STUDIES ON CRYOPRESERVATION OF FEMALE GAMETE IN BUFFALOES

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

MASTER OF SCIENCE
IN
DAIRYING
(LIVESTOCK PRODUCTION AND MANAGEMENT)

By
ARINDAM DHALI
B.V.Sc. & A.H.

DIVISION OF DAIRY CATTLE BREEDING
NATIONAL DAIRY RESEARCH INSTITUTE
(I.C.A.R.)
KARNAL - 132001 (HARYANA), INDIA
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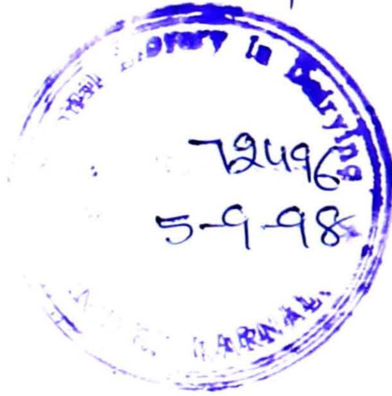

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BY

ARINDAM DHALI

A Thesis submitted to the National Dairy Research Institute
(Deemed University), Karnal
in partial fulfilment of
the requirements for the degree of

MASTER OF SCIENCE

IN

DAIRYING

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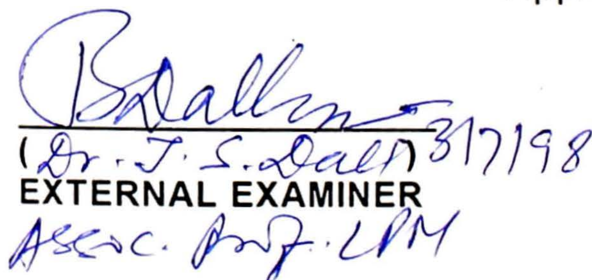
Approved by



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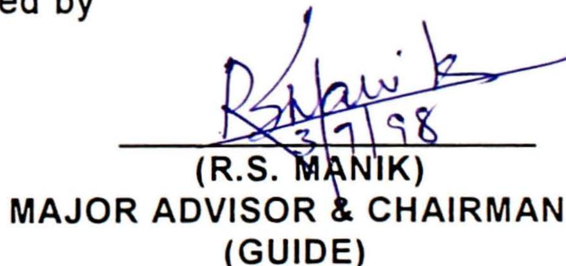

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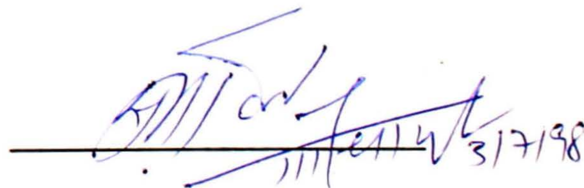

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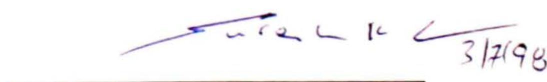

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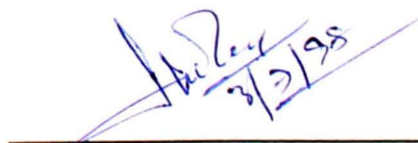

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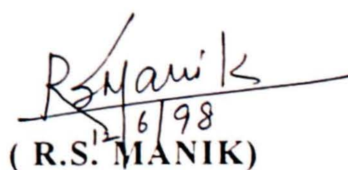
This is to certify that the thesis entitled **"STUDIES ON CRYOPRESERVATION OF FEMALE GAMETE IN BUFFALOES"** submitted by **ARINDAM DHALI** in partial fulfilment of the requirement for the award of the degree of **MASTER OF SCIENCE in DAIRYING (LIVESTOCK PRODUCTION AND MANAGEMENT)** of the National Dairy Research Institute (Deemed University), Karnal - 132 001 (Haryana), India, is a bonafide research work carried out by him under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.



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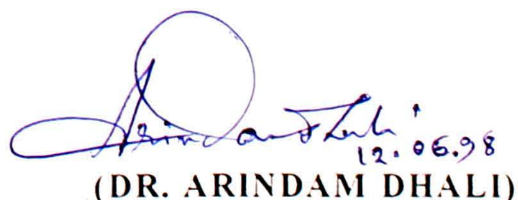

(DR. ARINDAM DHALI)

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CHAPTER - 1

INTRODUCTION

1. INTRODUCTION

The importance of animal production is enormous in India. Because bovines consume forages, therefore, are of special value, they convert feed that is inedible for human beings into edible animal products. A good index of efficacy in an animal production system is its ability to provide adequate amounts of affordable food for human consumption. There are four basic ways of achieving more efficient production : i) improvement in husbandry practices ii) genetic improvement of animals, iii) development of techniques for exploiting the full reproductive potential, and iv) to reduce the wastage of valuable genetic resources.

The reproductive capacity of males is being enhanced through the use of artificial insemination procedure. The impact of genetic improvement on milk yield in the country has been so far due to the realization of an reproductive potential of male. The semen collection and freezing was started long back and has now become a routine procedure. From a bull, normally 3 to 6 ml of semen can be obtained in each ejaculation. After collection and evaluation of sperm for density and motility, ejaculate can be extended to varying degree with a variety of buffered isotonic solution containing a carbohydrate energy source, protective protein, antibiotics and various cryoprotective agents and can be cryopreserved for future use. Male's genetic influence is determined by the number of doses frozen.

Exploitation of reproductive efficiency in female is also essential for maximum production in minimum time and lot of work has been carried out in same lines. Efforts to preserve embryos by freezing began at about the same time as semen freezing but no young ones were produced until the early 1970. The basic difference between freezing of semen and freezing of embryos is that a vial or straw with one killed embryo or oocyte cannot produce a pregnancy, whereas a vial or straw with millions of killed spermatozoa can.

Cryopreservation means to arrest all biological process and place the cell into suspended state of animation. It is an area of rapid scientific and technological advancement based on rapidly evolving concept of cryobiology, embryology and reproduction. Over last three decades major progresses have been made in techniques for cryopreservation of different cell types, more complex tissues and even organ.

The cryopreservation of embryos of most domestic species has become a routine practice as a part of embryo transfer technology and recently advances have been made in cryopreservation of mammalian oocytes. The ability to sustain viable oocytes and embryos from mammalian species at low temperature has important implication to livestock improvement. Recent advances in the study of physio-chemical behaviour of different cryoprotectants, use of various macromolecules, additives in cryoprotective solution, isolation and use of protein of plant and animal origin with antifreeze activity offers many new options for cryopreservation of oocytes and embryos of animal and human origin. At the same time rapidly developing methods of oocyte and embryo manipulation such as in vitro embryo production, embryo splitting, embryo biopsy for gene and sex determination, embryo cloning and the isolation of individual blastomeres create new challenges in cryopreservation.

The cryobanking of oocytes along with in vitro fertilization (IVF) technology and in vitro embryo production offers many advantages : i) the circumvention of the problem of timing of ovulation for artificial insemination, ii) the potential for producing more embryos than can be usually collected from stimulated donors, iii) the ability to make use of animals with certain types of infertility, such as endometritis or tubal obstruction etc., iv) a reduction in the number of viable sperm needed for in vitro fertilization as compared with the artificial insemination or natural breeding, v) the potential of salvaging genetic material from female animals after death, vi) the possible inclusion of pre-pubertal or pregnant

animals as oocyte donors, vii) increases the possibilities to reduce the transportation cost in trade both locally and internationally as compared to live animal transportation, and viii) the possible reduction in the spreading of infectious diseases through cryopreserved oocytes or embryos during migration as compared to live animal.

Therefore, the cryopreservation of oocytes would undoubtedly be the most practical way of preserving genetic material from female animals postmortem and from live animals using transvaginal procedures. But the current state of this technology for bovine oocyte indicates that less than 3% of all oocytes treated progress to blastocysts in vitro (Martino *et. al.*, 1996). Certainly, considerably more research will be necessary for feasible cryobanking of oocytes. Therefore, the present study was undertaken to investigate the following objectives:

1. To compare the different concentrations of vitrification solution for cryopreservation of oocytes.

2. To study the effect of different equilibration times for cryopreservation of oocytes.

3. To study the maturation rate of cryopreserved oocytes.

CHAPTER - 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

The cryopreservation of oocytes would undoubtedly be the most practical way of preserving genetic material from female animals. Investigations had been done to standardise the technique. Mainly two methods were adopted by various research workers, controlled freezing with the help of a biological freezer and vitrification. For the present study vitrification method was adopted which is not only very promising as indicated by several workers but also very easy to perform and requires less skill, which reveals its possible adoption at field level. However the current state of this technology for bovine oocytes indicates that considerably more research will be necessary before the feasibility of cryobanking of oocytes for domesticated bovine species. Unfortunately not many reports are available in the literature regarding the vitrification of oocytes in bovines especially buffalo. Therefore, the informations available in bovines as well as other species have been reviewed here. The retrospect of the present study has been reviewed under the various following heads :

2.1 Folliculogenesis

2.2 Retrieval of oocytes

2.3 Slow and rapid freezing of oocytes

2.4 Vitrification of embryos

2.5 Vitrification of oocytes.

2.6 In vitro maturation of oocytes

2.1 FOLLICULOGENESIS

In the buffalo ovaries the follicular population always remain in a continuous dynamic state and at any given time, follicles are present which progress from a stage of quiescence to one, characterised by volumetric growth and increased activity of the oocytes, granulosa and thecal layer (Guraya 1979; Danell 1987). They also reported that follicular growth starts in infancy and

continues during various phases of reproduction including periods of anestrous. Between right and left ovaries the distribution of follicles are binomial and there is no convincing evidence for alternative ovulation in buffalo. Guraya (1985) also reported that the factors regulating the selective growth of primordial follicles occupying the ovarian cortex are still poorly understood. Rate at which follicles leave the non-growing pool of primordial follicles varies with age. It is highest in young females and falls with attainment of maturity. The progress of separation of individual germ cells i.e. primordial oocytes associated with some pregranulosa cells progressively extends towards the periphery till fully developed fetus, all the ovigenous cord leaving behind primordial follicles. Factors regulating formation of meiotic oocytes from oogonia are still not known. Meiotic inducing substances believed to be formed by rete cells may be responsible for the induction of meiosis, oocyte maturation and follicle formation (Zamboni *et.al.*, 1980). During formation of primordial follicles from ovigenous cord, there is seen an extensive degeneration of germ cells, leaving behind relatively a small number of healthy primordial oocytes in buffalo, but degeneration of germ cells in developing ovary limits the number of primordial follicles.

Danell (1987) reported primordial follicles in the ovaries of buffalo (average number 11,384) were less than that in cattle (average number 50,000). Le-van-Ty *et. al.* (1989) compared the follicular population and the main characteristics of follicular development in the ovaries of 8 Swamp buffaloes and 4 cows from both Indonesia and Vietnam. The overall population of antral follicles in the buffalo was 20 per cent of the cow (47.5 $\pm$ 23.8 vs 233.0 $\pm$ 95.8). It appeared to be related to its low reproductive potential. Many factors have been assumed to be holding the oocytes at dictyate stage of arrest. The factors include gonadotrophin (Tsafriri *et. al.*, 1982; Fulka *et. al.*, 1985) and fluid (Guraya, 1985). At the puberty, the oocytes under the influence of gonadotrophin resume the meiotic process just before ovulation

and following LH surge the oocytes go through extensive changes both in cytoplasm as well as in nucleus (Staigmiller 1988). The final resumption of meiosis occurs when the sperm penetrates the ovum, resulting into formation of female pronucleus and extrusion of second polar body.

Follicular growth in buffalo occurs in wave like pattern and in general there was predominance of two wave follicular activity (Manik *et. al.,*1994a and Baruselli *et.al.*, 1996). The number of surface follicles of various size categories were not constant throughout the estrous cycle (Manik *et.al.*, 1998). Further there was significant difference in the number of follicles 5-6 mm and 7-10 mm in diameter but not in number of follicles 3-4 mm and > 11 mm. Spicer and Echternkamp (1986) also reported in cattle that the rate of growth of small follicles (1to 3 mm) in to large follicles increased as the estrous cycle progresses. Most of the large follicles (10 mm) persist on ovarian surface for day 5 or more. Donaldson and Hansel (1968) and Marino and Gier (1971) stated that the follicular growth is continuous and independent of the phase of the cycle. In one of the study Manik *et.al.*(1994b) reported, 50 per cent of the total ovaries examined using ultrasonography, were devoid of follicles of size 3 mm or more on day 10th of estrous cycle in buffalo.

2.2 RETRIEVAL OF OOCYTES

For successful in vitro fertilization and large scale embryo production the primary requirement is the availability of large number of good quality oocytes. An important aim of oocyte recovery method is to maximise the number of good quality oocytes per ovary which can be employed for in vitro maturation, in vitro fertilization and in vitro culture. There are three different techniques commonly employed to collect follicular oocytes in vitro. These are dissection, aspiration and slicing. Aspiration of the visible surface follicles is a common method of recovering the bovine oocytes.

Aspiration of more than 4600 buffalo ovaries provided only 0.42 good quality oocytes per ovary (Madan *et. al.*, 1994). Totey *et. al.*(1992) reported recovery of 0.43 non atretic (usable) oocytes per ovary after aspirating 12,263 ovaries. Das *et. al.* (1996) reported per ovarian recovery of 0.7±0.10 cumulus oocyte complexes with a total recovery of 1.7±0.18 oocytes through the aspiration of buffalo ovaries. However, Singla (1995) recovered on an average oocytes per ovary 1.57 and 1.55 for ovaries without corpora lutea and with corpora lutea, respectively, with an overall average of one oocyte per ovary of good quality. On the other hand, Nandi *et. al.* (1997) reported an average of 0.60 oocytes per ovary of grade A and B. In comparison in cattle, the number of oocytes per ovary is upto 10 (Gordon and Lu, 1990). The low recovery of oocytes per ovary in buffalo may be attributed to the low average number of primordial follicles in the ovaries.

2.3 SLOW AND RAPID FREEZING OF OOCYTES

Slow freezing is a conventional equilibrium methods using relatively slowly permeating cryoprotectants and slow controlled cooling and relatively fast warming rate. Conventional slow freezing procedures have the following distinctive steps (Palasz and Mapletoft, 1996).

1. Oocytes/embryos are exposed at room temperature to molar concentration of low molecular weight permeating cryoprotectants. Such as ethylene glycol, glycerol, dimethyl sulphoxide or propylene glycol until equilibrium is reached between cryoprotectant solution and oocytes or embryos.

II. Induction of ice crystal (seeding), at -5° to -7° C.

III. Controlled, slow cooling (0.2° to 2.0°C/min).

IV. Plunging into liquid nitrogen (-196°C) at -30° to -70°C and storage.

V. Removal of cryoprotectant at room temperature prior to culture or transfer.

Immature bovine oocytes were apparently sensitive to a sucrose or trehalose treatment than fertilized eggs, upon freezing and thawing, only very few oocytes (6%) could be matured in vitro (Hayman *et. al.,* 1986). Schroeder *et. al.* (1990) assessed the survival and developmental capacity of the cumulus cell enclosed mouse oocytes frozen at Germinal Vesicle (GV) stage. Survival rate was 69%, after insemination in vitro, 9% of frozen thawed GV stage oocytes cleaved to 2 equal blastomeres, but none developed to blastocysts.

Das (1993) cryopreserved immatured buffalo oocytes using 1,2, propanediol, glycerol and dimethyl sulphoxide as cryoprotectant, by slow freezing and found that normal recovery rate was 77.4 to 86.3 per cent, 72.72 to 80.48 percent and 77.7 to 89.6 per cent respectively. Hochi *et. al.* (1994) showed that immature equine oocytes mature to Metaphase-II (M-II) stage in vitro following freezing and thawing in ethylene glycol or 1,2 propanediol but not in glycerol.

Yang *et. al.* (1994) conducted the study to determine the developmental competence of cryopreserved bovine oocytes at GV stage as well as their subcellular damages. Cumulus oocyte complexes from antral follicles of bovine were frozen in 1.0 M glycerol and 1.2 M glycerol. The morphologically normal recovery of oocytes were significantly higher in 1.0 M glycerol (80.9%) than 1.2 M glycerol (78.4%). But in vitro fertilization rates were only 10.0 and 7.1 per cent in 1.0 M glycerol and 1.2 M glycerol, respectively. It was concluded that the developmental competence of cryopreserved GV stage oocytes were severely impaired because of extensive and severe ultrastructural damage and only a very small proportion can complete maturation, fertilization and cleavage in vitro.

