidly after activation within 2-3 hours and reaches its basal level around 4 hours post activation and then starts rising again after 6 hours post activation (Fig. 6.2)

The results imply that the MPF level stays for very short time at basal level. Hence the use of enucleated cytoplast as universal recipient is disappointing.

6.5- DISCUSSION

The results of experiments conducted to study the effect on development of mouse embryos reconstructed by nuclear transfer at different times after activation confirms that development to 2-cell is greatly influenced (p< 0.01) by the cell cycle stage of the recipient cells. The observations on the timing of the initiation of cleavage of non-enucleated and fragmentation of enucleated oocytes (Table: 6.1) indicated that fragmentation time in enucleated post activated oocytes is about 5.5 hours earlier than cleavage in the non-enucleated ones. One possible explanation of this early fragmentation in enucleated oocytes is that MPF activity returns after strontium activation. The early rise in the level of MPF during the time of pronuclear formation was unexpected (fig 6.2). It was anticipated that the level would remain low after strontium activation. The study of Jones et al., (1995) has demonstrated that the Ca^{2+} transients stimulated at fertilization continue until the formation of pronuclei in the one cell mouse embryo. The observations of early rise in MPF in present study may be the use of strontium activation protocol in this experiment,

which does not mimic adequate events during activation. Although it initiates early development, the stimulus is inadequate and suggests the need for longer exposure to strontium. The study of Bos-Mikich *et al.*, (1995) and Wakayama *et al.*, (1998) in mouse demonstrates the use of 10 mM of strontium for longer period of time. However, the combination of strontium and protein phosphorylation inhibitor (6-DMAP) was observed beneficial in parthenogenetically activating the metaphase II oocytes (chapter 5). The combination of Strontium and protein synthesis inhibitor was also shown to be effective in parthenogenetic activation of mouse oocytes (Bos-Mikich *et al.*, 1995). The study further demonstrates that strontium treatments leads to Ca^{2+}-dependent CSF destruction and cycloheximide leads to loss of MPF activity. The other reason may be due to the lack of feed back mechanisms in the absence of a nucleus. As observations on the level of MPF (Fig: 6.2) suggests that MPF starts rising after 6-8 hours post activation, this rise in the MPF level may induce cleavage-the nucleus may be involved in direction of cleavage plane. Lack of a nucleus may disrupt this leading to uncoordinated cleavage and fragmentation.

The results of experiment conducted to map the level of MPF through evaluating the nuclear morphology (Fig: 6.2) that MPF disappears within 2-3 hours post activation and development to morula/blastocyst (39%) was observed in nuclear transfer embryos reconstructed 2-3 hours post activation of cytoplasts. It is evident from the experiment conducted to map the cytoplasmic activity of MPF that the cytoplasmic activity of MPF reaches its basal level around 4 hours post activation (Fig: 6.2) and development to 2-cell after nuclear transfer is 50% but shows less development (29%) to morula/blastocyst stage. The enhanced activation by further release of Ca^{2+} transient with the NEBD of donor nuclei (Kono *et al.*, 1996) in embryos reconstructed at 2-3 hours post activation with strontium containing medium could be responsible for increased development. The low rate of development to morula/blastocyst in the embryos reconstructed at zero and 6-8 hours post activation by using 4-cell (G1) donor nuclei was observed as 8 and 6% respectively. The results of the studies by Collas *et al.,* (1992a) in rabbits and Cheong *et at.,* (1993) in mice demonstrated high rate of developments when embryos reconstructed by using G1 stage donor nuclei and metaphase II recipient cytoplasts. In this study the difference in the development to morula/blastocyst stage of the embryos reconstructed at different times post activation of the cytoplasts could be because donor nuclei were probably in S-phase and not G1 at the time of fusion and there was maximum activity of MPF and CSF at zero hours of activation and increased activity, after destruction, at 6 hour post activation. As a result of this MPF activity, NEBD and PCC occurred in donor nuclei and a lower rate of development to morula/blastocyst was observed.

The donor nuclei which were at late 2-cells or G2 stage, when fused to 6-8 hours pre-activated oocytes, developmental competence to morula/blastocyst stage (2%) was quite low, which is expected because of the aneuploidy/DNA damage of the chromosomes in the presence of MPF activity. The result could be correlated with the level of MPF , that level of MPF in enucleated activated cytoplasts stays at its basal level for short time and provides very small window of opportunity to use them as "Universal recipient". The level of MPF started rising again after 6-8 hours of activation, the donor nuclei which were at late 2-cells or G2 stage, when fused to 6-8 hours pre-activated oocytes, did not develop to blastocyst stage perhaps because of the aneuploidy/DNA damage to the chromosomes due to increasing activity of MPF activity.

When nuclei from 4-cell embryos were transferred to MII cytoplasts, the largest proportion of embryos developed morula (34%) and blastocyst (8%), when the donor nuclei were in early G1-phase. These result confirm earlier reports in which development to blastocyst was achieved with nuclei in G1-phase when embryos were reconstituted using metaphase II oocytes as recipient (Collas *et al.*, 1992a, Cheong *et al.*, 1993). The maximum development in this case is probably due to synchrony in the cell cycle stage of both the donor nuclei and recipient cytoplasm. The proportion of reconstituted embryos that developed to blastocyst following nuclear transfer and activation at the same time was lower than that reported in the literature (Kono *et al.*, 1991b; 1992; Cheong *et al.*, 1993), suggests the need for manipulation practice.

This study provides observations on the cell cycle co-ordination of donor and recipient cells. The Fragmentation in non-enucleated cytoplasts occurs earlier (13.5 hr) in the absence of nucleus. The results implies that the MPF level stays for short time at basal level, hence there is very small window of opportunity for using the cytoplasts as universal recipients in mouse. However, further experiments to achieve proper activation stimuli through prolonged exposure of MII cytoplast to strontium medium are suggested.

DNA Replication in 3rd and 4th cell cycle: Duration of the G1 phase

7.1: INTRODUCTION

The studies of Campbell *et al.,* 1993 in bovine in this laboratory suggested that normal development after nuclear transfer is expected only by maintaining the normal ploidy. The study of Otaegui, (1995 Ph.D. thesis) in mouse and other earlier findings demonstrates that development after nuclear transfer of reconstructed embryos is greatly influenced by the cell cycle phase of the donor nuclei (Smith *et al.,* 1988; Tsunoda *et al.,* 1989 and Kono *et al.,* 1991b). Increased development after nuclear transfer of donor nuclei at G1 phase of cell cycle has been reported by different researchers (Collas *et al.,* 1992a in rabbits; Cheong *et al.,* 1993 in mice).

The results obtained by Otaegui, (1995 Ph.D. thesis) demonstrate the use of G1 donor nuclei as nuclear donor to reconstruct the embryos by using MII or S-phase cytoplasts. It was further suggested that by using the permissive window of the G1-phase of the cell cycle, development after nuclear transfer could be maximized. The rate of development after nuclear transfer with the donor nuclei at G1 stage depends on the ability of the researcher to physically select the donor nuclei at G1-phase of the cell cycle. In the experiments presented in chapter 5, the number of nucleoli (9-12) were used as the basis to select donor nuclei at G1-phase of cell cycle. A short duration of the G1-phase has been reported by Otaegui, (1995 Ph.D. thesis) i.e. 45 minutes, however, 1 to 1.5 hours duration was reported by another study (Smith and Johnson, 1986).

G1 was originally defined as a time interval, a gap between the readily observed events of mitosis and DNA synthesis. The pre-DNA synthetic (G1) period is usually considered as the time in the cell cycle when a cell is signaled to proceed for another round of DNA synthesis and subsequent mitosis (Prescott, 1976). In cultured mammalian cells, the duration of G1 may range from 2 to 20 hours. There is also the example of a Chinese hamster cell line (V79) which is devoid of G1

period (Robbins and Scharff, 1967). The fact that mammalian cells without a G1 can be found suggests that this period (G1) is not very essential and can be eliminated without affecting the rest of the cell cycle. In those cells having a long G1 period, the initiators of DNA synthesis were reported to be made toward the end of this phase (Prescott, 1976). The cell fusion studies of Rao and Johnson (1970) showed that binucleate cells produced by fusion between two G1 cells entered S phase at about the same rate as mononucleate cells. However, the later study of Fournier and Pardee (1975) on Syrian hamster BHK21 / C13 cells indicated that binucleate cells, produced by treating mitotic cells with Cytochalasin B for 90 minutes, completed G1 period significantly faster than their mononucleate counterparts. To explain these results, they proposed a model, according to which there is a nuclear cooperation for the initiation of DNA synthesis, which is non-concentration dependent. They assumed that, among the multinucleate cells, each nucleus makes a critical substance during early G1 which is utilized during late G1, and the total amount, not the concentration, of the substance determines the duration of G1. The greater the amount of this substance available, the shorter is the G1 period. Because binucleate cells make twice the amount of this substance than the mononucleate cells they have shorter G1 period. This provides the clue that why the cell cycle in mouse is longer in the start of development to 2-cells and then become shorter as development proceeds and showed shorter duration of G1.

Cell proliferation markers are a prerequisite to study the initiation of DNA replication in normal cell cycle. Experiments are performed to localize the proliferating cell nuclear antigen (PCNA) which is a highly conserved protein expressed only in cells committed to DNA synthesis. PCNA plays an essential role in nucleic acid metabolism as a component of the replication and repair machinery (Kelman, 1997).

-      Proliferating cell nuclear antigen (PCNA) is a 36kD nuclear protein which has been demonstrated to be a co-factor for DNA polymerase δ (Bravo *et al.,* 1987) and is essential for the synthesis of DNA during the S-phase of cell division. PCNA is detectable immunocytochemically only at S-phase and may be used as a naturally occurring marker for cell proliferation (Galand and Degraef, 1989; Thaete *et al.,* 1989; Hall and Woods, 1990). Based on fixation procedures two forms of PCNA can be visualized in cultured cells (Bravo and MacDonald Bravo, 1987) 1) a soluble nucleoplasmic form, which persists throughout the cell cycle, and 2) an insoluble form, which appears during S-phase and is associated with the sites of DNA replication, exhibiting distinctive punctate staining patterns (Kill *et al.,* 1991).

A previous study of Otaegui, (1995 Ph.D. thesis) to determine the DNA synthesis timing was based on the immuno-detection of BrdU incorporation which indicated that BrdU incorporation starts at 165 minutes after release from synchronization. As the minimum period of 45 minutes of incubation with BrdU was required to determine the DNA synthesis in preimplantation embryos , it was hypothesized that perhaps the lower development after nuclear transfer could be improved by using a more accurate selection criterion of using donor nuclei at G1, based on the information of detection of PCNA localization and BrdU incorporation for the start of replication period.

The following experiments were designed to determine:

1) Length of G1 phase

2) Timing of the onset of DNA replication in the 3rd and 4th cell cycle of pre-implantation mouse embryos by employing the techniques of pulse labeling.

7.2 INITIATION OF DNA REPLICATION AND PCNA LOCALIZATION.

The aim of the experiment was to determine the timing of events of mouse preimplantation embryos, to answer the question of how long the G1-phase lasts and when DNA replication starts in 3rd and 4th cell cycle of mouse preimplantation embryos.

7.2.1 EXPERIMENTAL DESIGN

To determine the timing of events of mouse preimplantation embryos and the start of DNA replication , 2-cell embryos were recovered from F1 females 44-48 hours post hCG injections and mating. When 4-cell embryos were required, they were recovered 58 hours post hCG injections and mating. The embryos were exposed to 10 μM nocodazole in M16 at 37°C in 5% CO_2 in air for 9 hours for synchronization. Embryos were washed in many drops of M2 and sM16 to released them from synchronization and cultured in drops of sM16 under paraffin oil at 37°C in the atmosphere of 5% CO_2 in air.

Embryos started dividing after 30 minutes of their release from the synchronization medium and mitosis was completed within 90 minutes (Data not shown). The embryos were divided into batches of 10-15 embryos in each groups. Embryos were exposed to PCNA staining in batches starting from 60 minutes after their release from synchronization.

7.2.1.1- METHOD OF PCNA STAINING

The zona pellucidas of the embryos were removed by treating them with acid tyrodes. Because prolonged exposure to acid tyrodes is detrimental to embryos, the embryos were continuously observed during treatment and removed immediately as soon as the zona dissolved. Embryos were washed with PBS and placed on to clean coverslips with minimal amount of PBS (coverslips washed with 70% alcohol were used). The coverslips were dried briefly and fixed by using 3.7% formaldehyde at 4°C for 30 minutes. The coverslips were washed with PBS and then treated with 0.5% triton X 100 (BDH) for 2 minutes at room temperature.

It was learned here that different fixation method plays an important role in the detection of localization of PCNA. The coverslips were stored in the humidified chamber at 4°C till treated with anti-PCNA. The anti-PCNA/cyclin (Immuno concepts Cat: No. 2037) was diluted with PBS containing FCS (9.9 ml PBS + 100 μM FCS) with the ratio of 1:10 i.e. 450 μl of above diluent and 50 μl of anti-PCNA. The mixture (anti-PCNA) was spun at 1300 rpm for 30 seconds and drops of 50μl were placed on to the fixed embryos on coverslips. The coverslips were put in the humidified chamber at 4°C for over night then the coverslips were washed with PBS containing 1% FCS.

The Texas red (Vector Lab. Cat: No. TI-3000) was diluted with PBS containing 1% FCS with the ratio of 1:20 and drops of 50 μl dilution of Texas red were placed on the embryos on coverslips. The coverslips were incubated for 1 hour at room temperature in a humidified chamber in the dark. The coverslips were washed with 4 changes of PBS. The slides were cleaned and 5μl drop of vectashield with DAPI (Vector Lab. Cat:No. H-1200) was put on to the each slide and coverslips were mounted on them. The coverslips were sealed with nail varnish and stored at 4°C in the dark till scanning.

