CRYOPRESERVABILITY OF CAPRINE OOCYTES AND EMBRYOS BY CONVENTIONAL STRAW AND OPEN PULLED STRAW VITRIFICATION

RATHEESH BABU. M.

Thesis submitted in partial fulfilment of the requirement for the degree of

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Department of Animal Reproduction, Gynaecology and Obstetrics
COLLEGE OF VETERINARY AND ANIMAL SCIENCES
MANNUTHY, THRISSUR-680651
KERALA, INDIA

DECLARATION

I hereby declare that the thesis entitled **"CRYOPRESERVABILITY OF CAPRINE OOCYTES AND EMBRYOS BY CONVENTIONAL STRAW AND OPEN PULLED STRAW VITRIFICATION"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

07/06/06

RATHEESH BABU. M.

Mannuthy
07/06/06

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Certified that the thesis entitled **"CRYOPRESERVABILITY OF CAPRINE OOCYTES AND EMBRYOS BY CONVENTIONAL STRAW AND OPEN PULLED STRAW VITRIFICATION"** is a record of research work done independently by **Ratheesh Babu. M.**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

07/06/06

Dr. V. Vijayakumaran
(Chairman, Advisory Committee)
Associate Professor
Department of Animal Reproduction,
Gynaecology and Obstetrics
College of Veterinary and
Animal Sciences, Mannuthy

Mannuthy
07/06/06

CERTIFICATE

We, the undersigned members of the Advisory Committee of **Ratheesh Babu. M.,** a candidate for the degree of Master of Veterinary Science in Animal Reproduction, agree that the thesis entitled **"CRYOPRESERVABILITY OF CAPRINE OOCYTES AND EMBRYOS BY CONVENTIONAL STRAW AND OPEN PULLED STRAW VITRIFICATION"** may be submitted by Ratheesh Babu.M., in partial fulfilment of the requirement for the degree.

07/06/06

Dr. V. Vijayakumaran
(Chairman, Advisory Committee)
Associate Professor
Department of Animal Reproduction,
Gynaecology and Obstetrics
College of Veterinary and
Animal Sciences, Mannuthy

7/6/06

Dr. T. Sreekumaran
Associate Professor and Head
Department of Animal Reproduction,
Gynaecology and Obstetrics
College of Veterinary and
Animal Sciences, Mannuthy
(Member)

7/6/06

Dr. Metilda Joseph
Associate Professor
(Animal Reproduction)
College of Veterinary and
Animal Sciences, Pookot
(Member)

07/06/06

Dr. Syam K. Venugopal
Assistant Professor
Department of Veterinary
Surgery and Radiology
College of Veterinary and
Animal Sciences, Mannuthy
(Member)

7/6/06

(Dr. D. KATHIRESAN)

External Examiner

DIRECTOR OF CLINICS
TAMIL NADU VETERINARY AND
ANIMAL SCIENCES UNIVERSITY
CHENNAI-600 007.

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LIST OF SELECTED ABBREVIATIONS USED

AI	Artificial Insemination
BSA-V	Bovine serum albumin fraction V
CIDR	Controlled internal drug release
CL	Corpus luteum
COCs	Cumulus oocyte complexes
CS	Conventional straw
DM	Dilution medium
DMSO	Dimethyl sulfoxide
EFS	Ethylene glycol-Ficoll-Sucrose
EG	Ethylene glycol
FDA	Fluorescent diacetate
FGA	Fluorogestone acetate
GV	Germinal vesicle
GVBD	Germinal vesicle break down
hCG	Human chorionic gonadotrophin
HM	Holding medium
ICM	Inner cell mass
IU	International units
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
IVP	*In vitro* production
LH	Luteinising Hormone
M	Molar
mDPBS	modified Dubecco's phosphate buffered saline
MII	Metaphase II
MOET	Multiple ovulation and embryo transfer
oFSH	Ovine follicle stimulating hormone

OPS	Open pulled straw
pFSH	Porcine follicle stimulating hormone
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PMSG	Pregnant Mare Serum Gonadotrophin
PRCL	Prematurely regressed corpus luteum
PROH	Propanediol
SM	Sucrose medium
TCM	Tissue culture medium
TL-HEPES	Tyrode's lactate-hydroxy ethyl piperazine ethane sulfonic acid
VM	Vitrification medium
ZP	Zona pellucida
χ^2	Chi square
µ	Microns
µg	Microgram
µl	Microlitre

Introduction

1. INTRODUCTION

India ranks first among the countries of the world in goat population and it contributes about two percent to the total GDP of the country. A large diverse genetic resource of goat is found in India and among this, Malabari breed, a native of Kerala, is well known for its milk production and adaptability. It plays a major role in rural economy of the state but fragmentation of land due to population explosion and dwindling farming communities make it necessary to formulate a more suitable production system for these small ruminants whereby it can take a major role and contribute more towards the economy.

The future trends in goat production are expected to be dictated by the emerging technologies of reproduction such as Multiple Ovulation and Embryo Transfer (MOET), *in vitro* embryo production, cryopreservation and micromanipulation. Among this superovulation followed by transfer of embryos to timely synchronized recipient has proved to be an effective means of increasing the contribution of superior females to the genetic pool. But main limiting factor in application of embryo transfer is unpredictable ovarian response and embryo recovery and time lag for successful transfer to synchronized animal. One of the possible ways to alleviate the difficulties associated with these problems is through the establishment of embryo banks using *in vivo* or *in vitro* produced embryos.

There is an increased interest towards the *in vitro* production of embryos due to difficulty in getting the required number of embryos of the required stage of development through *in vivo* source and their exorbitant cost. An inevitable part of *in vitro* embryo production is the requirement of matured oocytes. *In vivo* matured oocytes obtained by either surgical or laparoscopic method are expensive and number of oocytes recoverable per animal is limited. An alternative and cheap source of embryo is by *in vitro* maturation and fertilization of immature oocytes from ovaries of recently slaughtered animal. However relatively short fertile life of mammalian oocyte in external environment is a

major limiting factor in the implementation of many *in vitro* methodologies, but this limitation could be overcome largely through cryopreservation

Though cryopreservation technique is applied extensively for male gametes, its application is very much limited in case of female gametes and embryos due to its low survival rate. The slow freezing was the widely used cryopreservation protocol for oocytes and embryos until recently. This procedure is time consuming and requires costly equipments like programmable Bio-freezer. A promising substitute in this field is cryopreservation by vitrification (Rall and Fahy 1985a). It has a potential advantage over conventional freezing as an inexpensive, rapid and user friendly method which avoids freezing damage considerably. Hence this method is well suited for routine field embryo transfer programme (Naitana *et al.,* 1995). However the embryo cryopreserved by vitrification may still be injured by toxicity of cryoprotectant, extracellular ice formation and adverse osmotic effects (Kasai *et al.,* 1996). These toxic effects are more severely affected to oocytes than multi cellular embryos. A recently developed modification to this new technology is Open pulled straw (OPS) vitrification (Vajta *et al.,* 1997b) which overcomes the draw back of conventional straw vitrification by accelerating the rate of cooling and warming beyond 16,700^{0}C/ minute as against 2500^{0}C per minute (Rall, 1987) for CS vitrification. Applying this method various researchers has successfully vitrified caprine, porcine and bovine embryos (El- Gayar and Holtz, 2001; Berthelot *et al.,* 2001 and Rizos *et al.,* 2001) and bovine and equine oocytes (Hurtt *et al.,* 2000; Men *et al.,* 2002 and Albarracin *et al.,* 2005). Perusal of literature reveals the paucity of such information from India regarding the caprine oocytes and embryos. The only published work from India as per the available literature on OPS is rabbit embryo vitrification as reported by Naik *et al.* (2005).

It was in this background that the present study was undertaken to assess the comparative efficiency of CS and OPS vitrification for the cryopreservation of caprine oocytes and embryos. The ultimate objective of this study is to identify an efficient and user-friendly method of cryopreservation for caprine oocytes and embryos.

Review of Literature

2. REVIEW OF LITERATURE

2.1 OOCYTES RETRIEVAL

2.1.1 Source of oocytes

Goat ovaries collected at slaughter provide an abundant source of oocyte at low cost and this was widely used either for freezing (Le Gal, 1996; Dutta *et al.*, 1998b; Agrawal, 1999 and Kharche *et al.*, 2005) or for *in vitro* maturation (Ongeri *et al.*, 2001; Rodriguez-Gonzalez *et al.*, 2003 and Bormann *et al.*, 2003). Graff *et al.* (1995) used laparoscopic aspiration procedure (LAP) and Transvaginal Ultrasound Guided Aspiration (TUGA) and Baldassarre *et al.* (2002) used Laparoscopic Ovum Pick-Up (LOPU) to collect oocyte from live animal. Later LOPU was successfully used by Begin *et al.* (2003) for collection of oocytes for vitrification and Koeman *et al.* (2003) for *in vitro* fertilization.

2.1.2 Media for ovary transport

To maintain the healthiness of caprine ovaries and its follicle during the transport from slaughterhouse to laboratory Pawshe *et al.* (1993), Pawshe *et al.* (1994), Malik *et al.* (1999) and Tajik and Esfandabadi (2003) used normal saline with or without antibiotic supplementation like Penicillin, Streptomycin or Gentamycin for keeping it free of bacterial contamination. Penicillin and streptomycin combination @ 100-200 IU and 100-200 µg respectively or gentamycin alone @ 50 µg/ml was added to the medium. Various others used Phosphate Buffered Saline (PBS) supplemented with antibiotics (Izquierdo *et al.*, 1998; Izquierdo *et al.*, 1999; Rodriguez-Gonzalez *et al.*, 2003 and Kharche *et al.*, 2005) for the transportation of caprine ovary.

2.1.3 Temperature of media

Sekine *et al.* (1992) reported that 31^{0}C to 34^{0}C temperature was the optimum for 1.5 h transport of bovine ovaries. Usually caprine ovaries were transported from abattoir to laboratory with in the temperature range of 25-30^{0}C (Pawshe *et al.*, 1993 and Bormann *et al.*, 2003) or 30-35^{0}C (Pawshe *et al.*, 1994; Keskintepe *et al.*, 1996 and

Rho *et al.*, 2001) or 35-37^{0}C (Mogas *et al.*, 1997; Izquierdo *et al.*, 1998; Izquierdo *et al.*, 1999; Rodriguez-Gonzalez *et al.*, 2002 and Rodriguez-Gonzalez *et al.*, 2003).

2.1.4 Oocyte retrieval methods

Pawshe *et al.* (1994) reported that recovery rate of the total number of goat oocytes per ovary was significantly higher by aspiration than by slicing or puncturing method (2.78 ± 0.15, 2.40 ± 0.12 and 2.28 ± 0.13 respectively) and suggested that a significantly more good quality oocytes enclosed with compact cumulus cells were obtained by slicing (0.9 ± 0.06) than by aspiration (0.5 ± 0.07) or by puncturing (0.5 ± 0.06) and the time required for processing of ovaries for oocytes recovery was significantly less using the slicing method than the puncture or aspiration method. Martino *et al.* (1994) concluded that the slicing technique yielded more oocytes per ovary than dissection or aspiration. Das *et al.* (1996a) reported that the average recovery of oocytes by slicing and puncturing method was 6.4 ± 0.78 and 4.14 ± 0.41 respectively. Later Bonde *et al.* (2000) performed aspiration followed by puncture of ovine and caprine ovaries and concluded that follicle puncture following aspiration of ovaries increase the oocyte recovery rate and this increase was higher for goat ovaries (4.25 ± 0.42) in comparison with sheep (2.22 ± 0.39).

2.1.5 Media used for oocyte retrieval

Pawshe *et al.* (1993) and Ongeri *et al.* (2001) dissected ovaries in TL-Hepes media supplemented with 2-4mg/ml BSA. Das *et al.* (1996b) used PBS supplemented with 10 per cent heat inactivated goat serum for retrieval of oocyte of caprine origin. Mogas *et al.* (1997), Izquierdo *et al.* (1998), Izquierdo *et al.* (1999) and Katska-Ksiazkiewicz *et al.* (2004) recovered oocytes from caprine ovaries by mincing at room temperature in TCM 199 supplemented with 10 per cent fetal calf serum. To avoid the coagulation of retrieval medium with the follicular fluid, heparin was added @ of 1 IU/ml or 11.1µg/ml to TCM (Malik *et al.*, 1999 and Rodriguez-Gonzalez *et al.*, 2003).

2.1.6 Morphological evaluation of oocytes

2.1.6.1 Grading

Leibfried and First (1979) classified bovine oocytes based on the layer of cumulus and this system of classification was followed later by Bonde *et al.* (2000) and Kumar *et al.* (2004) for grading the caprine oocytes. Sarkhel *et al.* (1997) classified caprine oocytes into Grade I, II, III and IV. Grade I included oocytes with more than three layers of compact cumulus cells, grade II included oocytes having partially thick 1-2 layers of cells around ZP, grade III was having broken or incomplete cumulus cells and grade IV included naked oocytes with ZP covering. Later Kalita *et al.* (2000) followed this classification for micrometric characterisation of oocytes in Assam local goats.

2.1.6.2 Morphometry of oocytes

Oocyte diameter is an important factor in acquiring meiotic competence Yong, *et al.* (1997). Sarkhel *et al.* (1997) reported that the overall diameter of oocyte without and with cumulus was 159.99 µm and 221.05 µm respectively. From grade IV to grade I there was a gradual increase in the diameter of the oocyte with cumulus whereas there was no such pattern noticed in the oocyte without cumulus (Crozet *et al.*, 2000). Kumar *et al.* (2004) showed that the average diameter of categories 1, 2, 3 and 4 oocytes with ZP was 172.59 ± 0.94 µm, 164.00 ± 0.78 µm, 157.08 ± 1.22 µm and 149.66 ± 1.31 µm respectively. This increase in diameter of oocytes from category four to one might be because the category one oocyte had more number of cumulus cell layers than the other categories.

2.2 CRYOPRESERVATION OF OOCYTE

Variable superovulatory responses and reduced embryo recovery had rendered *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) procedures an attractive alternative for the production of additional offspring in farm animals (Sirard *et al.*, 1988). Because of limited time during which an oocyte remains viable preservation techniques at the germinal vesicle stage would have a great impact on research and commercial application (Hashimoto *et al.*, 2003). Thus cryopreservation of mammalian

oocytes could be used to establish an ova bank for reproductive biotechnology (Kharche *et al.*, 2005).

But cryopreservation separately or cumulatively contribute damage to the plasma membrane, ZP, organelles or cytoskeltal arrangements (Parks and Ruffing, 1992) especially in matured oocyte (Rall, 1992). But the freezing of immature oocytes at the germinal vesicle stage might circumvent these problems associated with freezing (Le Gal, 1996) and generate a readily available source of oocytes for research.

2.2.1 Conventional freezing

Otoi *et al.* (1993) and Le Gal (1996) used Hepes buffered TCM and Schmidt *et al.* (1995), Im *et al.* (1997) and Lim *et al.* (1999) used PBS as the holding media (HM) for cryopreservation of bovine and caprine oocytes in glycerol, Propanediol (PROH) and DMSO. The straws were cooled at 1-2.5^{0}C / minute to -5 to -7^{0}C and were held at this temperature for seven to 15 minutes at which seeding was induced. The straws were cooled further at 0.3 to 0.6^{0}C/minute to -30^{0}C to -35^{0}C, held for 10 to15 minutes and plunged into liquid nitrogen. The straws were thawed at 37^{0}C water bath for 20-30 s. Cryoprotectant was then removed by one step or three steps dilution in sucrose washing in holding medium.

2.2.2 Conventional vitrification

Vitrification offered several advantage and high survival rate of oocyte because of faster and simplified freezing and thawing procedures. But the concentration of cryoprotectants required to achieve vitrification was very high which led to toxic effects on oocytes (Niemann, 1991). Hence cryoprotectant concentration and duration of exposure are of critical importance for oocyte vitrification.

2.2.2.1 Addition of cryoprotectants

Arav *et al.* (1993) and Kharche *et al.* (2005) directly exposed the immature bovine and caprine oocytes to the vitrification medium (VM) that consisted of 40 per cent PROH with 0.25 M trehalose for 10 minutes and reported that the PROH was the cryoprotectant of choice for immature bovine oocyte vitrification because of its fast

permeating rate and low cytotoxic effect than glycerol and DMSO. Dutta *et al.* (1998b) and Agrawal (1999) exposed immature and matured caprine oocytes in 10 per cent glycerol and 20 per cent PROH for 10 minutes at room temperature followed by vitrification in medium containing 25 per cent glycerol and 25 per cent PROH at 4^{0}C. According to Wani *et al.* (2004b) the oocytes reaching to M-II stage after vitrification in 7 M glycerol (23.5 %) were significantly lower than that of the oocytes vitrified in 7 M EG, PROH and DMSO (42.5, 40.4 and 40.3 per cent respectively) and reported that the poor survival and maturation rate of the oocytes cryopreserved in glycerol was due to the low permeability of the cells to glycerol. Park *et al.* (2005) equilibrated bovine oocytes in 20 per cent EG for 10 minutes at 22-24^{0}C followed by vitrification in EFS for one minute.

Chen *et al.* (1994), Hotamisligil *et al.* (1996) and Otoi *et al.* (1998) reported that VM containing both permeating and non permeating cryoprotectant was suitable for oocytes than that contain permeating cryoprotectant only.