Gal (1996) investigated the ability of immature goat oocytes to undergo in vitro maturation (IVM) and fertilization (IVF). Fully grown germinal vesicle stage goat oocytes were submitted to different variables of

cryopreservation: i) exposure to propanediol before maturation but without freezing to detect the level of damage attributable to propanediol alone ii) removal of cumulus cell to mimic damage attributable to osmotic stress during cryoprotectant exposure or freezing procedure, and iii) rapid freezing with propanediol. Maturation and fertilization rates were 82.1, 71, 65.3 and 23.7 per cent and 71.2, 40, 58.4 and 23.1 per cent for control, exposed, denuded and frozen oocytes, respectively. These results indicate that freezing sticto sensu (i.e. cooling and worming process) had detrimental effects on IVM of GV stage oocytes where as the reduced IVF rates of post thaw matured oocytes were imputable to a cryoprotectant effect.

The immature bovine oocytes at the GV stage were sensitive to direct chilling injury which causes irreversible damage, which appear shortly after exposure to low, but not freezing temperature and mostly its primary target being the cytoplasmic membrane (Zeron and Arav, 1996). The effect of butylated hydroxy toluene (BHT) on the survival of bovine GV stage oocytes exposed to low temperature of 4^0C was studied. Results revealed that BHT protects bovine oocytes from chilling injury only when cooling rate was slow ($< 1^0$C/min) and increasing the storage time from 4 to 18 hour resulted in very low maturation rate and no fertilization.

Suzuki *et. al.* (1996) studied the fertilization and cleavage rates of in vitro frozen thawed bovine oocytes at germinal vesicle stage (GV). Effect of various permeating cryoprotective agents (1.8 M ethylene glycol (EG), 1.3 M ethylene glycol monomethyl ether (EME) and 1.6 M 1,2-Propanediol (PROH), different concentrations of trehalose (T) and polyvinylpyrolidone (PVP) on post thaw developmental capacity were examined. When bovine GV stage oocytes were frozen slowly in a mixtures of 1.8 M EG plus 5% PVP and 0.05 M T, almost 80% of them developed to metaphase and 22% were degenerated after in vitro maturation and none of those cryopreserved underwent parthenogenetic activation. The fertilization rate was higher for oocytes frozen in a mixture of 1.8 M EG plus 0.05 M T or 0.1 M T.

In case of rapid freezing, a rapid cooling rate of 1250°C per minute is applied to the oocytes that have been partially dehydrated before cooling. A prerequisite for successful cryopreservation of oocytes by rapid freezing method is a cryoprotectant mixture composed of a 2 to 4.5 M solution of a permeating cryoprotectant such as glycerol, propanediol. dimethyl sulphoxide or ethylene glycol, and 0.25 to 0.5 M of a nonpermeating cryoprotentant such as sucrose, trehalose, lactose or galactose. After a short equilibration time (30 sec. to 180 sec.), oocytes in a partially dehydrated state are cooled to intermediate temperatures in liquid nitrogen vapour after which they are plunged into liquid nitrogen. In contrast to vitrification, under this condition intracellular ice formation is possible (Palasz *et. al.*, 1996).

Very few reports are available in the field of rapid freezing of oocytes. The best survival (58.3%) and fertilizability rate (19.0%) were obtained by holding the mouse oocytes with 2.0 M PG at -40°C for 30 min. and by storage at -196°C for one day. Thirty minute holding at -40°C reduced oocyte damage during the procedure but not significantly. This study demonstrated that mammalian oocytes could be cryopreserved in the presence of PG by utilizing a rapid freezing and thawing procedure. (Ko *et. al.*, 1988).

Rayos *et. al.*(1994) frozen unfertilized mouse oocytes by directly plunging them into liquid nitrogen vapour after equilibration in a freezing medium containing 3 M EG + 0.25 M sucrose or trehalose l^{-1} for 5 to 40 min. Higher fertilization rates were obtained with equilibration in 3 M EG + 0.25 M sucrose or trehalose l^{-1} for 20 and 40 min than with 5 and 10 min. in vitro.

2.4 VITRIFICATION OF EMBRYOS

Vitrification is a term used in the cryopreservation of cells, tissues, organ at low temperature by creating a completely vitreous (glass) state (i.e. ice crystal free freezing). In order to achieve vitrification at the practical cooling rates (direct plunge into liquid Nitrogen; -2500°C/min), multimolar

concentrations of commonly used permeating cryoprotectants which may also be toxic to cells are necessary. Theoretically vitrification can be achieved with a 1.5 M concentration of any cryoprotectant providing a cooling rate of 15,000°C/min. (Palasz and Mapletoft, 1996)

Shaw *et. al.* (1991) investigated the viability of 2-cell mouse embryos rapidly frozen in DMSO solutions with brief equilibration of 3 min. Cryoprotectant solution containing 4.5 M DMSO vitrified throughout on cooling but ice form on warming, 90 to 91 per cent of embryos formed blastocysts in vitro and 77 to 78 per cent form normal fetuses and no chromosomal rearrangements were found.

Tada *et. al.* (1992) vitrified mouse embryos in either 2.75 M DMSO+2.75 M PG+1.0 M sucrose or 2.75M DMSO+2.75M PG+0.16M raffinose vitrification solution. They found in vitro survival rate of 82% and 81% respectively. The vitrification solution containing 5.5 M EG l^{-1} and 1.0M sucrose l^{-1} was a good vitrifying mixture and was non toxic to mouse embryos when they were exposed upto 30 min. (Ali and Shelton, 1993).

Ishimori *et.al.* (1993) conducted the experiment in which embryos were exposed to an equilibration solution (50% VSED) containing 12.5% V/V ethylene glycol and 12.5% V/V dimethyle sulphoxide in modified dulbecco's phosphate buffered saline (m-PBS) with 4 mg/ml BSA for 1,2,5 minutes at room temperature. The embryos were then placed in a 15 μl vitrification solution (VSED) containing 25% V/V ethylene glycol and 25% V/V dimethyle sulphoxide in m-PBS with 4 mg/ml BSA and loaded into 0.25 ml plastic straws at room temperature. After 30 sec. straws were placed into liquid nitrogen vapour for 2 minutes and then plunged into liquid nitrogen and stored. Viability of embryos were assessed by the forming or reforming of the blastocele after 24 hour of culture. The in vitro survival rates (70-90%) of vitrified embryos were higher after 1 and 2 minute equilibration than 5 minute equilibration.

Mahmoudzadeh *et. al.* (1993) reported the effect of vitrification on in vivo produced bovine embryos using one step addition of different vitrification solutions. In experiment-I, embryos were vitrified in 2.6 M DMSO+2.6 M acetamide + 1.3 M propylene glycol+7.5 mM polyethylene glycol (30 sec. exposure), in experiment-II, embryos were vitrified in 3.4 M glycerol+3.4M propylene glycol (2 min. exposure) and in experiment-III, embryos were vitrified in 7.15 M ethylene glycol +2.5 mM ficoll+0.3 M sucrose (3 min. exposure). The survival rates were 3.6, 0 and 88.7 per cent in the three experiments respectively. In experiment-III cryoprotectant was diluted in 0.5 M sucrose. A low concentration of sucrose for dilution of ethylene glycol was also found to reduce the chance of possible osmotic injury due to dehydration.

In vivo derived bovine blastocyst can be successfully vitrified in a solution of EG and sucrose without macromolecule. Liu *et. al.* (1996) reported that fast freezing yielded significantly higher survival rate than controlled slow freezing and vitrification while the percentage of normal appearing post thaw embryos were higher in vitrification than other methods. The embryos and oocytes can also be cryopreserved using vitrification following centrifugation to separate the lipid droplets from cytoplasm. However, centrifuged porcine embryos and oocytes vitrified appeared to be less viable than delipated embryos and oocytes (Nagashima *et. al.*, 1996).

The ovine morulae and blastocysts were vitrified in propylene glycol and ethylene glycol + ficoll + sucrose. The developmental percentage was 58 and 54 per cent, respectively. The corresponding values for hatching percentage was 34 and 30 (Martinez *et. al.*, 1996). In vitro survival rate of 81.5, 73.8 and 86.6 and hatching rate of 81.8, 73.3 and 69.1 per cent were obtained using ethylene glycol based vitrification solution (Delval *et. al.*, 1996).

Leeuw *et.al.*(1997) conducted a field trial to obtain accurate pregnancy rate of day-7 bovine embryos after vitrification in a solution containing 6.5M glycerol and 60% BSA (W/V) and one step dilution in 1M sucrose compared with controlled slow freezing in 1.5M glycerol and three step dilution. The overall pregnancy rate was 44.5% for vitrified embryos and 45.1% for slowly frozen embryos.

The use of heat stable plant protein (HSPP) in an ethylene glycol based solution for the vitrification of in vitro derived day 7,8 and 9 embryos were examined (Palasz *et.al.*, 1996). The use of HSPP at a concentration of 25 mg/ml in the vitrification medium did not effect the post thaw embryo survival over that of no protein supplementation.

2.5 VITRIFICATION OF OOCYTES

Kola *et.al.* (1988) reported an increase in occurrence of aneuploidy at the first cleavage division in vitrified mouse oocytes. Wood *et.al.* (1991) observed a high incidence of post implantation loss in embryo derived from oocytes vitrified in a solution containing DMSO. Only DMSO depolymerises the microtubules of the meiotic spindle in mammalian oocytes, but normal segregation of chromosomes were seen after the resumption of meiosis in oocytes frozen in low concentration of DMSO.

Hamano *et.al.* (1992) conducted vitrification study on bovine oocytes. In vitro matured (IVM) cattle oocytes were vitrified in a mixture of 2.0M DMSO+1.0M acetamide+3.0M PG, dissolved in medium-199. After thawing, cryoprotectants were removed either in 1 step (2M sucrose) or in 12 steps of descending sucrose concentration. The removal of cryoprotectants in 1 step resulted in no development beyond 4-cell stage. For oocytes subjected to the 12-step removal of cryoprotectant, 88 of 90 inseminated oocytes developed to blastocyst stage. Three blastocysts were transferred to 3 recipient, resulting in 2 pregnancies.

Tachikawa *et.al.* (1993) conducted the study on in vitro matured cattle oocytes by using EFS, GFS and PFS vitrification solution. These solution contained 40 percent EG, GLY and PG, respectively diluted in modified PBS containing 30% ficoll+0.5M sucrose. After 1 or 2 minute exposure, the survival rate was 74 to 77 percent in EFS vitrification solution and 53 percent in PFS after one minute exposure.

Miyake *et.al.* (1993) vitrified mouse embryos and oocytes in an ethylene glycol based vitrification solution. At the GV stage, the morphologically normal oocytes were 36 to 39% in cumulus enclosed oocytes, where as in cumulus removed immature oocytes it was only 2 to 4 percent.

Yang *et. al.* (1994) vitrified cumulus enclosed bovine oocytes (COC) at GV stage. After thawing, COC were cultured in an atmosphere of 5% CO_2 in air, 39°C for 22 hour and fertilized in vitro. Morphologically normal rate of vitrified oocytes was 94.1 per cent and fertilization rate was 12.1 per cent.

Fuku *et.al.* (1994) showed that vitrification or ultra-rapid freezing has been used successfully for cryopreservation of mouse oocytes using a mixture of 2M DMSO, 1M acetamide, 3M propanediol and 10 per cent FCS in TCM-199 (DAP213). However, they reported that due to species differences and lower yields of viable embryos from in vitro procedures, the overall success of such techniques for bovine oocytes remained poor.

Saha, *et.al.* (1996) vitrified IVM and pronuclear oocytes with three types of vitrification solutions. Oocytes were vitrified in precooled (4^0C) VS containing 40% ethylene glycol+0.3 M trehalose+20% polyvinylpyrrolidone in Dulbecco's Phosphate Buffered Saline negative ($DPBS^-$), positive ($DPBS^+$) and sodium chloride supplemented with 10% superovulated cow serum (SCS) and 0.3% bovine serum albumin (BSA). The cleavage rates were 27%, 18% and 14% for IVM oocytes and 23%, 21% and 13% for pronuclear oocytes in three vitrification solutions, respectively after one week of storage.

Nag and Maurya (1997) studied the vitrification of buffalo oocytes. Compact cumulus oocyte complexes were collected from ovaries procured from local abattoir and were exposed to the cryoprotective agent (DMSO/EG/PG) in gradually increasing concentration from 0.6M to 2.4M for 3 min each before placing them in 3M concentration of the cryoprotectant in PBS supplemented with 0.25M sucrose and 4% BSA. The maturation rate was 34.0, 43.23 and 32.62 per cent of oocyte in 3 cryoprotectant groups, respectively.

2.6 IN VITRO MATURATION OF FOLLICULAR OOCYTES

Immature oocytes have been induced to mature in vitro either by culturing oocytes within the isolated follicles (intrafollicle culture) or by removing them from follicles before culturing (extrafollicular culture). The aspiration of oocytes from follicles is one of the most convenient method of harvesting oocytes. When the oocytes are removed from this follicular environment they undergo spontaneous resumption of meiosis. The literature has been scanned on maturation rate for both type of oocytes i.e. oocytes not subjected to freezing and oocytes subjected to freezing protocals and then thawed and matured.

It is known fact that successful fertilization whether in vitro or in vivo requires proper maturation of oocytes. Culture medium is one of the important factors in maturation of follicular oocyte in vitro. As a base medium, TCM-199 (Moor and Trounson, 1977) and TALP (Crister *et.al.*, 1984) have been extensively used for maturation either exclusively or in a modified way through chemical and biological supplementation. The oocytes with compact cumulus cells, have higher IVM rates, fertilization, cleavage capacity and developmental competence than that of partially denuded and denuded oocytes (Shioya *et.al.*, 1988; Madison *et.al.*, 1992; Suzuki *et.al.*, 1992; Blondin and Sirard, 1995).

Gonadotrophins may play an important role in the maturation of follicular oocytes. Addition of gonadotrophins (LH+FSH) and estradiol (E2) increased the maturation rate significantly in cattle (Fukui *et.al.*, 1982; Fukushima and Fukui, 1985).

The addition of FSH to maturation medium has been found to increase the maturation of buffalo oocytes (Singh *et.al.*, 1989). Corresponding improvement of maturation in Ham's F-10 and TC-199 media over control group was 23.19 and 53.0 per cent, respectively. Singh *et.al.* (1989) reported that when oocyte matured in Ham's F-10 or TC-199 with 10% FCS and FSH(10μg/ml), the maturation percentage was 83.4 and 86.0 for the two above medium, respectively. The addition of estrous cow serum (ESC) or fetal calf serum to medium significantly increased the percentage of bovine oocyte maturation over that Ham's F10 medium alone (Lambert *et.al.*, 1986).

Fetal bovine serum (FBS) added in maturation medium has been reported to contain gonadotrophin, estrogen and a number of growth factors (EGF and TGF-α) which have an important role in regulation of oocyte maturation via cumulus cell (Skinner, 1990). Totey *et.al.* (1991) reported that hormonal supplementation of gonadotrophins (FSH+LH) and estradiol (E_2) was essential for maturation of buffalo oocytes. They observed that maturation rate in these oocytes was 40 to 60 per cent.

Madan *et.al.* (1994) studied the maturation of buffalo COC oocytes in culture medium containing TCM-199 supplemented with 5% and 10% Buffalo estrus serum (BES) for 22 to 24 h. The percentage of oocyte maturation (M-II phase) were 64.77 and 75.87 for 5% BES and 10% BES, respectively. Das *et.al.* (1996) reported that there were no significant differences between the maturation rates obtained with FBS+FSH and buffalo follicular fluid (BFF) (20% and 40%). The result indicated that both FBS and FSH can be completely replaced by 20% BFF to obtain in vitro maturation rates, comparable to those obtained by supplementation of culture media with 10% FBS and 10% FBS+FSH.

The very few reports are available for maturation rate of frozen/ thawed oocytes. Schroeder *et. al.*(1990) reported that the mouse oocytes frozen at the GV stage were matured in vitro but their developmental capacity were severely impaired. Whereas, in vitro matured oocytes which were treated with FSH during maturation, greatly improved the capacity to cleave and developed to blastocyst after freezing and thawing.