7.2.2 - RESULTS

7.2.2.1 DNA SYNTHESIS IN 4-CELL EMBRYOS AS DETECTED THROUGH LOCALIZATION OF PCNA

The G1-phase is very short as determined from the immuno-detection of 1757 cells from 500 embryos through the localization of PCNA. DNA synthesis was detected as early as 60 minutes after the release of embryos from synchronization where 25±4.6% cell showed PCNA localization (Fig. 7.1). The standard errors were estimated from the usual expression for the variance of a
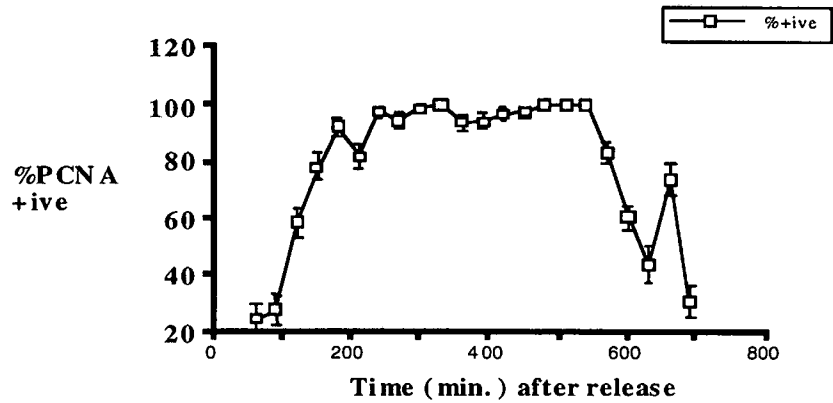
Fig: 7.1 Showing the start of replication in 4-cell embryos minutes after release from synchronization
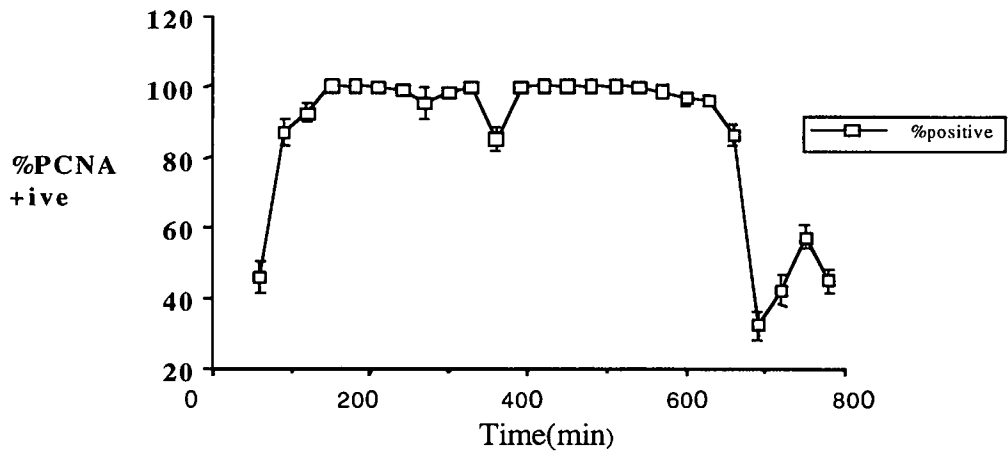
Fig: 7.2 Showing the start of replication in the 8-cell embryos after release from synchronization (percent±SE)

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binomial distribution and presented in fig: 7.1 in the form of mean±SE. DNA synthesis showed a linear trend as 58.68± 4.48% PCNA positive nuclei were observed within 2 hours after their release from synchronization. DNA synthesis in embryos reached its maximum after 330 minutes of their release from synchronization and all the nuclei of the cells of the embryos were PCNA positive. The proportion of PCNA positive nuclei remained high up to 540 minutes after release then started to decline and reached to 43.86±6.5% at 630 minutes after release. The proportion of the PCNA positive nuclei started increasing again (73.68±5.05) at 660 minutes after release but declined again and reached 30.98±5.4% at 690 minutes.

7.2.2.2 DNA SYNTHESIS IN 8-CELL EMBRYOS AS DETECTED THROUGH LOCALIZATION OF PCNA

The results of the experiments based on the observations of 3450 cell from the 484 embryos are presented in Fig: 7.2. The G1-phase is quite short in the 8-cell embryos as 45.83± 4.5% cells showed onset of DNA replication at 60 minutes after release from nocodazole. DNA replication increased abruptly as over 90% cells showed localization of PCNA at 120 minutes after release from the nocodazole. DNA synthesis reached its maximum (100%) by 150 minutes after release. This continued up to 630 minutes after release and then started to decline at 660 minutes where 86.05±3.05% cells showed PCNA localization. The PCNA localization further decreased at 690 minutes after release and only 32.31±4.1% cells showed DNA replication. The PCNA localization started rising again at 720 minutes after release from the nocodazole.

7.2.2.3 - CHANGES IN THE NUCLEAR DISTRIBUTION OF PCNA DURING S-PHASE IN CULTURED PRE-IMPLANTATION MOUSE EMBRYOS;

The technique of PCNA staining was employed to examine the distribution of PCNA localization during S-phase. Earlier studies have shown that during S-phase characteristic redistribution of PCNA localization occur and that similar if not identical patterns are observed for the sites of DNA replication. The embryos in 3rd and 4th cell cycle were used after their release from synchronization for PCNA staining and the accumulation and distribution of PCNA during S-phase was observed (Fig. 7.4 a&b.)

In these experiments, the patterns of PCNA staining in individual nuclei from one of the experiment were depicted in five types termed A-E (Fig. 7.3). In

Type A, a regular granular pattern was observed over the whole of the nucleus; in type B, the pattern showed very few foci of PCNA staining; in type C, the staining pattern was punctate but with predominant fluorescence over the perinuclear regions; the replication granules were completely excluded from nucleoli (visible as darker region Fig. 7.3 a &b) and later in this kinetic study, the nuclei showed significant fluorescence appeared over the perinuclear region including the perinuclear heterochromatin; in type D, extremely bright fluorescence was observed in smaller number of large granules and in type E, the diffused pattern of fluorescence was observed in nucleoli which may be the representative of G2 of the cell cycle.

The results of different types of nuclear distributions of PCNA localization in 4-cell pre-implantation mouse embryos are shown in Fig.7.4a. Type A pattern appeared 60 minutes post their release from synchronization and 50 % nuclei showed this pattern and reached to its maximum (61%) at 90 minutes. Type B pattern observed was 7% at 60 min. post release and maximum number (73%) of nuclei were observed at 210 min. after release. Type C was observed (34%) at 120 min. post their release and appeared to be dominant in among all patterns observed. The higher proportion of nuclei with pattern C was observed at 300 min. post their release from synchronization. Type D pattern was first showed at 330 min. and higher proportion was observed at 630 min. post release. Type D pattern was visible in some of the nuclei at 60 min. post release may be due to delay in completion of mitosis in some of the cells in the embryos. This pattern was again shown to be visible at the end of cell cycle. The results of different type of nuclear distributions of PCNA localization in 8-cell pre-implantation mouse embryos are depicted in Fig. 7.4b. The higher proportion of type A was observed at 60 min. post release from synchronization which decreased to 2% at 150 min. The varying proportions of type B patterns were observed in 8-cell embryos. However, type C pattern was observed in 35% nuclei at 90 min. post release and shown to be dominant in the 8-cell embryos. As compared to 4-cell, the nuclei showed higher proportion at 210 min. post their release. Type D pattern was first observed at 360 min. post release. In contrast, type E in 8-cell was observed early (450 min.) in the cycle as compared to 4-cell embryos which showed type E pattern near the time of mitosis.

7.2.3.- CONCLUSIONS

The G1-phase of the 3rd cell cycle is very short. More than 70% of embryos could be used as G1 nuclear donor if nuclear transfer was performed 60-90 minutes

Fig. 7.3 Different patterns of PCNA localization detected through pulse labeling technique

a) type A pattern of PCNA localization detected 60 min. After release from synchronization

b) type B pattern of PCNA localization detected after release from synchronization

c) type C pattern shows the perinuclear DNA replication and also replication on heterochromatin

d) type D pattern was detected in late stages of S-phase

e) type E shows the diffused pattern of PCNA localization on the nucleoli

A

B

C

D

E

135
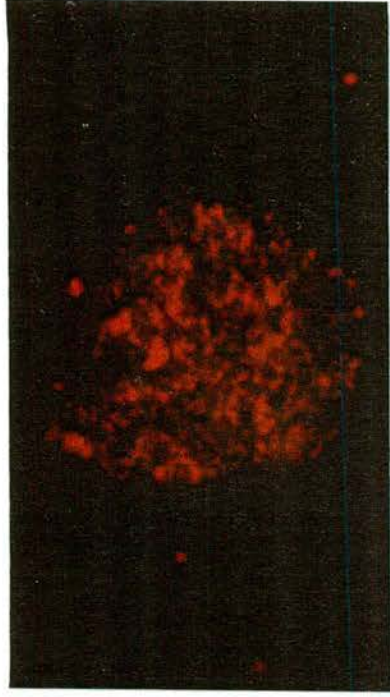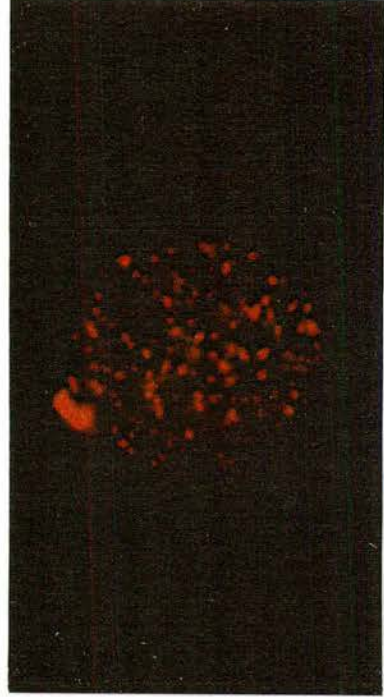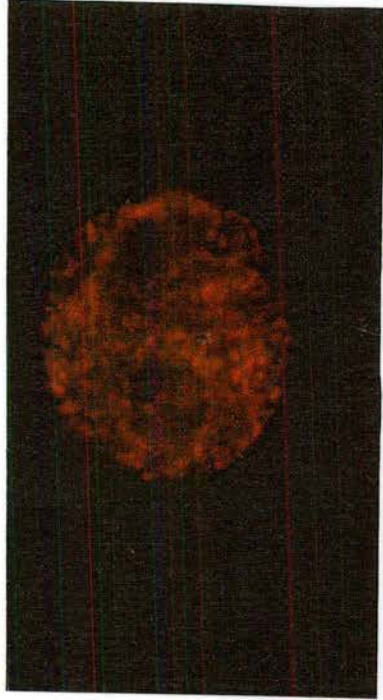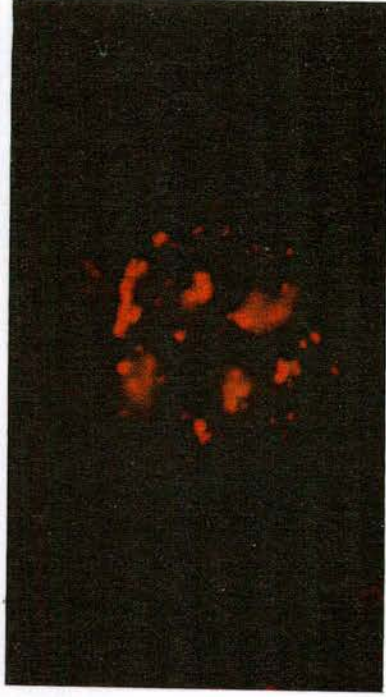
Fig: 7.4a Graph showing different types of PCNA localizations indicative of DNA replication in S-phase of 3rd cell cycle in pre-implantation embryos by pulsing technique
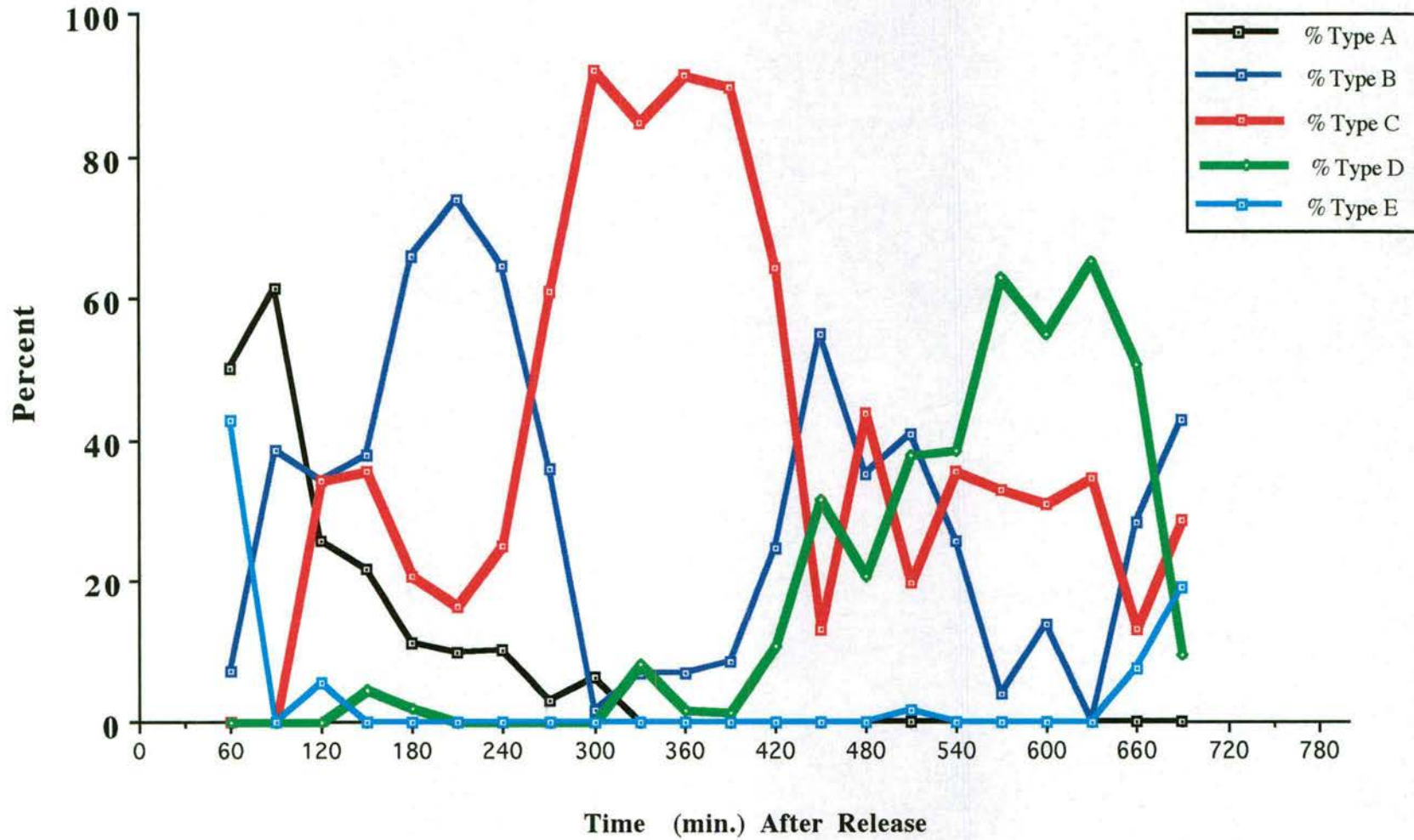
Fig: 7.4b Graph showing different types of PCNA localizations indicative of DNA replication in S-phase of 4th cell cycle in pre-implantation embryos by pulsing technique.
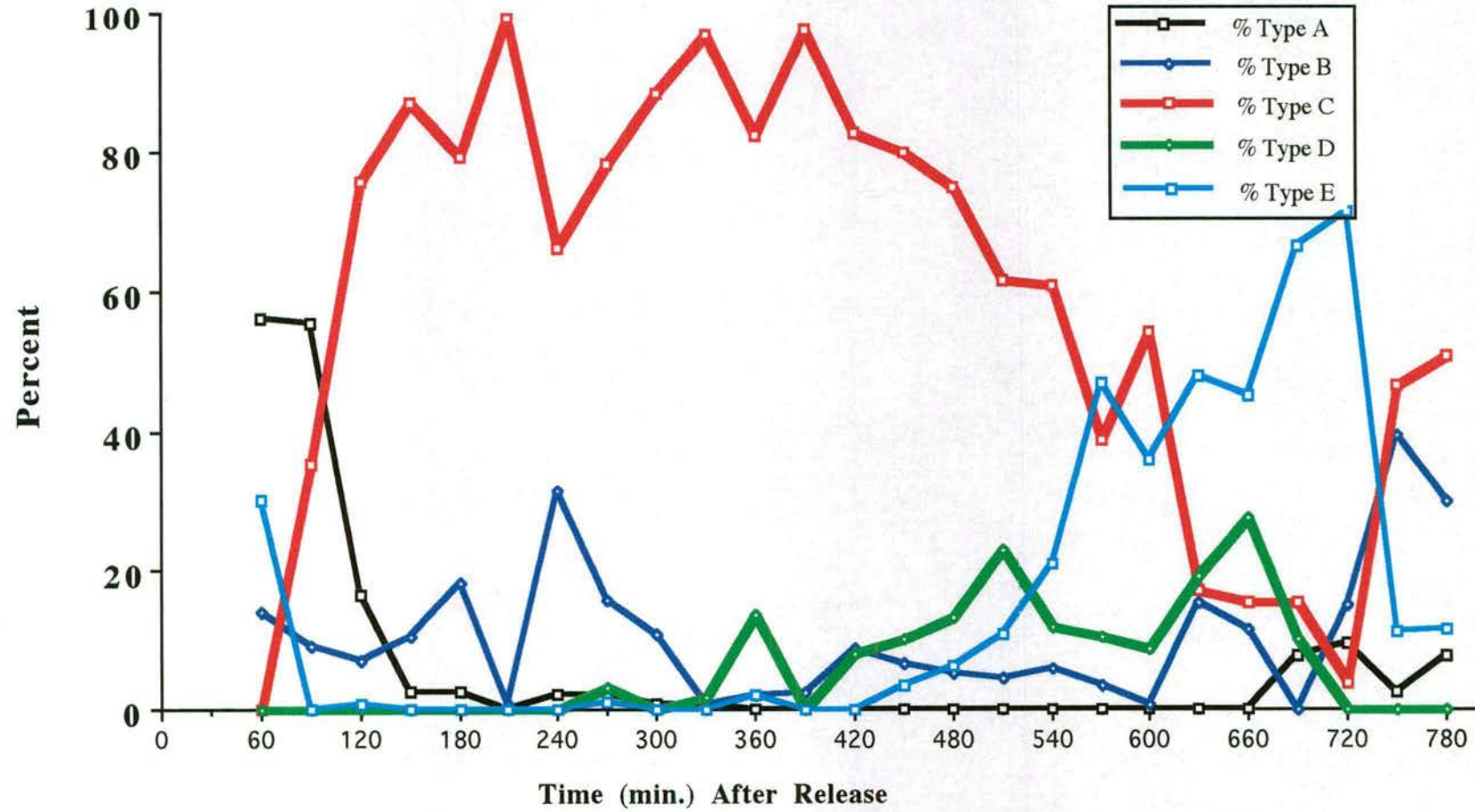
after their release from nocodazole. DNA replication reaches its maximum at 330 minutes after release. DNA replication declined after 9 hours but started rising at 11 hours after release. This suggests that perhaps cyclin production increases again.