2.2.2.2 Thawing and cryoprotectant removal

Arav *et al.* (1993) and Kharche *et al.* (2005) thawed the straw in 37^{0}C water bath for 40 s, mixed the straw content with 1 M sucrose for one minute followed by the addition of PBS drop by drop to reach the final sucrose concentration of 0.125 M within 10 minutes. But Hochi *et al.* (1998), Dhali *et al.* (2000a), Dhali *et al.* (2000b), Wani *et al.* (2004a), Wani *et al.* (2004b) and Park *et al.* (2005) thawed straw in 20-30^{0}C water bath for 20-30 s and cryoprotectant was removed in one step procedure with 0.5 M sucrose for 5-10 minutes or as three step procedure using 0.5, 0.25 and 0.1 M sucrose for five minutes each.

Nag and Maurya (1997) reported that prolonged the storage of vitrified oocytes from 10-30 h in liquid nitrogen resulted reduction in maturation and fertilization rates. Bautista *et al.* (1998b) reported that five minutes dilution time was insufficient for the removal of intracellular EG from mouse oocytes which led to low recovery of oocytes with normal morphology and suggested that 10 minutes dilution was beneficial to the cells. Agrawal (1999) thawed straws in water bath at 20^{0}C and the straws were mixed

by shaking and kept for 10 minutes at 4^0C. Then sucrose was gradually diluted in 1, 0.5 and 0.25 M sucrose or directly washed in the HM.

2.2.2.3 Morphological evaluation

Papis *et al.* (1995) recorded high per cent recovery of morphologically intact bovine oocyte (56 %) after vitrification in EG with sucrose than in DMSO-Acetamide-PROH or PROH-Glycerol-Sucrose or EFS or EG-butanediol-Sucrose (33, 0, 30 and 35 % respectively). After vitrification of immature caprine oocytes in glycerol-PROH medium Dutta *et al.* (1998b) recorded 73.33 per cent morphologically normal oocytes. Agrawal (1999) could recover 92.8 per cent of vitrified immature caprine oocytes in glycerol-PROH medium and reported that remaining per cent of oocytes exhibited ZP and ooplasm damage. Kharche *et al.* (2005) morphologically evaluated vitrified caprine COCs after thawing and reported that 18.6 per cent of oocytes had greatly reduced cumulus mass, clear cytoplasm and ruptured ZP with abnormal shape.

Dhali *et al.* (2000b) recovered 88 and 98.4 per cent morphologically intact buffalo oocytes after equilibration for one and three minutes respectively in 50 per cent of the VM followed by vitrification in EG and DMSO. Dhali *et al.* (2000a) recorded 92.6 and 92 per cent morphologically normal buffalo oocytes after vitrification in 4.5 M EG with 3.4 M DMSO and 3.5 M EG with 3.4 M DMSO respectively while 69, 36, five and three per cent of the damaged oocyte showed cracking of ZP, leakage of cellular content, change in shape and split into two halves respectively.

2.2.2.4 Staining

Agrawal (1999), Wani *et al.* (2004a), Wani *et al.* (2004b), Kharche *et al.* (2005) and Park *et al.* (2005) used Orcein stain and Dhali *et al.* (2000a) and Dhali *et al.* (2000b) used Giemsa stain to assess the status of nuclear maturation rate of vitrified matured caprine, buffalo or porcine oocytes.

2.2.2.5 Oocyte developmental stage and its cryotolerance

Miyake *et al.* (1993) and Wani *et al.* (2004a) reported that the presence of cumulus cells provided considerable protection to mouse oocytes during vitrification so

germinal vesicle (GV) stage COCs, rather than *in vitro* matured oocytes was considered as better for vitrification. Fuku *et al.* (1994) and Fuku *et al.* (1995) studied the ultra structural changes in the bovine immature and *in vitro* matured oocytes after vitrification and reported that GV stage oocytes were more sensitive to vitrification than matured oocytes.

2.2.3 Open pulled straw vitrification

Vitrification was proposed in the literature as an alternative for avoiding chilling injuries and ice crystal formation, but the viability of oocytes after vitrification in conventional straws was not encouraging (Fuku *et al.*, 1995). Martino *et al.* (1996) showed that reducing the volume of VM and its direct exposure to liquid nitrogen enabled to reduce the chilling injury of bovine oocytes more efficiently. Later in the OPS method, a narrow plastic tube carrier and low volume of medium (1μL) enabled to achieve rapid cooling rate of 20000^{0}C per minute (Vajta *et al.*, 1997b) which decreased the chilling injury dramatically, permitted the use of less concentrated cryoprotectants, and shortened the time of exposure with the final cryoprotectant both before cooling and after warming (Vajta, 2000).

2.2.3.1 Addition of cryoprotectants

Vajta *et al.* (1998), Men *et al.* (2002), Li *et al.* (2002) and Albarracin *et al.* (2005) equilibrated bovine oocytes in 10 per cent EG with 10 per cent DMSO for 30-45 s followed by vitrification in medium containing 20 per cent EG, 20 per cent DMSO and 0.5 M sucrose for 20-30 s before plunging into liquid nitrogen. All the manipulations were performed on a slide warmer at 40-41^{0}C in a warm room (28^{0}C). Later Chang *et al.* (2004) suggested that the developmental capacity of vitrified oocytes could be improved by increasing the duration of equilibration to three minutes in 10 per cent EG and 10 per cent DMSO. Lowering the concentration of cryoprotectant with extending the duration of equilibrium resulted in lower survival rate.

2.2.3.2 Thawing and cryoprotectant removal

Men *et al.* (2002), Li *et al.* (2002) and Albarracin *et al.* (2005) warmed oocytes by immersing the end of straw into 1.2 ml of 0.25 M sucrose prepared in HM for 1-5

minutes at 37^0 C to achieve a faster warming rate and all oocytes were transferred into 1.2 ml 0.15 M sucrose in HM for another five minutes followed by washing in HM.

2.2.3.3 Morphological evaluation

Chen *et al.* (2000) recovered higher per cent of morphologically intact mouse oocytes after vitrification in the conventional straw than OPS method (81 vs. 62). Hurtt *et al.* (2000) observed that OPS vitrified equine and bovine oocytes showed some cumulus expansion immediately after thawing and the cumulus cells from vitrified oocytes were easier to remove which indicated the damage of cumulus-oocyte junctions. Later Chen *et al.* (2001) recorded higher per cent of morphologically intact mouse oocytes after vitrification in the closed pulled straw and conventional straw (79 and 77 respectively) than open pulled straw and electron microscope grid (63 and 39 respectively) and reported that the direct contact with liquid nitrogen on grid or a portion of the oocytes in the OPS had a negative effect on oocyte survival.

2.3 SYNCHRONISATION OF OESTRUS

Oestrus synchronization is a valuable tool for controlled breeding of animals especially in species where oestrus detection is difficult. Goat is such type of animal (Perera *et al.,* 1978) because of high variability in oestrus intensity and cyclical duration. As MOET is concerned, the time of breeding during oestrus following superovulatory treatment should be precise to have maximum fertilization rate and to recover more number of good quality embryos. Several agents are generally used to achieve these goals through synchronisation of oestrus. Nuti *et al.,* 1992; Mellado *et al.,* 1994; Romano, 1998; Ahmed *et al.,* 1998 and Kanuya *et al.,* 2000 used prostaglandin alone or Freitas *et al.* (1997), Senthilkumar *et al.* (1999), Lee *et al.* (2000) and Senthilkumar *et al.* (2003) used combination of prostaglandin with progestagen for synchronisation of oestrus. Mellado *et al.* (2000) and Lee *et al.* (2000) used progestagen in the form of subcutaneous auricular implant and Amarantidis *et al.* (2004) and Lehloenya *et al.* (2005) used in the form of intravaginal sponges for oestrus synchronisation in goats. Progesterone in the form of silicone intravaginal device like CIDR or CIDR-G was used by Menchaca and Rubianes, (2001), Greyling *et al.,* (2002)

Bretzlaff and Madrid (1985) compared the synchronization efficiency of 6 mg and 3 mg norgestomet ear implant with 400 IU PMSG and 50 µg cloprostenol and reported that 3 mg norgestomet were equally effective and more economic than whole implant. Ishwar and Pandey (1992) reported that synchronization scheme also included the administration of prostaglandin prior to or at the time of progestin removal in the transitional period. Norgestomet ear implant available for the synchronization in cattle (Synchromate-B and Crestar) was successfully used for synchronisation in goat without the co-treatment of prostaglandin (Pendleton *et al.*, 1992a; Pendleton *et al.*, 1992b and Lee *et al.*, 2000). Freitas *et al.* (1996) reported that almost 97 per cent animals responded when treated with 3 mg of norgestomet or 45 mg of fluorogestone acetate (FGA) sponges with 50 µg of cloprostenol. Freitas *et al.* (1997) compared the efficiency of FGA or norgestomet ear implant in non-lactating adult goat with PMSG and $PGF_{2\alpha}$ and reported that goats treated with half of the implant came into oestrus (27.8 ± 5.0 h) earlier than those receiving vaginal sponges (33.0 ± 6.6 h) and concluded that oestrus synchronization with a norgestomet implant either as such or half-implant did not reduce the variability in the onset of oestrus and LH peak.

2.4 SUPEROVULATION

Superovulation is the process by which the ovaries are stimulated to produce more than the normal number of ovulatory follicles and release more ova (Doijode *et al.*, 1992) to obtain consistently higher number of transferable quality embryos. For the induction of superovulation Pregnant Mare Serum Gonadotrophin (PMSG) or Follicle Stimulating Hormone (FSH) or its combination or gonadotrophin with or with out ovulation inducing agents has been widely used. But there is no real optimal superovulatory treatment regimen established for goats due to greater ovarian variation (Greyling *et al.*, 2002).

2.4.1 PMSG

Pregnant mare serum gonadotrophin had been widely used as means of inducing superovulation with embryo transfer techniques (Armstrong *et al.*, 1983a) and this is a suitable gonadotrophin due to its easy availability, single dose schedule and lower cost

superovulatory response due to the prolonged half-life of PMSG (Mc Intosh *et al.*, 1975) and anti-eCG antibodies production in repeat treatments (Roy *et al.*, 1999 and Drion *et al.*, 2001) had limited its use.

2.4.2 FSH

Use of PMSG in the superovulation protocol was replaced by the introduction of FSH due to its superior response in terms of ovulation, fertilization and embryo quality and low rate of anovulatory follicle (Armstrong *et al.*, 1983a and Sasada *et al.*, 2001) and premature luteal regression (Pendleton *et al.* 1992a; Rosnina *et al.*, 1992 and Riesenberg *et al.*, 2001) than PMSG. Purified FSH with little or no LH content product such as Folltropin-V (Cognie, 1999) and Super-Ov (Graff *et al.*, 2000) are available. However repeated superovulation with pFSH is associated with the appearance of anti-FSH antibodies in goat. Fortunately the superovulatory response was maintained in goats treated several times with ovine FSH (Cognie, 1999) which was also having very low LH activity (Lee *et al.*, 2000).

In an attempt to approximate the type of stimulation obtained with the single PMSG injection (Armstrong *et al.*, 1983a), several protocols have been used for superovulation in goat with FSH such as multiple injection of pFSH at 12 h interval started 48 h before progestagen withdrawal for four days (Ocampo *et al.*, 1988; Pendleton *et al.*, 1992b and Lee *et al.*, 1997) or six divided step down doses for three days (Nowshari and Holtz, 1993; Suyadi *et al.*, 2000 and Sasada *et al.*, 2001).

2.4.3 Anovulatory follicle

Armstrong *et al.* (1983a) administered 1000 IU PMSG with or with out progestagens and reported 5.8 ± 1.9 and 14.5 ± 2.4 anovulatory follicles respectively while Pendleton *et al.* (1992a), Biswas *et al.* (2001) and Senthilkumar *et al.* (2003) observed 3.1 ± 1.8, 4.17 ± 0.28 and 10.00 ± 0.8 respectively with 750-1000 IU PMSG. Armstrong *et al.* (1983a), Nuti *et al.* (1987) and Pendleton *et al.* (1992a) recorded 4.4 ± 1.9, 8.1 ± 5.6 and 0.9 ± 0.8 anovulatory follicles respectively in goats using pFSH. Pintado *et al.* (1996) reported 0.9 ± 0.5 and 2.3 ± 0.9 anovulatory follicles respectively

for does with or without PRCL occurrence. With 133 mg pFSH (Folltropin-V) Deshpande *et al.* (1997) observed 6.33 anovulatory follicles per animal.

2.4.4 Superovulatory response

Armstrong *et al.* (1983b) reported that as a single subcutaneous injection of 750-1250 IU PMSG between eight to 18 days after the previous oestrus resulted in 10.8 ± 1.2 ovulations per animal. Pendelton *et al.* (1992a), Singh *et al.* (1993), Senthilkumar *et al.* (2003) and Espinosa-Marquez *et al.* (2004) observed 12.5 ± 1.8, 1.25 ± 0.95, 11.33 ± 2.67 and 17.7 ± 3.3 ovulation respectively with 750-1000 IU PMSG.

Armstrong *et al.* (1983b), Nuti *et al.* (1987), Pendelton *et al.* (1992a) and Suyadi *et al.* (2000) recorded 16.1 ± 0.8, 17.7 ± 7.8, 14.7 ± 2.5 and 10.4 ± 1.1 ovulation respectively in goats treated with 10-18 mg pFSH. When superovulated with 20-200 mg pFSH (Folltropin-V), Deshpande *et al.* (1997), Greyling *et al.* (2002), Lima-Verde *et al.* (2003) and Senthilkumar *et al.* (2003) respectively noted 17.6, 18, 11.5 ± 6.6 and 21.83 ± 1.99 ovulation. With a total dose of 8.8 mg oFSH Gonzalez-Bulnes *et al.* (2003) and Gonzalez-Bulnes *et al.* (2004) recorded 14.3 ± 0.5 and 16.5 ± 0.9 ovulations respectively in Mediterranean goats.

2.4.5 Variability in superovulatory response

Variation in the superovulatory response was depended on many factors such as age (Armstrong and Evans, 1983; Gootwine *et al.*, 1997 and Lee *et al.*, 2000), the type and amount of gonadotrophin preparations used (Armstrong *et al.*, 1983a and Pintado *et al.*, 1998), FSH:LH ratio in the product used (Mc Natty *et al.*, 1989 and Batt *et al.*, 1993), combination of different gonadotrophin preparations used (Akinlosotu and Wilder, 1993 and Saharrea *et al.*, 1998), repeated collection (Gootwine *et al.*, 1997), dose (Pintado *et al.*, 1998 and Lee *et al.*, 2000), breed (Gonzalez Bulnes *et al.*, 1999 and Greyling *et al.*, 2002) and status of follicular development of the animal at the time of initiation of superovulatory treatment (Gonzalez-Bulnes *et al.*, 2003). However an average of six to eight transferable embryos per donor can be produced in a successful goat MOET program (Cognie *et al.*, 2003).

2.4.6 Premature luteal regression of CL

Premature regression of corpora lutea was an undesired condition usually encountered during surgical collection of embryos on 6-8 days after the onset of oestrus at which the CL was appeared as white or pale or light pink coloured small structures, (Armstrong *et al.*, 1983a; Tervit *et al.*, 1983; Pendleton *et al.*, 1992a; Saharrea *et al.*, 1998; Tiwari *et al.*, 1999 and Joseph, 2003) having the size of ≤ 5 mm (Saharrea *et al.*, 1998).

Incidence of this condition was more commonly seen in PMSG treated animal than FSH (Armstrong *et al.*, 1983a; Pendleton *et al.*, 1992a and Riesenberg *et al.*, 2001) due to continuous recruitment of follicle and the possibility of endogenous secretion of PGF2α of either follicular or uterine origin due to the stimulation of oestradiol from these large anovulatory follicles. Even though the condition is more associated with PMSG it is also seen in FSH treated goats (Pendleton *et al.*, 1992a; Pintado *et al.*, 1996; Tiwari *et al.*, 1998; Tiwari *et al.*, 1999 and Senthilkumar *et al.*, 2003).

Armstrong *et al.* (1983b), Tervit *et al.* (1983) and Chemineau *et al.* (1986) reported that PRCL condition affected the embryo recovery rate and quality of embryo due to abnormal embryo transport as a result of endocrine abnormalities in PMSG or FSH treated animal. There are many methods to circumvent the low recovery of embryos in PRCL condition such as collection of embryo between day two and day four after the onset of heat (Armstrong *et al.*, 1983b), use of prostaglandin inhibitors (Battye *et al.*, 1988), administration of hCG (Saharrea *et al.*, 1998) and supplementation of progesterone from later half of day four of superovulatory heat until the day of embryo collection (Joseph, 2003).

2.5 EMBRYO COLLECTION

The first successful embryo collection and transfer in goat was reported by Warwick *et al.* (1934) with the intention to produce hybrid between goat and sheep and this method of collection became popular for recovery of embryos from goat.

2.5.1 Media

The flushing media is a well-balanced physiological solution used to retrieve embryos from uterus or oviduct and acts as a nourishing medium through out the procedure. The commonly used medium is PBS or DPBS with pH 7.2-7.4. It is enriched with 2-10 per cent fetal calf serum (Krisher *et al.*, 1994; Pintado *et al.*, 1996, Pintado *et al.*, 1998 and Senthilkumar *et al.*, 2003) or heat inactivated goat serum @ 2-20 per cent (Pereira *et al.*, 1998; Gogai *et al.*, 2001; Joseph, 2003 and El-Gayar and Holtz, 2005) as a source of certain essential amino acids and to prevent the sticking of embryos to the collection petridishes.

In order to prevent the bacterial and fugal growth, flushing medium was usually supplied with antibiotics and antimycotics (Nuti *et al.*, 1987; Bessoudo *et al.*, 1988 and Pendleton *et al.*, 1992a). Pereira *et al.* (1998) and El-Gayar and Holtz (2005) added 100 IU/ ml penicillin and 100 mg/ml streptomycin to DPBS medium containing two per cent heat-inactivated goat serum.