Das (1993) cryopreserved immature cumulus enclosed oocytes in three different cryoprotectant solutions 1,2, propanediol (2.0 M), DMSO (2.5M) and glycerol (10%) through controlled slow freezing. After thawing oocytes were cultured in TCM-199 supplemented with super ovulated estrus serum for 24 to 26 hrs. The maturation rates of 16.39, 14.41 and 12.06 per cent were found for the above three cryoprotectant solutions, respectively.

Yang *et.al.* (1994) vitrified bovine COC oocytes. They reported the maturation rate of 20 per cent and the fertilization rate of 12.1 per cent in post-thaw vitrified oocytes. They also reported that the developmental competence of cryopreserved GV stage oocytes were severely impaired because of the extensive and severe ultrastructural damage.

Nag and Maurya (1997) vitrified compact cumulus oocyte complexes in DMSO, ethylene glycol and propylene glycol. After thawing oocytes were cultured in TCM-199 supplemented with 10% FCS, ovine LH 10 μg/ml, ovine FSH 1 μg/ml and estradil-17ß 1 μg /ml. After 24 hour culture the maturation rates were 34.0, 43.23 and 32.62 percent for DMSO, ethylene glycol and propylene glycol, respectively.

CHAPTER - 3

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The work reported in the body of this dissertation regarding oocyte freezing using vitrification procedure involves many items/steps that are used for conventional embryo transfer and in vitro fertilization programme. The steps involved in oocyte freezing and culturing are entirely different from the steps of semen freezing and therefore essential requirements are in line with which are used for embryo transfer and in vitro fertilization programme.

3.1 SUPPLIES

All disposable materials like different kinds of petridishes, straws, vials, syringes and other containers used for embryo transfer are useful for oocyte vitrification and in vitro maturation. The handling of oocytes was performed by 10 μl unopettes (Becton-Dickinson Vacutainer Systems, NJ, USA) alongwith low pressure rolling pipetters (Micro-pipex, Germany) and glass mouth pipettes (Travenol Lab. Inc., McGraw Park IL, USA). The glasswares used during experiment were made of pyrex glass. The glasswares were cleaned with mild detergent and ultrapure water and sterilized in hot air oven. All plasticwares used were of tissue culture grade. Falcon products manufactured by Becton Dickinson Labware, Lincoln Park, NJ, USA were used throughout the study. These were made of specially formulated, high quality polystyrene. All the plasticwares were used once and discarded. The 0.22 μm and 0.45 μm filters were obtained from Millipore Corporation, Bedford, MA, USA.

The Ethylene glycol (EG) was obtained from Analytical Rasayan, S.D. Fine-Chem. Ltd., Boisar-401501. Dimethyl sulphoxide (DMSO), Sucrose and D-glucose were obtained from Sisco Research Laboratories Pvt. Ltd., Bombay-400 060, India. Methanol and glacial acetic acid were obtained from Qualigens Fine Chemicals, Glaxo India Ltd., Bombay-400 025.

Ready-made media in liquid form were readily available from Sigma chemicals Co., St. Louis, MO., USA. For TCM-199, a product of catalogue number of M-7542, with L-amino acid, EBSS, 25 mM HEPES and $NaHCo_3$, sterile filtered and endotoxin tested was procured. For Dulbecco's Modified Phosphate Buffered Saline (DPBS), product of catalogue number D-4031, sterile filtered, endotoxin tested with sodium pyruvate, streptomycin sulphate, kanamycin monosulphate, glucose and calcium chloride was procured.

Fetal bovine serum used in the study was procured from Sigma chemicals Co., USA., catalogue number F-2442. The FSH-P was obtained from Schering-Plough Animal Health Corp., Kenilworth, NJ 07033. Bovine serum albumin, (lyophylized), was obtained from Sigma chemicals Co., USA, catalogue number A-8551.

3.2 PREPARATION OF SOLUTIONS FOR OOCYTE COLLECTION AND CULTURE

3.2.1 NORMAL SALINE

0.9 % sodium chloride in distilled water-sterilized.

3.2.2 ESTRUS SERUM

Blood was collected from the estrus buffalo under sterilized conditions and serum was collected after clotting and centrifugation. Serum was heat inactivated at 58°C for 30 min, filtered through 0.45 μm filter and then preserved at -20°C in aliquots of 10 ml.

3.2.3 ASPIRATION/OOCYTE COLLECTION MEDIA

Composition	-	**200 ml**
TCM-199 (HEPES buffered)	-	95 ml
DPBS	-	100 ml
BES/FBS	-	5 ml
Bovine serum albumin, lyophylized (BSA)	-	0.6 g

3.2.4 WASHING MEDIA

Composition	-	60 ml
TCM-199	-	54 ml
BES/FBS	-	6 ml

3.2.5 OOCYTE CULTURE MEDIA

Composition	-	20 ml
TCM-199	-	17 ml
FBS	-	3 ml
FSH-P	-	20 μl

During the preparation of above three media, all the components were mixed well and incubated in a CO_2 incubator at 38.5°C and 5 per cent CO_2 for one hour for stabilization of pH and temperature before further use. All the media were filtered through 0.22 μm filter just before use.

3.3 PREPARATION OF SOLUTIONS FOR VITRIFICATION

3.3.1 VITRIFICATION SOLUTION-1 (VS-1)

VS-1 consist of 4.5 M EG,3.4 M DMSO, 0.33 mM sodium-pyruvate, 5.56 mM glucose and 4 mg/ml BSA in DPBS.

Composition	-	50 ml
DPBS	-	25 ml
EG	-	12.5 ml
DMSO	-	12.5 ml
BSA	-	0.2g
Glucose	-	0.05 g
Sodium pyruvate	-	1.81 mg

3.3.2 VITRIFICATION SOLUTION-2 (VS-2)

VS-2 consist of 3.5 M EG, 3.4 M DMSO, 0.33 mM sodium pyruvate, 5.56 mM glucose and 4 mg/ml BSA in DPBS.

Composition	-	**50 ml**
DPBS	-	27.73 ml
EG	-	9.77 ml
DMSO	-	12.5 ml
BSA	-	0.2 g
Glucose	-	0.05 g
Sodium pyruvate	-	1.81 mg

During preparation of vitrification solutions first required volume of DPBS was taken into a glass beaker. Add the amount of glucose, sodium pyruvate and BSA into the same beaker and wait for 10 minutes and allowed them to dissolve completely into DPBS. Then add required volume of EG and DMSO into the solution and the beaker was kept at 4°C. The solutions were filtered through a 0.22 μm filter just before use. Fresh vitrification solutions were prepared during every trial.

3.3.3 DILUTION MEDIA

0.5 M sucrose solution was used as dilution media to remove cryoprotectants from oocytes after thawing.

Composition	-	**25 ml**
DPBS	-	25 ml
Sucrose	-	4.27 g

3.4 PREPARATION OF SOLUTIONS FOR OOCYTE FIXATION AND STAINING

3.4.1 HYPOTONIC SOLUTION

0.9 per cent tri-sodium citrate used as hypotonic solution.

Composition	-	**10 ml**
Distilled water	-	10 ml
Tri-sodiumcitrate	-	0.09 g

3.4.2 FIRST FIXATIVE

First fixative contained methanol, glacial acetic acid and distilled water in the ratio of 5:1:4, respectively.

Composition	-	**10 ml**
Methanol	-	5 ml
Glacial acetic acid	-	1 ml
Distilled water	-	4 ml

3.4.3 SECOND FIXATIVE

Second fixative contained methanol and glacial acetic acid in the ratio of 3:1, respectively.

Composition	-	**4 ml**
Methanol	-	3 ml
Glacial acetic acid	-	1 ml

3.4.4 GIEMSA STAIN

Modified Giemsa stain from Sigma Chemicals was used for staining. Two per cent Giemsa stain was used for oocyte staining.

Composition	-	**50 ml**
Giemsa stain	-	1 ml
Distilled water	-	49 ml

3.5 COLLECTION AND TRANSPORTATION OF OVARIES

Ovaries were collected from old Delhi slaughter house which is 130 km away from the laboratory. Ovaries were collected from water buffalo randomly, irrespective of their body condition and reproductive status just after slaughter. After removing extra tissues they were washed thrice with warm normal saline (32°-37°C), containing 400 IU/ml penicillin and 500 μg/ml streptomycin. Washed ovaries were then put into a thermoflask in warm normal saline (32°-37°C), containing same antibiotics at the same dose level. The collected ovaries were transported to the laboratory within 4-5 hours.

3.6 ASPIRATION OF OOCYTES

Ovaries were washed in the laboratory 2-3 times with warm normal saline at 37°C and then dried with sterilized blotting paper. A 5 ml sterilized glass syringe attached to a 19 gauge needle was used for oocyte aspiration. Oocytes were aspirated from all visible surface follicles of 3-8 mm diameter. One ml of aspiration media was taken into the syringe and needle inserted into the surface follicle from back side and the oocytes along with the follicular fluid were aspirated. The aspirated fluid alongwith the oocyte collection media was collected in a sterilized oocyte searching dish.

3.7 SEARCHING OF OOCYTES

The oocytes were searched from the dish with the help of a zoom stereomicroscope (Nikon, Japan). All the oocytes were picked up from the dish with the help of a 10 μl unopettes alongwith a low pressure rolling pipetters and placed in a 35 mm culture petri dish containing 4 ml of washing media. All the oocytes were then washed 5-6 times with washing media.

3.8 GRADING OF OOCYTES

Gradation of oocytes were made on the basis of their morphology and integrity of cumulus cells as described below.

Grade A: Cumulus oocyte complexes with 4 or more layers of compact cumulus cells surrounding the zona pellucida and having an evenly granulated cytoplasm.

Grade B: Cumulus oocyte complexes with 1 to 3 layers of cumulus cells surrounding the zona pellucida and having an evenly granulated cytoplasm.

Grade C: Oocytes partially devoid of cumulus mass or having expanded cumulus mass.

For the present study, only above mentioned quality oocytes, irrespective of grade were taken.

3.9 VITRIFICATION PROTOCOL

Two vitrification solutions were used for the study. Vitrification solution-1 (VS-1) contained 4.5 M EG, 3.4 M DMSO, 0.33 mM sodium pyruvate, 5.56 mM glucose and 4 mg/ml BSA in DPBS. Vitrification solution-2 (VS-2) contained 3.5 M EG, 3.4 M DMSO, 0.33 mM sodium pyruvate, 5.56 mM glucose and 4 mg/ml BSA in DPBS. Fifty per cent of each vitrification solution (VS-1 and VS-2) in DPBS was used as equilibration medium. Vitrification procedure was same for both the vitrification solutions and has been described in the Fig.1. Good quality aspirated oocytes (Grade A, Grade B and Grade C) were taken and washed properly with washing medium. After proper washing, oocytes were equilibrated in the equilibration medium (50% vitrification solution) either for one minute or three minute. Then oocytes were put into a 15 μl drop of vitrification solution (100%) and immediately loaded into a 0.25 ml French straw containing 150 μl of 0.5 M sucrose solution in DPBS. The loading was done as indicated in the Fig.1. Four to five oocytes were loaded in each straw. After loading, the straws were sealed with hot forceps and precooled by keeping the straw in liquid nitrogen vapour

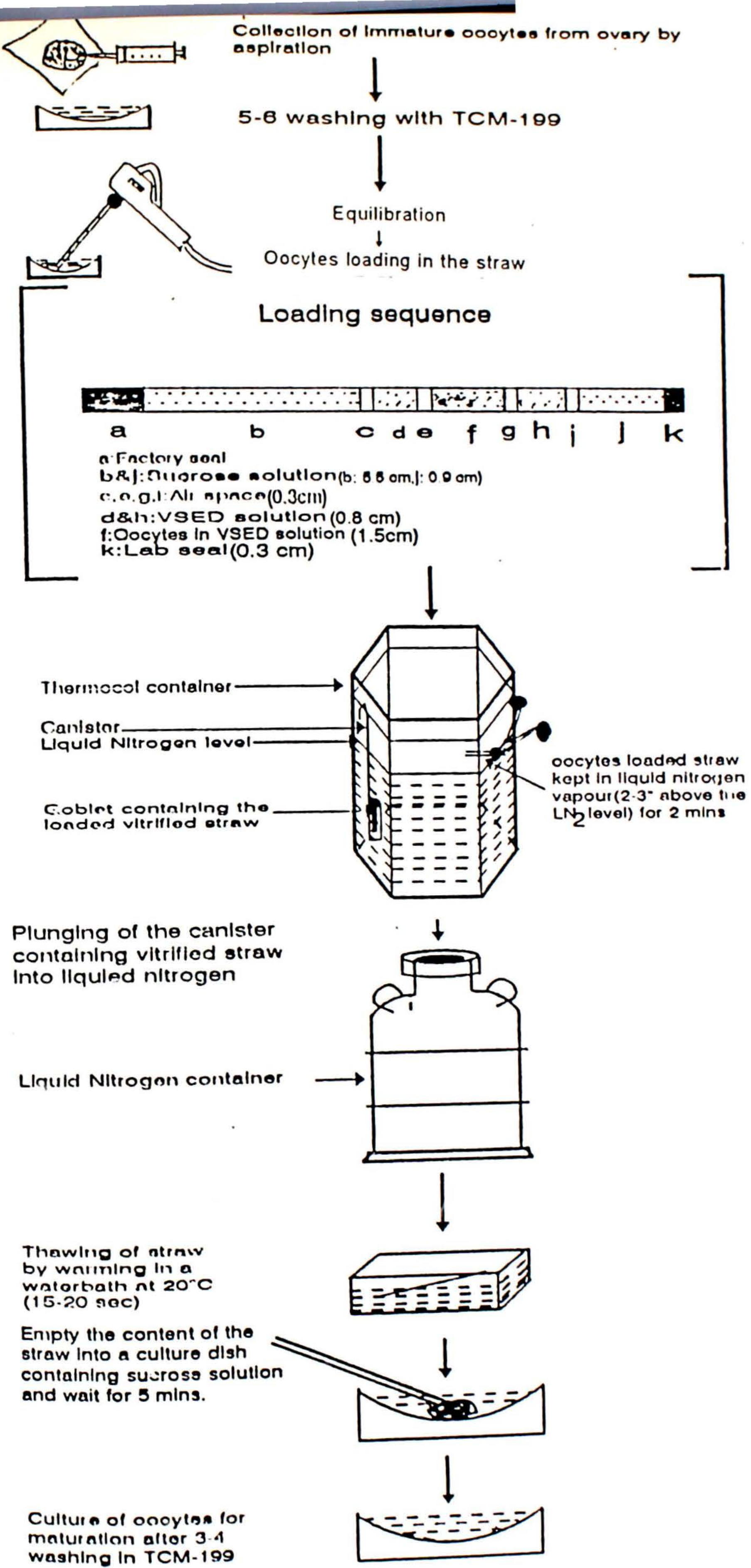

Fig:1 Schematic representation of the different steps of the vitrification procedure and thawing.

for 2 minutes at the height of about 5 cm from the liquid nitrogen level and then the straws were plunged into liquid nitrogen and stored for minimum of seven days before thawing.

3.10 THAWING

Straws were taken out from liquid nitrogen container with the help of forceps and transferred to a water bath at 28°C for 20 seconds.

3.11 REMOVAL OF CRYOPROTECTANT

The contents of the straws were expelled into a empty plastic dish and the vitrified oocytes were allowed for a five minute equilibration with 0.5 M sucrose + DPBS dilution solution. After five minutes oocytes were transferred into washing media.

3.12 POST-THAW EVALUATION OF OOCYTES

The oocytes were evaluated for their morphology and freezing injury as described below.

Normal : Oocytes with spherical and symmetrical shape with no sign of lysis, membrane damage, swelling, vacuolization, degeneration or leakage of the cellular content.

Abnormal : Oocytes exhibiting a ruptured zona pellucida or ruptured vitelline membrane, with sign of degeneration and fragmented cytoplasm.

3.13 CULTURE OF OOCYTES

Normal oocytes recovered after thawing were taken for culturing. The oocytes were put into a 50 μl drop of culture medium in a 35 mm plastic culture dish and covered with sterilized preincubated (at 38.5°C and 5 per cent CO_2) mineral oil to check the media evaporation and microbial contamination. Ten to fifteen oocytes were taken in a single culture drop. The oocytes were incubated in a CO_2 incubator (Forma-Scientific Model 3194) at 38.5°C under 5 per cent CO_2 and high humidity for 26 hours. Immature non vitrified oocytes were also cultured as control.