The G1-phase of the 4th cell cycle is very short which provides less chance to use donor nuclei at G1 stage of the cell cycle. The proportion of cells showing PCNA localization even at 60 minutes after release is larger than the proportion observed in 4-cells embryos which suggests that as the embryos advanced in their development, the G1 permissive window shortened which creates more difficulties using of 8-cell donor nuclei at G1 stage of the cell cycle. The increase in the localization of PCNA at 720 minutes after the decreasing trend Leads to the conclusion that cyclin production resumed again at the end of the cell cycle.

7.3 - DNA SYNTHESIS AND BROMODEOXYURIDINE INCORPORATION

Bromodeoxyuridine (BrdU) has been used as an alternative to tritiated thymidine ($[^3H]$TdR) as a tracer for studying DNA replication. The introduction of a monocolonal antibody which recognized BrdU in single stranded DNA (Gratzner, 1982) has enabled BrdU to be used for studying cell cycle kinetics in a similar manner to ($[^3H]$TdR). Bromodeoxyuridine (BrdU) is a thymidine analog and is specifically incorporated into DNA during DNA synthesis. Anti-Bromodeoxyuridine monocolonal antibody is used to identify cells that have incorporated BrdU.

Otaegui, (1995 Ph.D.) studied DNA replication in preimplantation embryos and showed that the technique for the incorporation of BrdU required at least 45 minutes of incubation. This incubation time might be misleading in concluding the timing of initiation of DNA replication in the preimplantation embryos.

Recently a modified technique of rapid pulsing of BrdU incorporation has been introduced in starfish *Asterina pectinifera* (Nomura *et al.,* 1993), which has provided the opportunity to test this in other species.

The aim of the present experiment was to study the cell cycle kinetics through the detection of BrdU incorporation by using the rapid pulsing technique of Nomura *et al.,* (1993) with some modifications.

7.3.1 EXPERIMENTAL DESIGN

To study the cell cycle kinetics and the timing of initiation of DNA replication in 4-cell embryos, 2-cell embryos were recovered from F1 females 44-48 hours post hCG injections and mating. The embryos were synchronized by using the 10 μM of nocodazole in sM16 for 9 hours at 37°C under paraffin oil in an atmosphere of 5% CO_2. The embryos were released from synchronization medium and were washed with M2 and sM16 through several changes. They were cultured in sM16 under paraffin oil at 37°C in an atmosphere of 5% CO_2. The embryos started dividing after 30 minutes of their release from synchronization.

7.3.1.1. RAPID PULSING METHOD OF BrdU INCORPORATION.

The zona pellucida of the embryos were removed by exposing them to the acid tyrodes solution. The embryos were continuously observed during their exposure to acid tyrodes and washed quickly with PBS as soon as the zona disappeared. The group of 10 embryos (8-cells) were exposed to the M16 medium containing 10 mM BrdU and 0.125% triton X 100 for 5-8 minutes. After 5-8 minutes of labelling, the embryos were put on to the clean coverslips air dried and fixed later by: (i) treating with methanol for 10 minutes at -20°C, (ii) then with 3.7% formaldehyde at 4°C for 20 minutes and washed with PBS.

7.3.1.1.2 IMMUNOCYTOLOGICAL PROCEDURE

The embryos were stained for the incorporation of BrdU with anti-BrdU-fluorescein (Boehringer Mannheim Cat: No. 1202 693). The embryos on the coverslips were incubated at 37°C in 4 N HCl for 2 hours to denature the DNA. They were washed with PBS for at least for 5 minutes with 3 changes. The excessive PBS was drained off by touching the edge of the coverslip onto the tissue and stored in a humidified chamber. The embryos were covered with 50-100 μl of solution containing 50μg/ml anti-Bromodeoxyuridine-fluorescein diluted in PBS with 0.1% (w/v) BSA. The coverslips were incubated over night in a humidified chamber at 4°C. The next morning coverslips were washed with PBS with 3 changes over a period of 10 minutes. The slides were cleaned and a 5μl drop of vectashield with DAPI (Vector Lab. Cat:No. H-1200) was put on to the each slide and coverslips were mounted on them. The coverslips were sealed with nail varnish and stored at 4°C in the in the dark till scanning.

7.3.2. RESULTS

The results were based on the observation of 1447 cells from 420 embryos for detection of BrdU incorporation are presented in Fig: 7.6. Data obtained by using the rapid pulsing technique of BrdU incorporation showed that DNA synthesis in 4-cell mouse embryos initiated at 90 minutes post release from synchronization. The standard errors were estimated from the usual expression for the variance of a binomial distribution and presented in the form of mean±SE%. The embryos released from synchronization showed DNA synthesis 32.86±5.5% at 90 minutes. DNA synthesis in embryos was observed with an increasing trend in relation to time and reaches its maximum 90.28±3.49% at 660 minutes post their release from synchronization. However, the end point was not studied in this experiment.

7.3.3. CONCLUSIONS

The rapid pulsing technique of BrdU incorporation provides opportunities for early detection of DNA replication in the preimplantation embryos (Fig. 7.5). This technique could be used to determine the timing of the initiation of DNA replication in preimplantation embryos and the embryos could be used as donor nuclei at G1 stage of cell cycle. Though the pattern of BrdU incorporation showed the linear trend of DNA replication in the embryos, the technique needs more refinements to be used on regular basis.

7.4 - COMPARISON OF PCNA AND BrdU PULSE LABELING TECHNIQUES IN THE DETECTION OF INITIATION OF DNA REPLICATION.

The results of the above mentioned experiment for detection of initiation for DNA replication in 4-cell mouse embryos are depicted in Fig. 7.6. The comparison showed that PCNA localization occurred just after their release as about 25% nuclei were observed PCNA positive at 60 min. In contrasts, the detection of initiation of DNA replication was first observed at 90 min. post their release from synchronization. After removing the categories of type A and B pattern from the data, the result presented in fig 7.7 shows that even the pattern type C (7%) could be detected at 60 minutes post the release of embryos from synchronization. The results shows that the PCNA pulse labelling technique is more efficient than the technique of BrdU for the detection of DNA replication (S-phase) in early embryos.

Fig. 7.5 Detection of BrdU incorporation using rapid pulsing

a) BrdU incorporation detected 390 minutes after release

b) BrdU incorporation detected 420 minutes after release

c) BrdU incorporation detected 510 minutes after release

A

—— 25 μm

B

—— 5 μm

C
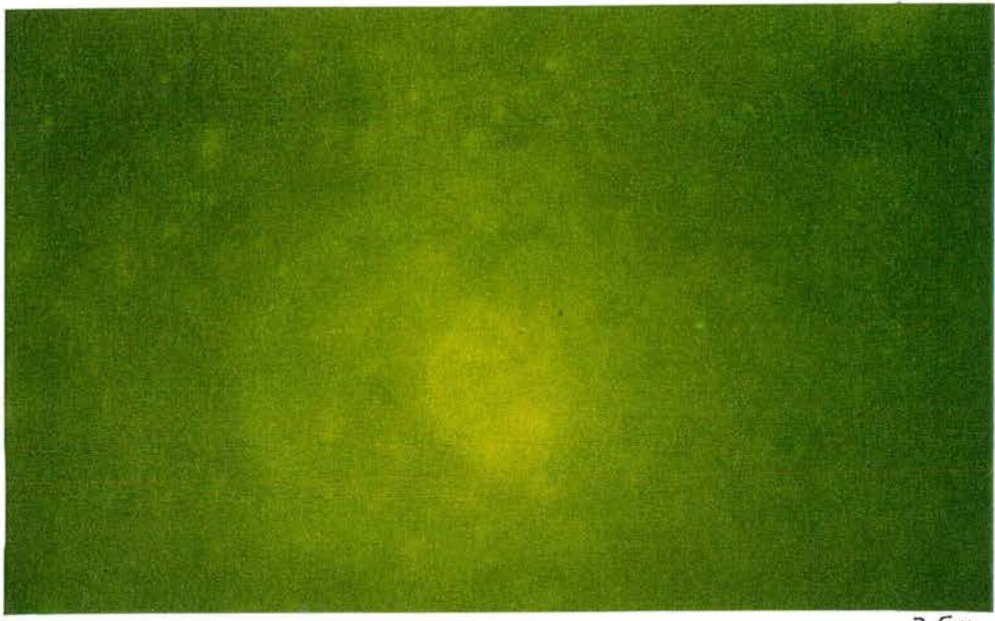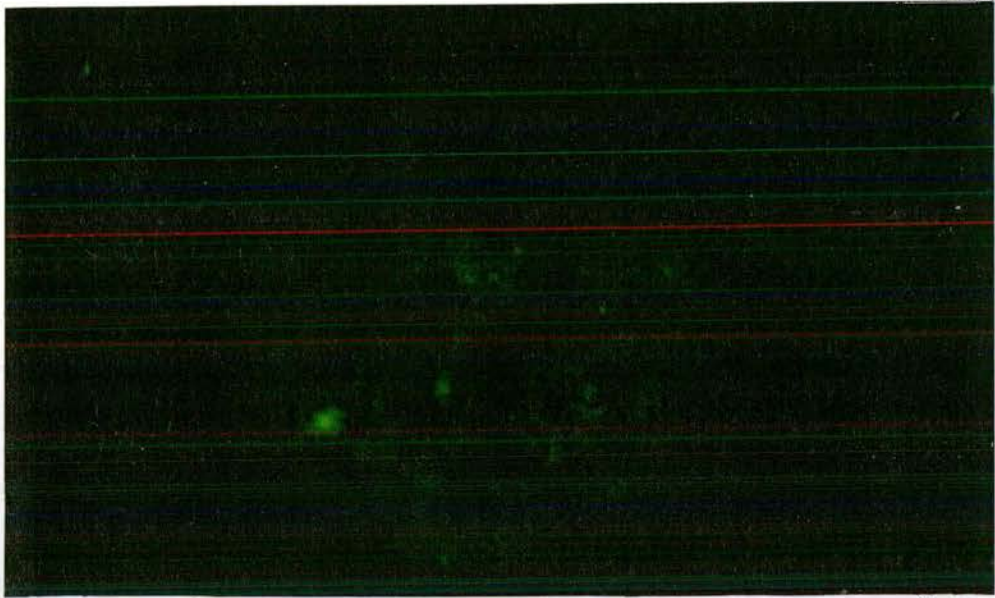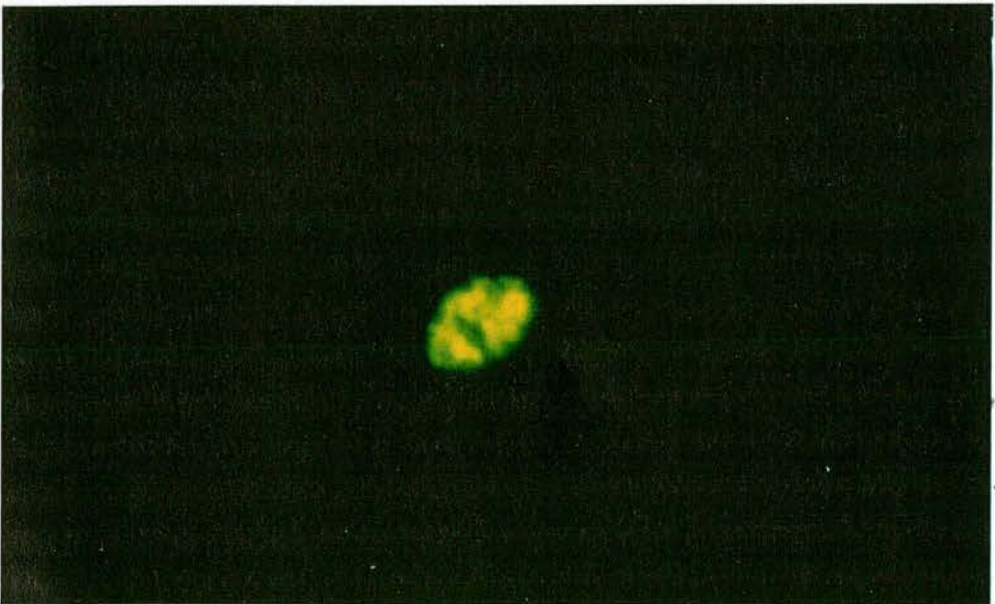
—— 25 μm