2.5.2 Anaesthesia

William *et al.* (1994) reported that the quality of xylazine-ketamine combination was excellent in terms of duration of anaesthesia, muscle relaxation and exteriorization of uterus. Joseph (2003) had given 0.2 mg of xylazine as IM injection 10 minutes before the administration of ketamine IM @ 10mg/kg. During the operation anaesthesia was maintained by administering ketamine @ 5mg/kg body weight.

2.5.3 Technique

2.5.3.1 Surgical

Three techniques involving laparotomy have been described for the collection of embryos, all of which involves exteriorization of uterus through a mid ventral incision and examination of the ovaries to determine the response to superovulatory treatment. Utero-oviductal embryo collection by Armstrong *et al.* (1983b), Chemineau *et al.* (1986) and Pendleton *et al.* (1992a), oviductal collection by Nuti *et al.* (1987), Goel *et al.* (1993), Krisher *et al.* (1994), Deshpande *et al.* (1997), Lee *et al.* (2000), Senthilkumar *et al.* (2003) and Freitas *et al.* (2003) and uterine collection by Tiwari *et*

al. (1999), Dhandapani *et al.* (2001), Greyling *et al.* (2002) and Espinosa-Ma´rquez *et al.* (2004) are the usual methods of embryo collection followed in relation to the age of embryo.

2.5.3.2 Non surgical

Non surgical collection via laparoscopy (Dhandapani *et al.*, 2001) and transcervical method (Lima-Verde *et al.*, 2003) in goat was less expensive and less traumatic which prevented the formation of post surgical adhesions and enables to use genetically superior donor repeatedly for embryo collection.

2.5.4 Embryo recovery rates

The embryo recovery rate was usually influenced by day of collection (Armstrong *et al.*, 1983b and Sarmah *et al.*, 1996a), avascular corpora lutea (Armstrong *et al.*, 1983b), ovulation rate (Gootwine *et al.*, 1997; Gogai *et al.*, 2001; Terblanche *et al.*, 2001 and Hasin *et al.*, 2004), type of gonadotrophin (Senthilkumar *et al.*, 1999), administration of ovulation inducers (Biswas *et al.*, 2001), site of recovery and technique (Gogai *et al.*, 2001 and Dhandapani *et al.*, 2001) and season (Terblanche *et al.*, 2001).

Pargaonkar *et al.* (1992) reported that the per cent recovery of ova/embryo and embryo were 82.75 ± 2.12 and 76.35 ± 7.8 respectively on day five after the breeding from superovulated goats. Terblanche *et al.* (2001) obtained 12.5 ± 6.3 ova and embryo from the uterine flushing six days after AI.

Nuti *et al.* (1987) could recover 67 per cent ova/embryo by uterine flushing on day seven after the mating, out of which 51.85 per cent were fertilized ova. Gonzalez-Bulnes *et al.* (2003) recovered 11.3 ± 0.5 embryos per goat with a mean recovery rate of 78.6 ± 1.9 per cent uterine flushing of Murciana-Granadina goats on day eight following sponge removals.

2.6 MORPHOLOGICAL EVALUATION OF EMBRYOS

2.6.1 Grading

Linder and Wright (1983) classified bovine embryos as excellent, good, fair and poor. Later this system of classification was widely accepted for assessing the viability of caprine embryos (Pendleton *et al.*, 1992a; Pintado *et al.*, 1996; Pereira *et al.*, 1998; Pintado *et al.*, 1998; Tiwari *et al.*, 1999; Suyadi *et al.*, 2000 and Hasin *et al.*, 2004).

Nowshari and Holtz (1993) and Nowshari and Holtz (1995) classified caprine embryos as good, fair and poor and they considered that embryo with any imperfection, irregular shape, too light or too dark colour were not suitable for cryopreservation. According to Senthilkumar *et al.* (1999) the excellent and good quality embryos were considered as transferable whereas Selvaraju *et al.* (2003) considered excellent, good and fair quality embryos as transferable.

2.6.2 Developmental stages of embryo

Shea, (1981) reported that the most extensively used criteria to evaluate bovine embryos are whether an embryo has attained an appropriate stage of development. According to Pintado *et al.* (1996) the mean number of unfertilized oocytes, 2-16 cell, morula, early blastocysts and blastocysts recovered on day six after the onset of oestrus were 2.0 ± 0.4, 1.0 ± 0.4, 6.0 ± 0.8, 0.5 ± 0.3 and 0.4 ± 0.4 respectively. But on contrast Dutta *et al.* (1998a) recorded high number of blastocysts than morula after the uterine flushing on day six of post oestrus. Biswas *et al.* (2001) recovered 53.08 per cent morula, 28.39 per cent early blastocysts, 12.39 per cent 2-8 cell stage and 6.19 per cent degenerative embryos during the collection on day six of superovulatory response. Joseph (2003) obtained 100 per cent morula from prepubertal as against 96.30 per cent morula and 3.7 per cent blastocyst from adult Malabari goats on day six after the onset of oestrus.

Collection on day seven after mating Nuti *et al.* (1987) recovered 36 eggs, out of which one was four celled, 12 were morula, 15 were blastocysts and eight were unfertilized eggs. Sasada *et al.* (2001) reported that collection on 6-8 days post coitus yielded morula stage and on 8-10 days blastocysts stage. Joseph (2003) reported that on

day seven after the onset of heat both morula and blastocysts could be recovered from both prepubertal and adult Malabari goats and on eighth day all were blastocysts stage.

2.7 CRYOPRESERVATION OF EMBRYOS

Since the first report of normal offspring from cryopreserved mouse embryos (Whittingham *et al.*, 1972), the cryopreservation of mammalian embryos has became an integral part of methods to control animal reproduction. Advancement in cryobiology, cell biology and domestic animal embryology have enabled the development of embryo preservation methodologies for sheep and goat (Dobrinsky, 2002). As the part of development in cryobiology, several cryopreservation methods such as conventional slow freezing, rapid freezing and vitrification have been put forward to preserve embryos of many species which resulted in the birth of live offspring.

2.7.1 Conventional freezing

The conventional freezing and thawing procedures of caprine embryo included initial exposure to an equilibriation with cryoprotectants (in one step or three step), cooling to subzero temperature (1°C/minute from 26°C to -7°C or directly to alcohol bath precooled to -5°C), seeding for 10 minutes, slow cooling (0.3°C/minute to -32 or -36°C), stored in liquid nitrogen, thawing at 30-35°C for 40 s to two minutes and finally dilution and removal of cryoprotectants in one step or three step (Wang *et al.*, 1988; Nowshari and Holtz, 1993; Nowshari and Holtz, 1995 and El-Gayar and Holtz, 2001).

In the first report of successful transfer of frozen caprine embryos by conventional slow freezing technique, Bilton and Moore (1976) reported that glycerol was the better cryoprotectant than DMSO. Le Gal *et al.* (1993) showed that EG is a better cryoprotectant than glycerol. Nowshari and Holtz (1995) cryopreserved either freshly collected blastocysts or blastocysts cultured from morula by conventional slow freezing in 1.4 M glycerol and showed that frozen thawed freshly collected blastocyst was superior in terms of pregnancy rate and embryo survival rate (83 and 67 % respectively) than cultured blastocyst from morula (54 and 41 % respectively). Traldi *et al.* (2000) compared the conventional freezing and vitrification of caprine embryos

(morula to hatched blastocyst) and reported that slow freezing in EG was superior to vitrification with glycerol and EG combination.

2.7.2 Conventional vitrification

The slow freezing protocols that are widely used to cryopreserve embryos from different species require an expensive biological freezer and the procedures are time consuming. The successful cryopreservation of embryos by vitrification was first reported by Rall and Fahy (1985a). The main advantage of vitrification was it obviates the need for a freezing machine and reduced the time required for cryopreservation compared to traditional methods (Ishimori *et al.*, 1993; Szell and Windsor, 1994; Mahmoudzadeh *et al.*, 1994 and Ptak *et al.*, 1999). Baril *et al.* (2001) suggested that the use of ultra rapid technique such as vitrification might help to reduce a part of the cost in ovine MOET.

2.7.2.1 Principles of freezing

Rall (1987) explained that a shrink-swell change occurred when embryo were placed in 25 per cent VM. The initial shrinkage resulted from the osmotic dehydration of the cytoplasm of the embryo due to the exposure to hypertonic solution. After reaching the osmotic equilibrium embryos would gradually swell as the cryoprotectant permeated into the cytoplasm. When embryos transferred into more concentrated VM at 4^0C they shrink again due to osmotic dehydration and remain shrunken. Eventually cytoplasm became sufficiently concentrated to be capable of vitrifying upon cooling to low temperature. Ali and Shelton (1993c) stated that during cooling, vitrification was evidenced by the formation of a transparent glass and during warming; medium that did not devitrify was transformed from solid clear state to the liquid state without the evidence of a milky appearance. Ali and Shelton (1993c) reported that volume and surface area affected the response of solutions to cooling and warming. Martinez *et al.* (1998) indicated that the combination of cryoprotectants was important, but the number of steps and the concentrations of the cryoprotectants during equilibriation before the VM appeared to be more important.

2.7.2.2 Holding media

Kuwayama *et al.* (1992) showed that TCM 199 could be successfully used for the vitrification of *in vitro* produced bovine embryos. Vajta *et al.* (1996b), Vajta *et al.* (1997a), Nagashima *et al.* (1999), Pugh *et al.* (2000) and Lazar *et al.* (2000) used Hepes buffered TCM supplemented with 20 per cent calf serum or 0.4 per cent BSA because of the simplicity of using the same base medium in the *in vitro* maturation, vitrification and culture steps. Ptak *et al.* (1999), Nguyen *et al.* (2000), Baril *et al.* (2001), Martinez *et al.* (2002) and Al-Katanani *et al.* (2002) used phosphate buffered saline containing 3-4 mg/ml BSA or 10-20 per cent Fetal calf serum as the HM for the vitrification procedures of embryos.

2.7.2.3 Cryoprotectant

Kasai *et al.* (1992b) suggested that inclusion of one or more cryoprotectant additives in the freezing medium was essential for successful cryopreservation of mammalian embryos in liquid nitrogen. Ali and Shelton (1993b) reported that the major role of VM wass to induce dehydration in embryos thereby greatly increasing the intracellular concentration to allow intracellular and extracellular glass during cooling and the choice of the most appropriate cryoprotectant and its concentration was very important since the efficiency of vitrification is directly proportional to the cryoprotectant concentration (Bautista and Kanagawa, 1998).

2.7.2.3.1 Permeating cryoprotectant

Ethylene glycol

Szell *et al.* (1989) reported that day six sheep and day seven cattle embryos are more permeable to EG than to glycerol. Ali and Shelton (1993c) showed that EG is least toxic, which was followed in the order of increasing toxicity by methanol, glycerol, DMSO, propylene glycol and butylene glycol. Mahmoudzadeh *et al.* (1993) and Bautista and Kanagawa (1998) stated that the molecular weight of the EG is lower than that of glycerol, propylene glycol and DMSO. Therefore sufficient permeation of EG into embryos for vitrification and its removal during dilution taken place in a

shorter time than that for other cryoprotectants resulting in a decrease in the rate of embryo toxicity.

EFS medium was successfully used for the vitrification of rabbit morula (Kasai *et al.*, 1992a), bovine compact morula and expanded blastocysts (Mahmoudzadeh *et al.*, 1993; Mahmoudzadeh *et al.*, 1994 and Mahmoudzadeh *et al.*, 1995). Later Martinez and Matkovic (1998) showed that EFS medium was more effective than glycerol-propylene glycol for the vitrification of ovine embryos in terms of pregnancy rate (40.0 vs. 28 %).

Glycerol

Ali and Shelton (1993a) suggested that toxicity of VM was probably positively related to the amount of glycerol present. By using 6.5 M glycerol and six per cent BSA, Rall and Wood (1994) demonstrated that mouse embryo could be vitrified with widest range of cooling and warming condition, Dobrinsky *et al.* (2000) produced live healthy piglet from vitrified embryos. Nedambale *et al.* (2004) vitrified *in vitro* produced bovine embryos more effectively than the conventional slow freezing method.

Propylene glycol

Ali and Shelton (1993c) reported that propylene glycol was a good vitrifying agent but its toxicity did not favour its use in vitrification of embryos. Szell and Windsor (1994) showed that embryos vitrified in mixtures containing glycerol and EG required cryoprotectants at the level of 4.5 M concentration each to achieve a survival rate of 43 per cent comparable to that obtained with 3.5 M glycerol and 3.5 M propylene glycol (48 %) there by suggested that the higher toxicity of propylene glycol could be partially offset by its effectiveness at a lower concentration.

Dimethyl sulfoxide

Ishimori *et al.* (1992a) formulated a VM containing 25 per cent EG and 25 per cent DMSO and succeed in the vitrification of bovine embryos. This method was later adopted by Ishimori *et al.* (1992b) and Ishimori *et al.* (1992c) for vitrification of mouse

morula and blastocysts, Ishimori *et al.* (1993) and Vajta *et al.* (1996a) for bovine morula and blastocysts and Vicente and Garcia-Ximenez (1996) for rabbit embryos.

Ali and Shelton (1993c) reported that DMSO was having greater glass forming characteristics, which improves vitrification. Vicente and Garcia-Ximenez (1994) reported that DMSO is usually regarded as less permeable but in combination with EG the permeability of the two cryoprotectants seems to be even higher than that of EG alone.

2.7.2.3.2 Non-permeating cryoprotectant

Szell and Shelton (1986) reported that sugars contributed to the dehydration of the cells prior to permeation by the cryoprotectant. Liebermann *et al.* (2002) indicated that the incorporation of non-permeating compounds into the vitrifying medium and the incubation of the cells in this medium before vitrification helps to withdraw more water from the cells and lessens the exposure time of the cells to the toxic effects of the cryoprotectants.

Kasai (1992a), Ali and Shelton (1993a) and Ali and Shelton (1993b) showed that addition of 1 M sucrose allowed the concentration of EG to be reduced with out the loss of vitrifying capacity. Kasai (1996) reported that sucrose present in the EFS medium promoted shrinkage and reduced intracellular concentration of cryoprotectant in embryo before dilution.

According to Kasai *et al.* (1992b) and Martinez *et al.* (2002) sucrose at the level of 0.75 M to 1 M had toxic effect on mouse embryos and bovine blastocysts respectively. Ali and Shelton (1993b) reported that day six sheep blastocysts did not survive when they were subjected to dehydration with sucrose before equilibration and vitrification.

Valdez *et al.* (1991) and Valdez *et al.* (1992) showed that 1 M trehalose in a single step dilution could be effectively used to dilute the embryos out of a highly concentrated VM after thawing. Donnay *et al.* (1998), Kaidi *et al.* (1998), Baril *et al.* (2001) and Kaidi *et al.* (2001) used 0.8 to 1.7 M galactose dissolved in HM.

Macromolecules

According to Kasai *et al.* (1992b), Kasai (1996), Martinez *et al.* (1998) and Nguyen *et al.* (2000) macromolecules like Ficoll assisted the formation of stable glasses, allowed to reduce the cryoprotectant concentration and improved the capacity of embryos to tolerate the cryoprotectant. Macromolecule such as Polyvinylpyrrolidone (Titterington *et al.,* 1995), Polyvinylalcohol (Naitana *et al.,* 1997) and Polyethylene glycol (Ohboshi *et al.,* 1997a) were effective for vitrification.

2.7.2.4 Addition of cryoprotectant

One strategy that controls the extent of cryoprotectant permeation and produce necessary degree of cytoplasmic dehydration was to use a stepwise equilibration procedure (Rall, 1987). Kasai *et al.* (2002) showed that when embryos were vitrified with EFS 40 for 10 s at 25^{0}C without equilibration, none of the embryo was survived which indicated that they were injured by insufficient permeation of EG. Pugh *et al.* (2000) demonstrated that for a simple two-step method of cryoprotectant addition and in-straw dilution, VM that contained two or three permeating cryoprotectants was superior to that contained a single cryoprotectant.

2.7.2.4.1 One step method

Kasai *et al.* (1990) and Mahmoudzadeh *et al.* (1993) reported that one step vitrification with EFS was highly efficient for embryos in terms of survival rate.

2.7.2.4.2 Two step method

Ishimori *et al.* (1993) equilibrated bovine embryos in 12.5 per cent DMSO and 12.5 per cent EG for one or two or five minutes at room temperature and reported that the survival rate of the vitrified blastocysts after one and two minutes equilibrations (85 and 73 % respectively) were higher than five minutes equilibriation (20 %). But Vajta *et al.* (1996a), Vajta *et al.* (1996b), Vajta *et al.* (1997a) and Markkula *et al.* (2001) performed vitrification by equilibration of embryos with 12.5 per cent EG and 12.5 per cent DMSO at 20-25°C for 60 s, then with 25 per cent EG and 25 per cent DMSO at 4°C for another 60 s.

2.7.2.4.3 Three step method

At room temperature Agca *et al.* (1994) equilibrated and vitrified bovine blastocysts in 10 per cent glycerol for five minutes, 10 per cent glycerol and 20 per cent EG for five minutes and finally in the VM consisting of 25 per cent glycerol and 25 per cent EG for 30 s before plunging into liquid nitrogen. Later this procedure widely used by Agca *et al.* (1998), Donnay *et al.* (1998), Martinez *et al.* (1998), Baril *et al.* (2001), Al-Katanani *et al.* (2002) and Okada *et al.* (2002a) for vitrification of bovine and sheep embryos.