3.14 EVALUATION OF OOCYTE AFTER CULTURE

Oocytes were evaluated after culturing for cumulus expansion and then maturation was confirmed by staining of oocytes.

3.15 PREPARATION OF OOCYTES FOR STAINING

3.15.1 DENUDATION OF OOCYTES

After 26 h of culturing, oocytes were completely freed from cumulus cells mechanically by vigorous pipetting the cumulus oocyte complexes in and out through a mouth pipette which had an inner diameter slightly larger than the diameter of an oocyte in 35 mm culture petri dish containing preincubated oocyte culture medium (at 38.5°C and 5 per cent CO_2). Care was taken to avoid the rupture of zona pellucida.

3.15.2 FIXING OF OOCYTES

After complete denudation, oocytes were fixed on clean slides. Oocytes were first transferred to 100 μl droplet of hypotonic solution (0.9 per cent tri-sodium citrate in distilled water) and kept for 10 minutes at normal room temperature. Oocytes were then transferred in a 50 μl droplet of chilled fixative I (Methanol:Acetic acid:Distilled water:: 5:1:4) for 5 minutes. Then chilled fixative II (Methanol:Acetic acid::3:1) was applied drop-wise. Soon after the first drop was placed on the slide, the oocytes disappeared and as the fixative began to evaporate, they reappeared and were soon covered by another drop of fixative II. In this manner, about 8-10 drops of fixative II were applied and the last drop was allowed to dry completely resulting in sticking of the oocytes on the slide.

3.15.3 STAINING OF OOCYTES

The slides were stained with 2 per cent Giemsa stain. In a coplin jar, one ml Giemsa stain and 49 ml distilled water were taken and mixed well.

Slides were then put into the jar for 30 min. After 30 min, the slides were taken out, washed with distilled water and air dried. The air dried slides were then mounted with DPX-mountent.

3.16 OOCYTE EVALUATION FOR MATURATION

After fixation and staining of oocyte, nuclear maturation was evaluated with a compound research microscope (Microphot-FXA, Nikon) under oil immersion lens. Oocytes which were in telophase I and metaphase II were considered as matured oocytes.

Telophase I Two equally spread groups of chromosomes.

Metaphase II Contracted metaphase chromosomes and the first polar body.

3.17 STATISTICAL ANALYSIS

Available data for normal recovery and maturation rate after thawing were subjected to arcsin transformation and ANOVA.

CHAPTER - 4

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

4.1 PRESENSE OF VISIBLE SURFACE FOLLICLES

The number of ovaries having surface follicles is a prerequisite to get good number of oocytes for various purposes like cryopreservation, in vitro fertilization, etc. This stress the need to study the number of ovaries with and without surface follicles. The ovaries obtained from slaughter house of Delhi were trimmed off the extra tissues and immediately carried to Karnal in a sterile normal saline. The number of ovaries with and without surface follicles were counted and recorded during each trial. The perusal of Table 1 suggests that in all 883 ovaries were handled during 8 trials. Out of the total ovaries handled, 284 did not had surface follicles, that made 32.17 per cent of total ovaries. On an average only 67.83 per cent of total ovaries had surface follicles, whereas they ranged from 60 to 80 per cent during different trials. The per cent ovaries not having any surface follicles ranged from 20 to 40(Table 1).

There are few studies which have dealt extensively with ovarian follicular development during bovine estrous cycle. On the basis of histological evaluation of the ovaries, Danell (1987) reported an average number of 11,384 primordial follicles in cyclic buffalo heifer, which is much lower than 50,000 previously reported in cattle. He observed more atresia in buffalo follicles (66.66%) than in bovine follicles (50%). Le Van Ty *et. al.* (1989) recorded the number of antral follicles in Swamp buffalo, they found only 20% of those observed in cattle under similar conditions (47.5±23.8 vs 233.0±95.8). Ovarian follicular population develops in a wave like pattern in cattle (Savio *et. al.* 1988; Sirois and Fortune, 1988) andwa buffalo (Manik *et. al.*, 1994a; Baruselli *et. al.* 1997) and the number of small follicles recruited per wave is lower in buffalo than in cattle. Fifty per cent of the total ovaries were devoid of surface follicles of size 3 mm or more on day 10 of estrous cycle in buffalo (Manik *et al.*; 1994b) observed using ultrasound technique. The number of

Table 1. Presence of visible surface follicles (S.F.) among ovaries obtained for oocyte aspiration

Trial No.	*Total No. of ovaries*	*Ovaries with visible S.F. (%)*	*Ovaries without visible S.F. (%)*
1	105	75 (71.43)	30 (28.57)
2	110	76 (69.09)	34 (30.91)
3	100	.80 (80.00)	20 (20.00)
4	100	70 (70.00)	30 (30.00)
5	116	71 (61.21)	45 (38.79)
6	125	75 (60.00)	50 (40.00)
7	116	81 (69.83)	35 (30.17)
8	111	.71 (63.96)	40 (36.04)
Total	**883**	**599 (67.83)**	**284 (32.17)**

surface follicles of various size categories was not constant throughout the estrous cycle and was is less in buffalo as compared to cattle (Manik *et. al.*, 1998). In present study also, about 1/3rd of the ovaries were not having visible surface follicles. This may be attributed to lower number of primordial follicles in buffalo ovaries as suggested by various workers. Thus, there is an urgent need to understand the machanism which controls the recruitment and development of follicles.

4.2 RETRIEVAL OF OOCYTES

Oocyte, in general mean the entity that begins as a single cell and is released from the ovarian follicle at ovulation, later to become fertilized and developed into an embryo. What is ovulated in most mammals is a secondary oocyte, with the first polar body emitted and the chromosomes arranged on the second maturation spindle. However, in in vitro collection of oocytes, ovaries were brought from the slaughter house and individual buffalo ovaries having surface follicles of size 3-8 mm were aspirated for oocytes using a needle of 19 gauge and a glass syringe. It was also assessed to know the potential of these ovaries, in terms of oocyte yield both with cumulus and without cumulus cells.

A total number of 883 ovaries were collected during 8 visits to slaughter house and only 599 ovaries were eligible for oocyte aspiration. The total number of oocytes recovered with and without cumulus cells were 571 and 248, respectively, thus making a total of 819 (Table 2). The total ovaries handled during each trial for this purpose ranged from 70 to 81. Oocytes retrieved with cumulus cells (Figs. 2 and 3) and without cumulus cells (Fig. 4) ranged from 23 to 148 and 10 to 65, respectively and collectively it ranged from 33 to 198. Further on an average only 69.72 per cent of the total oocytes retrieved were with cumulus cells, where as they ranged from 57.58 to 80.25 per cent during different trials. The percentage of oocytes not having any cumulus cells ranged from 19.75 to 42.42, with an overall average of 30.28 per cent(Table 2).

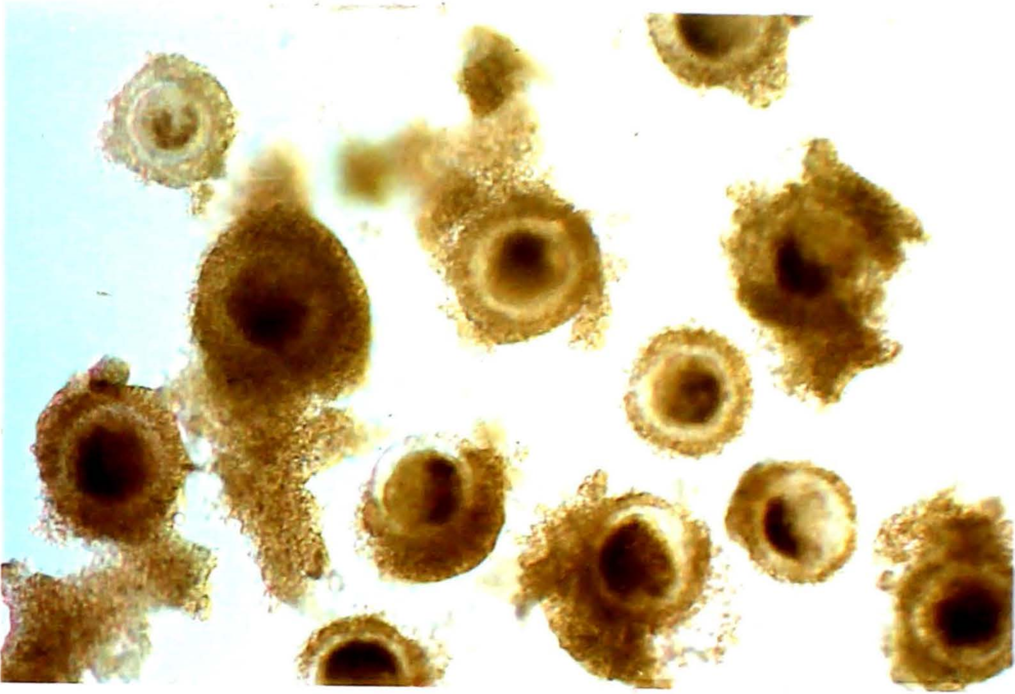

Fig 2 Buffalo cumulus oocyte complexes of grade A, B and C

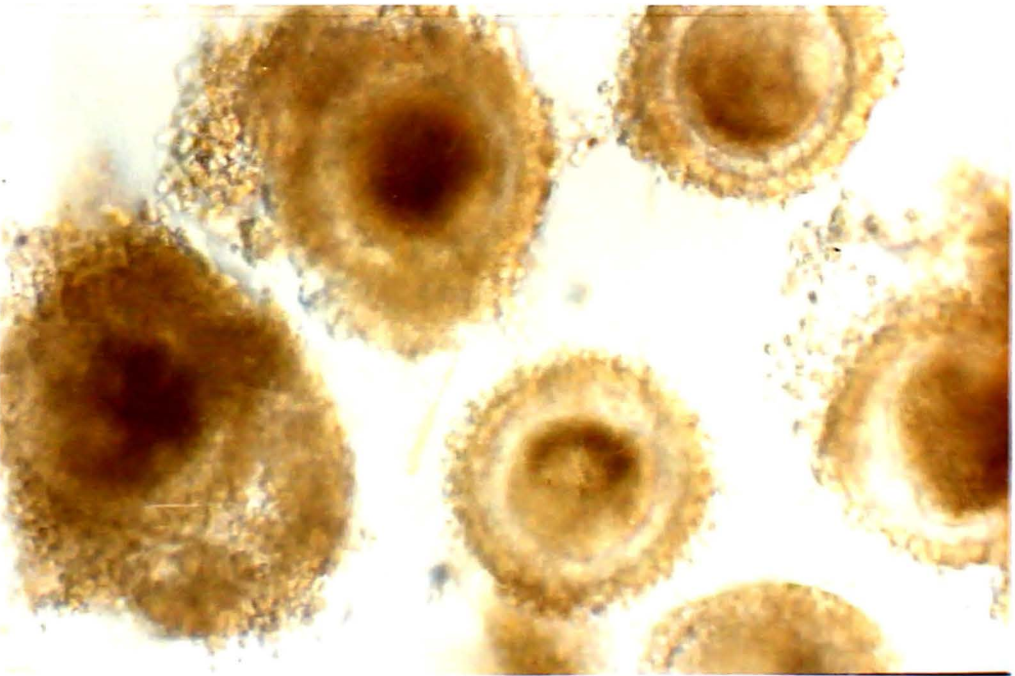

Fig 3 Buffalo cumulus oocyte complexes of grade B

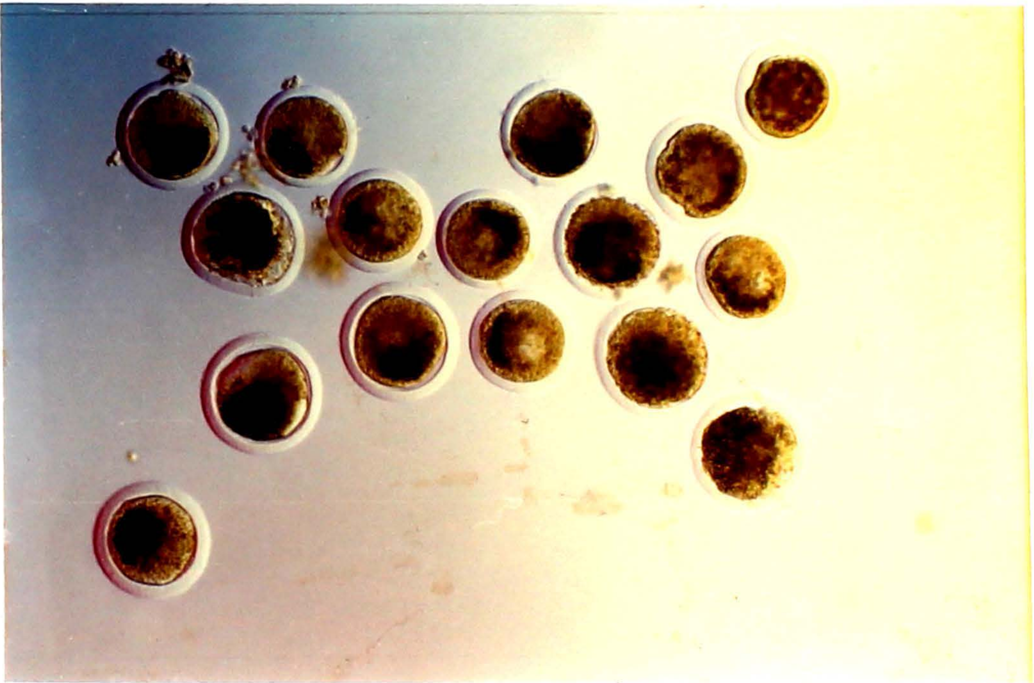

Fig 4 Buffalo oocytes completely devoid of cumulus mass

Table 2. Number and quality of oocytes recovered through the aspiration of visible surface follicles

Trial No.	*No. of ovaries aspirated*	*Oocytes*		*Total*
		With cumulus cells (%)	*Without cumulus cells* (%)	
1	75	23 (69.70)	10 (30.30)	33
2	76	65 (80.25)	16 (19.75)	81
3	80	38 (57.58)	28 (42.42)	66
4	70	58 (64.44)	32 (35.56)	90
5	71	60 (76.92)	18 (23.08)	78
6	75	148 (74.75)	50 (25.25)	198
7	81	65 (69.15)	29 (30.85)	94
8	71	114 (63.69)	65 (36.31)	179
Total	**599**	**571 (69.72)**	**248 (30.28)**	**819**

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Singla (1995) reported that 46.3 per cent of the total oocytes aspired were of grade I and grade II, whereas Suzuki *et. al.* (1992) and Jain *et. al.* (1995) have reported 9.5 and 11.1 per cent of grade I oocytes, respectively. The same authors have reported grade II oocytes to the tune of 17.0 and 37.8 per cent, respectively. Similarly grade III oocytes as reported by Suzuki *et. al.* (1992), Jain *et al.* (1995) and Singla *et: al.* (1995) were 55.2, 51.1 and 27.7 per cent, respectively. The results presented by these workers cannot be directly compared with the present findings, due to the fact that in grade III oocytes, they have taken both denuded and partially denuded oocytes but we have taken only partially denuded oocytes in this category. But in the present study the overall oocyte recovery was lower than that reported by Das *et. al.* (1996), 1.70 $\pm$ 0.18, whereas the recovery of cumulus oocyte complexes 0.7 $\pm$ 0.10 reported by the same workers was lower than the result of the present study. However, results of above workers provide enough indications that our recovery of about 70 per cent oocytes with cumulus cells is in line with the literature reports.

The data were further subjected to calculate per ovarian availability of total oocytes and oocytes with and without cumulus cells (Table 3). The overall oocyte recovery per ovary was 1.37 $\pm$ 0.28 with the range of 0.44 to 2.64 during different trials (Table 4). Oocytes retrieved with cumulus cells ranged from 0.31 to 1.97 per ovary with an average of 0.96 $\pm$ 0.19. The corresponding values for oocytes without cumulus cells was 0.13 to 0.92 and 0.41 $\pm$ 0.09 (Table 4).