Fig: 7.6 Graph showing the difference in recognition of the start of DNA replication through PCNA and BrDu techniques
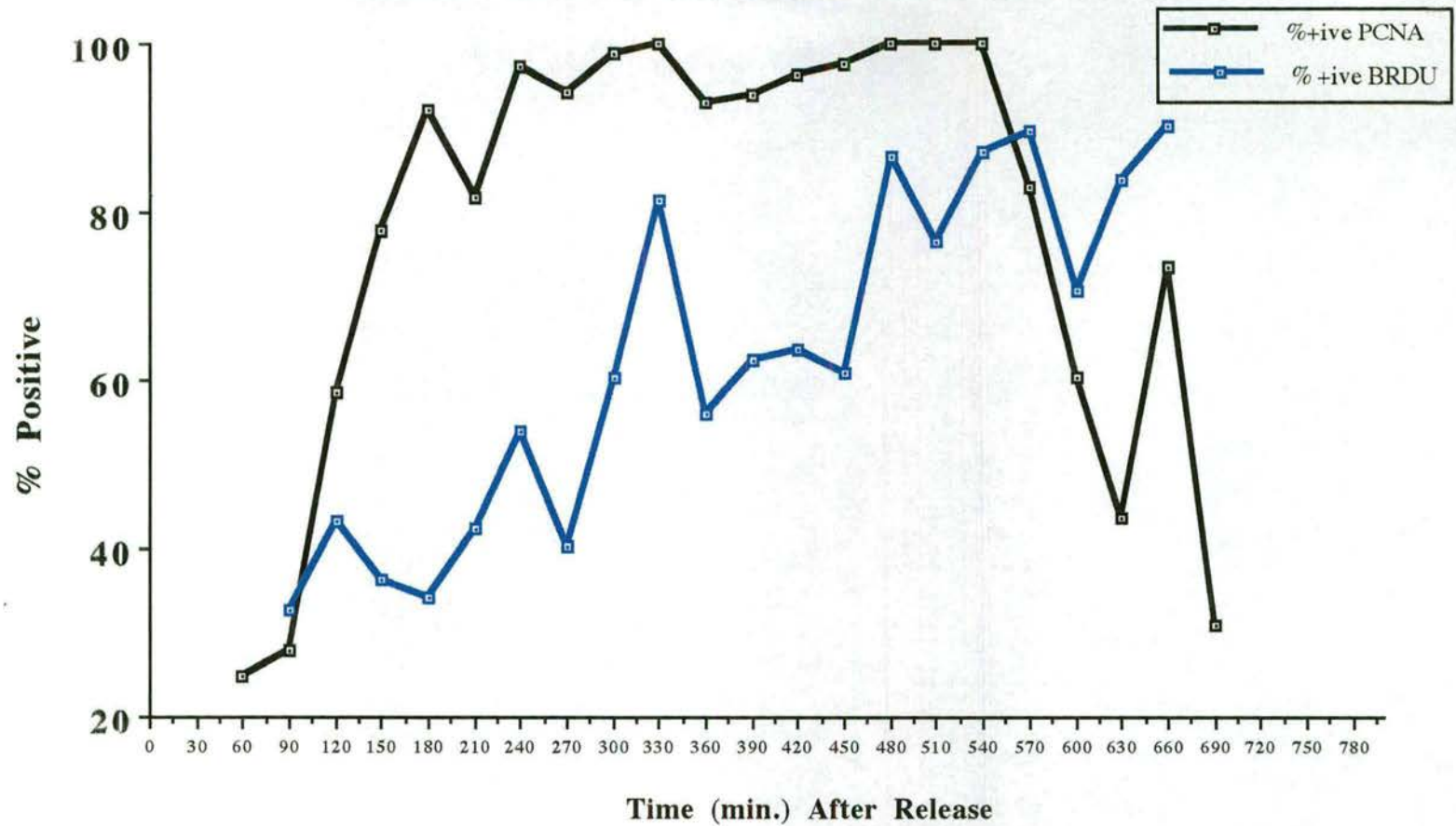
Fig: 7.7 Graph showing the difference in recognition of the start of DNA replication through PCNA (CDE types) and BrdU techniques
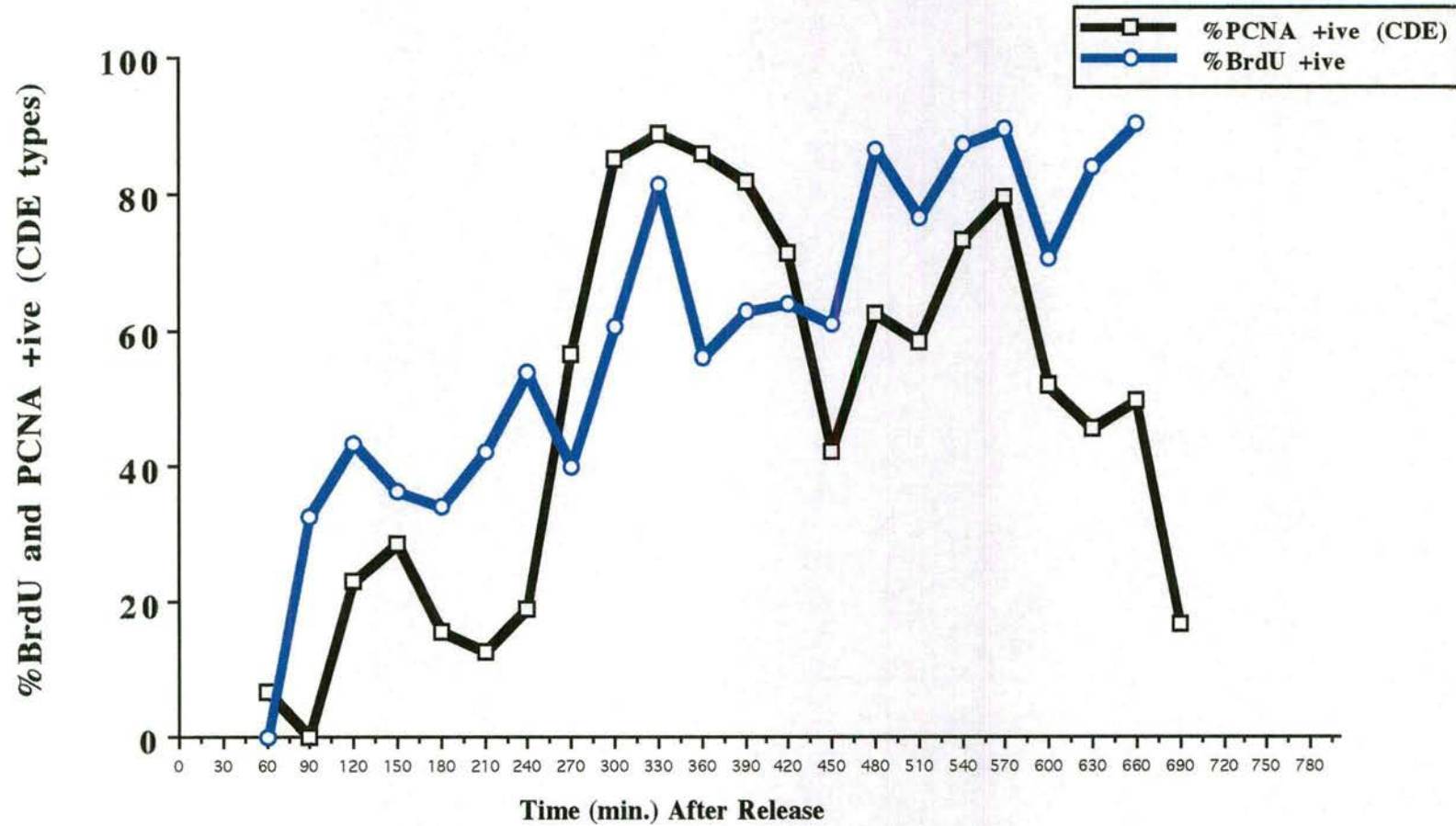
7.5 - DISCUSSION

The results of the experiments presented in this chapter clearly showed the difference in the ability of the pulse labeling techniques.

Of the two techniques used for detection of the initiation of DNA synthesis in 4-cell were employed, the PCNA pulse labeling technique proved more effective in the detection of initiation of DNA replication. The observation obtained through the detection of localization of PCNA suggested that DNA replication starts just after the release of embryos i.e. 60 min. post release. In contrast, rapid pulsing technique BrdU incorporation first detected initiation of DNA replication 90 min. post release. The chain elongation as measured by the rapid pulsing technique of BrdU incorporation might be due to the low level of BrdU incorporation in early S-phase which was below the limits of detection.

By using double indirect immunofluorescence to compare the timing of the appearance of PCNA foci with in replicon clusters and their relative distributions within S-phase nuclei, the study of Kill *et al.*, (1991) shows that PCNA accumulates at the sites of DNA replication some time before any DNA synthesis can be detected at those sites. It is further demonstrated that although PCNA is located at the sites of active DNA synthesis, it appears to accumulate at these sites in a preinitiation complex up to fifteen minutes before replication starts. The results of present study in comparing the efficiencies of two techniques used to detect the timing of the start of DNA replication in 4-cell mouse embryos, shows that PCNA localization occurs some time before the detection of BrdU incorporation in mouse embryos. This implies that PCNA is organized into pre-initiation complex that is modified before use.

The results contradicts the earlier findings of Otaegui, (1995 Ph.D. thesis) who reported the initiation of DNA replication 165 min. post release from synchronization. The difference in the detection of the initiation of DNA replication is due to the difference in the pulse labeling technique of BrdU incorporation.

The technique employed by Otaegui, (1995 Ph.D. thesis) required at least 45 min. for the incorporation of BrdU. In contrast, the rapid pulsing technique used in this study required only 5-8 min. The results of this study for the detection of initiation of DNA synthesis by detection of PCNA localization suggests that the G1 duration is very short in early pre-implantation mouse embryos. This could be correlated with the results of earlier experiments (Chapter 6&8) with nuclear transfer using G1 donor nuclei based on the informations of G1 duration of 1.0 to

1.5 hours (Smith and Johnson, 1986). Previous reports have indicated that PCNA is required for eukaryotic DNA replication and have suggested that active DNA synthesis occurs at the sites of PCNA localization (Wong *et al.,* 1987; Jaskulski *et al.,* 1988; Bravo and MacDonald-Bravo, 1987).

The results of present experiment that G1 duration is very short, demonstrated the use of donor nuclei as early as possible to catch the G1 stage of the cell cycle. Hence, the nuclear transfer experiment were conducted using donor nuclei 15 minutes after their division (chapter 5). The donor nuclei at G1 stage were selected on the basis of the number of nucleoli in these experiments. All those nuclei containing more than 6 nucleoli were considered at G1 stage of cell cycle immediately after mitosis and enhanced development after nuclear transfer was achieved. But the nuclei with more than 6 nucleoli as studied in these experiments for PCNA localization showed positive staining confirmed the earlier report (Kill *et al.,* 1991) that the localization of PCNA occurred at the sites of DNA replication as pre-initiation complex.

Five patterns of PCNA distribution were observed in the present study i.e. Type A-E (Fig. 7.3). Type A and B pattern of PCNA localization are considered to be related with the pre-initiation complex but Type C and D relates to the DNA synthesis. Type A and B pattern were shown to be related to one of the pattern reported earlier (Bravo and MacDonald-Bravo, 1987). Out of five Types reported in this study, three patterns of PCNA distribution observed in the pre-implantation mouse embryos agrees with studies of different patterns of PCNA distribution in fibroblasts grown in culture (Nakamura *et al.,* 1986; Bravo and MacDonald-Bravo, 1987; Fox *et al.,* 1991).

The pattern observed in this study appeared to be a temporal sequence of replication in which euchromatin and nucleolar DNA is replicated before heterochromatin (Bravo and MacDonald-Bravo, 1987; Nakayasu and Berezney, 1989). The results are in agreement with the study of Nakayasu and Berezney, (1989) who demonstrated that perinuclear synthesis occurs before the internal heterochromatin regions are synthesized. Another defused pattern (Type E) of PCNA localization was also observed in this study, this looks to be the staining of nucleoli in late cell cycle, however, the fluorescence is not bright, or might be the soluble nucleoplasmic form of PCNA. The results show that the G1 period in pre-implantation mouse embryos is very short. The PCNA localization occurs in early pre-initiation complex of DNA replica clusters. The temporal sequential changes occurs in PCNA distribution pattern during DNA replication.

CHAPTER 8

DEVELOPMENT AFTER NUCLEAR TRANSFER

8.1 INTRODUCTION

The technique of nuclear transfer for the reconstruction of mammalian embryos has been extensively used to investigate the nucleo-cytoplasmic interactions during early development (McGrath and Solter, 1986). There are two ways in which a transferred nucleus may be able to support development, either the donor cells may be undifferentiated or the nucleus may be differentiated but be amenable to reprogramming by the recipient cell (Wilmut *et al.,* 1992. The experiments of nuclear transfer in mouse revealed that the ability of donor nuclei to support development to blastocyst stage *in vitro* and to term depends on the cell cycle stage of the of the recipient cytoplast to which it is fused. Previous studies of nuclear transplantation in mice and rats have shown limited preimplantation development of embryos reconstructed by using enucleated zygote and late 2-cell or more advanced donor nuclei (McGrath and Solter, 1984; Robl *et al.,* 1986; Tsunoda *et al.,* 1987; Kono and Tsunoda, 1988). However, development after nuclear transfer by reconstructing the embryos using donor nuclei from the early stage of 2-, 4- and 8-cell embryos with MII oocytes as recipient cytoplasts was achieved (Cheong *et al.,* 1993).

The stage of cell cycle of the donor nucleus and recipient cytoplasm are important factors for successful development of reconstituted embryos. On the other hand this could be explained in a sense that the stage of the cell cycle of the donor nucleus at the time of reconstruction be in a state which could be reprogrammable by the recipient cytoplasm i.e. metaphase II oocyte. As the cell cycle of the recipient oocytes is restarted by activating them with electric pulse or by ethanol or strontium chloride (Whittingham, 1980), altering the timing of oocyte activation with respect to the time of fusion of donor nuclei provides a number of possible approaches to reconstruct the embryos for developmental studies.

It has been reported by previous studies that the fusion of interphase donor nuclei with metaphase II ooplasm resulted in chromatin undergoing premature chromosome condensation (PCC) (Czolowska *et al.*, 1984; Szollosi *et al.*, 1988; Collas *et al.*, 1992; Cheong *et al.*, 1994). The PCC and NEBD are induced by the maturation promotion factor (MPF), a complex of $p34^{cdc2}$ and cyclin (Choi *et al.*, 1991; Kubiak *et al.*, 1993; Whitakar, 1996). The study of Campbell and colleagues (1993) demonstrates that normal development from nuclear transfer could be expected by maintaining the ploidy of the reconstructed embryos. Two main approaches to maintain the normal ploidy were suggested. If the cytoplast has a high level of MPF then a diploid nucleus should be transferred, while pre-activation creates a recipient cytoplast able to support development of embryos reconstructed by using donor nuclei at any stage of cell cycle.