2.7.2.5 Temperature

Rall and Fahy (1985b), Rall (1987) and Szell and Windsor (1994) exposed the embryos to VM at 4^0C to eliminate the cryoprotectant toxicity by reducing its permeation. Vajta *et al.* (1996a) reported the advantage of low-temperature dehydration by relatively long incubation (up to one minute including loading and sealing) that improved *in vitro* viability of ovine embryos (Martinez and Matkovic, 1998).

Kasai *et al.* (1992b) showed that at normal room temperature of 20-25^{0}C, only 0.5 minute of exposure to EFS medium was enough for a high post-vitrification survival rate. Later Martinez and Matkovic (1998) indicated that EFS medium had the advantage that it did not require cooling 4^0C, so made it more suitable for cryopreservation of embryos under field condition. Ishimori *et al.* (1993) reported that using an EG/DMSO mixture, cooling was not required to achieve high survival rates, and this was regarded as a major advantage for on-farm conditions. de Paaz *et al.* (1994) reported that sheep morula and blastocysts showed limited *in vitro* viability when they were vitrified in the medium containing 25 per cent glycerol and 25 per cent propylene glycol at room temperature.

2.7.2.6 Time

Valdez *et al.* (1990) reported that high survival rate of mouse embryos was achieved after 10 minutes equilibration than five or 20 minutes exposure in glycerol and propylene glycol. Ishimori *et al.* (1993) carried out equilibration of bovine embryos in EG and DMSO for one, two and five minutes at room temperature and reported that

high survival rates were reduced after five minutes equilibration. Kasai (1996) indicated that if the exposure is too short, the permeation of the cryoprotectant would not be sufficient to reduce intracellular ice formation. Therefore the optimal exposure time for the successful vitrification must be a compromise between preventing the toxic injury and preventing the intracellular ice formation.

2.7.2.7 Method of loading embryos into the vitrification medium

de Paaz *et al.* (1994) reported that addition of air bubble adjacent to the column of VM have the advantage that it avoids both the devitrification due to sucrose contamination and the breakage of straw during thawing. Szell and Windsor (1994) filled the straw by aspiration with fraction containing two air bubbles separating the VM. Vitrification medium containing embryo were separated by two air bubbles from two columns of 0.5 M sucrose medium (SM) was reported by Naitana *et al.* (1995), Naitana *et al.* (1996), Ito *et al.* (1999) and Dattena *et al.* (2000).

2.7.2.8 Method of plunging straws into the liquid nitrogen

In order to prevent bursting of the straw caused by the rapid freezing of the solution Rall (1992), Ishimori *et al.* (1993), Szell and Windsor (1994), Naitana *et al.* (1997), Ito *et al.* (1999) and Markkula *et al.* (2001) cooled the straw first in liquid nitrogen vapour for 1-2 minutes (cooling rate 200^0C/minute) followed by direct plunging into liquid nitrogen. Pollard and Leibo (1994) observed a slightly higher survival rate of cryopreserved morula when the cooling rate was increased and suggested that extreme chilling injury of embryos at the early stages could be overcome sufficiently by rapid cooling. In order to achieve the rapid cooling rate de Paaz *et al.* (1994), Naitana *et al.* (1996), Ptak *et al.* (1999), Dattena *et al.* (2000), Leoni *et al.* (2001) and Martinez *et al.* (2002) directly plunged the straw into the liquid nitrogen vapour. Kasai (1996) suggested that fracture damage occurs during both cooling and warming, but the vitrification in vapor appeared to prevent sample fracture that could damage embryos or the ZP.

2.7.2.9 Warming

Rall (1992), Bautista *et al.* (1997), van Wagtendonk-de Leeuw *et al.* (1997) and Baril *et al.* (2001) warmed the straw in air for 5-10 s and then immersed in water 18-20^{0}C for 10-20 s or until all ice had disappeared. de Paaz *et al.* (1994), Szell and Windsor (1994), Naitana *et al.* (1995), Ito *et al.* (1999) and Markkula *et al.* (2001) directly thawed the straw in water bath at 20-22^{0}C for 8 to 15 s. Saha *et al.* (1996), Kaidi *et al.* (1998), Ptak *et al.* (1999), Dattena *et al.* (2000), Martinez *et al.* (2002), Al-Katanani *et al.* (2002) and Okada *et al.* (2002a) thawed straws in water bath maintained at temperature of 30-37^{0}C directly for 10-20 s or after six seconds in air.

2.7.2.10 Cryoprotectant removal

Ishimori *et al.* (1993), Ali and Shelton (1993a) and Ali and Shelton (1993c) suggested that sucrose dilution was beneficial for the survival of embryos after exposure to the VM for 10 minutes or longer. Mahmoudzadeh *et al.* (1993) suggested that a low concentration of sucrose for dilution of EG was found to reduce the chance of possible osmotic injuries of bovine embryos. Vajta *et al.* (1995) reported that direct in-straw rehydration in the HM with out sucrose dilution was also possible and after thawing bovine embryos survived in straw storage for up to 30 minutes, which affords sufficient time to make this method applicable for on-farm use. Later Markkula *et al.* (2001) followed this method for vitrification of bovine embryos for *in vitro* studies.

Ishimori *et al.* (1993), de Paaz *et al.* (1994), Szell and Windsor (1994), Dutta *et al.* (1998a), Kaidi *et al.* (1998) and Baril *et al.* (2001) carried out dilution by simple mixing of VM with 1 M sucrose or 0.5-1.7 M galactose kept for 5 to 10 minutes followed by washing in sucrose or galactose free HM for another 5 to 10 minutes.

After thawing Agrawal *et al.* (1994), Martinez *et al.* (1998), Martinez *et al.* (2002) and Al-Katanani *et al.* (2002) diluted embryo in 1 M SM, then in 0.5 M SM, and finally in 0.25 M SM (five minutes in each step at room temperature) before the embryos were washed in PBS. Ptak *et al.* (1999), Dattena *et al.* (2000) and Okada *et al.* (2002a) diluted embryo in 0.5 M SM for 3-5 minutes followed by 0.25 M SM and finally 0.125 M SM each for additional 3-5 minutes.

2.7.2.11 Viability assessment

2.7.2.11.1 Morphological evaluation of vitrified embryo

Kasai (1992a) and Kasai (1996) reported that 3.6 per cent rabbit morula and 1.6 per cent mouse morula were having ZP damage in EFS40. Agrawal *et al.* (1994) reported that 21.43 per cent thawed caprine embryos (morula to blastocysts stage) after vitrification showed ZP damage.

Saha *et al.* (1996) indicated that the inner cell mass (ICM) from frozen blastocysts were partially distorted and the cell-to-cell contact was less tight than that from fresh blastocysts, which indicated that the cell-to-cell contact was lost during freezing and thawing. Agrawal and Polge (1989) recovered 88.4 and 96.6 per cent morphologically normal vitrified mouse embryos after one step and three-step dilution procedure respectively. Dutta *et al.* (1998a) reported that after vitrification and warming 33.57 and 51.43 per cent morula and blastocysts respectively retained normal morphology. Nakao *et al.* (1998) reported that approximately 88 per cent of mouse blastocysts were morphologically normal and Kito *et al.* (2003) reported that 87 per cent 2-cell mouse embryo and 92 per cent 4-8 cell stage embryos were morphologically normal after vitrification.

Nguyen *et al.* (2000) achieved 97 and 96 per cent morphologically normal *in vitro* produced bovine blastocysts after vitrification in 60 per cent EG and EFS medium respectively at 4^0C which showed the efficiency of vitrification with partial dehydration in EG medium. According to Okada *et al.* (2002a) after the vitrification and warming of 29 ovine embryos (22 morula and 7 blastocysts), 69 per cent embryos retained normal morphology (13 morula and 7 blastocysts). Han *et al.* (2003) and Hredzak *et al.* (2005) reported that 100 and 77.3 per cent rat and mouse embryos respectively retained the normal morphology after vitrification in EFS medium.

Baril *et al.* (2001), Berthelot *et al.* (2003) and Menezo (2004) reported that morphology, whatever be the stage, was not a good criterion of embryo quality because it might change significantly due to recovery from cold, osmotic and toxic shock.

Selection based on strict morphology may result in the elimination of many viable embryos.

2.7.2.11.2 Staining

Many staining techniques are available in the assessment of embryo viability. Saha *et al.* (1996) and Bautista *et al.* (1998a) counted the mean cell number of vitrified cultured mouse and bovine blastocysts respectively with Giemsa stain. Vajta *et al.* (1997a) used Orcein staining to assess the total number cells of day 8 blastocysts. Ohboshi *et al.* (1997b) and Donnay *et al.* (1998) stained the vitrified cultured bovine embryo with Hoechst 33342 stain for determination of total cell number.

2.7.3 Open pulled straw vitrification

2.7.3.1 Principle of freezing

Recent progress in the cryopreservation of mammalian oocytes and IVP embryos includes vitrification characterized by an ultra-rapid cooling rate. Vajta *et al.* (1997b) reported an alternative method of ultra-rapid cooling that utilized open pulled plastic straws (OPS) for vitrification of bovine *in vitro* produced embryos.

To achieve high cooling rate by decreasing the volume of the final cryoprotectant, Vajta *et al.* (1997b) pulled 0.25 ml Cassou straw over a hot plate. The inner diameter and wall thickness was reduced from 1.7 mm to 0.8 mm and 0.15 to 0.07 mm respectively, which improved rapid heat transfer during cooling in liquid nitrogen. Vajta *et al.* (1998) measured the average cooling rate of liquid column in OPS as 16,700°C/minute between 0 and -195°C and those in normal-sized and heat-sealed control straws were 2,438°C/minute. Vajta *et al.* (1998) and Vajta *et al.* (2000) mentioned that OPS vitrification decreased the chilling injury dramatically (Begin *et al.*, 2003 and Tominaga and Hamada, 2004), which permited the use of less concentrated cryoprotectant solutions (Lopez-Bejar and Lopez-Gatius, 2002; Berthelot *et al.*, 2003 and Naik *et al.*, 2005) and reduce the time of exposure to the cryoprotectant (Beebe *et al.*, 2002 and Lopatarova *et al.*, 2002).

2.7.3.2 Holding media

Vajta *et al.* (1998), Vajta *et al.* (1999b), Lazar *et al.* (2000), El- Gayar and Holtz (2001), Berthelot *et al.* (2001) and Lopatarova *et al.* (2002) used TCM with 10-20 per cent calf serum or goat serum as the HM for the vitrification of bovine, caprine, ovine and porcine embryos. Vajta *et al.* (1999a) and Vajta *et al.* (1999c) reported that there was no difference in the survival and developmental rate when complex TCM 199 was replaced with simple PBS and serum was replaced with bovine serum albumin. Later Okada *et al.* (2002b), Cho *et al.* (2002), Dattena *et al.* (2004) and Naik *et al.* (2005) used PBS containing 5-20 per cent calf serum for vitrification of ovine, rabbit and bovine embryos.

2.7.3.3 Temperature of the cryoprotectant

Vajta *et al.* (1998) and El- Gayar and Holtz (2001) carried out all processing on a heated stage at 39^{0}C in a warm room so that the final temperature of the medium was 34-36^{0}C. Vajta *et al.* (1999c) showed that decreasing the temperature from 35^{0}C either at equilibration or during rehydration decreased both survival and developmental rate of vitrified warmed blastocysts. But Berthelot *et al.* (2001) and Dattena *et al.* (2004) could successfully performed vitrification of porcine and ovine embryos respectively at room temperature (22-24^{0}C).

2.7.3.4 Addition of cryoprotectant and duration of exposure

Vajta *et al.* (1998) and Lazar *et al.* (2000) incubated bovine embryos in 7.5 per cent EG and 7.5 per cent DMSO for three minutes and then transferred to an approximately 1-2 µL of 16.5 per cent EG, 16.5 per cent DMSO and 0.5 M sucrose. The time between the contact of embryo and cooling did not exceed 25 s. El- Gayar and Holtz (2001), Berthelot *et al.* (2001), Rizos *et al.* (2001), Cho *et al.* (2002) and Isachenko *et al.* (2003a) incubated caprine, ovine, bovine or porcine embryos in 10 per cent EG and 10 per cent DMSO for 1-3 minutes and then transferred to an approximately 1-2 µL of 16.5-20 per cent EG with 16.5-20 per cent DMSO and 0.4-0.6 M sucrose/trehalose or with out sucrose/trehalose. The time between the contact of embryo and cooling was within 20 s to one minute. Isachenko *et al.* (2003a) reported that OPS vitrification of ovine embryos with EG, DMSO and sucrose combination

produced better pregnancy rate than glycerol and EG (60 % vs. 25 %) but on contrast Dattena *et al.* (2004) recorded almost similar pregnancy rate of 50.0 vs. 51.7 per cent after the transfer of ovine embryos vitrified with EG-DMSO-Sucrose and glycerol-EG media respectively.

2.7.3.5 Warming

Vajta *et al.* (1998), Lazar *et al.* (2000), El- Gayar and Holtz (2001), Rizos *et al.* (2001), Lopatarova *et al.* (2002) and Cho *et al.* (2002) warmed embryos by immersing the end of the straw, into 1.2 ml of 0.25-0.33 M sucrose prepared in HM, for one or five minutes and then all embryos were transferred into 1.2 ml 0.15-0.2 M sucrose prepared in HM for another 1-5 minutes and then washed in HM. The temperature of the media used for warming was 35-37°C.

2.7.3.6 Morphological Assessment

After conventional slow cooled, OPS and cryoloop procedure, Oberstein *et al.* (2001) recorded 12.5, 18 and 12.5 per cent fractured embryos respectively for equine embryos. Further they observed a decrease in diameter of embryos from 232 µm to 184µm after OPS. Lopez-Bejar and Lopez-Gatius (2002) reported that morphologically normal rabbit embryo recovered after vitrification with 25 per cent EG and 25 per cent glycerol medium in the normal straw and modified OPS were 81.2 and 89.5 per cent respectively and that with 25 per cent EG and SM were 76.5 and 86.8 per cent respectively. Begin *et al.* (2003) reported that 72 and 100 per cent caprine 2-4 cell embryos have showed no loss of membrane integrity after Solid surface and Cryoloop vitrification respectively. Naik *et al.* (2005) observed 81, 58 and 91 per cent morphologically normal rabbit embryos respectively after programmed freezing, CS and OPS vitrification.

2.7.3.7 Disadvantage

Vajta *et al.* (1998), Bielanski *et al.* (2000) and Dattena *et al.* (2004) reported that the only disadvantage of OPS method was the potential hazard of contamination as the embryo HM was directly in contact with liquid nitrogen. But the risk may be lowered using filtered liquid nitrogen for cooling the straw and subsequent covering of

the open end of the straw with a sealed protective straw (Vajta *et al.,* 1998) or using sealed OPS (Lopez-Bejar and Lopez-Gatius, 2002) or repeated washing of thawed embryo in several drops of culture medium (Lopatarova *et al.,* 2002 and Hochi *et al.,* 2004). However Lazar *et al.* (2000) and Hochi *et al.* (2004) did not encounter any contamination of the culture medium through out the OPS vitrification despite the use of non-sterile liquid nitrogen. Kong *et al.* (2000) indicated that although the OPS method was simple to use, the straw floated during cooling and storage in liquid nitrogen, which complicated the long-term storage using traditional goblet systems.

2.7.3.8 Modifications

In order to increase the cooling rate during vitrification, new techniques were widely used such as microscope grid (Park *et al.,* 1999), Glass micro pipette (Kong *et al.,* 2000 and Cho *et al.,* 2002), Microdroplet (Misumi *et al.,* 2003), Solid surface vitrification (Begin *et al.,* 2003), Cryoloop (Begin *et al.,* 2003 and Hochi *et al.,* 2004), Super finely pulled straws (Isachenko *et al.,* 2003b and Cuello *et al.,* 2004b), Gel loding tips (Tominaga, 2004 and Hochi *et al.,* 2004) and Cryoptop (Hochi *et al.,* 2004). These recently used techniques were having the advantage of smaller volume of frozen sample and high heat conductivity. However Cuello *et al.* (2004a) indicated that increasing the cooling rate by the SOPS or Vit-Master-SOPS procedures above $20,000^{0}$C/minute did not enhance the efficiency of *in vitro* development of morula and blastocysts after warming.

Materials and Methods

3. MATERIALS AND METHODS

3.1 OVARY COLLECTION AND OOCYTE RETRIEVAL

3.1.1 Source of ovary

Ovaries required for the harvest of oocytes were collected from freshly slaughtered Malabari goats/ it's cross-bred from Corporation slaughter house, Kuriachira, Thrissur located 6 km away from the lab.

3.1.2 Collection and transportation of ovary

Ovaries dissected out from the goats of unknown age, reproductive status, body condition etc. were transported to the laboratory within 2 h of slaughter in a thermo flask containing modified Dulbecco's Phosphate Buffered Saline (mDPBS) enriched with Penicillin and Streptomycin at 35^0-37^0C.

3.1.3 Pre-retrieval processing of ovary

Ovaries were washed with the transportation medium repeatedly to remove the excess blood clots and other debris. The extraneous tissues were excised off and then washed with mDPBS three to four times. The ovaries were then kept in mDPBS maintained at 35-37^0C in a water bath until they were subjected to oocyte retrieval processing.

3.1.4 Oocyte retrieval

Before the oocyte retrieval, the final washing of the ovaries were done with TL-HEPES media prepared as per Gordon (2003). The oocytes were retrieved from the ovary by slicing method as per Pawshe *et al.* (1994) into TL-HEPES media enriched with 0.6 per cent BSA and Heparin @ 0.1 mg/ml. Medium containing oocytes were transferred into separate sterile 90 X 10 mm petridishes for identification of oocytes.

3.2. GRADING OF OOCYTES

The oocytes in the sterile petridishes were examined under stereo-zoom microscope at 20-40X magnification and all the oocytes were classified into four grades

based on ooplasm character and investment of cumulus cells.