Overall yield (1.37) and usable oocytes (0.96) were low in buffalo, as reported in present findings, compared to cattle in which number of usable oocytes recovered per ovary were upto 10 (Gordon and Lu, 1990). This significant difference may be attributed to the smaller number of primordial follicle reserve in buffalo (12000-19000; Samad and Nasseri, 1979) compared with that of cattle (133000; Erickson 1966). Further, it has been established

Table 3. Oocyte recovery per ovary through the aspiration of visible surface follicles

Trial No.	*With cumulus cells*	*Without cumulus cells*	*Total*
1	0.31	0.13	0.44
2	0.86	0.21	1.07
3	0.47	0.35	0.82
4	0.83	0.46	1.29
5	0.84	0.25	1.09
6	1.97	0.67	2.64
7	0.80	0.36	1.16
8	1.60	0.92	2.52

Table 4. Average number of oocytes per ovary recovered through the aspiration of visible surface follicles

Trial No. and range	*No. of ovaries collected*	*No. of ovaries aspirated*	*Oocytes per ovary*		
			Total	*With cumulus cells*	*Without cumulus cells*
8	883	599	1.37 ± 0.28	0.96 ± 0.19	0.41 ± 0.09
Range	100-125	70-81	0.44-2.64	0.31-1.97	0.13-0.92

that buffalo ovaries contains 20% less antral follicles than cattle in pre-ovulatory follicular phase (Le Van Ty *et al.*, 1989), therefore fewer follicles are recruited in each cycle in buffalo, contributing to a lower recovery of oocytes. The total number of oocytes and oocytes with cumulus cells retrieved per ovary in present investigation were higher (1.37 and 0.96) than reported by Totey *et. al.* (1992) (0.73 and 0.43); Nandi (1997) (1.17 and 0.60) and were comparable to Singla (1995) (1 per ovary usable). In most of the trials (6/8) the oocytes per ovary were more than 1.0 and only during two trials figure was less than one and was 0.44 and 0.82. The variation in the type of oocytes recovered i.e. with cumulus and without cumulus was of almost similar magnitude and the variability from trial to trial can be ascertained to the difference in the quality of ovaries obtained in each trial.

4.3 CRYOPRESERVATION OF OOCYTES

Oocytes were cryopreserved using vitrification procedures. Successful vitrification has three distinctive features: i) no ice formation during cooling, storage or warming, ii) cells are osmotically dehydrated prior to cooling by controlled equilibration in high concentrated solutions of cryoprotectants, and iii) a characteristic sequence of changes occur in osmotic volume of cell during the cryopreservation process. As vitrification offers considerable promise, because it is simple and requires no controlled rate freezing equipment, but, on the other hand, oocytes are characaterised by several unique features, which cause their susceptibility to freezing damages, due to the higher molar concentrations of cryoprotective agents. Therefore, in present investigation, two vitrification solutions i) 4.5 M EG + 3.4 M DMSO + 0.33 mM sodium pyruvate + 5.56 mM glucose + 4 mg/ml BSA in DPBS (VS-1) and ii) 3.5 M EG + 3.4 M DMSO + 0.33 mM sodium pyruvate + 5.56 mM glucose + 4 mg/ml BSA in DPBS (VS-2) and two equilibration timings, i.e., one minute and three minute were experimented. A total number of 522 oocytes with cumulus cells were vitrified in various experiments.

4.3.1 VITRIFICATION OF OOCYTES IN VS-1

In vitrification solution-1 (VS-1), 122 and 140 oocytes were cryopreserved under one and three minute equilibrarion time, respectively (Table 5.). Out of the total oocytes (122) handled with one minute exposure time, 96 were recovered morphologically normal, that made 78.69 $\pm$ 6.55 per cent of total oocytes. The figure ranged from 67.85 to 93.54 per cent, during different trials . On an average, 9.84 $\pm$ 1.19 per cent oocytes were damaged, whereas they ranged from 6.45 to 11.76 per cent in different trials. Further, the oocytes were also lost during handling, that made 11.47 $\pm$ 5.48 per cent on an average, but, ranged from 0.00 to 21.42 per cent in different trials. Out of the total oocytes (140) handled with 3 minute equilibration time, overall 83.57 $\pm$ 4.78 per cent were morphologically normal with a range of 78.94 to 100 per cent during different trials. 3.57 $\pm$ 2.61 per cent oocytes were damaged with the range of 0.00 to 11.11 per cent in different trials. Eighteen oocytes were lost during handling in this experiment, that made 12.86 $\pm$ 5.26 per cent in individual trials (Table 5; Figs.5 and 6).

4.3.2 VITRIFICATION OF OOCYTES IN VS-2

In vitrification solution-2 (VS-2), an equal number of oocytes (130) were handled in both the equilibration time. In one minute equilibration, 84.62 $\pm$ 3.52 per cent oocytes were recovered morphologically normal after thawing, with the range of 80.76 to 94.44 in various trials. On an average 6.92 $\pm$ 2.42 per cent oocytes were damaged, whereas they ranged from 0.00 to 11.53 per cent in different trials. Eleven oocytes were lost during handling, which was 8.46 $\pm$ 2.40 per cent on an average and ranged from 0.00 to 11.11 per cent in individual trials. The recovery of morphologically normal oocytes after thawing, under 3 minute equilibration time was 88.46 $\pm$ 3.96 per cent with a range of 83.33 to 97.05 per cent in different trials. The damaged oocytes were to the tune of 7.69 $\pm$ 2.21 per cent, ranging from 2.95 to 10.00

Table 5. **Effect of vitrification solution and equilibration time on normal recovery of oocytes**

Vitrification solution	*Equilibration time*							
	1 minute				*3 minute*			
	Oocytes vitrified	*Post thaw normal recovery (%)*	*Lost during handling (%)*	*Damage (%)*	*Oocytes vitrified*	*Post thaw normal recovery (%)*	*Lost during handling (%)*	*Damage (%)*
VS-1	122	96 (78.69±6.55)	14 (11.47±5.48)	12 (9.84±1.19)	140	117 (83.57±4.78)	18 (12.86±5.26)	5 (3.57±2.61)
VS-2	130	110 (84.62±3.52)	11 (8.46±2.40)	9 (6.92±2.42)	130	115 (88.46±3.96)	5 (3.85±2.52)	10 (7.69±2.21)

VS-1: 4.5 M EG + 3.4 M DMSO + 0.33 mM Sodium Pyruvate + 5.56 mM Glucose + 4 mg/ml BSA in DPBS
VS-2: 3.5M EG + 3.4 M DMSO + 0.33 mM Sodium Pyruvate + 5.56 mM Glucose + 4 mg/ml BSA in DPBS

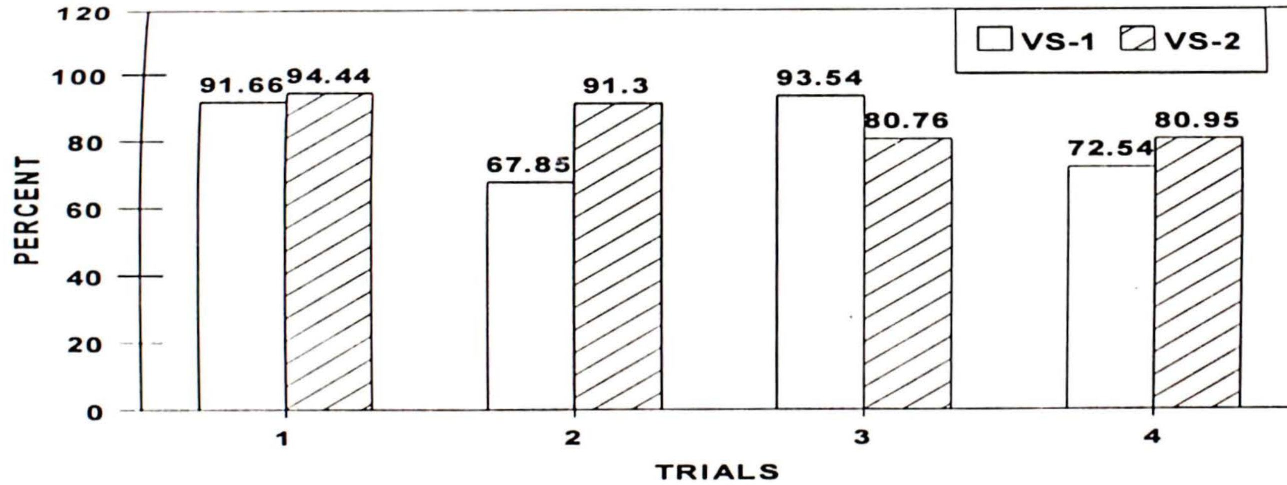

Fig.5 : Recovery of morphologically normal oocytes in one minute equilibration time with VS-1 and VS-2

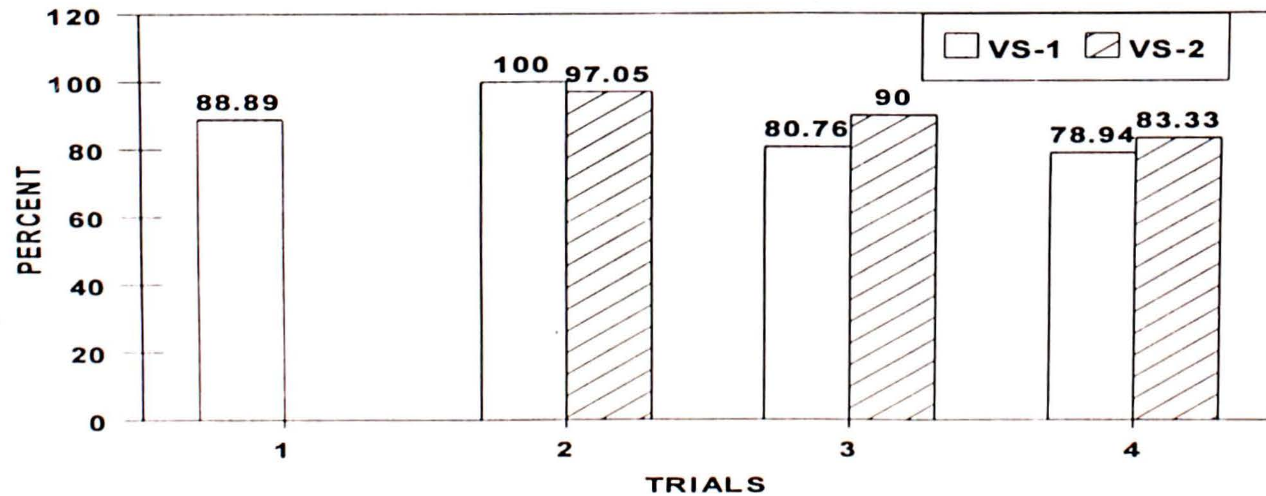

Fig.6 : Recovery of morphologically normal oocytes in three minute equilibration time with VS-1 and VS-2

per cent. Five oocytes were lost while handling, that was minimum of all the experiments, i.e., 3.85 ± 2.52 per cent with the range of 0.00 to 7.57(Table 5; Figs.5 and 6).

From the present investigation, it is clear that the mean post thaw morphologically normal recovery rate was maximum in VS-2 with three minute equilibration (88.46 ± 3.96 %) followed by V.S-2 with one minute equilibration (84.62 ± 3.52 %), VS-1 with three minute equilibration (83.57 ± 4.78 %) and VS-1 with one minute equilibration (78.69 ± 6.55 %). To detect the level of significance for these differences, the data were subjected to statistical analysis and the results revealed that the differences were statistically non-significant. However, these results suggest that VS-2, where the concentration of one of the cryoprotective agent ethylene glycol (EG) was lower, gave the higher post thaw normal recovery rate, irrespective of equilibration time. On the other hand, three minute equilibration time gave better results than ore minute.

Cryopreservation through vitrification requires a solution which is cyroprotectant, will vitrify on cooling at an easily achievable and repeatable rate, will remain vitreous on warming and is non toxic during the period of exposure before vitrification and during warming. Good vitrification may be obtained with high concentration of a single cryoprotectant, but, these concentrations are frequently toxic. Mixture of cryoprotectants generally vitrify at lower concentrations and are likely to be less toxic. Further, the cryoprotective quality of permeating compound is due to their ability to reduce the amount of water which freezes as ice at any sub zero temperature. So, these protectant reduces the amount of ice that forms in the cell suspension during freezing. This has the effect of reducing the exposure of cell to elevated salt and solute concentrations (Ali and Shelton, 1993).

In present investigation, a combination of mainly two low molecular weight, permeable cryoprotectant (EG and DMSO) alongwith energy source (sodium pyruvate and glucose) and BSA were tried. We have experimented one lower concentration of EG to minimize the toxic effect and to find out whether it vitrifies or not and the effect has been measured in terms of normal recovery rate as already discussed. Normal recovery rate ranging grom 72.72 to 89.6 percent has been reported, in slow freezing of immatured buffalo oocytes using 1,2, propanediol, glycerol and dimethyl sulfoxide as cryoprotectants (Das, 1993). Similary Yang *et. al.* (1994) reported morphologically normal recovery of post thaw oocytes to the tune of 80.9 per cent in 1 M glycerol and 78.4 per cent in 1.2 M glycerol, during slow freezing. However, a higher percentage (94.1) of morphologically mormal oocytes have been reported by same workers, when these bovine oocytes were cryopreserved using vitrification procedure. Contrary to this low survival rate of 74 to 77 per cent in ethylene glycol + ficoll + sucrose (EFS) and 53 percent in proplyene glycol + ficoll + sucrose (PFS) has been reported (Tachikawa *et. al.*, 1993) for cattle oocytes. Hence, the results reported in present investigation are in line with the literature reports and suggests that both ethylene glycol and dimethyl sulfoxide are agents highly capable of permeating an oocyte, thus, lower concentration of EG is better in terms of normal recovery rate and it vitrifies also. Further, three minute equilibrium in 50 per cent VSED allowed good intracellular conditions for vitrification.

After thawing the removal of intracellularly present cryoprotectant is necessary. The cell respond osmotically during removal of cryoprotectant (Schneider and Mazur, 1984), swelling of the cell during removal of a permeating cryoprotectant can be reduced or even converted into shrinkage if a non-permeating substance such as sucrose is added in the medium (Leibo and Mazur, 1978). Sucrose acts as an osmotic counterforce to arrest water movement across the membranes. As a consequence, the oocytes shrinks progressively, while the cryoprotectant leaves the cells. The oocyte then gains its normal volume when transferred into sucrose free isotonic medium.

We have also added 0.5 M sucrose in dilution media. In earlier reports in which vitrification solutions described by Rall and Fahy (1985) or by Massip *et al.* (1986) were used, dilution was usually carried out in 1 M sucrose solution for 10 minutes (Smorag and Gajda, 1991;. Szell *et al.*, 1990) in order to prevent osmotic injuries due to the swelling of blastomeres. In contrast, we used a low concentration of sucrose (0.5 M) and in short period of time (5 minutes) and found satisfactory results. This may be due to the low molecular weight of ethylene glycol which makes dilution at low concentration of sucrose and in a short period of time possible and which may have advantageous effects on the survival of thawed oocytes. One such report (Dobrinsky *et al.*, 1992) also demonstrated the relationship between concentration of sucrose and duration of dilution with survival rate in embryos.

4.4 FREEZING DAMAGES IN VITRIFIED/THAWED OOCYTES

The preservative action of the cryoprotectant is brought about through a sum of chemical and physiological processes which involves the microenvironmental changes in and around the oocytes, once exposed to different cryoprotectants. The physical nature of each will influence the migration of chemical unit through the oocytes and also transfer of cryoprotectant in and out of the cell. The movement is influenced by the concentration of the cryoprotectant, its solubility, ionic strength, pH and the rate of diffusion forces in the oocyte. The permeability of zona pellucida to the cryoprotectant under these circumstances deeply effect the preservability. However, concentrated solutions of permeating cryoprotectant are required for successful cryopreservation of oocytes when rapid cooling and warming rates are used. However, on the other hand, when lower concentration of cryoprotectant are used, despite apparent vitrification, membrane destruction is unavoidable (Rubinsky *et.al.*, 1991).