The aim of present series of experiments was to study the interactions of different stages of second and third cell cycle donor nuclei with enucleated metaphase II oocytes with respect to fusion and activation at different time. In this study two different protocols were initially used. The donor nuclei were fused with enucleated recipient oocytes as follows: (i) the donor nuclei were fused with enucleated oocytes and activated at the same time, (ii) the donor nuclei were fused to enucleated and pre-activated oocytes.

Previous report on nuclear transfer experiments with G2-phase donor nuclei into metaphase II oocytes demonstrates that the donor nuclei undergo premature chromosome condensation (PCC) (Kono *et al.*, 1991b, 1992) due to the high level of MPF (Kubiak *et al.*, 1993). After artificial activation, normal diploid nuclei were reported following karyokinasis (Kono *et al.*, 1991b, 1992). The lower rate of development to term from late 2-cell donor embryos was also reported (Kono et al., 1993). It was suggested that oocytes receiving a G2-stage nucleus do not develop a normal spindle. The study of Kwon and Kono, (1996) demonstrated the production of identical sextuplet mice from 4-cell metaphase nuclei through serial nuclear transfer. The study suggested the beneficial use of prolonged exposure of donor nuclei to cytoplasmic environment (MII) before activation.

Thus the experiments were also performed using the method of delayed activation of reconstructed embryos which gave rise to another type of protocol i.e. (iii) the donor nuclei were fused to enucleated oocytes which were activated later on (delayed activation).

8.2 Effect of interaction of donor nuclei cell cycle phase with recipient cell cycle phase on the development of embryos reconstructed by nuclear transfer from 2-cell.

The aim of present experiment was to study the effect of interaction of 2-cell donor nuclei at G2 stage of cell cycle with the two types of recipient cytoplast with respect to the level of MPF on the development of reconstructed embryos.

8.2.1 EXPERIMENTAL DESIGN

To study the interaction of 2-cell donor nuclei at G2 stage with two different types of cytoplast recipients, the F1 female mice were superovulated following the super ovulation regime (section 3.3.2). The oocytes were recovered 14 hours post hCG injections. The donor 2-cell embryos at G2 stage of cell cycle were recovered 40 hours post hCG injection from the mated F1 females. The oocytes were denuded from cumulus and enucleated using micromanipulation procedures (Section 3.4.4). All the micromanipulation were performed in M2 medium containing 10 μM nocodazole, 7.5 μg/ml Cytochalasin B and 10 % FCS. The embryos were reconstructed at zero hours of activation (enucleation $\Rightarrow$ fusion/activation) by using 2-cell donor nuclei at G2 stage of cell cycle at the time of enucleation. The recipient cytoplasts at this stage were considered to be with high level of MPF (see 7.2.2) and the donor nuclei were exposed to partial exposure to MPF

The experiments were also conducted to reconstruct the embryos by using 2-cell donor nuclei at G2 stage of cell cycle 4 hours after the activation of enucleated metaphase II oocytes (enucleation/activation $\Rightarrow$ fusion). The activation stimuli in these experiments were achieved by exposing the enucleated oocytes or reconstructed embryos to 25 mM concentration of strontium chloride in M16 for one hour. The zero hour was considered when they were first exposed to strontium chloride. The pre-activated cytoplasts used in this experiment were considered to be with basal level of MPF (see 7.2.2). Two control groups were also made to compare the effect of activation stimuli and auto-activation of the oocyte with any other physical or chemical stimuli. The Sendai virus mediated method was used for fusion of reconstructed embryos.

8.2.1.1 STATISTICAL ANALYSIS

The data on development after nuclear transfer was analyzed using Generalized Linear Mixed Model (Breslow and Clayton, 1993) looking at the effect

of treatment whilst allowing for differences between days. The treatment effects were tested using a Wald statistics (distributed as chi-squared), and, if significant, differences between means were assessed by an approximate t-test. Means declared to differ, differ at the 5% level or better.

8.2.2- RESULTS

Development after nuclear transfer has been shown to be affected by the cell cycle stage of the recipient cytoplast (p< 0.001). The results presented in table: 8.1 shows that when the embryos were reconstructed using 2-cell donor nuclei at G2 stage of cell cycle at the time of enucleation of metaphase II oocytes by using enucleation, fusion/activation at the same time, the development beyond 2-cell is greatly effected. Though 38% embryos showed pronuclear formation only 26 % developed up to 2-cell stage and 2% reached to morula/blastocyst stage. When the embryos were reconstructed by using the pre-activated metaphase II oocyte (4 hpa) using enucleation/activation $\Rightarrow$ fusion protocol, 77% showed pronuclear formation (p< 0.001) and 58% developed to 2-cell stage. The development to morula/blastocyst stage in embryos reconstructed by using pre-activated enucleated MII oocytes was high (p<0.001) as compared to the embryos reconstructed at the time of enucleation/activation (16% Vs 2%). The parthenogenetic development in activation control group (+ive control) shows the ability of activation stimuli. However, no difference in development to 2-cell was found when compared with the embryos reconstructed by using pre-activated cytoplast (87% Vs 77%) respectively.

8.2.3 CONCLUSIONS

The results of the present experiment shows that the development after nuclear transfer is greatly affected by the cell cycle stage of the donor nuclei and recipient cytoplast. The development of embryos reconstructed by using the G2 stage of 2-cell embryos with recipient cytoplast by using enucleation/fusion and activation at the same time protocol, is minimum (2%). The partial exposure of G2 stage donor nuclei to high level of MPF may not be able to completely reprogram the donor nuclei. The disturbance in the normal ploidy as NEBD and PCC is expected in the reconstituted embryos in the presence of high level of MPF at the time of reconstruction and re-replication of the already replicated chromosomes at G2 stage may leads to lower development of reconstituted embryos to morula/blastocyst stage. In contrast when the embryos were reconstructed by using pre-activated recipient cytoplasts, the development of reconstituted embryos to

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Table: 8.1 DEVELOPMENT AFTER NUCLEAR TRANSFER OF EMBRYOS RECONSTRUCTED BY TRANSFERRING 2-CELL (G2/M) DONOR NUCLEI INTO ENUCLEATED METAPHASE II OOCYTES (0 hpa) AND PRE-ACTIVATED RECIPIENT CYTOPLASTS (4 hpa).

Treatments (recipients)	NUCLEAR DONOR	REPLICATES	TOTAL	FUSED/ TOTAL(%)	PN/ FUSED(%)	2-cell/ FUSED(%)	Morula/ Blast.(%)
Enuceation fusion/activation 0 hours*	2-Cell (G2)	5	98	87/98(89)	33/87(38)	23/87(26)	2/87(2)
Enucleation activation ⇒ fusion 4 hours*	2-Cell (G2)	7	117	86/117(77)	66/86(77)	50/86(58)	14/86(16)
+ive Control	--	4	88	--	77/88(88)	68/88(77)	26/88(30)
-ive Control	--	4	47	--	3/47(6)	2/47(4)	0/47(0)

*= Hours post activation. The non-enucleated oocytes in activation control group (+ive) were activated at the same time. Both of the control groups were kept under paraffin oil in M2 medium in the dark at room temperature till treatments.

morula/blastocyst stage is shown to be restricted to 16%, may be due to cell cycle stage effect or may be some cytoplasmic activity of MPF causing NEBD and PCC, leading to disturbance in the normal ploidy of the reconstructed embryos.

8.3 The effect of interaction of different phases of donor cell cycle with cytoplast cell cycle phase on the development of the embryos reconstructed by nuclear transfer from 4-cell embryos.

The present experiment was aimed to investigate the effect of interaction of different phases of 4-cell donor nuclei with different cell cycle phases of recipient cytoplasts on the development of embryos reconstructed by nuclear transfer.

8.3 EXPERIMENTAL DESIGN

To investigate the effect of interaction of different phases of donor nuclei with different cell cycle phases of recipient cytoplasts, the 2-cell embryos were recovered 44-48 hours post hCG injection from the mated females. The donor nuclei were synchronized for at least 9 hours using 10 µM nocodazole in sM16. The embryos were released from synchronization by washing through several drops of M2 and sM16 and cultured in microdrops of sM16 medium under paraffin oil at 37°C in the atmosphere of 5% CO_2 in air. The 2-cell embryos after their recovery from the females were stored at low temperature of 4°C under paraffin oil in M2 till synchronization so that they could be used at preferred hours during day time and desirable cell cycle stage of 4-cell embryos be captured. In experiments where 4-cells at G1/S and S-phase of the cell cycle stage were required, the 2-cells were released in batches from the synchronization medium. The embryos started dividing 30 minutes after their release from nocodazole. The embryos were reconstructed by using G1/S 4-cell donor nuclei 75-90 minutes after their release. To reconstruct the embryos by using the S-phase of 4-cell donor nuclei, the 2-cell embryos were released from synchronization 4 hours prior to the reconstruction of embryos. On the other hand when the 4-cell donor nuclei at G2 or G2/M phase were required, the 4-cell embryos were recovered 58 hours post hCG injection from the mated females. The 4-cell embryos were then synchronized by using 10 µM nocodazole for at least 6 hours to be used as 4-cell nuclear donor at G2/M stage of cell cycle. The embryos in these experiment were reconstructed by depositing the donor nuclei into perivitalline space of zona pellucida using micromanipulation procedures (section 3.4.4). The fusion method used was sendai mediated (section 3.4.4.3.1). By using mainly two approaches: (i) the embryos were reconstructed at the time of

enucleation i.e. at 0 hours of activation (Enucleation, fusion/activation) and (ii) they were reconstructed by using enucleated and pre-activated metaphase II oocytes i.e. 3-4 hours post activation (Enucleation, activation/ fusion). Because of the limitation in the co-ordination of different stages of donor and recipient cell cycle, the embryos were reconstructed by using various combinations on each day. The different combinations were: the G1 and S-phases of donor nuclei with enucleated metaphase II recipient at 0 hours of activation, the donor nuclei at G1 or S-phase into pre-activated metaphase II oocytes (3-4 hours post activation), the G2/M donor nuclei into enucleated metaphase II recipient (0 hours of activation) and G2/M donor nuclei into pre-activated enucleated oocytes (3-4 hours post activation). Two control groups were also made to compare the effect of activation stimuli and auto-activation of the oocyte with any other physical or chemical stimuli. The reconstructed embryos or recipient cytoplasts and activation control groups were activated by using strontium activation media containing 25 mM strontium chloride concentration in M16 (see Chapter 3).

8.3.1.1- STATISTICAL ANALYSIS

The analysis of the data was done using the method as stated earlier (see section 8.2.2)

8.3.2 RESULTS

The development after nuclear transfer in mouse is greatly affected (p< 0.001) by the interaction of the cell cycle stages of donor nuclei and recipient cell cycle and the data is presented in table 8.2. The development of reconstructed embryos to blastocyst stage is shown to be greatly effected (p< 0.001) by the cell cycle stage of the donor nuclei. The development of embryos to blastocyst stage (41% and 46%) was better (p<0.001) when donor nuclei at G1 or G1/S phase were transferred into metaphase II at the time of enucleation and activation (0 hpa) and into pre-activated recipient cytoplasts (3-4 hpa) to morula/blastocyst respectively. It was shown as greatly affected when embryos were reconstructed by transferring 4-cell S-phase and G2/M phase donor nuclei into metaphase II recipient (0 hpa) and into pre-activated recipient cytoplast (3-4 hpa). Though the development to 2-cell in embryos reconstructed by transferring S-phase donor nuclei into metaphase II recipient (0 hpa) and pre-activated (3-4 hpa) recipient cytoplasts is 26% and 21% but no development (0%) to morula or blastocyst was observed in former group as compared to the 7% development in later group. The same trend is shown in

Table: 8.2 DEVELOPMENT AFTER NUCLEAR TRANSFER OF RECONSTRUCTED EMBRYOS USING 4-CELL DONOR NUCLEI FROM DIFFERENT STAGES OF CELL CYCLE WITH 2 CYTOPLAST RECIPIENTS.

PROTOCOL USED	NUCLEAR DONOR		REPLICATES	TOTAL	FUSED/ TOTAL(%)	2-CELL/ FUSED(%)	(MORULA/BLAST)/ FUSED(%)
Enuceation	4-Cell	(G1/S)	3	45	29/45(64)	17/29(59)	12/29(41)
fusion/activation	4-Cell	(S)	3	36	23/36(64)	6/23(26)	0/23(0)
0 hours*	4-Cell	(G2/M)	3	62	50/62(81)	2/50(4)	0/50(0)
Enucleation	4-Cell	(G1/S)	5	53	39/53(74)	23/39(59)	18/39(46)
activation ⇒ fusion	4-Cell	(S)	3	44	29/44(66)	6/29(21)	2/29(7)
3-4 hours*	4-Cell	(G2/M)	3	39	29/39(74)	24/29(83)	2/29(7)
Act. Control	--		9	101	--	80/101(79)	31/101(31)
-ive Control	--		9	99	--	3/99(3)	1/99(1)**

* hours post activation. **Developed upto morula.

embryos reconstructed by using the G2/M stage donor nuclei with metaphase II oocytes (0 hpa) and pre-activated recipient cytoplast (3-4 hpa).

8.3.3 CONCLUSIONS

The development after nuclear transfer is greatly effected (p< 0.001) by the interaction of cell cycle phases of donor nuclei and recipient cytoplasts. The development to morula/blastocyst after nuclear transfer of donor nuclei at G1 or G1/S stage is high (p< 0.001) when embryos were reconstructed by using recipient cytoplasts with high level of MPF at the time of enucleation/fusion and activation and by using pre-activated recipient cytoplasts with basal level of MPF. When embryos were reconstructed by transferring donor nuclei at S-phase and G2/M phase of cell cycle into metaphase II recipient cytoplast, with high level of MPF, no embryo developed beyond 2-cell stage, may be due to aneuploidy in donor nuclei caused by the high level of MPF. On the other hand the development of the embryos reconstructed by transferring S-phase and G2/M phase into pre-activated recipient cytoplasts where MPF level supposed to be at basal level, is mainly restricted to 2-cell and lower proportion reaches to morula/blastocyst stage. The reason may be due to some cytoplasmic activity of level of MPF which stays at its basal level for short period of time (see 7.2.2). The overall results of the experiment concludes that the development after nuclear transfer is effected by the cell cycle phase of the donor nuclei and its interaction with recipient cytoplasts.

8.4 - The effect of delayed activation protocol on the pronuclear formation and development after nuclear transfer

Is there any beneficial effect of exposing reconstituted embryos to high level of MPF? The present series of experiments aimed to answer this question.

8.4.1- EXPERIMENTAL DESIGN

8.4.1.1- The effect of delayed activation and Cytochalasin B exposure on the pronuclear formation of reconstructed embryos.