Grade A:

Morphologically normal oocytes having more than three complete layers of compact cumulus mass around the zona pellucida with evenly granulated ooplasm.

Grade B:

Morphologically normal oocytes with 1-2 complete layers of cumulus cells around zona pellucida with evenly granulated ooplasm.

Grade C:

Morphologically normal oocytes with broken or incomplete layers of cumulus cells with evenly granulated ooplasm.

Grade D:

Degenerated oocytes with apparently normal morphology enclosed by zona pellucida only.

3.3 MORPHOMETRY OF OOCYTES

The morphometry of oocytes was measured before the vitrification procedures using ocular micrometer at 60X magnification under stereo-zoom microscope. Cumulus having largest width from ZP is taken as cumulus thickness and largest width of oocyte including cumulus mass was considered as total diameter. While doing this, the oocytes were kept in fresh drops of TL-Hepes media maintained at 35-37^{0}C. Only grade A and B oocytes were used for this study.

3.4 VITRIFICATION OF OOCYTES

Total of 139 oocytes having at least one complete layer of cumulus (Both grade A and B) were randomly divided into 85 and 54 for Conventional straw (CS) and Open Pulled Straw (OPS) vitrification procedure respectively.

PLATE 1: QUALITY GRADES OF OOCYTE

A. Grade A

B. Grade B

C. Grade C

D. Grade D

PLATE 2: DEVELOPMENTAL STAGES OF EMBRYO

A. Morula

B. Compact morula

C. Early blastocyst

D. Blastocyst

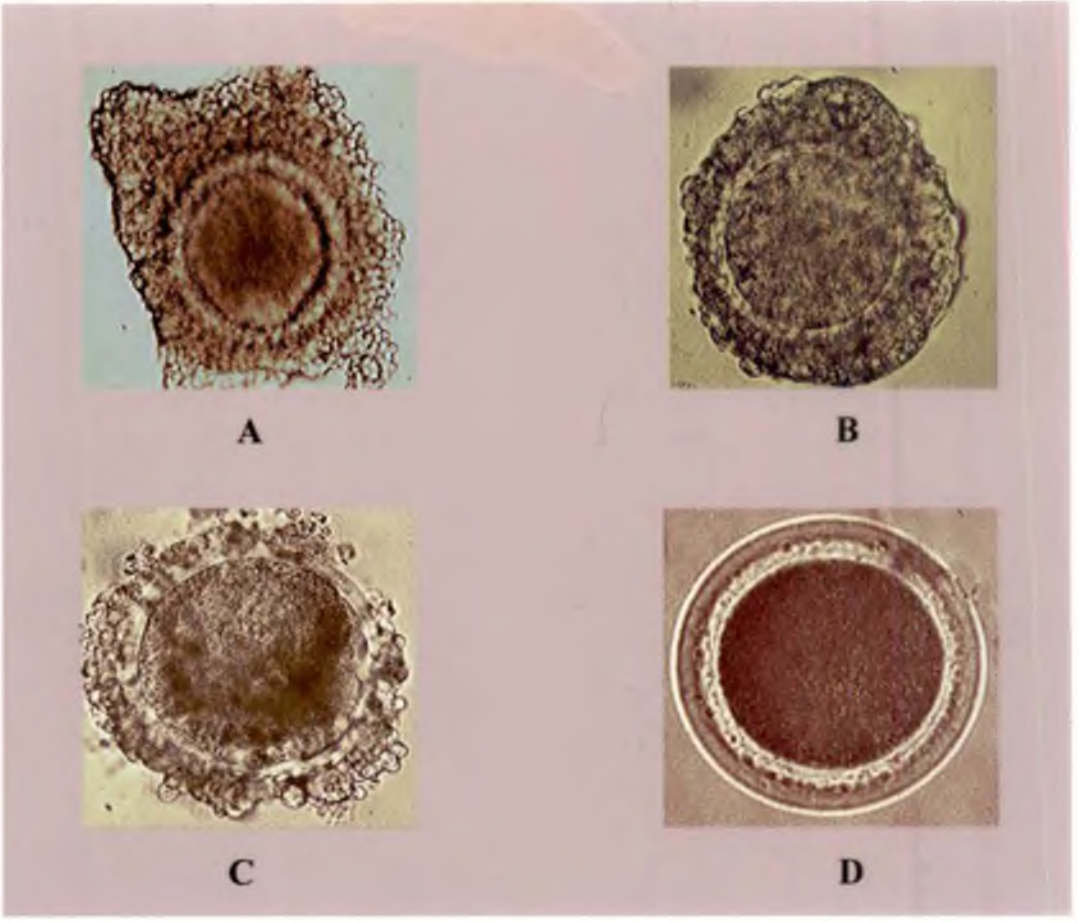
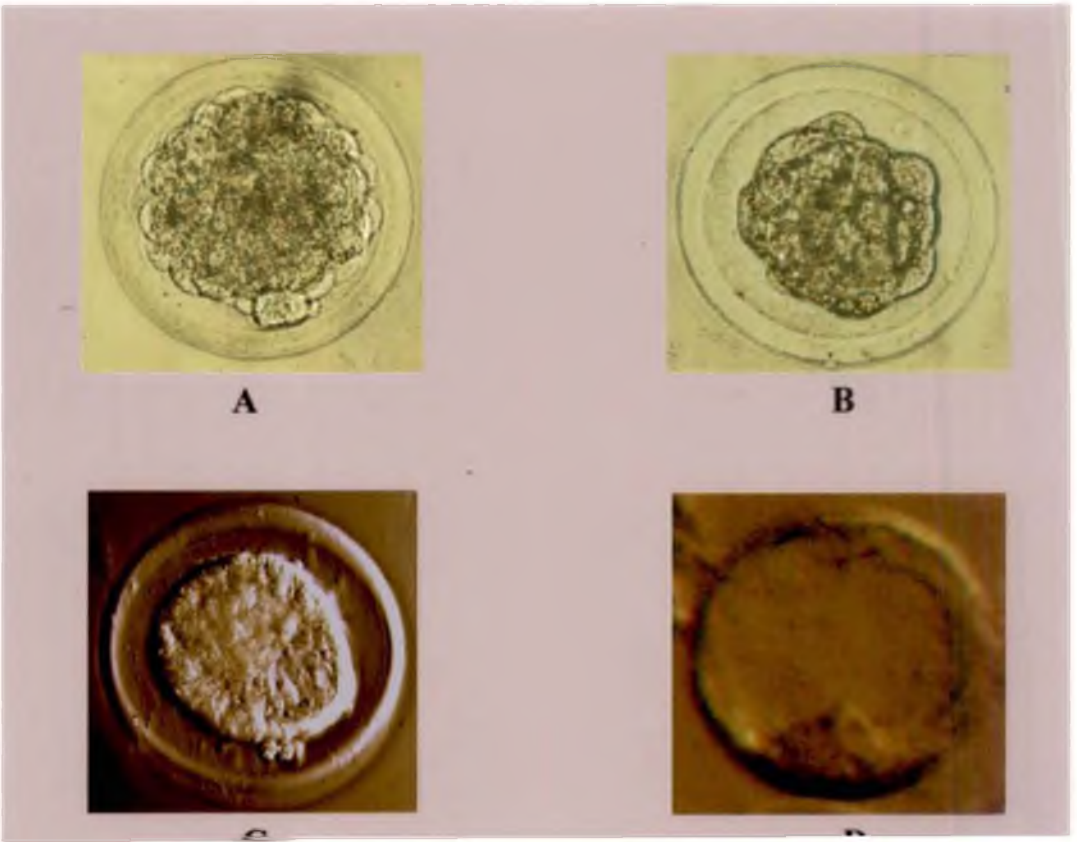


PLATE 1



3.4.1 Conventional straw vitrification of oocytes

3.4.1.1 Exposure to cryoprotectant

The oocytes were equilibrated and vitrified as described by Agrawal (1999) with minor modifications in the type of cryoprotectant and the method of plunging straw into liquid nitrogen.

The holding medium (HM) consisted of mDPBS supplemented with Zeitz filtered heat inactivated goat serum @ 20 percent. The whole medium was filtered through a 0.22 µ syringe filter before use. The final pH of the medium was adjusted to 7.2-7.4. The oocytes were equilibrated in 10 per cent glycerol with 20 per cent EG in HM for 10 minutes followed by the exposure to vitrification medium (VM) containing 25 per cent glycerol and 25 per cent EG in HM.

3.4.1.2 Loading and plunging of straw

Aspirated 120 µl of 1 M sucrose in HM into a 0.25 ml Cassou straw (IMV-France) which was connected to a tuberculin syringe through an embryo filter adaptor system (Minitub-Germany), followed by 3 mm air bubble. Then 20 µl of VM containing oocytes (one oocyte/ straw) was aspirated followed by a 3 mm air bubble. The remaining part of the straw was filled with the same sucrose medium (SM) and was heat-sealed before direct plunging into liquid nitrogen. The time taken from the contact of oocytes with the VM up to the plunging into liquid nitrogen did not exceed 30-35 seconds.

3.4.1.3 Thawing and stepwise removal of cryoprotectant

After a storage period of more than 10 days, the straws were taken out from the liquid nitrogen and thawed for 10 s in the water bath maintained at 20^0C. After thawing, contents of the straw were mixed by shaking and emptied in a petridish (60 X 10 mm). The temperature of the medium was maintained at 4-12^0C by placing the petridish over a freezer coolant pack. After 10 minutes, the oocytes were transferred sequentially to 0.5 M and 0.25 M SM for five minutes each followed by two times washing in HM. Then the oocytes were subjected to assessment of morphometry and viability.

3.4.2 Open pulled straw vitrification of oocytes

3.4.2.1 Exposure to cryoprotectant

Open pulled straws were prepared and oocytes were equilibrated and vitrified by the procedure mentioned by Vajta *et al.* (1998) with minor modification in the concentration of cryoprotectant and duration of exposure. Oocytes were equilibrated in 7.5 per cent EG with 7.5 per cent DMSO in HM (TCM-199 with 20 per cent heat inactivated goat serum) for 3 minutes followed by the exposure to VM containing 16.5 per cent EG and 16.5 per cent DMSO dissolved in HM containing 0.5 M sucrose. All the procedures were carried out on a stage warmer at 41^{0} C (Linkam-England) fixed to an inverted microscope (Leica DM IL).

3.4.2.2 Loading and plunging of straw

After loading, the Open pulled straw with 1.8 µl of VM containing oocyte (one oocyte/ straw) was directly plunged into liquid nitrogen. The liquid nitrogen for this purpose was temporarily stored in a thermocol box. The time from the exposure of oocytes with VM up to the plunging into liquid nitrogen did not exceed 20-25 seconds.

Then the OPS straw containing oocyte was introduced into a precooled 0.5 ml Cassou straw (IMV-France), which was held in the liquid nitrogen. This 0.5 ml straw was heat sealed with hot forceps, put into plastic tubes having screw caps and then immersed into liquid nitrogen tank for storage.

3.4.2.3 Thawing and stepwise removal of cryoprotectant

After a storage period of more than 10 days in liquid nitrogen, the straws were warmed and the cryoprotectant was removed as described by Vajta *et al.* (1998). The morphometry and viability of vitrified oocytes were assessed while it was in the HM.

3.5 VIABILITY ASSESSMENT OF OOCYTES

3.5.1 Morphological examination

Immediately after thawing and cryoprotectant removal, oocytes were examined for morphological characteristics under inverted microscope at 400X magnification.

PLATE 3: VITRIFICATION DEVICES

A. Tuberculin syringe with embryo filter adaptor system and 0.25 ml Cassou straw.

B. OPS vitrification unit (Locally fabricated).

C. Cassette and OPS.

D. 0.25 ml straw Cassou straw and OPS.

A

B

C

D

Oocytes with shrinkage and degenerated ooplasm, zona pellucida crack, damage and degenerated cumulus cells were discarded and oocytes without these abnormalities were considered as morphologically viable.

3.5.2 Staining

Morphologically viable vitrified oocytes were stained with Fluorescein diacetate (FDA) to assess the membrane integrity of ooplasm as described by Smith (1993). A stock solution of 5 mg / ml FDA in acetone was prepared and stored at -20^{0}C. Just before the use, 0.5 µl of stock solution was mixed with 1ml mDPBS to prepare a working solution having a final concentration of 2.5 µg FDA/ml. Oocytes were incubated in this solution for one minute at room temperature after which they were washed several times in mDPBS. After incubation at room temperature for 10 minutes, oocytes were examined under fluorescent microscope (Leica DM IL) using a blue filter block (I3) under 450-490 nm excitation range. Oocytes with uniform bright green fluorescein were considered as viable as they possessed good membrane integrity.

3.6 SUPEROVULATION AND EMBRYO COLLECTION

3.6.1 Experimental animals

Twelve healthy Malabari goats maintained in the Embryo Transfer Lab, Department of Animal Reproduction belonging to two pre-assigned categories (fresh and repeatedly superovulated) were utilised for superovulation and embryo collection. The fresh animal group included six regularly cyclic goats in the age group of 18 to 30 months with body weight of 23 to 34 kg. Repeatedly superovulated group included six regularly cyclic animals belonging to the age group of three to four and half years with 30 to 38 kg body weight, which were exposed to at least one superovulation and embryo collection procedures earlier. All the goats were maintained under the same agroclimatic and managemental conditions.

3.6.2 Synchronisation and superovulation treatment

Crestar ear implants (Intervet-Holland) containing 3 mg of synthetic progesterone-norgestomet was divided into two equal halves. One half of the implant was inserted subcutaneously in the middle third of the outer surface of the ear of each

PLATE 4: HORMONES, MEDIA AND CRYOPROTECTANTS

A. Crestar ear implant and injection

B. pFSH (Folltropin-V)

C. Prostaglandin (Lutalyse)

D. mDPBS

E. TCM-199

F. Sucrose

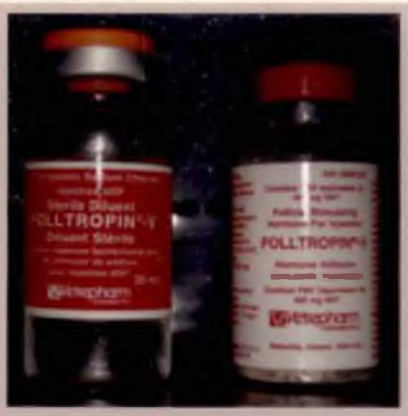
G. Ethylene glycol

H. Glycerol

I. Dimethyl sulfoxide


CRESTAR

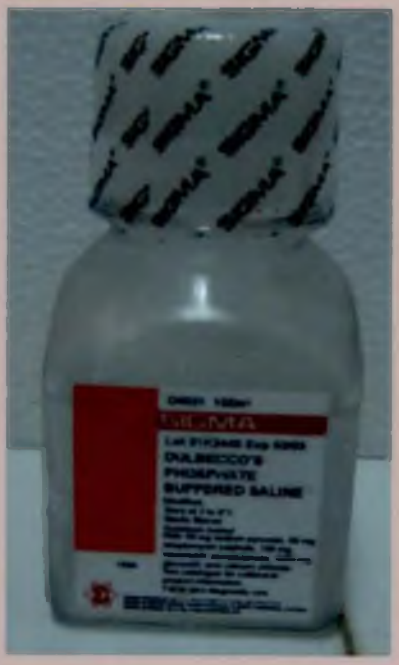
A


FOLLTROPIN

B


5 mg/ml

C


SIGMA
DULBECCO'S
PHOSPHATE
BUFFERED SALINE

D

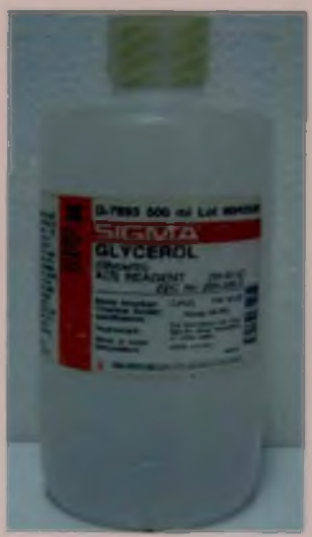

SIGMA
MEDIUM 199
10x

E

F


SIGMA
ETHYLENE
GLYCOL

G


SIGMA
GLYCEROL

H


SIGMA

I

animal by using an applicator. At the time of implant insertion all goats were administered 0.5 ml Crestar injection containing 1.25 mg oestradiol valerate and 0.75 mg norgestomet. The day of ear implantation was considered as day '0' of the treatment. The inserted implant was left *in situ* for 10 days and was removed on day 11 in the evening. A total of 10 mg of Prostaglandin $F_2\alpha$ analogue dinoprost (Lutalyse, Pharmacia-Upjohn, Belgium) was administered intramuscularly (IM) as two equal divided doses at 12 h interval on the day of implant removal. For inducing superovulation, animals were administered with 133 mg pFSH (Folltropin-V, Vetrepharm, Canada) in eight divided step down doses (24-24, 18-18, 12.5-12.5 and 12-12 mg) subcutaneously, at 12 h interval starting on day ninth of the implant insertion. All the animals were observed for estrum and were inseminated with freshly collected buck semen at 12 h interval commenced from 36 h after the implant removal till the end of oestrus.

3.6.3 Assessment of ovarian response through laparotomy

On day seven after the onset of oestrus (onset of oestrus = 0 day) laparotomy was carried out under general anaesthesia and the superovulatory response was evaluated by observing the ovaries. Preoperative preparations, surgical procedure and postoperative care were carried out as per the method followed by Joseph (2003). The goats were sedated using xylazine @ 0.2 mg per kg body weight IM. Ten minutes later ketamine hydrochloride @10 mg per kg body weight was administered as IM injection.