In present investigation, cryoprotectants were removed by allowing the oocyte for 5 minute equlibration with 0.5 M sucrose solution. The oocytes were transferred into culture medium and observed under inverted microscope to detect morphological freezing damages. The percent oocytes in VS-1, VS-2, both for one and three minute equilibration time has been presented in Table.5. Various morphological abnormalities observed after thawing were viz., zona crack (Figs.7 and 8), split into two halves (Figs.9 and 10), change in normal shape (Fig.11), empty zone (Fig.12) and leakage of cellular content. Different kinds of damages found after vitrification in VS-1 and VS-2 at two different equlibration time has been depicted in Tables 6 and 7. From the critical analysis of the tables, it is evident that zona crack was the most common in both the vitrification solutions and at both the equlibration times. Das (1993) reported morphological abnormalities in cryopreserved (controlled slow freezing) buffalo immature oocytes. The common abnormalities were breakage of zona, empty zona, leakage of cellular content, degenerated oocytes, oocytes loosing spherical shape, vacuolization inside the cell and fragmented cytoplasm. Parkening and Chang (1977) and Ko and Threlfall (1988) also reported similar kinds of morphological abnormalities as reported in present investigation. In few zona cracked oocytes, complete disconnection between oocyte and cumulus cells was noticed. Similar type of observation was also reported by Yang *et al.* (1994).

4.5 IN VITRO MATURATION OF OOCYTES

Vitrification avoids potential risk of cellular injury caused by the formation of intracellular ice, but chemical toxicity and osmotic damage may result through exposure to the high concentration of cryoprotectants required for vitrification. Disruption of the cytoskeleton during cooling, warming or exposure to cryoprotectants has been of major concern in mammalian oocyte preservation and post thaw developmental capacity. An oocyte even after thawing must be morphologically normal and should have all the prerequisite

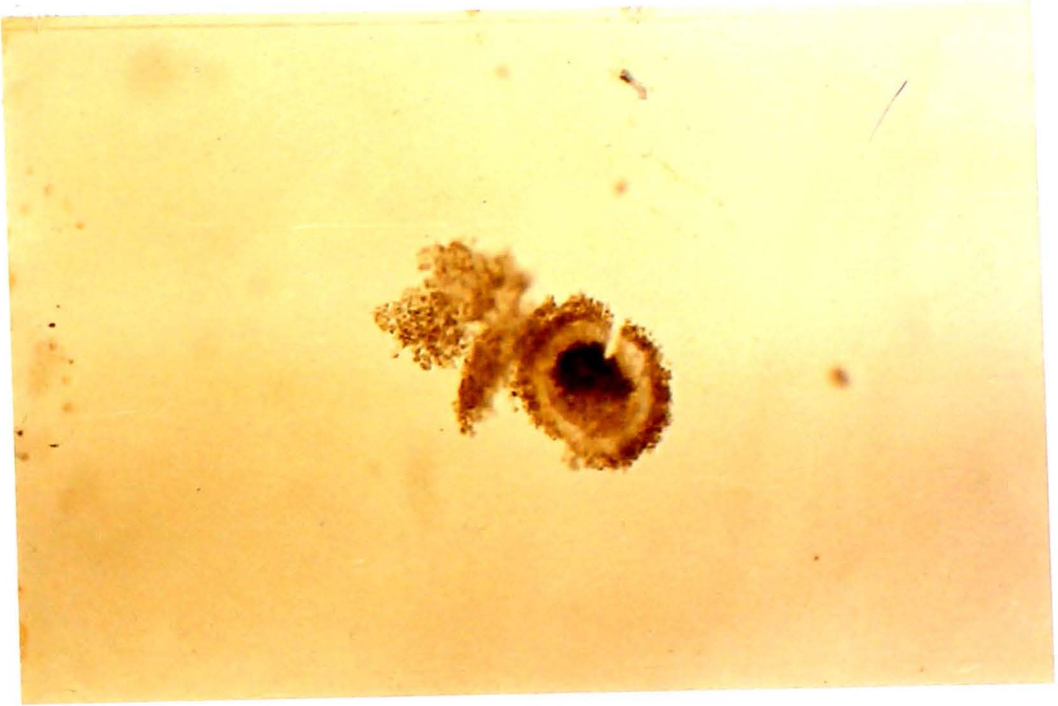

Fig.7 Oocyte with zona crack at lower magnification

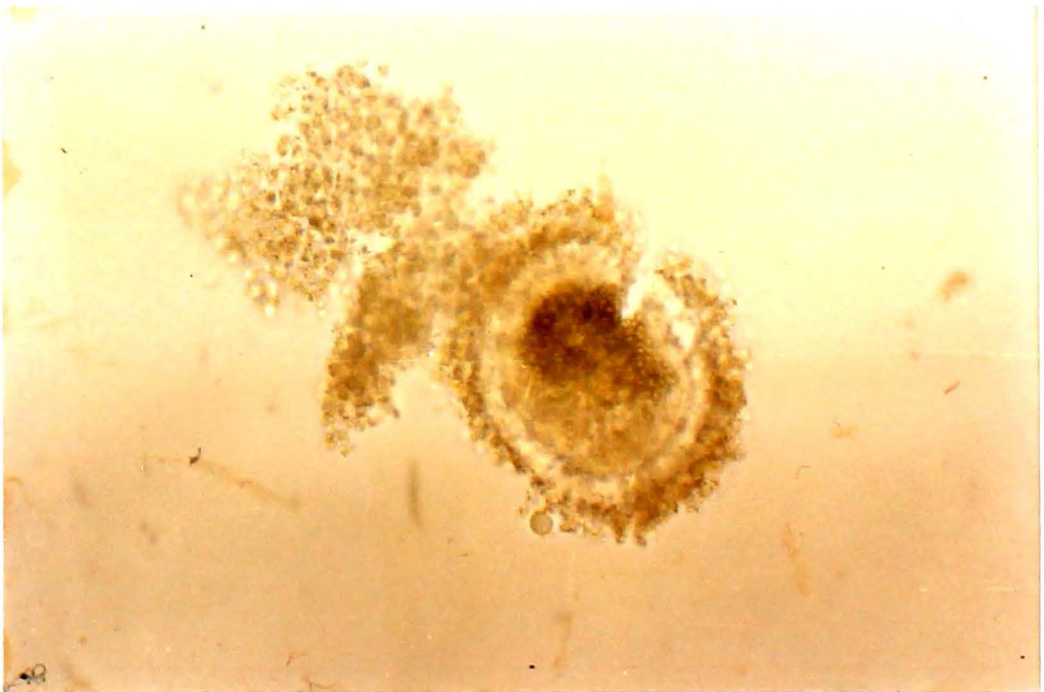

Fig.8 Oocyte with zona crack at higher magnification

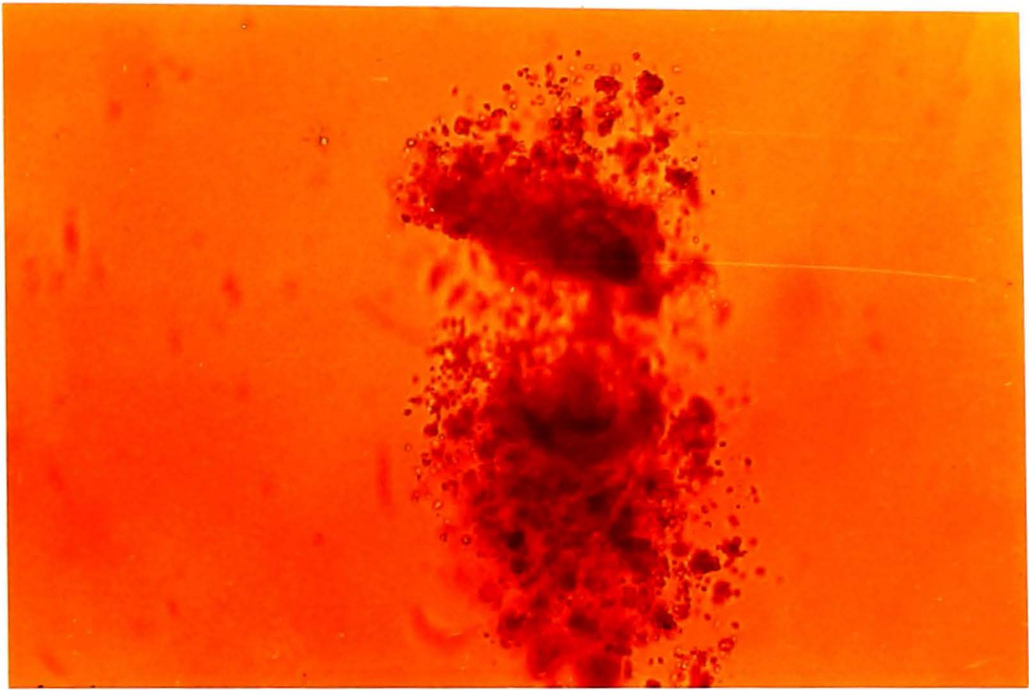

Fig.9 Oocyte splits after vitrification and thawing

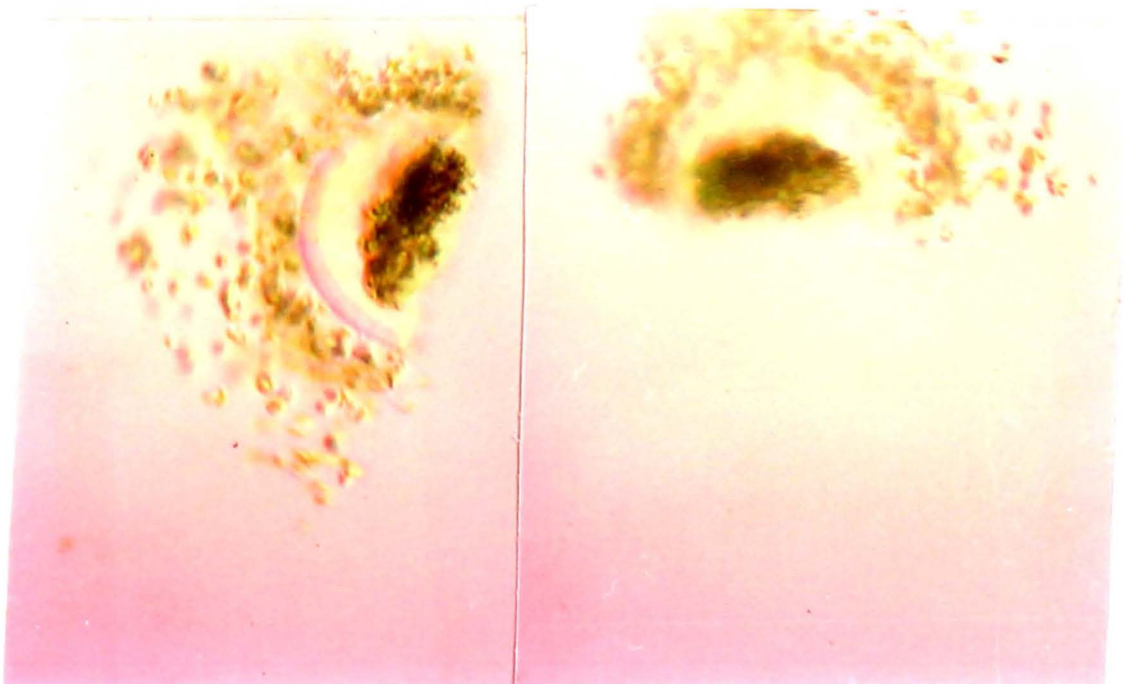

Fig.10 Oocyte splits into nearly two equal halves after vitrification and thawing

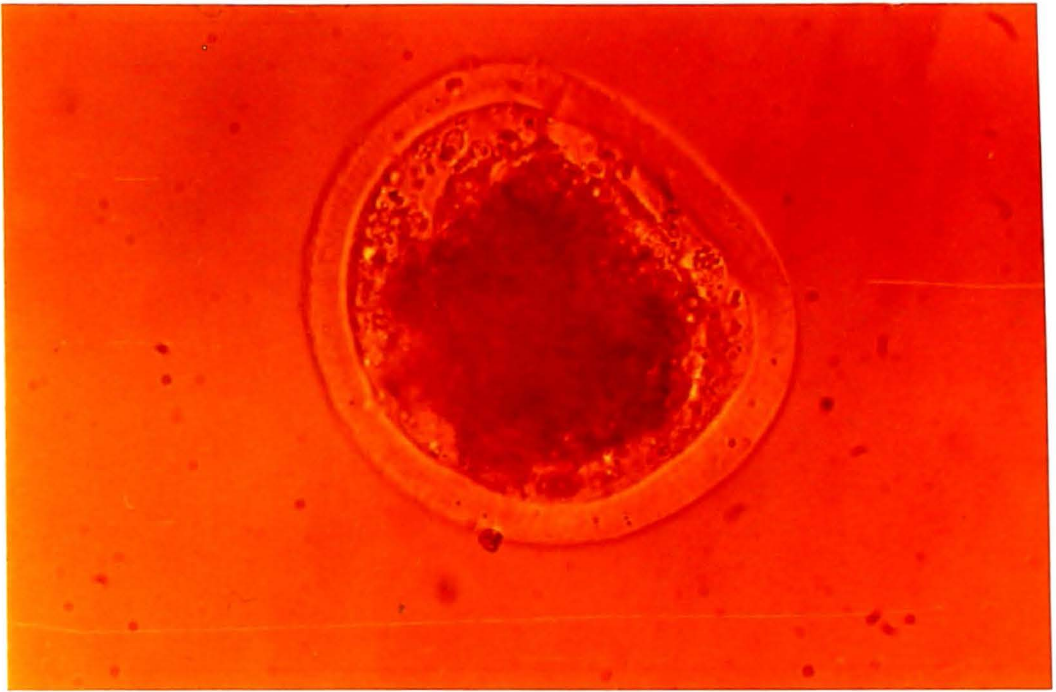

Fig.11 Oocytes looses its normal spherical shape and cumulus mass

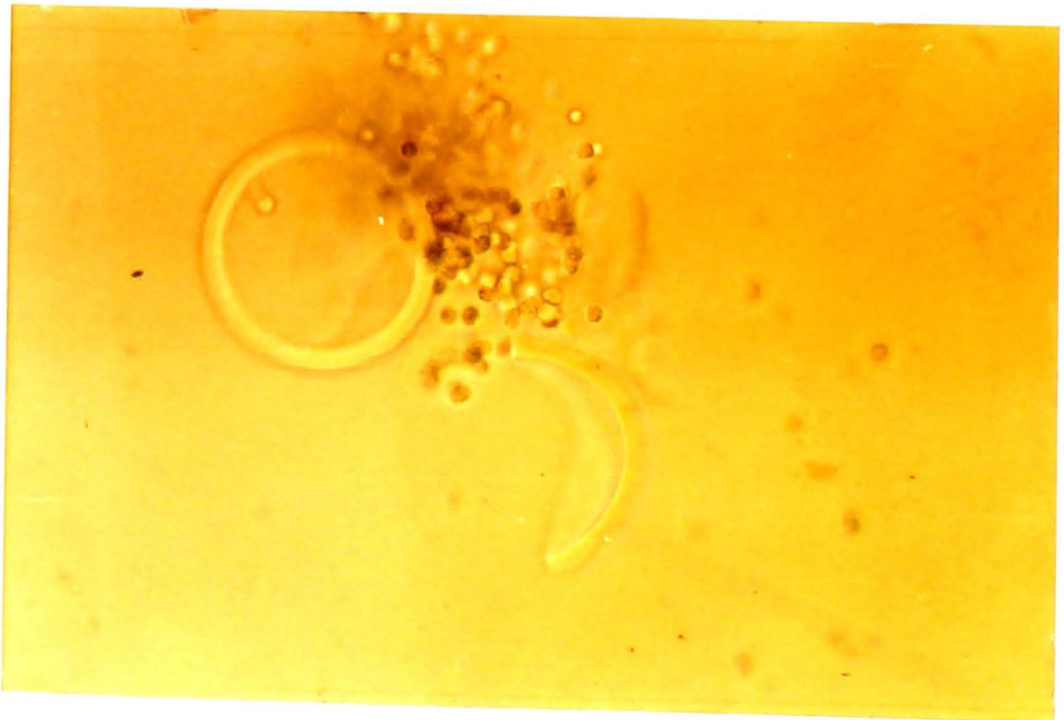

Fig.12 Drainout of cellular content leaving the empty zona

Table 6. **Different types of damage after vitrification in VS-1**

Equilibration time (Min.)	*No. of Oocytes damaged*	*Types of Damage*				
		Zona crack	*Split into two halves*	*Change in normal shape*	*Empty zona*	*Leakage of cellular content*
3	2	2	-	-	-	-
3	0	-	-	-	-	-
3	0	-	-	-	-	-
3	3	2	-	-	1	-
1	1	1	-	-	-	-
1	3	2	-	1	-	-
1	2	1	-	-	1	-
1	6	5	-	-	-	1

VS-1: 4.5 M EG+3.4 M DMSO + 0.33 mM sodium pyruvate + 5.56 mM glucose + 4 mg/ml BSA in DPBS

Table 7. **Different types of damage after vitrifiction in VS-2**

Equilibration time (Min.)	*No. of Oocytes damaged*	*Types of Damage*				
		Zona crack	*Split into two halves*	*Change in normal shape*	*Empty zona*	*Leakage of cellular content*
3	-	-	-	-	-	-
3	1	-	1	-	-	-
3	3	3	-	-	-	-
3	6	4	-	1	1	-
1	1	1	-	-	-	-
1	0	-	-	-	-	-
1	3	2	-	-	1	-
1	5	2	-	-	3	-

VS-2: 3.5M EG + 3.4 M DMSO + 0.33 mM sodium pyruvate + 5.56 M glucose + 4 mg/ml BSA in DPBS

qualities which are essential for fertilization in vitro. Hence, the present investigation was aimed to know the maturation of post thaw oocytes besides in vitro survival rate and damages as discussed previously. After thawing and removal of cryoprotectants, oocytes were cultured (Fig.13) in Co2 incubator at 38.5°c and 5% Co2 for 26 hrs. and cumulus expansion (Figs.14 and 15) were indicative of their maturation. This oocytes were further stained after removal of cumulus mass (Fig.16) and screened to know the exact maturation rate. The oocytes reached to the stage of telophase-I (Fig.17) and metaphase-II (Figs.18 and 19) were considered as matured for the present study. A total number of 367 oocytes were screened to evaluate the maturation rate in various vitrification solutions and equillibration time.