To investigate the effect of delayed activation post fusion for different length of time and subsequent exposure to Cytochalasin B (The cytoskeleton inhibitor) on the pronuclear formation of reconstructed embryos, the oocytes recovered 14 hours

post hCG injections were enucleated and fused with 4-cell donor nuclei. The enucleation/fusion and delayed activation protocol was used in this experiment. The 4-cell embryos recovered from F1 mated females, 58 hours post hCG injection were synchronized for 6 hours to use them at G2/M phase of cell cycle. In contrast the 2-

cell embryos recovered from mated females, 44-48 hours post hCG injection were synchronized for at least 9 hours. They were released from synchronization and used as 4-cell S-phase donor to reconstructed the embryos. The embryos reconstructed by transferring 4-cell donor nuclei at S-phase and G2/M phase of cell cycle with enucleated MII oocytes using micromanipulation procedures (see Chapter 3) were activated 2, 4, and 6 hours post fusion were used. The embryos which were reconstructed by using 4-cell at S-phase of cell cycle were activated 2 hours post fusion only. The reconstructed embryos after their activation with 25 mM Strontium Chloride in M16 were exposed to Cytochalasin B treatment (7.5 μg/ml) in microdrops of sM16 for 6 hours at 37 °C and 5% CO_2 in air in the incubator. At the end of the treatment the reconstructed embryos were washed, put on the slide and holded under the coverslip. The reconstructed embryos were fixed with Methanol: Acetic acid (3:1) for over night and stained with 2% Aceto Orcene. The data on the formation of pronuclei were obtained by scanning the slides under the microscope and were analyzed using method described in section 8.2.2.

8.4.1.2- RESULTS

The results of the experiment presented in table 8.3 shows highly significant difference (p<0.001) in the formation of 2 pronuclei. When the reconstructed embryos were activated 2 hours post fusion, all the embryos reconstructed by using 4-cell S-phase donor nuclei underwent multiple pronuclei formation (> 2PN). Similarly, 95% of embryos reconstructed using 4-cell donor nuclei at G2/M stage of cell cycle showed more than 2 pronuclei. By delaying the time activation for 4 hours, 80% of the embryos reconstructed using 4-cell at G2/M phase of cell cycle showed 2 pronuclei formation (Fig. 8.1) and 15% 1PN+1PB formation, while only 3% were observed with multiple pronuclei. The proportion of embryos showing 2PN increased to 91% when the reconstructed embryos were activated 6 hours post fusion.

8.4.1.3 - CONCLUSIONS

The exposure of reconstructed embryos to high level of MPF for 2 hours leads to the formation of multiple pronuclei. The shorter exposure of donor nucleus.

Fig. 8.1 Formation of two pronuclei

The embryos reconstructed were activated 4 hours after fusion and then exposed to 7.5 µg/ml of Cytochalasin B.
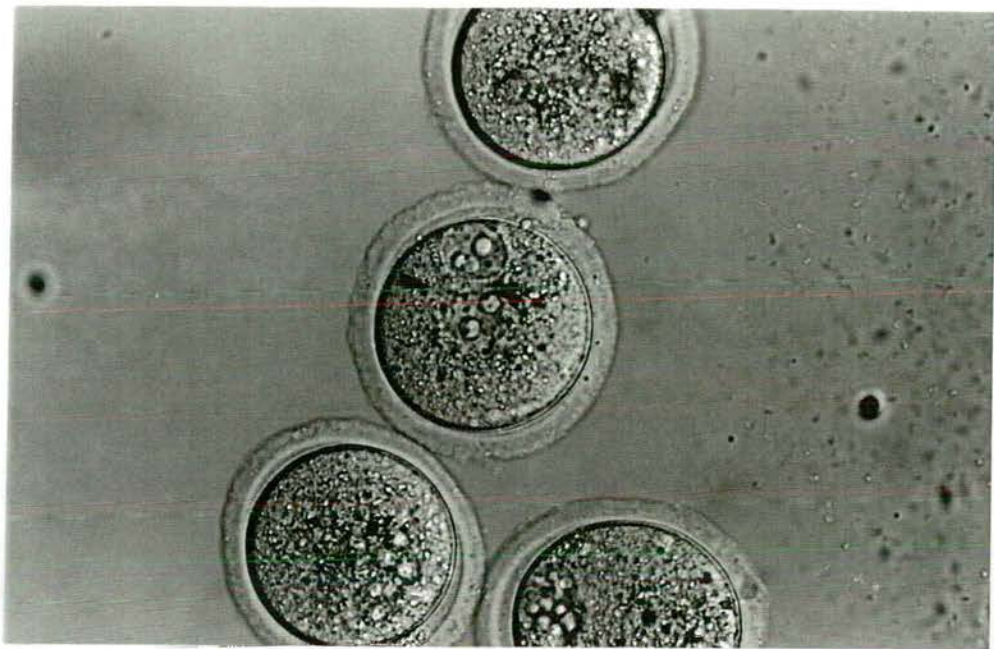
Table: 8.3 THE EFFECT OF DELAYED ACTIVATION AND CYTOCHALASIN B EXPOSURE ON THE PRONUCLEAR FORMATION OF RECONSTRUCTED EMBRYOS.

Treatment	Donor Nuclei	Total	Fused (%)	1PN+1PB (%)	2 Pronuclei (%)	> 2PN (%)
Enucleation						
fusion ⇒ activation	4-cell (S)	27	21/27(78)	0/21(0)	0/21(0)	21/21(100)
(delayed activation)*	4-cell (G2/M)	25	19/25(76)	0/19(0)	1/19(5)	18/19(95)
Enucleation						
fusion ⇒ activation	4-cell (G2/M)	97	74/97(76)	11/74(15)	59/74(80)	2/74(3)
(delayed activation)**						
Enucleation						
fusion ⇒ activation	4-cell (G2/M)	33	23/33(70)	2/23(9)	21/23(91)	0/23(0)
(delayed activation)***						

*The reconstructed embryos were activated 2 hours post fusion. ** The reconstructed embryos were activated 4 hours post fusion.
***The reconstructed embryos were activated 6 hours post fusion.

to high level of MPF may not be helpful in complete PCC which resulted into more than 2PN formation. It is obvious from the results that when the donor nuclei at G2/M stage of cell cycle were exposed for 4 hours to high level of MPF, only 3% showed > 2PN formation

8.4.2- Effect of delayed activation protocol on the development of embryos reconstructed by transferring 2-cell donor nuclei at G2/M stage of cell cycle into MII cytoplasts.

The present experiment was conducted to investigate the effect of delayed activation and Cytochalasin B exposure on the development of embryos reconstructed by transferring 2-cell donor nuclei at G2/M stage of cell cycle into MII cytoplast.

8.4.2.1- Experimental Design

To study the effect of delayed activation and subsequent exposure to Cytochalasin B on the development of reconstructed embryos, the oocytes recovered 14 hours post hCG injection from the F1 females were enucleated using micromanipulation techniques (section 3.4.4). The embryos were reconstructed by transferring 2-cell donor nuclei at G2/M of cell cycle. For this purpose the 2-cell embryos were recovered from the mated F1 females 44 hours post hCG and mating. The embryos were exposed to 10 μM nocodazole till their use for nuclear transfer. The embryos were reconstructed by depositing donor nuclei with small amount of Sendai virus inside the perivitalline space of the zona pellucida of the enucleated MII oocyte using micromanipulation techniques in the micromanipulation chamber. The reconstituted embryos were then washed and cultured in the microdrops of sM16 in the incubator at 37°C and 5% CO_2 in air. After 4 hours, the reconstructed embryos were activated by using 25 mM Strontium Chloride in M16 (see Appendix) for 1 hour. The embryos were washed through several changes in M2 and sM16 and transferred to microdrops of sM16 containing Cytochalasin B (7.5 μg/ml) in a separate dish and cultured for further 4 hour. Two control groups were also made to compare the effect of activation stimuli and auto-activation of the oocyte with any other physical or chemical stimuli. At the end of Cytochalasin B treatment, the reconstructed embryos were washed through several changes of M2 and sM16 and cultured for further development. The observations on the developmental pathway were recorded and the data was analyzed as described in section 8.2.2

8.4.2.2- RESULTS.

The reconstructed embryos after their release from Cytochalasin B treatment extruded their extra polar body and 86% showed 1 pronucleus (Table: 8.4) . The pronounced effect of delayed activation protocol was observed as 82% embryos developed to 2-cell and 68% reached morula/blastocyst development. When development to morula/blastocyst was compared with activation control group, the development was better (p< 0.01) with delayed activation protocol.

8.4.2.3 - CONCLUSIONS

The exposure of donor nuclei to high level of MPF for 4 hours is beneficial in the development of embryos reconstructed by using the 2-cell donor nuclei at G2/M stage. The high level of MPF could be involved in the PCC and also the reprogramming of the donor nucleus. The extrusion of extra polar body may provides embryos with diploid genome and correct ploidy is maintained.

8.4.3- Effect of delayed activation protocol on the development of embryos reconstructed by transferring 4-cell donor nuclei at different stages of cell cycle into MII cytoplasts.

This experiment was aimed to study the effect of delayed activation protocol on the development of embryos reconstructed by transferring 4-cell donor nuclei at G1 or G1/S, S, and G2/M stage of cell cycle into MII cytoplasts.

8.4.3.1 - EXPERIMENTAL DESIGN

To investigate the effect of delayed activation on the development of reconstructed embryos, the oocytes recovered 14 hours post hCG injection were denuded from cumulus and enucleated using micromanipulation technique. In contrast, the 2-cell embryos were recovered from the mated females 44-48 hours post hCG injections. They were synchronized with 10 µM of Nocodazole for at least 9 hours. The embryos were released in groups from synchronization by washing through many drops of M2 and sM16. The embryos started dividing to 4-cell 60 minutes after their release from synchronization and were used as G1 or G1/S donor to reconstruct the embryos 75 minutes after their release. When the 4-cell at S-phase donor nuclei were required, the embryos were reconstructed 4 hours post release. In contrast, when 4-cell donor nuclei at G2/M stage of cell cycle were

Table: 8.4 DEVELOPMENT AFTER NUCLEAR TRANSFER OF EMBRYOS RECONSTRUCTED BY TRANSFERRING 2-CELL (G2/M) DONOR NUCLEI INTOENUCLEATED METAPHASE II OOCYTES FOLLOWING ENUCLEATION/FUSION AND DELAYED ACTIVATION PROTOCOL.

Treatments (recipient)	Donor Nuclei	Replicates	Total	Fused Total (%)	PN/ Fused(%)	2-cell/ fused(%)	Morula/ Blast.(%)
Enucleation fusion ⇒ activation (delayed activation)*	2-Cell (G2/M)	3	34	22/34(65)	19/22(86)	18/22(82)	15/22(68)
+ive Control**	--	2	38	--	33/38(87)	30/38(79)	12/38(32)
-ive Control***	--	2	22	--	0/22(0)	0/22(0)	0/22(0)

*The reconstructed embryos were activated 4 hours post fusion of the couplets. **The activation control group (+ive) received same activation stimulus at the same time. *** The control group (-ive) received no activation stimulus and cultured at the same time. Both of the control groups were kept under paraffin oil in M2 medium in the dark at room temperature till treatments.

required, the 4-cell were recovered from the mated females 58 hours post hCG injections and synchronized with 10 μM of Nocodazole for 5 hours. The embryos were reconstructed by transferring donor nuclei inside the perivitalline space of zona pellucida of the cytoplasts with small amount of Sendai virus in the micromanipulation chamber using micromanipulation techniques (section 3.4.4). Most of the fusion occurred inside the micromanipulation chamber. The reconstructed embryos were then washed with several drops of M2 and sM16 to remove the effect of drugs and cultured in microdrops of sM16 in the incubator at 37°C and 5% CO_2 in air for 4 hours. The reconstructed embryos were then activated using 25 mM Strontium Chloride in M16 for 1 hour. Two control groups were also included to compare the effect of activation stimuli and auto-activation of oocytes with any physical stimuli. The reconstructed embryos after their activation were washed and cultured in microdrops of sM16 containing Cytochalasin B (7.5 μg/ml) for further 4 hours. At the end of Cytochalasin B treatment, the reconstructed embryos were washed and cultured in the microdrops of sM16 for development. The observations recorded on the developmental pathway and the data was analyzed using method described in section 8.2.2.

8.4.3.2.- RESULTS

The development after nuclear transfer is shown (Table: 8.5) to be greatly effected by the donor cell cycle stage when development to 2-cell was compared. When the embryos were reconstructed using 4-cell at S-phase of cell cycle, no embryo developed even to 2-cell stage due to donor nuclei exposure to cytoplasmic environment with high level of MPF. In contrast, when the embryos were reconstructed using donor nuclei from G1 or G1/S and G2/M stage of cell cycle, the development to 2-cell is shown to be 50% and 56% respectively. The development to morula/blastocyst in the embryos reconstructed by using donor nuclei at G2/M stage of cell cycle with delayed activation protocol is 38% which is indistinguishable from the development of embryos reconstructed by using donor nuclei at G1 or G1/S stage of cell cycle was used (38% vs. 42%).

8.4.3.3.- CONCLUSIONS

The exposure of donor nuclei to high level of MPF for 4 hours is beneficial in the development of embryos reconstructed by using the 4-cell donor nuclei at G2/M stage. The high level of MPF could be involved in the PCC and also the reprogramming of the donor nucleus. The results of the experiments demonstrates that the development after nuclear transfer by using delayed activation protocol is.

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Table: 8.5 DEVELOPMENT AFTER NUCLEAR TRANSFER OF RECONSTRUCTED EMBRYOS USING 4-CELL DONOR NUCLEI FROM DIFFERENT STAGES OF CELL CYCLE WITH CYTOPLAST RECIPIENTS USING ENUCLEATION/FUSION AND DELAYED ACTIVATIO PROTOCOL.

PROTOCOL USED	NUCLEAR DONOR	REPLICATES	TOTAL	FUSED TOTAL	2-CELL/ FUSED	(MOR./BLAST.)/ FUSED
Enucleation fusion ⇒ activation (delayed activation)*	4-Cell (G1/S)	3	30	26/30(87)	13/26(50)	11/26(42)
	4-Cell (S)	3	34	27/34(79)	0/27(0)	0/27(0)
	4-Cell (G2/M)	4	49	32/49(65)	18/32(56)	12/32(38)
Act. Control**	--	3	72	--	56/72(78)	21/72(29)
-ive Control***	--	3	30	--	2/30(7)	0/30(0)

*The reconstructed embryos were activated 4 hours post fusion of the couplets. **The activation control group (+ive) received same activation stimulus at the same time. *** The control group (-ive) received no activation stimulus and cultured at the same time. Both of the control groups were kept under paraffin oil in M2 medium in the dark at room temperature till treatments.

only obtained by using donor nuclei at G1 or G1/S and G2/M stage of cell cycle. In contrast no development is expected when S-phase donor nuclei are used to reconstruct the embryos because of high level of MPF which leads to chromosomes abnormalities

8.5 - DISCUSSION

The results of the present findings indicates the role of controlling cell cycle stage of donor nuclei and recipient cytoplasm for successful development of embryos reconstructed by nuclear transfer. The experiments were conducted to study the effect of interaction of donor nuclei and recipient cytoplasm on the development of embryos reconstructed by nuclear transfer. After activation of recipient cytoplasm, the cytoplasmic environment varies in relation to their MPF level. Freshly ovulated MII oocytes contain high levels of MPF activity and causes NEBD and PCC of the donor nuclei if reconstructed at this point (Czolowska et al., 1984; Szollosi et al., 1986a, b. and Szollosi et al., 1988) . In contrast, NEBD and PCC is not observed in the embryos reconstructed after the disappearance of the cytoplasmic activity of MPF. The present experiments were performed by reconstructing the embryos at the time of activation (0 hpa) of MII cytoplasts and using pre-activated cytoplasts (3-4 hpa). Moreover, the experiments were also performed using the protocol of delayed activation. The results of present series of experiments confirmed the earlier reports on the effect of interaction of donor nuclei and recipient cytoplasm on the development of reconstructed embryos.