Ovaries of the donor animals were examined immediately after laparotomy and the number of functional corpora lutea, prematurely regressing white avascular corpora lutea and follicles of more than 5 mm diameter were recorded. The superovulatory response was assessed by counting the number of functional as well as prematurely regressed corpora lutea.

3.6.4 Embryo collection

After observing the ovarian response, embryo flushing was carried out from the uterine horn in orthograde manner as per the method of Tervit and Havik (1976) with minor modifications (Plate 5C). A 24-gauge needle of scalp vein set connected to a 20

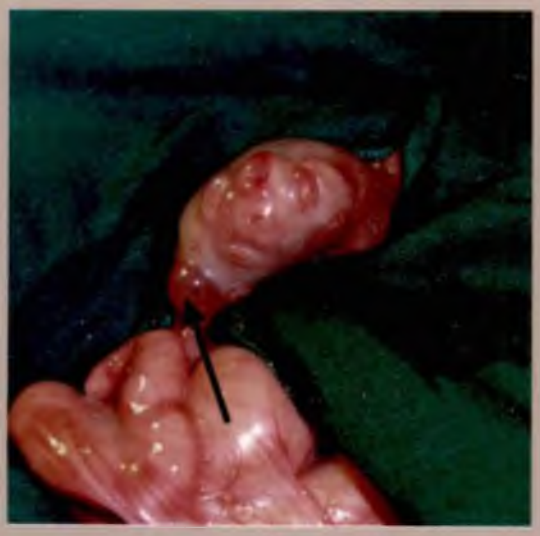
PLATE 5: SUPEROVULATORY RESPONSE AND SURGICAL EMBRYO COLLECTION

A. Anovulatory follicle.

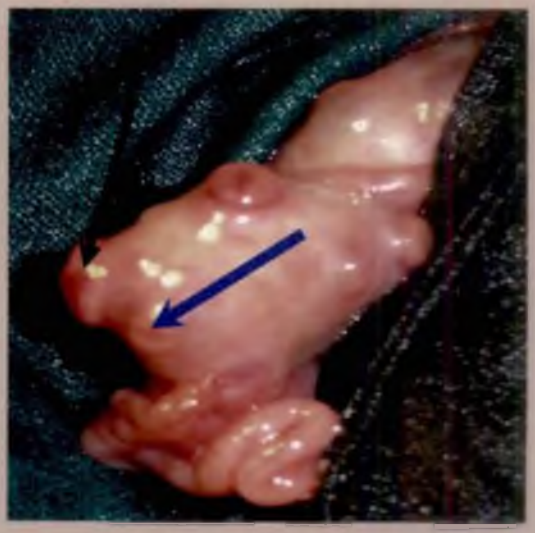
B. Developed CL (Black arrow) and PRCL (Blue arrow).

C. Surgical embryo collection by uterine flushing.

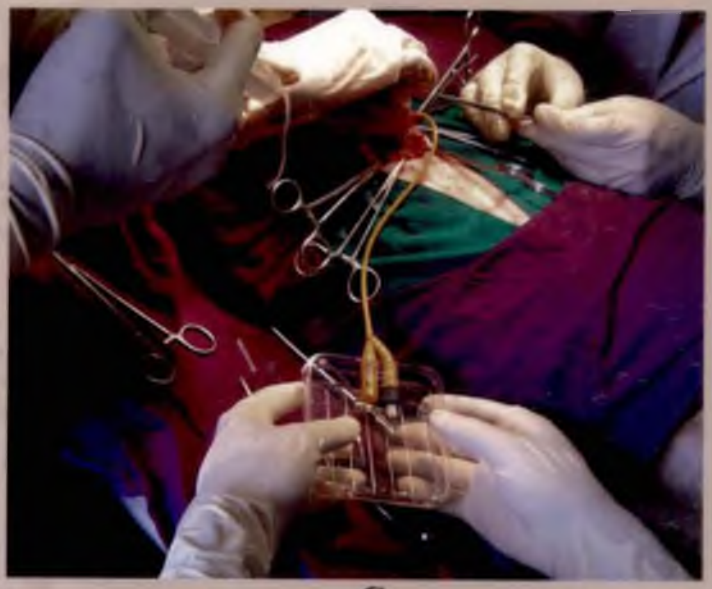
D. Adhesion of reproductive tract after repeated embryo collection.



A



B



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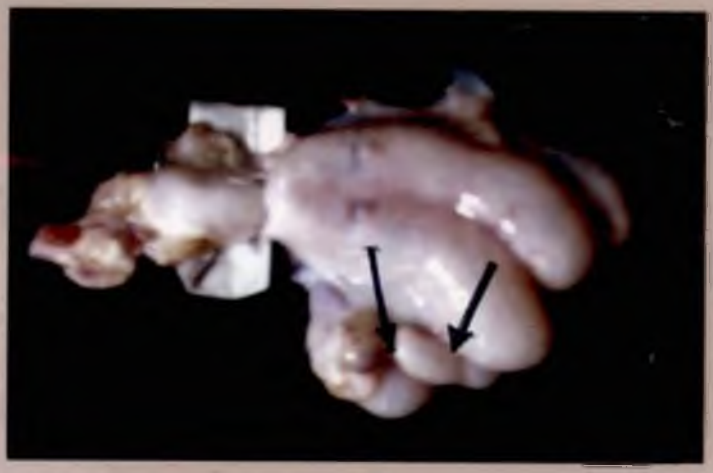

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ml syringe was inserted at the utero-oviductal junction towards the uterine horn for infusion and a double way Foley catheter of size-Ch 08 (Rusch-Germany) was inserted 3-4 cm anterior to the uterine bifurcation for collection of flushing media. The flushing was carried out as two step procedure. Initially a 30 ml media was infused and completely recovered out by massaging of uterine horn towards the catheter. Then the same procedure was repeated again with 30 ml of flushing media. The other horn was also flushed in the same pattern.

3.6.4.1 Embryo flushing media

Flushing medium consisted of mDPBS supplied by Sigma chemicals, USA (Cat. No. D-6650) containing 10 per cent heat inactivated goat serum. The whole medium was filtered through a cellulose acetate filter of pore size 0.45 microns (Millipore Cat.No. HAWP 047 00) and was warmed to body temperature before use. The final pH of the medium was adjusted to 7.2-7.4.

3.6.4.2 Embryo recovery

Flushing medium from each horn was separately collected in a sterilized petridish (100x15 mm square style with 13 mm grid) and the total number of embryos and unfertilised ova were assessed under stereo zoom microscope at 20-40X immediately after flushing. The identified embryos were recovered using unopette (Unopette – 5888 Becton Dickinson, USA). The embryos from each grided petridish were transferred to another sterile petridish (35 X 10 mm), containing two ml fresh HM consisting of mDPBS supplemented with 20 per cent heat inactivated goat serum. The HM was filtered using 0.22-μ syringe filter (Millex Cat.No. SLGS 025 0S) before use. The embryos were washed six times by serially transferring them into fresh drops of HM and were held in the medium until vitrification at room temperature (20-25^{0}C). The morphological grading and morphometry studies of embryos were carried out during this holding period.

3.7 GRADING AND MORPHOMETRY OF EMBRYOS

All the embryos from both fresh and repeatedly superovulated goats were

mass and width of perivitelline space was measured under stereo zoom microscope using an ocular micrometer at 60X magnification. Morphological appearance of embryos in HM was examined at 400X of inverted microscope and was graded as per Linder and Wright (1983) as excellent, good, fair and poor. Both excellent and good quality embryos were considered as transferable and assigned for vitrification procedures. The fair and poor quality embryos were graded as non transferable and so discarded.

3.8 VITRIFICATION OF EMBRYOS

Thirty four transferable embryos from both fresh and repeatedly superovulated animals were pooled together and equally divided for CS and OPS vitrification within 3-6 hours after the collection.

3.8.1. Conventional straw vitrification of embryos

3.8.1.1 Exposure to cryoprotectant

Embryos were equilibrated and vitrified as described by Baril *et al.* (2001) with minor modifications such as the base medium used for vitrification was mDPBS supplemented with 20 per cent heat inactivated goat serum. 1 M sucrose was used for the one step cryoprotectant removal of embryos instead of 0.8 M galactose.

3.8.1.2 Loading and plunging of straw

The loading and plunging of straw into liquid nitrogen was carried out as described for the CS vitrification of oocytes.

3.8.1.3 Thawing and stepwise removal of cryoprotectant

After 10-days storage in liquid nitrogen, the straws were thawed in a water bath maintained at 37^0C. Then the straws were gently shaken to allow mixing of the VM with 1 M sucrose. The whole contents were then directly emptied into a petridish maintained at room temperature. After the removal of cryoprotectant by incubation for five minutes in 1 M sucrose, the embryos were washed two times in the HM for five minutes each. The vitrified embryos in the HM were later examined for morphometry and viability.

3.8.2 Open pulled straw vitrification of embryos

3.8.2.1 Exposure to cryoprotectant

The embryos were equilibrated and vitrified by the procedure mentioned by El-Gayar and Holtz (2001) with minor modification in the concentration of cryoprotectant and duration of exposure. The embryos were equilibrated in HM (TCM-199 with 20 per cent heat inactivated goat serum) containing 7.5 per cent EG with 7.5 per cent DMSO for three minutes followed by the exposure to VM containing 16.5 per cent EG and 16.5 per cent DMSO dissolved in HM and 0.5 M sucrose. All the procedures were carried out at 37^0 C on a stage warmer (Linkam-England) fixed with an inverted microscope (Leica DM IL).

3.8.2.2 Loading and plunging of straw

The loading and plunging of straw into liquid nitrogen was carried out as described for the OPS vitrification of oocytes.

3.8.2.3 Thawing and stepwise removal of cryoprotectant

After 10 days storage in liquid nitrogen, the straws were warmed as described by Vajta *et al.* (1998). In the HM the morphometry and viability of embryos were assessed.

3.9 VIABILITY ASSESSMENT OF EMBRYOS

3.9.1 Morphological examination

Immediately after thawing and cryoprotectant removal, the embryos were examined for morphological characteristics under inverted microscope at 400X magnification. Morphologically good looking embryos without any abnormalities such as degeneration, shrinkage and protrusion of blastomeres, crack or thinning of zona pellucida were considered as morphologically viable.

3.9.2 Staining

The vitrified embryos morphologically assessed as viable were stained with Fluorescein diacetate (FDA) as per the method of Mohr and Trounson (1980) for

mouse embryos. FDA stain was prepared as described for the oocyte. After one minute incubation in the FDA stain, the embryos were washed with mDPBS for another one minute followed by the exposure under fluorescent microscope using the same filter for the oocyte examination. All the blastomeres having bright green fluorescein were considered as viable.

3.10 DETAILS OF RESEARCH MATERIALS AND EQUIPMENTS

Detailed list and specification of research materials and equipments used in this study are furnished in Annexure.

3.11 STATISTICAL METHOD

Data were statistically analysed using Chi-square Test (Snedecor and Cochran 1985).

Results

4. RESULTS

4.1 RECOVERY OF OOCYTES

The total number of oocytes recovered, average yield per ovary and yield of various grades of oocytes per ovary are furnished in table 1.

4.1.1 Average yield of oocytes

After slicing, 374 oocytes were recovered from 73 ovaries. The average yield of oocytes per ovary was 5.12.

4.1.2 Morphological grading of oocytes

Recovered oocytes were graded based on ooplasm character and number of layer of cumulus cells. Out of the 374 oocytes recovered, 36.9, 18.18, 13.1 and 31.82 per cent were A, B, C and D grade respectively as shown in table 1. Average yield of grade A, B, C and D oocytes per ovary were 1.89, 0.93, 0.67 and 1.63 respectively. The total recovery of good quality oocytes and its average yield per ovary were 206 and 2.82 respectively (A and B grade oocytes were pooled together).

4.2 OOCYTE CRYOPRESERVATION

4.2.1 Recovery rates of oocytes after vitrification

The number and percentage recovery of oocytes after vitrification is furnished in the table 2. Out of 85 oocytes vitrified in the Conventional straw (CS) vitrification method, 57 were recovered with the percentage recovery rate of 67.06. Recovery rate was recorded for OPS vitrification method was 94.44 per cent; 51 were recovered out of 54 oocytes.

4.2.2 Viability of oocytes after vitrification

Followed by vitrification and thawing, the oocytes were assessed for its morphological quality based on the intactness of cumulus, zona pellucida and ooplasm. Total and percentage recovery of morphologically viable and damaged oocytes are furnished in table 2 and 3 respectively. The number and percentage of oocytes that developed different types of damages are presented in table 3.

4.2.2.1 Morphological viability of vitrified oocytes

Out of 57 oocytes recovered, 24 oocytes (42.11 %) retained normal morphology after CS vitrification while 36 oocytes (70.59 %) out of 51 were having normal morphology after OPS vitrification method.

4.2.2.2 Non-viable vitrified oocytes

Oocytes with degenerated or damaged cumulus, ooplasm-showing shrinkage or degeneration and thinning or crack in zona pellucida were considered as non-viable. Out of the 57 oocytes recovered after CS vitrification, 33 (57.89 %) were damaged and that after OPS vitrification was 29.41 per cent; out of 51 oocytes recovered, 15 were damaged.

4.2.2.3 Types of damages due to vitrification

Different types of damage affected the oocytes are shown in the table 3. Cumulus or zona pellucida damage either alone or together was not noticed in any of the oocytes after vitrification by CS or OPS vitrification methods. In the CS group itself combined cumulus and ooplasm damage out of recovered oocytes were higher (35.08 %) than ooplasm damage alone (17.5 %) followed by both ooplasm and zona damage and cumulatively all the three types of damage together (3.5 % and 1.75 % respectively). It was observed that if the cumulus was completely damaged or stripped off, the ooplasm also would be damaged.

In the OPS vitrification, out of the recovered oocytes ooplasm damage alone was higher (15.68) than cumulus and ooplasm damage together (9.8 %). 1.96 per cent of the recovered oocytes were observed with the damage of both ooplasm and zona pellucida and a similar percentage of oocytes were observed to be affected with all the three types of damages together.

4.2.2.4 FDA viability of vitrified oocytes

After assessing the morphological viability, oocytes were stained with FDA to reassess its ooplasm integrity. Total and percentage of FDA viable and FDA non-viable

Table.1 Yield and quality of oocytes retrieved by slicing technique.

No. of ovaries sliced	Total no. of oocytes recovered	Average oocytes yield per ovary	Morphological grades of oocytes and its yield per ovary				Average yield of good quality oocytes per ovary
			Grade A	Grade B	Grade C	Grade D	
73	374	5.12	1.89 (36.9)	0.93 (18.18)	0.67 (13.1)	1.63 (31.82)	2.82 (55.08)

Figures in parenthesis denote the percentage

Table.2 Recovery rate of morphologically normal oocytes after vitrification

Method of cryopreservation	No. of oocytes vitrified	No. of oocytes recovered after vitrification	Oocyte recovery rate	Recovery rate of morphologically normal oocytes	
				No.	(%)
Conventional straw vitrification	85	57	67.06%	24	42.11
Open pulled straw vitrification	54	51	94.44%	36	70.59

Any value in the same column between two groups differ non-significantly (χ^2 test)

Table.3 Type of morphological damages of vitrified oocytes

Method of vitrification	No. of oocytes recovered	Recovery rate of damaged oocytes		Types of morphological damages (No. and percentage)							
				Ooplasm damage alone		Combined damage of cumulus and ooplasm		Combined damage of ZP and ooplasm		Combined damage of cumulus, ZP and ooplasm	
		No.	(%)	Out of damaged oocyte	Out of recovered oocyte	Out of damaged oocyte	Out of recovered oocyte	Out of damaged oocyte	Out of recovered oocyte	Out of damaged oocyte	Out of recovered oocyte
Conventional straw	57	33	57.89	10 (30.3)	17.5%	20 (60.6)	35.08%	2 (6.06)	3.5%	1 (3.03)	1.75%
Open pulled straw	51	15	29.41	8 (53.3)	15.68%	5 (33.33)	9.8%	1 (6.67)	1.96%	1 (6.67)	1.96%

Any value in the same column between two groups differ non-significantly (χ^2 test)
None of the oocyte showed cumulus damage alone, ZP damage alone or combination of both the type after both method of vitrification
Figures in parenthesis denote percentage

oocytes and percentage recovery of morphologically normal FDA viable oocytes in terms to the total number of oocytes recovered are presented in table 4.

Out of 24 conventionally vitrified morphologically normal oocytes stained, 18 (75 %) were retaining the uniform fluorescence. Out of 36 OPS vitrified oocyte 27 (75 %) were retained the fluorescence uniformly. There was a higher recovery of morphologically normal FDA viable oocytes (52.94 %) after OPS vitrification than that after the CS vitrification method (31.58 %). Out of 24 and 36 morphologically normal oocytes after CS and OPS vitrification respectively, six and nine oocytes respectively lost the stain and was appeared as either non-fluorescence or with light fluorescence.

4.2.2.5 Effect of vitrification on morphometric characteristics of oocytes

Morphometry of a representative number of oocytes (total diameter and cumulus thickness) recorded before and after vitrification is presented in table 5. After the CS vitrification, the mean total diameter of oocytes was increased for the value of 109.86 ± 12.77 μ, which is higher than in the OPS vitrification methods 35.07 ± 6.61 μ. Similarly the cumulus cell layer thickness also showed a higher increase after CS vitrification than OPS (56.69 μ vs. 17.25 μ).

4.3 RESPONSE TO SYNCHRONIZATION

All goats from both fresh and repeatedly superovulated groups were responded positively to synchronization treatment, which is presented in table 6.

4.3.1 Signs of estrum

Most of the goats in the repeated groups showed wagging of tail as the predominant sign for oestrus. Vulval edema and presence of discharge on speculum examination was also observed.