4.5.1 MATURATION RATE OF OOCYTES VITRIFIED IN VS-1

In vitrification solution-1 (VS-1), 82 and 104 oocytes were viewed under the microscope after staining for one and three minute exposure, respectively (Table 8). Perusal of this table reveals that 28.04 ± 10.37 per cent oocytes were matured in one minute equilibration, with the range of 10.00 to 50.00 per cent in individual trial (Figs. 20 and 21). The respective percentage for three minute equillibration time was 31.73 ± 1.06 and 28.57 to 33.33. This indicates that variation in three minute equillibration time was less as compared to one minute in different trials, hence speaks about the consistent and repeatable results with 3 minutes exposure.

4.5.2 MATURATION RATE OF OOCYTES VITRIFIED IN VS-2

In vitrification solution-2 (VS-2), 85 and 96 oocytes were examined under the microscope, after staining for one and three minute equilibration time, respectively. On an average 23.53 ± 3.48 per cent oocytes were matured (Table 8), with the range of 17.07 to 33.33 per cent in different trials (Figs. 20 and 21), with one minute of equilibration. The corresponding figures for 3 minute equilibration time were 33.33 ± 9.26 and 25.00 to 53.84. The variation

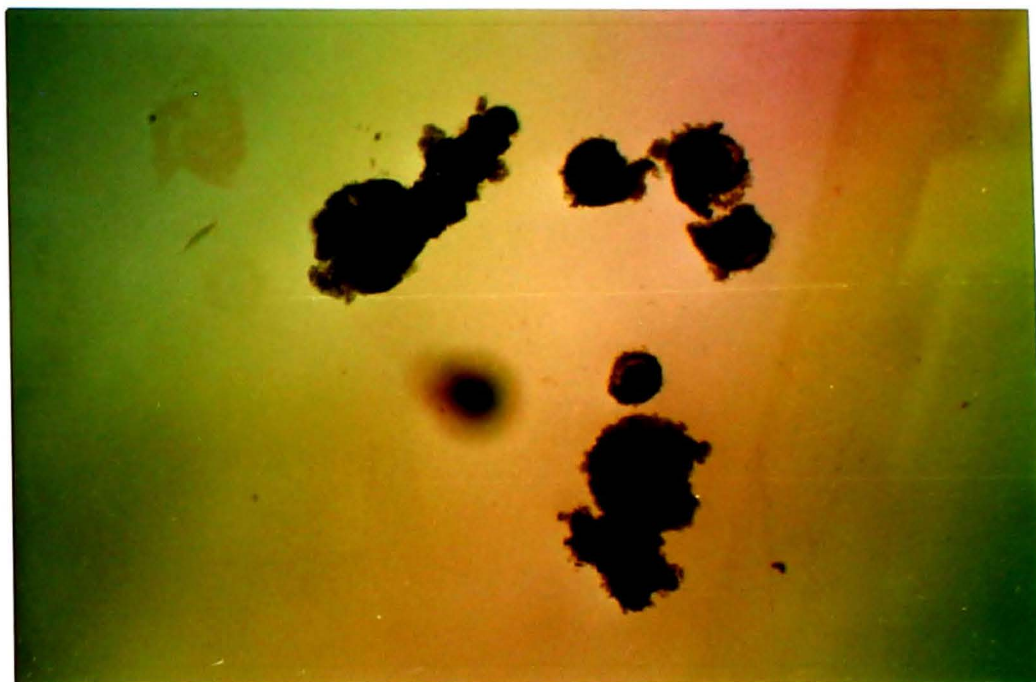

Fig. 13 Buffalo cumulus oocyte complexes into culture drop

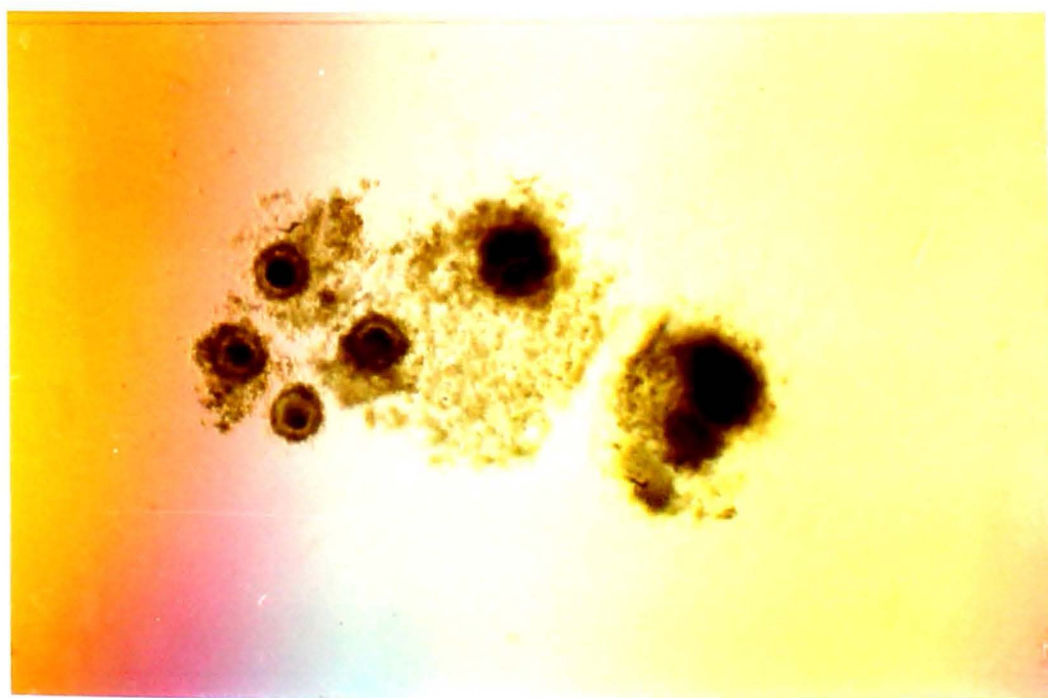

Fig. 14 Same oocytes as in Fig. 13 showing different degrees of cumulus expansion after 26 hr. culturing at lower magnification

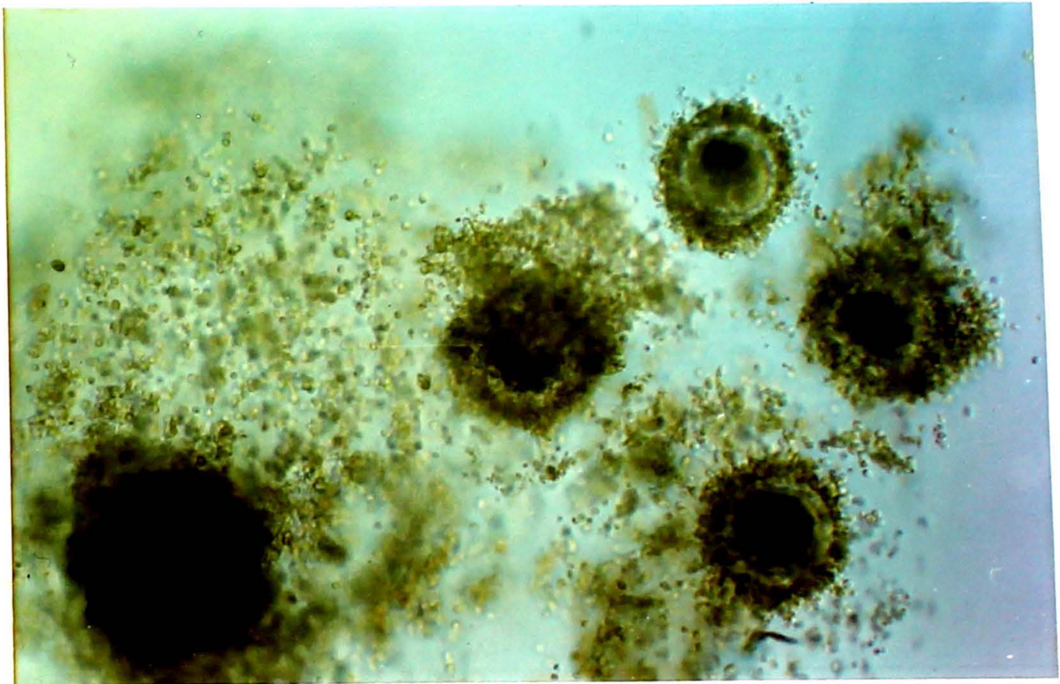

Fig. 15 Same oocytes as in Fig. 14 showing different degrees of cumulus expansion after 26 hr. culturing at higher magnification

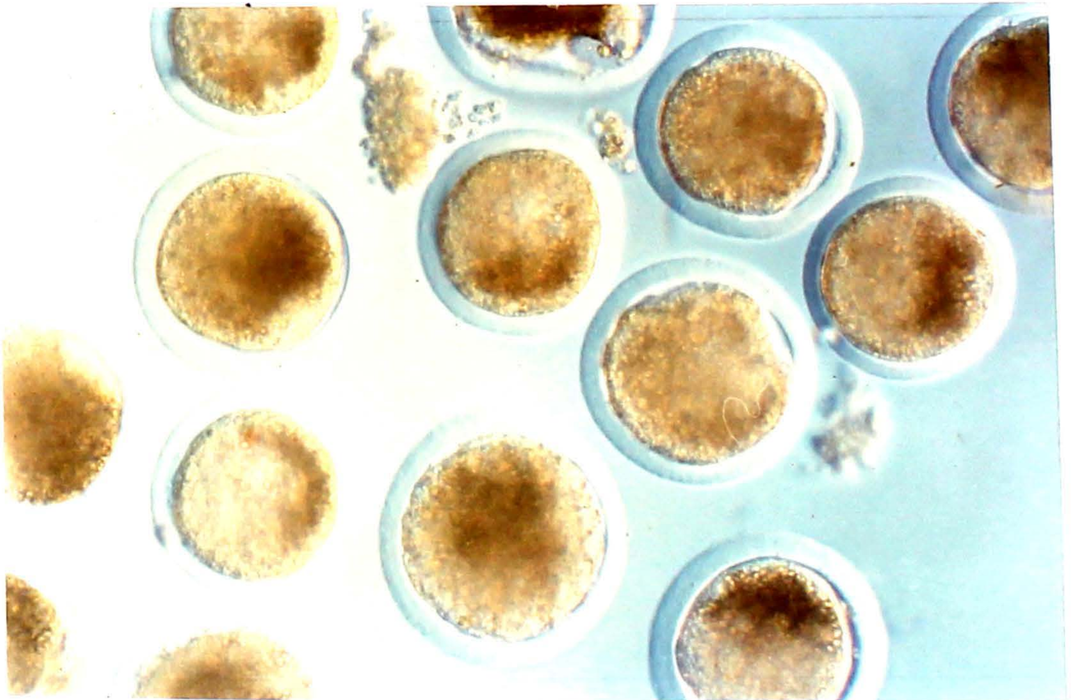

Fig. 16 Post thaw buffalo oocytes after maturation and removal of cumulus mass for staining

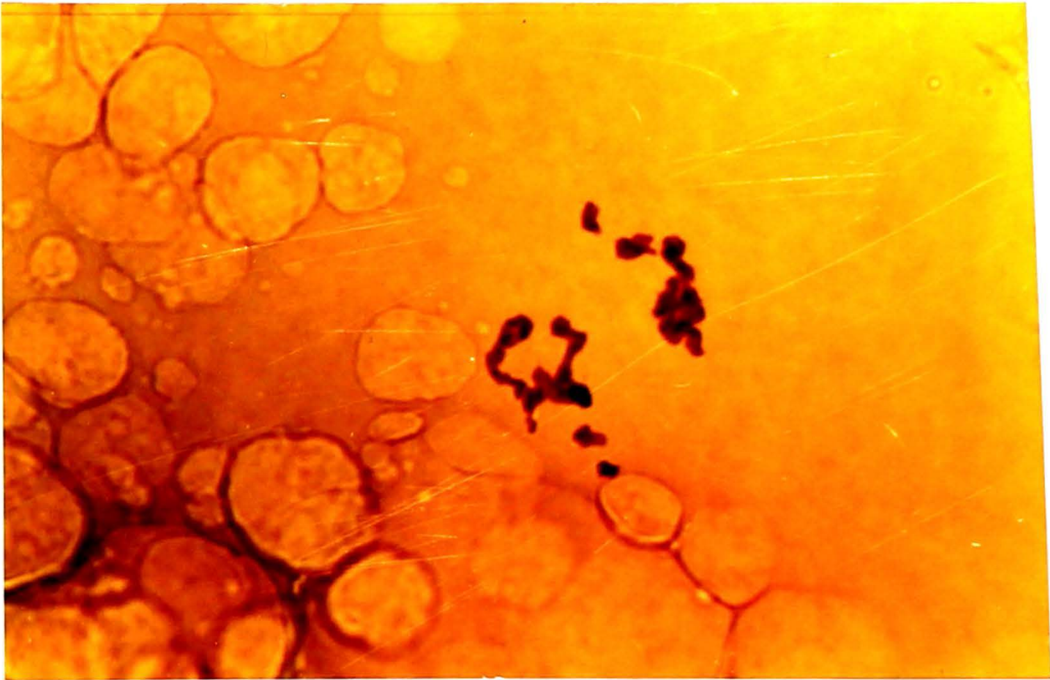

Fig.17 Telophase-I : The chromosomes are arranged in two equally spread groups

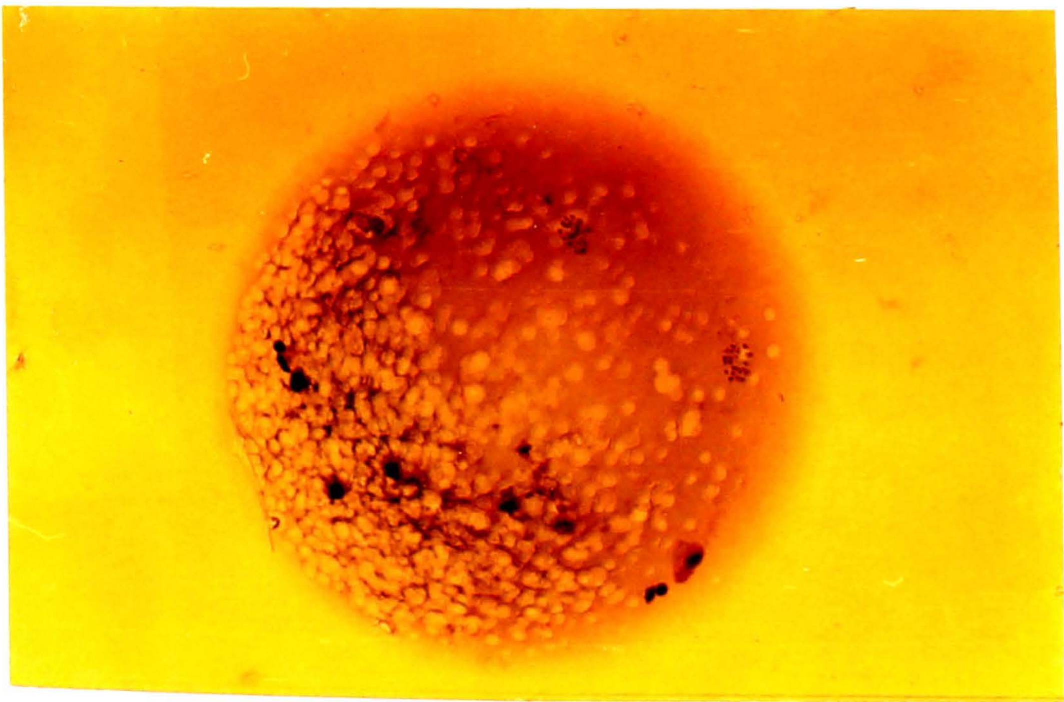

Fig.18 Metaphase-II : Showing two sets of chromosomes pertaining to oocyte and polar body