In this study, when the embryos were reconstructed by transferring 2-cell donor nuclei at G2 stage of the cell cycle into different cytoplasmic environments, the development to morula/blastocyst remained restricted mainly to pre-activated cytoplasts. The lower development in embryos reconstructed at the time of enucleation/ fusion/ activation (0 hpa) at the same time may be due to the partial exposure of high level of MPF which was unable to cause proper chromatin condensation necessary for reprogramming of the donor nuclei. This idea is supported by the results of previous studies with amphibians (DiBerardino, 1988) and mammals (Collas and Robl, 1991; Collas et al., 1992a, b) which demonstrated that the exposure of donor nucleus to an cytoplasmic environment resulting chromatin condensation are helpful for nuclear reprogramming and have beneficial effects on the development of embryos reconstructed by nuclear transfer.

The higher proportions of development to morula/ blastocyst stage is observed when the embryos were reconstructed by transferring 4-cell donor nuclei

from G1 stage of cell cycle into MII cytoplasts (41%) and into pre-activated cytoplasts (46%). Present results confirmed the earlier findings (Collas *et al.,* 1992; Cheong *et al.,* 1993) which demonstrated development to blastocysts by transferring G1 donor nuclei into MII cytoplasts. The results (Table 8.2) development (46%) of embryos reconstructed by transferring G1 donor nuclei into pre-activated cytoplasts after the disappearance of MPF are in line with the study of Campbell *et al.,* (1993) which demonstrated that the normal ploidy in embryos reconstructed from all stages of cell cycle is expected, if reconstructed after the disappearance of MPF in the cytoplasts. The lower rate of development in embryos reconstructed by using G2 and S-phase donor nuclei in these experiments by using pre-activated cytoplasts demonstrated some cytoplasmic activity of MPF still present at the time of reconstruction of embryos, it stays for a very short time at basal level (referred to Chapter 6 fig: 6.2), causing damaging effect on the chromosome by pulverization (S-Phase donor) and re-replication of previously replicated chromosomes (G2 donor).

In present study the experiments conducted to prolong the exposure of donor nuclei to cytoplasmic environment with high level of MPF using method of delayed activation enhances the developmental potential of reconstructed embryos. The embryos reconstructed by using 2-cell donor nuclei at G2 stage of cell cycle with delayed activation protocol showed considerably higher (68%) proportion of development to morula/blastocyst (Table: 8.4) than fusion and activation at the same time (Table: 8.1). The exposure of donor nuclei to MII cytoplasts before activation provides cytoplasmic environment with high level of MPF. Since these experiments were completed, a benefit in delaying activation has also been described after transfer of cumulus cell nuclei to MII oocytes (Wakayama *et al.,* 1998). The mechanism of this effect is not understood.

The effect of prolonged exposure to cytoplasmic environment in non-activated MII cytoplasts for different period of time on the pronuclear formation was studied in embryos reconstructed by G2 donor nuclei which suggested minimum exposure of 4 hours. The shorter exposure resulted in the formation of multiple pronuclei indicated duration time was not good enough for complete formation of the metaphase plate necessary for the formation of 2PN. The experiments conducted to prolong the exposure of 4-cell donor nuclei from different stages of the cell cycle to cytoplasmic environment enhances the developmental potential of reconstructed embryos. The development of embryos reconstructed using G2 donor nuclei was found to be comparable to the embryos reconstructed using G1 donor suggested the beneficial effects of exposing to cytoplasmic

environment with high level of MPF for reprogramming. No development in embryos reconstructed by using 4-cell donor nuclei at S-phase stage of cell cycle and prolong exposure to non-activated cytoplasmic environment of MII cytoplasts is hypothesised due to high level of MPF causing PCC which led to chromatin pulverisation. The report of previous studies (Rao and Johnson, 1974; Haneen and Rohme, 1982) showed the chromatin pulverisation of PCC by passaging of 8-cell nucleus through MII cytoplasm. In this study the reconstructed embryos were exposed to Cytochalasin B for 4-6 hours. The embryos reconstructed by using G2/M donor nuclei into MII cytoplast environment when exposed to Cytochalasin B for 4 hours, 80% showed 2PN formation. This proportion increased to 91% by increasing the Cytochalasin B exposure to 6 hours. These reconstructed embryos when further cultured after washing, showed cone formation immediately and extrude a polar body showing only 1PN in the embryos. This extrusion of polar body in this case may be the extrusion of 1PN, leaving embryo with diploid chromosome and hence development from the G2 donor nuclei was achieved.

In conclusion, the result of present study confirm that development after nuclear transfer is affected by the cell cycle stage of the donor nucleus and recipient cytoplasm and their interactions. Provided the cytoplasmic environment is suitable, the donor nuclei at early and late phases of cell cycle support development to morula and blastocyst stage. The observation of greater development with donor nuclei in G2 and G1 phases suggests that there may be differences in chromatin structure during these periods which allow reprogramming of the gene expression.

CHAPTER 9

GENERAL DISCUSSION

All experiments presented in this thesis were carried out on mouse. The mouse is the most commonly used laboratory mammal. Due to its small size and high fecundity it is relatively cheap to breed, buy and maintain. Recent major advances in technique of nuclear transplantation allow manipulation of genetic constitution of mouse eggs or embryos by the removal or addition of pronuclei or nuclei. Such procedures have made possible the investigation of a number of important areas in development: the developmental contribution of maternal and paternal pronuclei; nuclear-cytoplasmic interactions; the developmental potential of nuclei transferred to eggs from later stage embryos, and the feasibility of 'cloning'; production of transgenic animals and chimaeras; reproduction in mammals by parthenogenesis.

The results presented in this thesis emphasize the importance of cytoplasmic environment responsible for reprogramming of the transferred nucleus and confirmed the importance of cell cycle co-ordination in the development of reconstructed embryos. The results demonstrate the importance of controlling donor and recipient cell cycle stages for appropriate co-ordination to enhance development in the embryo reconstructed by nuclear transfer.

The results presented in table 8.2 demonstrated the significant effect of interaction of cell cycle stage of donor nuclei and recipient cytoplasts. When the embryos were reconstructed using donor nuclei from different stages of the cell cycle in the presence of high MPF activity, the G1 donor proved to be the best donor. These results confirmed the earlier finding of donor cell cycle stage effect (Collas *et al.*, 1992a). Inappropriate co-ordination of cell cycle stages may lead to aneuploidy or chromosomal damage. The lower development of embryos reconstructed by transferring S-phase and G2 phase donor nuclei into pre-activated cytoplasts could be related to the unexpected cytoplasmic activity of MPF (see section 6.2.2) which reached its basal level around 4 hours post activation, but still caused NEBD and

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Table 9.1: Different protocols for nuclear transplantation and their success rates.

Source	Donor Kayyoplast	Nuclear Transplantation Procedures	Morula (%)	Blastocyst (%)
Table 5.5	G1/S (4-cell)	Enucleation/Activation (Sr 1 hr $\Rightarrow$6-DMAP 3 hr) $\Rightarrow$Fusion (4 hpa)	33/57 (85)	31/39 (79)
Table 5.5	S (4-cell)	Enucleation/Activation (Sr 1 hr $\Rightarrow$6-DMAP 3 hr) $\Rightarrow$Fusion (4 hpa)	19/47 (40)	11/47 (23)
Table 5.5	G2 (4-cell)	Enucleation/Activation (Sr 1 hr $\Rightarrow$6-DMAP 3 hr) $\Rightarrow$Fusion (4 hpa)	23/49 (47)	13/49 (27)
Table 5.7	G1/S (4-cell)	Enucleation/Activation (Sr 1 hr $\Rightarrow$6-DMAP 3 hr) $\Rightarrow$Fusion (4 hpa)	41/45 (91)	35/45 (78)
Table 5.7	G1/S (4-cell)	Enucleation/Activation (Strontium 1 hr) $\Rightarrow$Fusion (4 hpa)	21/36 (58)	21/36 (58)
Table 8.1	G2 (2-cell)	Enucleation/ Fusion /Activation (Sr 1 hr) (0 hpa)	2/87 (2) (Mor./Blast.)	
Table 8.2	G2 (2-cell)	Enucleation/Activation(Sr 1 hr) /Fusion (3-4 hpa)	14/86 (16) (Mor./Blast.)	
Table 8.2	G1/S (4-cell)	Enucleation/ Fusion /Activation (Sr 1 hr) (0 hpa)	12/29 (41) (Mor./Blast.)	
Table 8.2	S (4-cell)	Enucleation/ Fusion /Activation (Sr 1 hr) (0 hpa)	0/23 (0) (Mor./Blast.)	
Table 8.2	G2/M (4-cell)	Enucleation/ Fusion /Activation (Sr 1 hr) (0 hpa)	0/50 (0) (Mor./Blast.)	

Table 8.2	G1/S (4-cell)	Enucleation/ Activation (Sr 1 hr) ⇒Fusion / (3-4 hpa)	18/39 (46) (Mor./Blast.)
Table 8.2	S (4-cell)	Enucleation/ Activation (Sr 1 hr) ⇒Fusion / (3-4 hpa)	2/29 (7) (Mor./Blast.)
Table 8.2	G2/M (4-cell)	Enucleation/ Activation (Sr 1 hr) ⇒Fusion / (3-4 hpa)	2/29 (7) (Mor./Blast.)
Table 8.4	G2/M (2-cell)	Enucleation/ Fusion ⇒ Activation (Sr 1 hr) (3-4 hpf)	15/22 (68) (Mor./Blast.)
Table 8.5	G1/S (4-cell)	Enucleation/ Fusion ⇒ Activation (Sr 1 hr) (3-4 hpf)	11/26 (42) (Mor./Blast.)
Table 8.5	S (4-cell)	Enucleation/ Fusion ⇒ Activation (Sr 1 hr) (3-4 hpf)	0/27 (0) (Mor./Blast.)
Table 8.5	G2/M (4-cell)	Enucleation/ Fusion ⇒ Activation (Sr 1 hr) (3-4 hpf)	12/32 (38) (Mor./Blast.)
Wakayama*	G0/G1(cumulus)	Enucleation/ Fusion/ Activation (Sr 6hr) (0 hpa)	61/153 (17) (Blastocyst)
Wakayama*	G0/G1(cumulus)	Enucleation/ Fusion ⇒ Activation (Sr 6hr) (1-3 hpf)	277/474 (58) (Blastocyst)
Wakayama*	G0/G1(cumulus)	Enucleation/ Fusion ⇒ Activation (Sr 6hr) (3-6 hpf)	101/151 (67) (Blastocyst)

*Wakayama *et al.*, (Nature 394, 369-374, 1998)

NEBD & PCC in 41% ± 4.22 and 10.25% ± 3.7 donor nuclei respectively. However, it is suggested that experiments should be performed to evaluate the level of H1 kinase activity to map the level of MPF. It is further suggested that further experiments using prolonged exposure to strontium containing medium should be performed to properly activate the metaphase II cytoplasts as demonstrated by Bos-Mikich *et al.*, 1995, Wakayama *et al.*, (1998).

Different nuclear transfer procedures were employed to study the cell cycle co-ordination in nuclear transfer experiment and the results are summarized in table 9.1. Direct comparison of different activation timings have been made with 4-cell nuclei at three different stages of the cell cycle (table 9.2)

Summary of effect of different activation protocols on development to morula/blastocyst (%) after nuclear transfer from 4-cell embryos

Nuclear Donor	Pre-activation*	Simultaneously**	Delayed activation***
G1/S	46	41	42
S-phase	7	0	0
G2/M	7	0	38

*Enucleation/activation ⇒ fusion (4 hpa). ** Enucleation /fusion/activation at the same time. ***Enucleation/fusion ⇒ activation (4 hpf).

Several different combinations give similar development to morula/blastocyst stage. Similar development with donor nuclei in G1/S was obtained with all three recipient cell cycle stages. By contrast, very poor development was obtained with donor nuclei in S-phase. Development from nuclei in G2/M was high only after delayed activation. Several factors probably contribute to these differences. In recent years greater development was obtained when oocytes were used as recipient cytoplasts

(Cheong et al., 1994) rather than enucleated zygote (McGrath and Solter, 1984). This difference may reflect an influence of reprogramming factor present in the oocytes at MII, but absent in the zygote after pronuclear formation. Factors able to reprogramme transferred nuclei may also be used in pronuclear formation and so no longer be available during nuclear transfer to an enucleated zygote.

The cytoplasmic environment appears to be conditioning the chromatin during the process of condensation and making it amenable for reprogramming. During the condensation process in mitosis most of the nuclear proteins, except for histones, are removed from the nucleus (Nagl, 1985; Albert *et al.,* 1989). Cytoplasmic proteins now have free access to chromosomes and may be passively trapped by the telophase nucleus as the nuclear envelope reforms (Swanson and McNeil, 1987). Similar events occurring during the completion of a meiotic maturation in activated oocytes may be necessary for reprogramming. Formation of a mature pronucleus may also require the active transport of proteins via the nuclear envelope: those supplied by an oocyte, and those which appear as new after egg activation (Dreyer, 1987; Howlett *et al.,* 1988). It is not yet confirmed whether the cytoplasmic activity of MPF or some other cytoplasmic factors played their role in the reprogramming of the transferred nucleus. The results presented in table 5.5 confirms that in the absence of high level of MPF and by maintaining the integrity of the nuclear envelope, the universal recipients support development of the reconstructed embryos from any stage of cell cycle to blastocyst stage. Under these circumstances some reprogramming factors must pass through the nuclear membrane. The reprogramming of the donor nuclear gene expression is an important step in enhancing the developmental potential of reconstructed embryos (Kono, 1997). It was suggested that the birth of lambs using donor cultured cell at G0 stage is the result of effective reprogramming (Campbell *et al.,* 1996). However, further studies are suggested to identify the nature of this reprogramming factor.

In experiments where the embryos were reconstructed using delayed activation protocol, the reconstructed embryos were exposed for 4 hours to Cytochalasin B. The embryos reconstructed using G2 donor nuclei when released from the Cytochalasin B exposure extruded a pronuclear like polar body and then began development. It is suggested that experiments should be conducted to evaluate the karyotype of these early embryos to confirm the ploidy. However, no polar body extrusion was observed in embryos reconstructed by using donor nuclei from G1 and S-phase of cell cycle. The present results contrast with earlier studies of Kono *et al.,*(1992) and Cheong *et al.,* (1993) who reported the extrusion of polar body like

structure containing DNA derived from the transferred nucleus. The difference might be due to the use of Cytochalasin B in this experiment. The extrusion of one of the pronuclear like structures in embryos reconstructed using G2 donors demonstrates that perhaps there is presence of some cytoplasmic mechanism which sense one of the pronuclear as polar body and extrudes it. The results confirms the earlier findings that window of opportunity exists from G2 through mitosis (Kwon and Kono, 1996) to G1. The state of universal recipient (Campbell *et al.,* 1993) in mouse was difficult to achieve as level of MPF was at basal level for a very short period (see section 6.2.2).