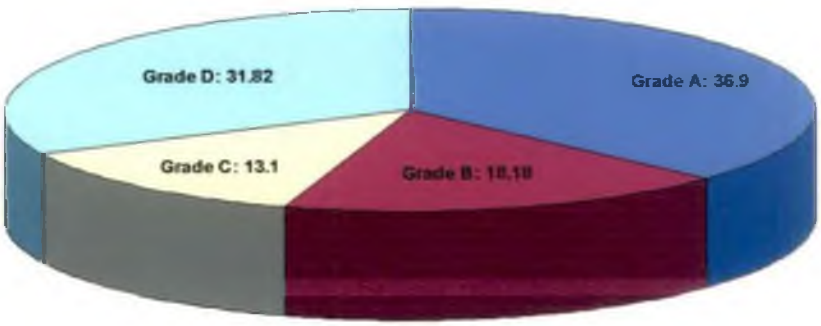
All fresh goats showed physical signs of heat such as presence of discharge and wagging of tail where as well-dilated cervical opening was more prominent in fresh goats than repeated groups.

Table.4 Viability assessment by FDA staining of vitrified morphologically normal oocytes

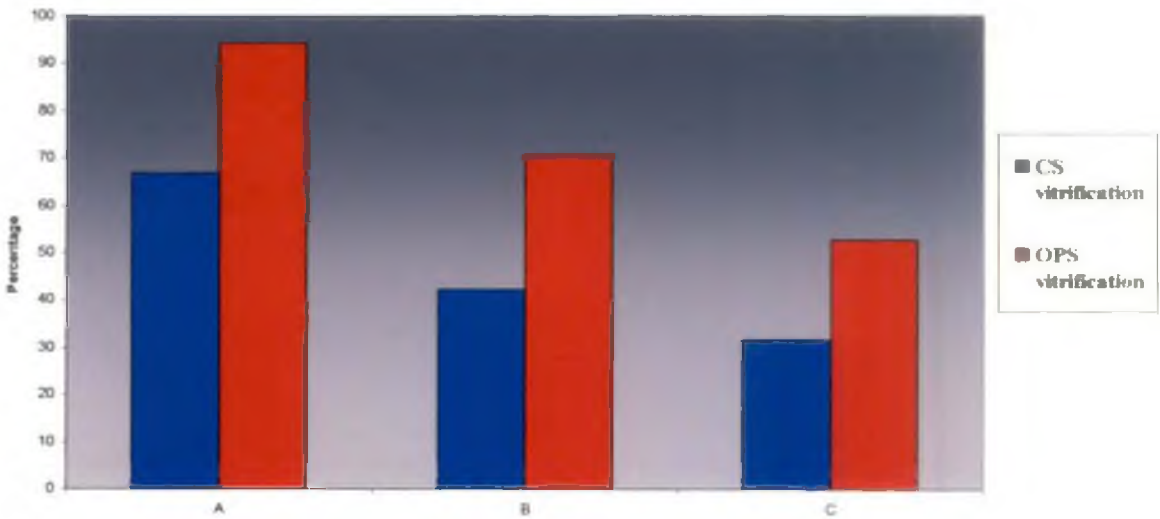
Method of cryopreservation	No. of oocytes recovered after vitrification	No. of morphologically viable vitrified oocytes subjected to FDA staining	FDA viable with normal morphology		FDA non-viable with normal morphology		Overall recovery of FDA viable oocyte
			Total	(%)	Total	(%)	
Conventional straw vitrification	57	24	18	75	6	25	31.58
Open pulled straw vitrification	51	36	27	75	9	25	52.94

Any value in the same column between two groups differ non-significantly (χ^2 test)

Percentage recovery of various grade of oocytes



Recovery rate of oocytes after CS and OPS vitrification



A: After thawing; B: Morphologically viable; C: FDA viable

PLATE 6: VIABLE OOCYTE UNDER DIFFERENT STEPS OF CS VITRIFICATION

A. Oocyte in HM before vitrification.

B. Oocyte in HM after vitrification.

C. Denuded oocytes in HM after vitrification.

D. FDA stained vitrified oocytes.

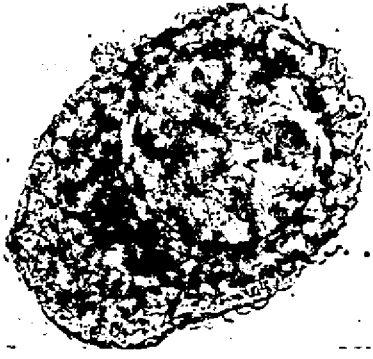
PLATE 7: NON-VIABLE OOCYTE UNDER DIFFERENT STEPS OF CS VITRIFICATION

A. Oocyte in HM before vitrification.

B. Oocyte in HM after vitrification.

C. Denuded oocytes in HM after vitrification.

D. FDA stained vitrified oocytes (Arrow points to shadow).



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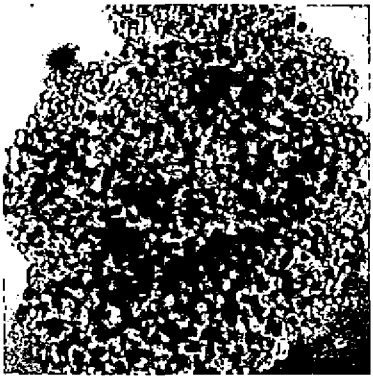

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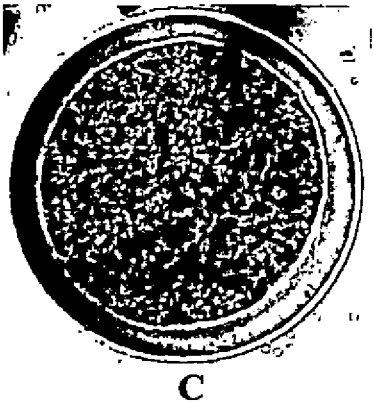

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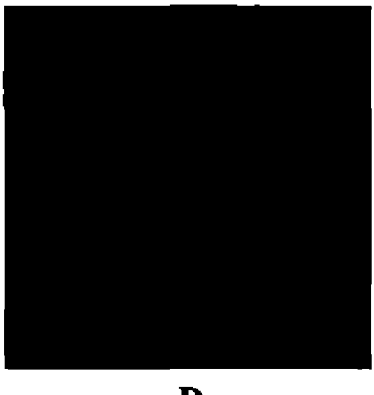

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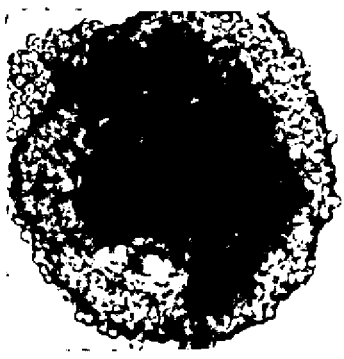
PLATE 6



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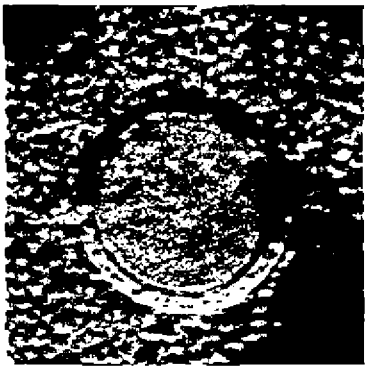

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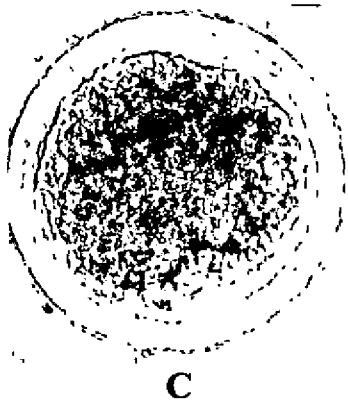

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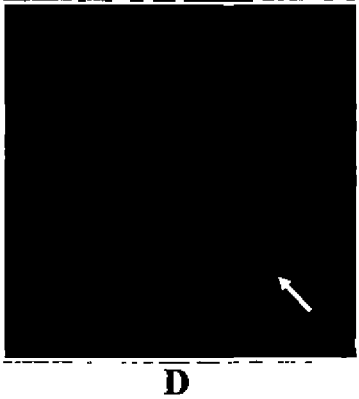

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PLATE 7

PLATE 8: VIABLE OOCYTE UNDER DIFFERENT STEPS OF OPS VITRIFICATION

A. Oocyte in HM before vitrification.

B. Oocyte in 0.25 M SM after vitrification.

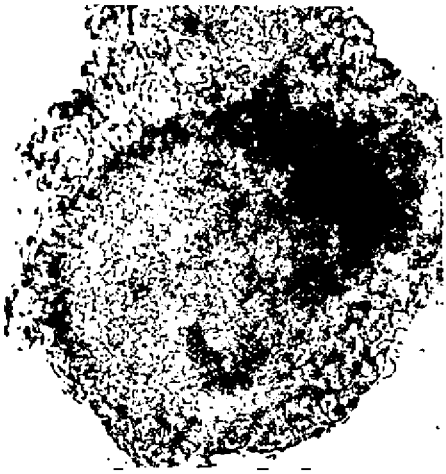
C. Oocyte in 0.15 M SM after vitrification.

D. Oocyte in HM after vitrification.

E. Denuded oocyte in HM.

F. FDA stained vitrified oocyte.

A



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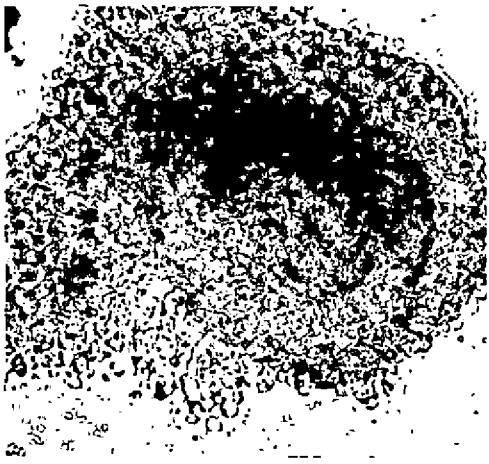

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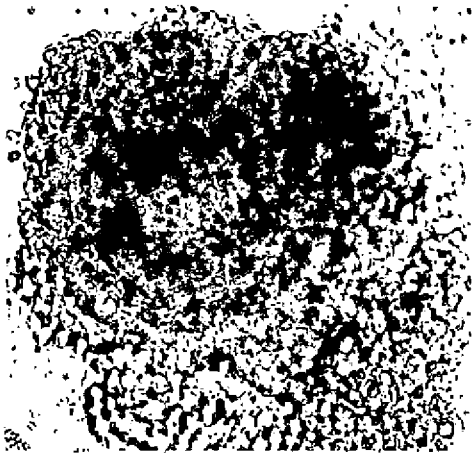

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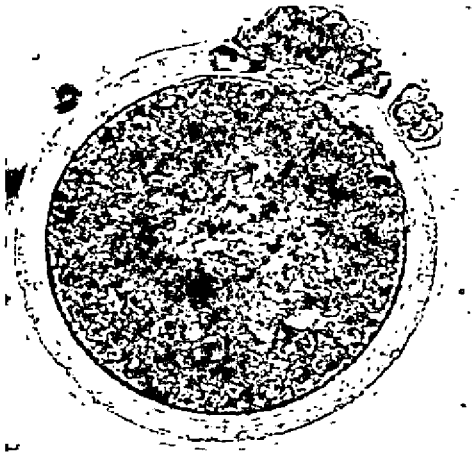

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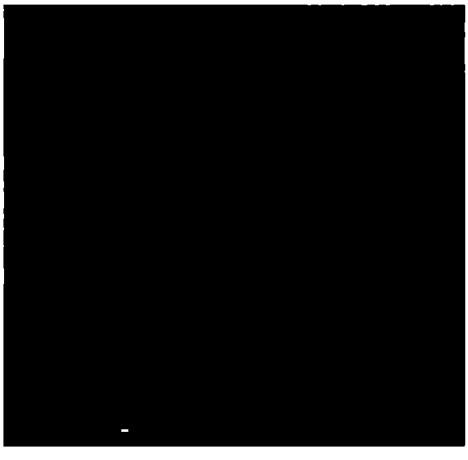

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PLATE 8

PLATE 6: VIABLE OOCYTE UNDER DIFFERENT STEPS OF CS VITRIFICATION

A. Oocyte in HM before vitrification.

B. Oocyte in HM after vitrification.

C. Denuded oocytes in HM after vitrification.

D. FDA stained vitrified oocytes.

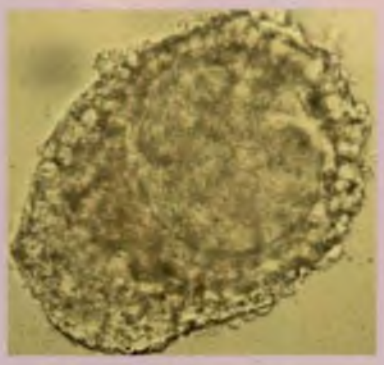
PLATE 7: NON-VIABLE OOCYTE UNDER DIFFERENT STEPS OF CS VITRIFICATION

A. Oocyte in HM before vitrification.

B. Oocyte in HM after vitrification.

C. Denuded oocytes in HM after vitrification.

D. FDA stained vitrified oocytes (Arrow points to shadow).



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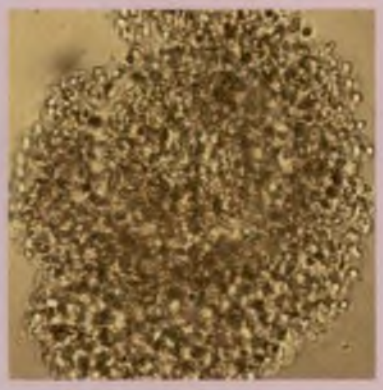

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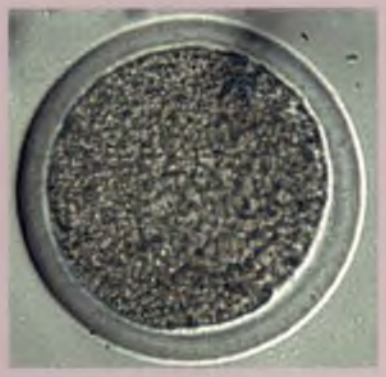

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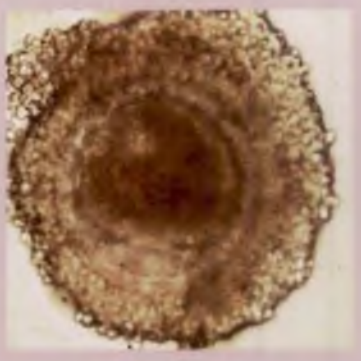

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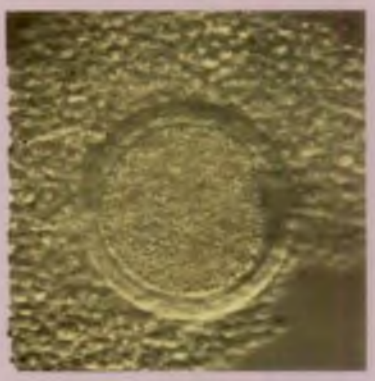

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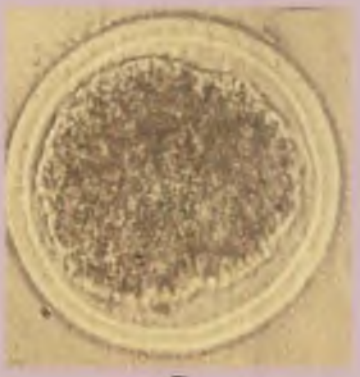

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PLATE 8: VIABLE OOCYTE UNDER DIFFERENT STEPS OF OPS VITRIFICATION

A. Oocyte in HM before vitrification.

B. Oocyte in 0.25 M SM after vitrification.

C. Oocyte in 0.15 M SM after vitrification.

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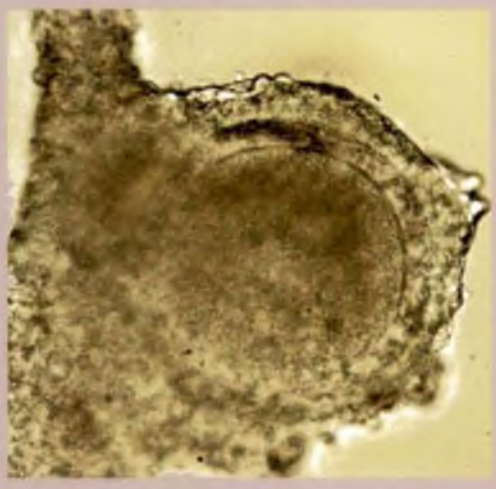

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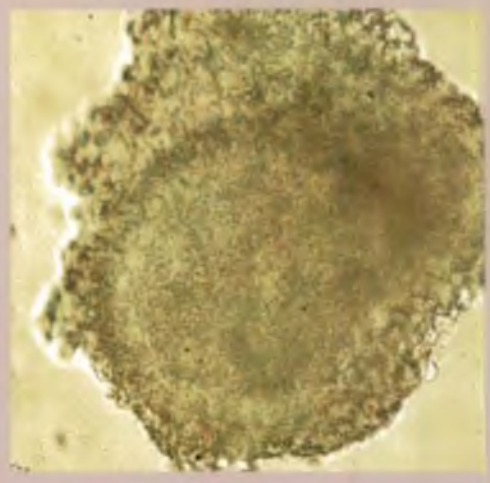