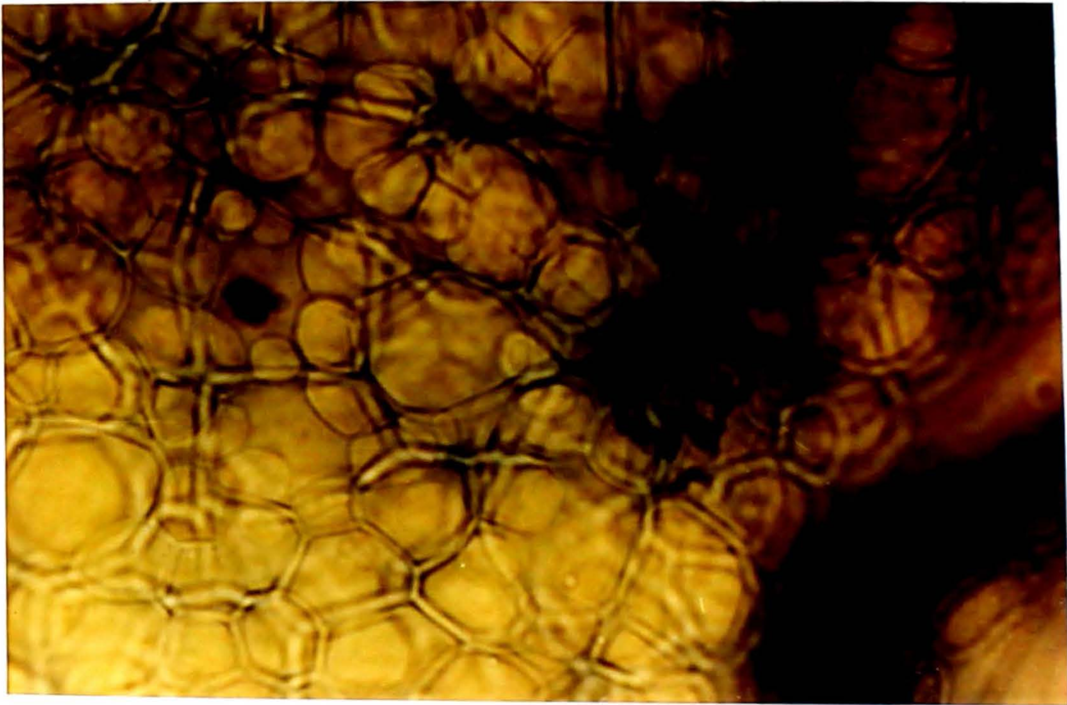

Fig.19 Metaphase-II : The polar body chromosomes are tightly packed, whereas the metaphase chromosomes are spread.

Table 8. Effect of vitrification solution and equilibration time on maturation of oocytes

Vitrification solution	*Equilibration time*							
	1 minutes				*3 minute*			
	Oocytes matured				*Oocytes matured*			
	Oocytes screened	*M-II (%)*	*Telophase-I (%)*	*Total (%)*	*Oocytes screened*	*M-II (%)*	*Telophase-I (%)*	*Total (%)*
VS-1	82	22 (26.83)	1 (1.21)	23 (28.04±10.37)	104	30 (28.85)	3 (2.88)	33 (31.73±1.06)
VS-2	85	19 (22.35)	1 (1.18)	20 (23.53±3.48)	96	27 (28.12)	5 (5.21)	32 (33.33±9.26)

VS-1: 4.5 M EG + 3.4 M DMSO + 0.33 mM Sodium Pyruvate + 5.56 mM Glucose + 4 mg/ml BSA in DPBS
VS-2: 3.5 M EG + 3.4 M DMSO + 0.33 mM Sodium Pyruvate + 5.56 mM Glucose + 4mg/ml BSA in DPBS

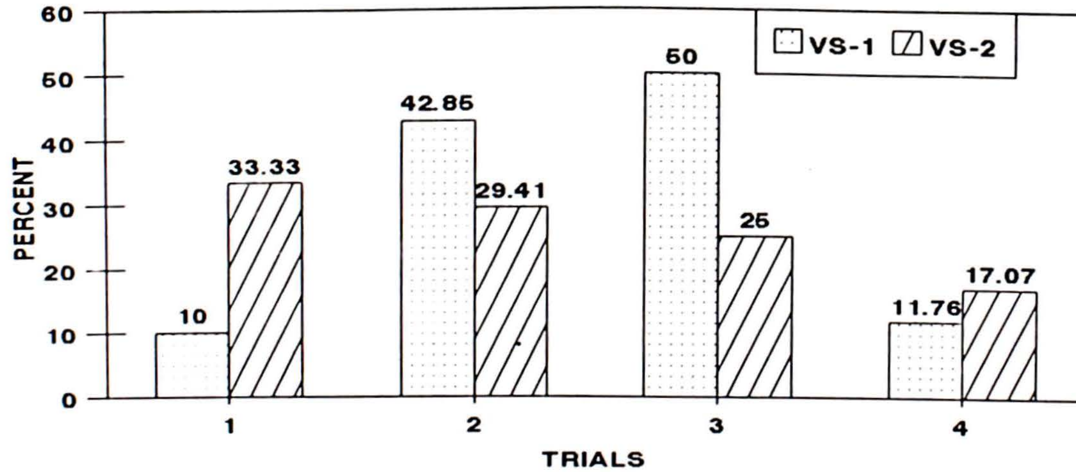

Fig.20 : Maturation rate in one minute equilibration time with Vs-1 and VS-2

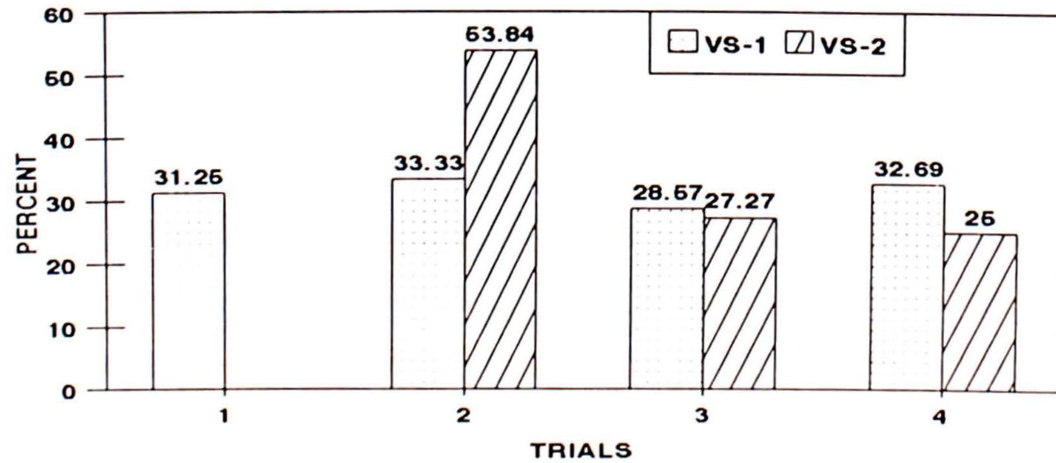

Fig.21 : Maturation rate in three minute equilibration time with Vs-1 and VS-2

among the trials, which is obvious was there in VS-2 also but was of less magnitude, and on contrary to VS-1, it was more in three minutes equilibration time.

4.5.3 MATURATION RATE OF NON VITRIFIED CONTROL GROUP

In non vitrified control group, 49 cumulus cell enclosed oocytes were cultured in different trials. These oocytes were stained with 2% Giemsa stain to see the nuclear maturation. After staining, 46 oocytes were screened under microscope and among them 31 oocytes (67.39%) were found as matured.

From these findings, it is revealed that the average maturation rate of oocytes, after thawing and culturing, was maximum in VS-2 (33.33 ± 9.26 per cent) with three minute equlibration followed by VS-1 (31.73±1.06 per cent) with three minute equilibration, VS-1 (28.04 ± 10.37 per cent) and VS-2 (23.53 ± 3.48 per cent) in one minute equilibration. The level of significance for these variations were statistically analysed and found to be non significant. Similar to the results obtained in post thaw normal recovery, the maturation rate was maximum in a group where ethylene glycol (EG) concentration was lower i.e. VS-2, but only with three minute equilibration time. This can be attributed to the fact that VS-2 contained lower concentration of EG that might have resulted in lower state of toxicity to oocytes.

Further it has also been noticed that in both the vitrification solutions 3 minute equilibration time has given good result than 1 minute equilibration. It may be due to the fact that 3 minute exposure time provided the optimum equilibration between oocytes and vitrification solutions. It has also been found that the fluctuation in maturation rate is higher in the VS-2 than in VS-1 with three minute exposure. It may be due to excess aspiration of 50 per cent VSED along with oocytes while placing them into 15-20 μl, drop of 100 per cent VSED, which reduces the solute concentration of the vitrification coloumn in which oocytes were ultimately vitrified. This reduced solute concentration

sometime leads to apparent vitrification (partial ice formation) which might have happened in VS-2. This may damaged the ultrastructure of oocytes, hence resulted in lower maturation rate in some of the trials, thus leading to more variation. But the particular incident did not create any problem in case of VS-1 as its solute concentration was higher than the VS-2 and the results were consistent. On the other hand the fluctuation of maturation rate in 1 minute equilibration in both the solutions i.e. VS-1 and VS-2 may be mainly due to the insufficient equilibration between oocyte and vitrification solution and apparent vitrification in case of VS-2.

It has been reported that presence of granulosa cell complex is the most important factors for in vitro maturation of follicular oócytes (Fukui and Sakuma, 1980). The maturation of oocytes to its M-II stage needs at least 80 per cent of its growth prior to start of nuclear maturation to be completed, which only occur when the oocytes are cultured with cumulus complex (Thibault *et. al.*, 1987). Schroeder *et al.* (1990) used isobutyl methyl xanthene (IBMX) an meiosis arresting agent in isolation medium to inhibit germinal vesicle break down stage (GVBD) reversibly during the processing steps for freezing. GVBD was initiated immediately upon liberation from follicular environment and was completed virtually in all cumulus enclosed oocytes after 75 minutes. This may be a particularly sensitive period, since there is movement and rearrangement of various organelles and microtubules within ooplasm (Calarlo *et. al.*, 1972). Schroeder *et al.* (1990) further reported that though frozen GV stage mouse oocytes were matured in vitro but their developmental capacity was severelly impaired. But in vitro matured oocyte in FSH supplemented media greatly improved the capacity to cleave and developed to blastocyst. In the present study, the maturation rates in both vitrification solutions and equilibration times were higher than that reported by Yang *et al.* (1994). However, Nag and Maurya (1997) reported a higher maturation rate of 34 per cent in DMSO and 43.23 per cent in ethylene glycol (EG) in vitrified cumulus

enclosed buffalo immature oocyte. But they reported a maturation rate of 32.62 per cent in propylene glycol (PG) which was lower than the maturation rate of the present study, i.e. 33.33 ± 9.26 per cent in VS-2 with 3 minute equilibratiaon time. These differences may be attributed to different mixture and concentration of cryoprotectants used for various experimentation.

CHAPTER - 5

SUMMARY AND CONCLUSION

5. SUMMARY AND CONCLUSION

Availability of artificial insemination procedure of mammalian semen using cryopreservation has permitted the expanded genetic improvement of the livestock. But similar advances in preservation of female gamete have not progressed rapidly. The cryopreservation of embryos began around 1970 and is currently widespread. Though cryopreservation of oocytes, which no doubt will be the most practical way to preserve genetic material from female animals, yet, it had very limited success, thus demands further research in this area. This investigation, therefore, was undertaken to assess the cryopreservabilty of oocytes, using vitrification procedure, which requires no controlled rate freezing equipment. The ovaries were brought from Delhi slaughter house under sterilized conditions and oocytes aspirated from 3-8 mm surface follicles using 19 gauge needle. The salient findings can be summarized as:

5.1 On the basis of number of ovaries with or without surface follicles, it was found that, on an average, 67.83 per cent of the total ovaries had surface follicles, thus were eligible for oocyte aspiration.

5.2 The overall oocyte recovery was 1.37 ± 0.28 per ovary, with an average of 0.96 ± 0.19 per ovary with cumulus cells and 0.41 ±0.09 without cumulus cells. Hence, only 70 per cent of the total oocytes retrieved were with cumulus cells and variations among trials were of almost similar magnitude.

5.3 In vitrification solution-1 (4.5 M EG + 3.4 M DMSO + 0.33 mM sodium pyruvate + 5.6 mM glucose + 4 mg/ml BSA in DPBS), a total number of 122 and 140 oocytes with cumulus cells were cryopreserved for one and three minute equilibration time, respectively. The average normal recovery rate of oocytes after thawing was 78.69 ± 6.55 and

83.57 ± 4.78 per cent in two equilibration time, respectively. The percentage of oocytes apparently damaged due to freezing injuries was 9.84 ±1.19 and 3.57 ± 2.61.

5.4 In vitrification solution-2 (3.5 M EG + 3.4 M DMSO + 0.33 mM sodium pyruvate + 5.6 mM glucose + 4 mg/ml BSA in DPBS), an equal number of oocytes (130) were cryopreserved in both the equilibration time. 84.62 ± 3.52 per cent oocytes were recovered morphologically normal after thawing in one minute equilibration and 88.46 ± 3.96 per cent in three minute equilibration. The corresponding values for per cent oocytes damaged was 6.92 ± 2.42 and 7.69 ± 2.21.

5.5 A concentration of 0.5 M sucrose in dilution media for a period of 5 minutes to remove intracellularly present cryoprotectants and to avoid swelling of the oocytes gave satisfactory results with respect to normal post thaw recovery.

5.6 Cryopreserved oocytes examined morphologically after thawing, showed deformities like zona crack, split into two halves, change in normal shape, empty zona and leakage of cellular content. However, zona crack was the most common among observed abnormalities.

5.7 In vitrification solution-1 (VS-1), a total number of 82 and 104 post thaw oocytes were screened for maturation under the microscope after culturing and staining, in one and three minute equilibration time, respectively. It was found that 28.04 ± 10.37 per cent oocytes were matured in one minute and 31.73 ± 1.06 per cent in three minute equilibration time, respectively.

5.8 In vitrification solution-2 (VS-2), a total number of 85 and 96 oocytes were examined for maturation under the microscope after culturing and staining, in one and three minute equilibration time, respectively. The corresponding values for maturation rate were 23.53 ± 3.48 and 33.33 ± 9.26 per cent.

The above findings revealed that both post thaw normal recovery and maturation rate was higher in VS-2 for 3 minute equilibration time. However, these differences were statistically non-significant. Vitrification solution-2 (VS-2) had lower concentration of ethylene glycol, it might have resulted in lower toxicity and ultrastructural damages, thus gave better post thaw normal recovery and maturation rate. Three minute exposure provided the optimum equilibration between oocytes and vitrification solutions. Finally, it can be concluded that female gamete can be cryopreserved using vitrification procedure, which is simple and does not require any freezing equipment. The technique has its importance for gene preservation and the establishment of the oocyte banks and salvaging the genetic material from female animals even after death.

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