The results presented in table 5.5, 5.7 demonstrate the use of 6-DMAP in preparation of cytoplasts as universal recipients and by using this activation protocol it has been possible for the first time to obtain similar development with 4-cell blastomeres with donor nuclei at any stage of the cell cycle. The key to success in these experiments was the maintenance of nuclear envelope which helped in the maintenance of normal ploidy in reconstructed embryos. However, it is suggested that further experiments should be planned to evaluate the effectiveness of this activation protocol (chapter 5) on development to term.

The rate of higher development with G1 donor nuclei reported in 5.5 and 5.7 could be related to the criteria used to select the donor at G1 stage. The criteria introduced during the experiments presented in chapter 5 was the number of nucleoli. Donor nuclei after mitosis containing more than 6 nucleoli were considered to be at G1 stage of cell cycle. The second reason for higher rate of development might be that the reconstructed embryos were fused immediately using electrofusion technique. However, it is suggested that this might be a useful criterion and an experiment should be conducted to assess development after transfer of nuclei with different number of nucleoli.

The results presented in chapter 7 demonstrated that initiation of DNA replication, in 3rd and 4th cell cycle as investigated by pulse labelling technique for detecting the PCNA localization, starts 60 min. after release from synchronization. The lower development of embryos reconstructed by using donor in G1 stage might be due to the fact that G1 duration is very short and the proportion of development as reported in chapter 8 and earlier is the proportion which was actually in G1. The timing of G1 duration is contradictory with that reported by Otaegui (1995, Ph.D. Thesis). The reason might be the difference in the use of techniques for the detection of the start of DNA replication in 3rd cell cycle. The BrdU technique reported in this

thesis is based on the rapid pulse labeling technique of (Nomura *et al.*, 1993) which required only 5-8 min. for BrdU incorporation. In contrast, the original technique involved incubation of at least 45 minutes.

It is suggested that the further studies might be focused to use the donor nuclei from established culture cell lines after inducing them into different stage of the cell cycle including quiescent (G0). The birth of mice following nuclear transfer from cumulus cells (Wakayama *et al.*, 1998) also suggests that also as a second approach nuclei should be transferred from a variety of differentiated cells.

Different Media and Stock Solutions

The composition of different media and their preparation from different stock solutions used in the different experiments are detailed below. Moreover, the appendix consisted of the details of stock solutions of chemical compounds/drugs and antibodies.

1.1 - Culture media M16, pM16 and sM16.

Table A 1.1 - Composition of M16, pM16 and sM16

Compound	MW	g/litre (mM)		
		M16	pM16	sM16
NaCl	58.450	5.533 (94.66)	4.700 (80.40)	4.700 (80.40)
KCl	74.557	0.356 (4.78)	0.356 (4.78)	0.356 (4.78)
$CaCl_2$ 7 H_2O	147.200	0.252 (1.71)	0.252 (1.71)	0.252 (1.71)
KH_2PO_4	136.091	0.162 (1.19)	0.162 (1.19)	0.162 (1.19)
$MgSO_4$ 7 H_2O	246.500	0.293 (1.19)	0.293 (1.19)	0.293 (1.19)
$NaHCO_3$	84.020	2.101 (25.00)	2.101 (25.00)	2.101 (25.00)
Sodium pyruvate	110.000	0.036 (0.33)	0.040 (0.36)	0.040 (0.36)
Glucose	179.860	1.000 (5.56)	0.350 (2.78)	0.350 (1.95)
EDTA	372.200	0	0.037 (0.1)	0.037 (0.1)
Sodium lactate (60% syrup)	112.100	4.349 (23 28)	4.349 (23 28)	4.349 (23 28)
L-Glutamine		0.000	0.000	0.146
Bovine serum albumin (BSA)		4.000	4.000	4.000
Penicillin G (K salt) (final conc. 100 units/ml)		0.060	0.060	0.060
Streptomycin sulphate (final conc. 50μg/ml)		0.050	0.050	0.050
Phenol Red		0.010	0.010	0.010
MEM non-essential amino acid solution 100x		0.000		10.0 ml (v/v)
MEM amino acids solution 50x				20.0 ml (v/v)
Water (Molecular water)		up to 1 litre	up to 1 litre	up to 1 litre.

Osmolarity : M16 ≈ 280; pM16 ≈ 265; sM16 ≈260 mOsmoles.

The compounds used in the preparation of those media, unless otherwise mentioned, were of cell culture tested and from Sigma (Poole, Dorset, UK).

Procedure - The media were prepared from the stock solutions mentioned in appendix 1.3. Various amounts of stock solutions were used depending upon the required volume of media. The stock D was always introduced in the end. All the stocks and BSA were dissolved in small amount of water in the volumetric flask then the volume was made up to desired volume. The required volume supplemented with BSA was then filter-sterilized into small appendorfs and stored at 4°C.

1.2 - Manipulation media M2.

Table A 1.2. - Composition of M2.

Compound	mM	MW	g/litre
NaCl	94.66	58.450	5.533
KCl	4.78	74.557	0.356
CaCl$_2$ 7 H$_2$O	1.71	147.200	0.252
KH$_2$PO$_4$	1.19	136.091	0.162
MgSO$_4$ 7 H$_2$O	1.19	246.500	0.293
NaHCO$_3$	4.15	84.020	0.349
HEPES	20.85	238.300	4.969
Sodium lactate	23.28	112.100	4.349 of 60% syrup
Sodium pyruvate	0.33	110.000	0.036
Glucose	5.56	179.860	1.000
L-Glutamine		0.000	0.146
Bovine serum albumin (BSA)			4.000
Penicillin G (K salt) final conc. 100 units/ml			0.060
Streptomycin sulphate (final conc. 50µg/ml)			0.050
MEM non-essential amino acid solution 100x			10.0 ml (v/v)
MEM amino acids solution 50x			20.0 ml (v/v)
Phenol Red			0.010
Water (Molecular water)		up to 1 litre.	

Osmolarity   M2 ≈ 280 mOsmoles.

(Sterile, endotoxin tested MEM non-essential amino acid solution (Cat no. M7145 SIGMA) and MEM amino acids solution without L-Glutamine (Gibco, BRL Cat no. 11130-036) were used in preparing the sM16 medium

1.3- Preparation of M2, sM16 and activation media from stocks.

Table A 1.3 - Composition of stock solutions

Stock Mg free A	Components	g/100ml (For M2)	g/100ml (For sM16)
(10 x concentration)	NaCl	5.533	4.700
	KCl	0.356	0.356
	KH_2PO_4	0.162	0.162
	Sodium lactate (60% syrup)	4.349	4.349
	Glucose	1.000	0.350
	Penicillin G	0.060	0.060
	Streptomycin sulphate	0.050	0.050
	EDTA	0.00	0.037

Stock B	Component	g/100ml (For M2)	g/100ml (For sM16)
(10 x concentration)	$NaHCO_3$	0.349	2.101
	Phenol Red	0.010	0.010

Stock C	Component	g/10ml (For M2)	g/10ml (For sM16)
(100 x concentration)	Sodium pyruvate	0.036	0.040

Stock D	Component	g/10ml (For M2)	g/10ml (For sM16)
(100 x concentration)	$CaCl_2$ 7 H_2O	0.252	0.252

Stock E	Component	g/10ml (For M2)	g/10ml (For sM16)
(10 x concentration)	HEPES	4.969	0.000
	Phenol Red	0.010	0.000

Stock Mg	Component	g/10ml (For M2)	g/10ml (For sM16)
(100 x concentration)	$MgSO_4$ 7 H_2O	0.293	0.293

Stock Glutamine	Component	g/10ml (For M2)	g/10ml (For sM16)
(100 x concentration)	L-Glutamine	0.146	0.146

Stock Sr	Component	g/10ml (For Activation Media)
(100 x concentration)	$SrCl_2$ 6 H_2O	0.452

Storage - At 4°C Stocks A Mg-, D, E, Mg and Sr were kept up to 3 months at 4°C, but stocks B and C were change every three weeks and stock glutamine was prepared freshly all the times.

Table A 1.4 - Procedure to prepare M2, sM16 and calcium magnesium free M16 plus strontium from stocks.

Stock	M2	sM16	Ca Mg free M16 +Sr
Mg free A	10.0 ml	5.0 ml	2.50 ml
B	1.6 ml	5.0 ml	2.50 ml
C	1.0 ml	0.5 ml	0.25 ml
D	1.0 ml	0.5 ml	0.0 ml
E	8.4 ml	0.0 ml	0.0 ml
Mg	1.0 ml	0.5 ml	0.0 ml
L-Glutamine	1.0 ml	0.5 ml	0.0 ml
Non-essential a.a.	0.0 ml	0.5 ml	0.0 ml
Min.Essential a.a.	0.0 ml	1.0 ml	0.0 ml

Sr	0.0 ml	0.0 ml	3.67 ml
Water	Up to 100 ml	Up to 50 ml	Up to 25 ml

A 1.5 - Phosphate Buffered Saline (PBS).

Calcium-magnesium free PBS was prepared by dissolving pre-formulated tablets (Flow Laboratories, Rickmansworth, Hertfordshire, UK) in distilled water into 500 ml bottles. PBS was then sterilized by autoclaving and stored at room temperature.

A 1.6 - Acidic Tyrode solution for removing zona pellucida.

A fresh 100 ml solution was prepared every two months and kept at room temperature. The pH was adjusted with Analar HCl. It was sterilized by filtration. Its composition in g/100 ml was:

NaCl	0.800
KCl	0.020
$CaCl_2$ $2H_2O$	0.024
$MgCL_2$ $6H_2O$	0.010
Glucose	0.100
Polyvinylpyrrolidone (PVP)	0.400

The PVP was added to increase the viscosity and reduce embryo stickiness.

A 1.7 - Hank's Balanced Salt solution (HBSS).

This solution was used as diluent through the production of Sendai virus. For some of the procedures glucose was omitted in its composition, in those cases it was denominated Hank's balanced salt solution without glucose (HBSSG-). The whole composition was: 0.14 g/l of $CaCl_2$; 0.40 g/l of KCl; 0.06 g/l of KH_2PO_4 ; 0.0977 g/l of $MgSO_4$; 8.00 g/l of NaCl; 0.048 g/l Na_2HPO_4 ; 1.00 g/l of glucose and 0.01 g/l of phenol red.

A 1.8 - Chemical stocks

1.8.1 - Anti-bromodeoxyuridine antibody

Mouse anti-bromodeoxyuridine (anti-BrdU) monoclonal antibody conjugated to fluorescein (Boehringer, cat no. 1202, Lewes, East Sussex, UK.) was diluted to 50 µg/ml in PBS supplemented with 0.1% BSA. Fifty µl aliquots were made and kept at - 20°C.

1.8.2 - Anti- proliferating cell nuclear antigen

. The anti-PCNA/cyclin (Immuno concepts Cat: No. 2037) was diluted with PBS containing FCS (9.9 ml PBS + 100 µM FCS) with the ratio of 1:10 i.e. 450 µl of above diluent and 50 µl of anti-PCNA.

1.8.3 - Bromodeoxyuridine

Bromodeoxyuridine (BrdU) (5-bromo-2'deoxyuridine 5'-triphosphate, Sigma code No. B-0631; Poole, Dorset, UK) was dissolved in water at 1 mg /ml concentration. Aliquots of 10 mM were made and kept at - 20°C.

1.8.4 - Cytochalasin B

Cytochalasin B, from Helminthosporium dematioideum (C_{29} H_{37} NO_5; Sigma code No. C-6762; Poole, Dorset, UK.) was dissolved in dimethyl sulphoxide at 5mg/5ml and stored at - 20 °C. The stock solution was used several times after freezing and thawing without any apparent lost of potency. The aliquots containing 10 µg/10 µl were made and stored at 4°C. The working solution of 10 µg/ml was made by adding one ml of medium in the aliquot.

1.8.5   6-Dimethylaminopurine (DMAP)

A stock solution of 6-dimethylaminopurine (N^6,N^6-Dimethyladenine ($C_7H_9N_5$);Sigma code No. D-2629; Poole, Dorset, UK.) (Sigma) was prepared by dissolving with the molecular water and the aliquots were made containing 2.5 mM/20 µl concentration. The aliquots were stored at -20°C. This solution was diluted with M16 or strontium activation media depending upon the type of treatment and the final concentration of 2.5mM/ml or 2.0 mM was achieved.

1.8.6 - Hoechst dye.

Hoechst no. 33342, bisbenzimide (2'-[4-Ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole; Sigma code no B-2261; Poole, Dorset,

UK.) was dissolved in distilled water at 1mg/ml . A working concentration of 1.0 µg/ml was used to stain the chromosomes for enucleation purposes.

1.8.7 - Hyaluronidase

Hyaluronidase, Type IV-S from bovine testes lyophilized powder containing approx. 90% protein ; balance primarily buffer salts. (Hyaluronoglucosaminidase; Hyaluronate 4-glycanohydrolase ; Sigma code no. H-3884; Poole, Dorset, UK.) was used. The stock solution was prepared by dissolving 30 mg/1 ml of distilled water. The aliquots of 10 µl were made and stored at -20 °C. A working concentration of 300 µg/ml of M2 was used in all the experiments to denuded the oocytes.

1.8.8   Ionomycin

The stock solution of ionomycin calcium salt from streptomyces conglobatus ($C_{41}H_{70}O_9Ca$); Sigma product no. I 0634 Poole, Dorset, UK) was prepared by dissolving 1mg of ionomycin calcium salt with 1338.5 µl of DMSO to the concentration of 1mM/1ml. The aliquots were made with the concentration of 5mM/10µl by dissolving the stock solution with molecular water and stored at -20°C. The ionomycin activation medias having concentration of 5µM was prepared by adding one milliliter of M2 medium in the aliquots.

1.8.9 - Nocodazole

Nocodazole, (methyl (5-[2-thienylcarbonyl]-1H-benzimidazol-2-yl) carbamate; Sigma code no. M-2759; Poole, Dorset, UK.) was dissolved in dimethyl sulphoxide at 2 mg/ml and kept at 4 °C. The stock solution was used several times without any apparent lost of potency. To get 10 mM concentration of nocodazole 1.5 µl/ml was used in the experiments.

1.8.10   Taxol (Paclitaxel)

Taxol is a trade mark of Paclitaxel. Paclitaxel, ($C_{47}H_{51}NO_{14}$); Sigma product no. T1912; Poole, Dorset, UK.)was dissolved in water at 1 mg/1.17 ml of water to give rise concentration of 1mM/1µl. The stock solution was aliquoted

1.8.11 - Texas Red

The Texas red (Vector Lab. Cat: No. TI-3000) was diluted with PBS containing 1% FCS with the ratio of 1:20.

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