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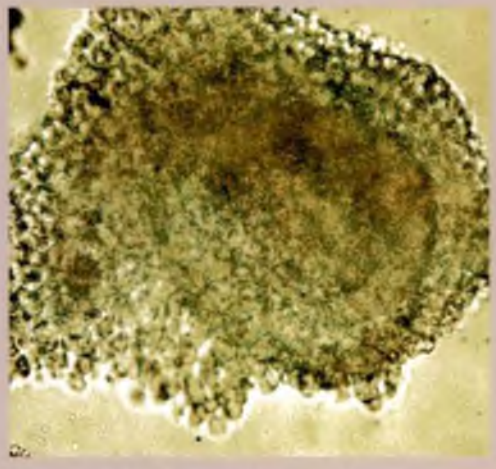

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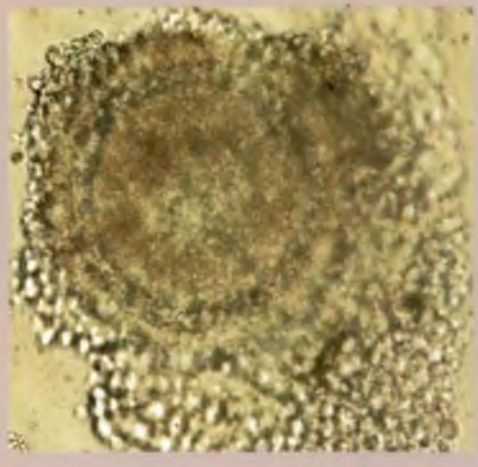

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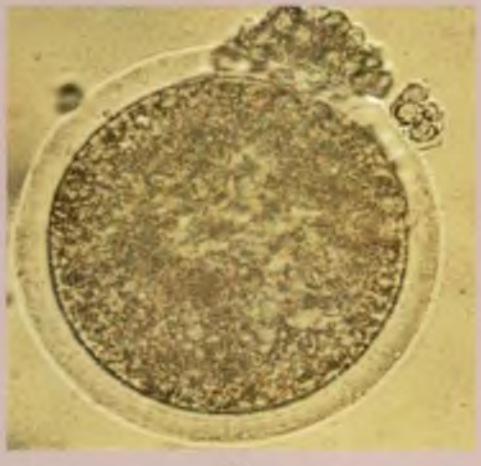

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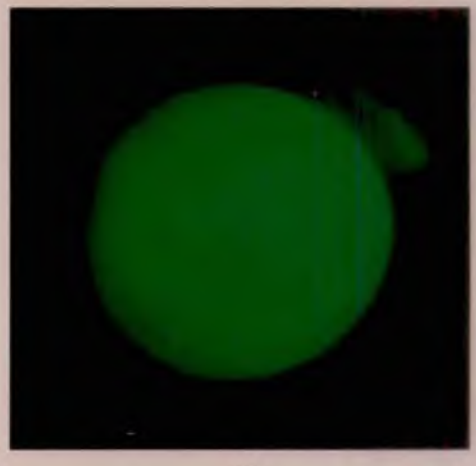

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PLATE 9: NON-VIABLE OOCYTE UNDER DIFFERENT STEPS OF OPS VITRIFICATION

A. Oocyte in HM before vitrification.

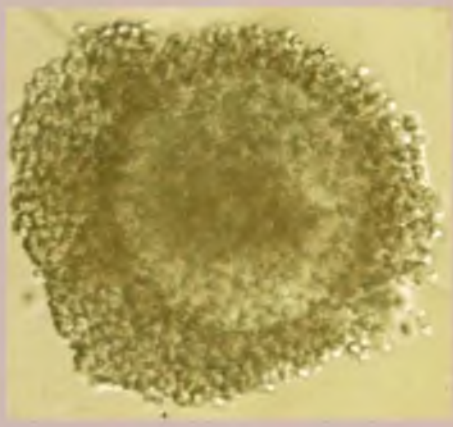
B. Oocyte in 0.25 M SM after vitrification.

C. Oocyte in 0.15 M SM after vitrification.

D. Oocyte in HM after vitrification.

E. Denuded oocyte in holding medium.

F. FDA stained vitrified oocyte (Arrow points to shadow).



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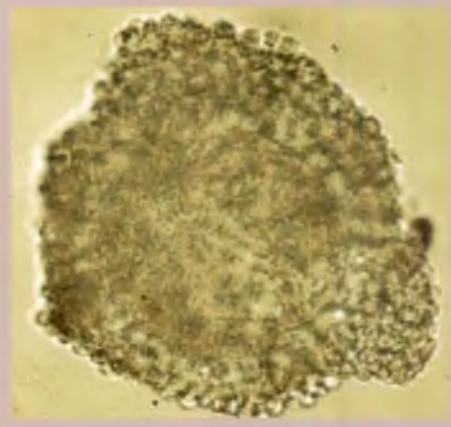

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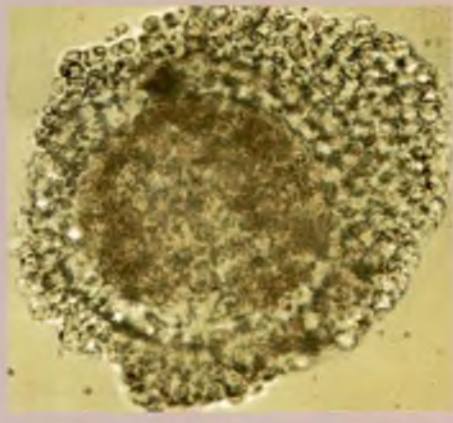

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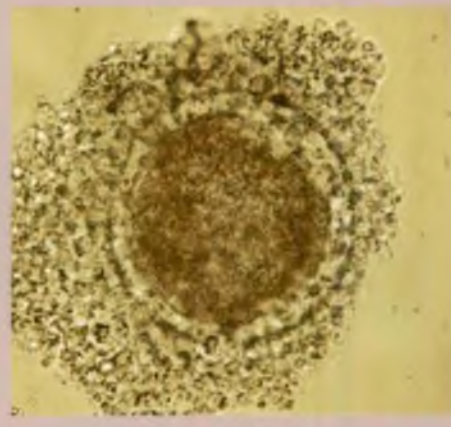

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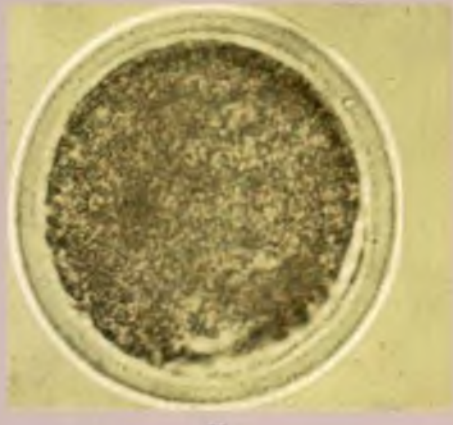

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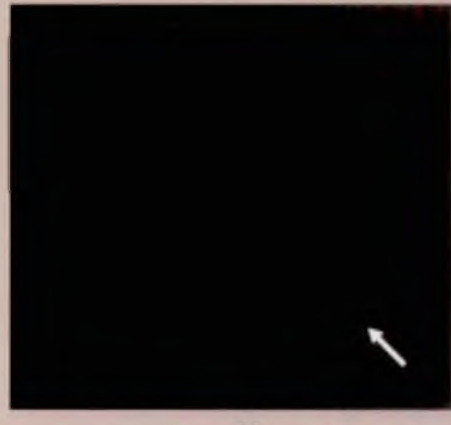

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Table.5 Morphometry of vitrified morphologically normal oocytes before and after vitrification

Method of cryopreservation	No. of oocytes vitrified	Mean total diameter (μ)			Mean thickness of cumulus layer (μ)		
		Before freezing	After freezing	Difference	Before freezing	After freezing	Difference
Conventional straw vitrification	14	302.72±14.53	412.58±16.1	109.86±12.77	104.02±12.9	160.71±17.01	56.69±12.08
Open pulled straw vitrification	14	231.42±10.69	266.49±11.94	35.07±6.61	43.83±8.16	61.08±9.01	17.25±3.03

Table.6 Synchronization and superovulation response

Treatment Group	No. of goats treated	No. and percentage of goats responded to synchronisation	No. and percentage of goats responded to superovulation
Fresh goats	6	6 (100)	6 (100)
Repeatedly superovulated goats	6	6 (100)	6 (100)

Figures in parenthesis denote percentage

4.4 OVARIAN RESPONSE TO SUPEROVULATION

The ovarian response was assessed by counting the number of corpora lutea (both functional and regressing) and anovulatory follicles of > 5 mm diameter separately for both right and left ovarics. Number and percentage of goats responded to synchronization and superovulation treatment is furnished in table 6 and the degree of superovulatory response in fresh and repeatedly superovulated goats are showed in table 7.

4.4.1 Anovulatory follicles

The average number of anovulatory follicles of > 5 mm diameter noted in fresh goats and repeatedly superovulated goats was 7.17 ± 2.0 and 5.17 ± 1.08 respectively. In fresh goats, the number of anovulatory follicles was higher in left ovary (24) than right ovary (19) while right ovary exhibited more anovulatory follicle (19) than left ovary (12) in repeatedly superovulated goats.

4.4.2 Superovulatory response

Data in table 7 revealed that the total and average number of ovulation in fresh goats was 111 and 18.5 ± 2.19 respectively and that for repeatedly superovulated goats was 66 and 11 ± 2.74 respectively. Number of ovulation observed in right ovary for fresh and repeatedly superovulated goats were 59 and 40 respectively and that for left ovary was 52 and 26 respectively. Functional CL noted in fresh animal and repeatedly superovulated group from left ovaries were 41 and 16 respectively.

4.4.3 Prematurely regressed CL (PRCL)

Premature regression of corpus luteum was predominantly noticed in this study. The functional corpus lutea appeared as cherry red colour and was prominently protruded from the surface of the superovulated ovaries. Whereas the regressing corpora lutea were white avascular structures with less diameter and the protrusion was not prominent as that of functional CL. Some of the goats showed corpora lutea in varying stages of regression whereas in certain other goats regression was almost complete. These goats had one or two large follicles in their ovaries. The incidence of PRCL was furnished in table 8.

Table.7 Ovarian response in fresh and repeatedly superovulated goats

Treatment group	No. of goats treated	Average No. of anovulatory follicle				Average No. of CL					
		Right ovary	Left ovary	Total	No. per goat	No. of functional CL		No. of prematurely regressed CL		Total	No. per goat
						Right ovary	Left ovary	Right ovary	Left ovary		
Fresh goats	6	19	24	43	7.17±2.0	50	41	9	11	111	18.5±2.19
Repeatedly superovulated goats	6	19	12	31	5.17±1.08	27	16	13	10	66	11±2.74

Any value in the same column between two groups differ non-significantly (χ^2 test)

Four goats (66.67 %) from fresh group and three goats (50 %) from repeatedly superovulated groups exhibited PRCL. Average number of PRCL per exhibited goats for fresh group was 5 ± 2.45 and that for repeatedly superovulated goats was 7.67 ± 2.4, but the average number of PRCL per treated animal was 3.33 ± 1.87 and 3.83 ± 2.02 for fresh and repeatedly superovulated goats respectively.

4.5 EMBRYO RECOVERY RATE.

Embryo collection was carried out by uterine flushing (Plate 5C). The total number, percentage recovery in terms of superovulatory response and average recovery per goat of unfertilized ova, embryo and both are presented in table 9.

4.5.1 Total Ova and Embryo Recovery

The total ova and embryo recovery rate was significantly lower ($P<0.05$) in repeatedly superovulated goats 28.79 per cent compared to fresh goat 76.57 per cent. The average total ova and embryo yield per goat from fresh goats and repeatedly superovulated goats were 14.17 ± 2.55 and 3.17 ± 0.87 respectively. In repeatedly superovulated animals, out of six goats one did not yield any unfertilized ova or embryo whereas in fresh group all goats contributed.

4.5.2 Unfertilized ova

Total and average number of unfertilized ova was significantly higher ($P<0.05$) in fresh goats (29 and 4.83 ± 2.12 respectively) than in repeatedly superovulated goats (15 and 2.5 ± 1.05 respectively). But the percentage recovery of unfertilized ova from repeatedly superovulated goats was 78.94 and that in the fresh goats was 34.12. Two goats each from both fresh and repeatedly superovulated groups did not yield any unfertilized ova.

4.5.3 Embryo recovery

The embryo recovery rates were significantly higher ($P<0.05$) in fresh goats (65.89 %) compared to repeatedly superovulated group (21.05 %). Out of six goats in the fresh group, one goat did not contribute any embryo. Out of six repeatedly superovulated goats, two goat yield four embryos and others did not. The total embryo

Table.8 Incidence of premature regression of CL in fresh and repeatedly superovulated goats

Treatment group	No. of goats treated	No. and percentage of goats exhibited PRCL	No. of PRCL			Average No. of PRCL per exhibited goat	Average No. of PRCL per treated goat
			Right ovary	Left ovary	Total		
Fresh goats	6	4 (66.67)	9	11	20	5±2.45	3.33±1.87
Repeatedly superovulated goats	6	3 (50)	13	10	23	7.67±2.4	3.83±2.02

Any value in the same column between two groups differ non-significantly (χ^2 test)
Figures in parenthesis denote percentage

Table.9 Embryo recovery rate in fresh and repeatedly superovulated goats

Sl. No	Treatment group	No. of goats treated	Superovulatory response		Embryo and unfertilised ova recovery		Unfertilised ova		Embryo recovery	
			Total ovulations (n)	Average response per goat	Total (%)	Average recovery per goat	Total (%)	Average recovery per goat	Total (%)	Average recovery per goat
1	Fresh goats	6	111	18.5±2.19	85[a] (76.57)	14.17±2.55	29[a] (34.12)	4.83±2.12	56[a] (65.89)	9.33±3.4
2	Repeatedly superovulated goats	6	66	11±2.74	19[b] (28.79)	3.17±0.87	15[b] (78.94)	2.5±1.05	4[b] (21.05)	0.67±0.49

Values bearing different superscripts in the same column differ significantly ($P<0.05$) - χ^2 test
Figures in parenthesis denote percentage

and its average recovery per goat (56 and 9.33 ± 3.4 respectively) were significantly higher ($P<0.05$) in fresh group compared to repeatedly superovulated group (4 and 0.67 ± 0.49 respectively).

4.6 DEVELOPMENTAL STAGES AND MORPHOMETRY OF EMBRYOS

The total recovered embryos from both fresh and repeatedly superovulated goats were pooled and were graded based on the developmental stages as shown in the table 10. Morphometry of embryos (Total diameter, zona pellucida thickness and diameter of embryo mass) were measured and presented in table 12.

4.6.1 Developmental stages

Out of 60 embryos recovered on day 7, 1.67, 18.33, 41.66, 35 and 3.33 per cent respectively were 8-16 cell stage, morula, compact morula, early blastocysts and blastocysts.

4.6.2. Morphometry of embryos

4.6.2.1 Total diameter of embryos

The total diameter of various developmental stages of embryos (Mean ± SE) ranged from 177.95 ± 2.38μ to 196.36 ± 0.0μ. For compact morulae the total diameter was 177.95 ± 2.38μ, which was lower than 8–16 cell stage (180) morulae (182.45 ± 2.13μ) and early blastocysts (185.41 ± 2.73μ). But an increasing tendency was observed for the total diameter of blastocyst (196.36 ± 0.0μ)

4.6.2.2 Thickness of Zona pellucida

Almost similar zona pellucida thickness was recorded for all the stages of embryos ranging from 13.19 ± 0.23 μ to 13.63 μ, except blastocyst, which were having a lower value of 10.25 ± 0.0 μ.

4.6.2.3 Diameter of inner cell mass

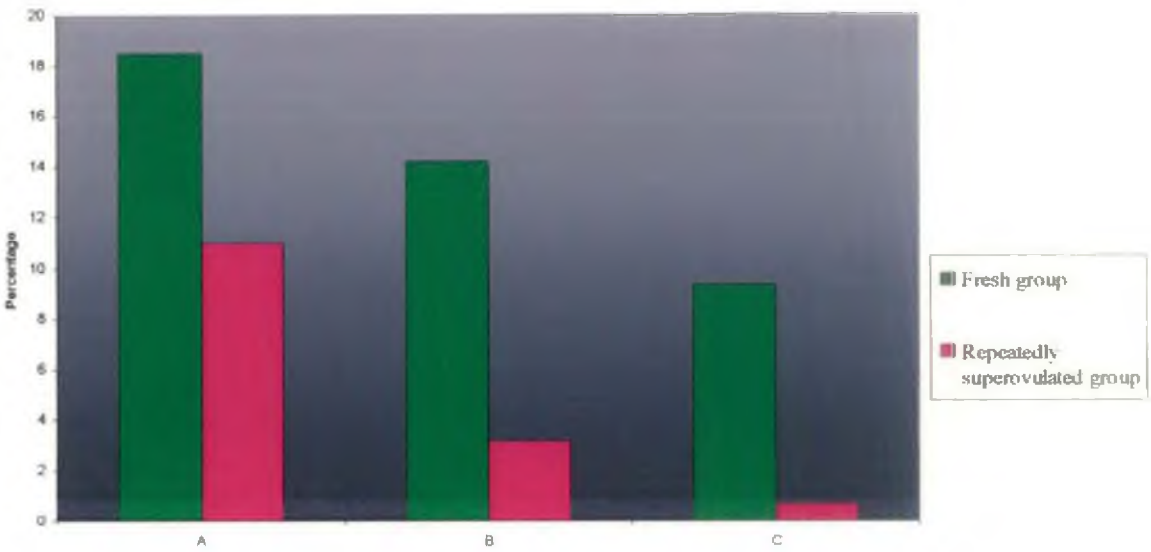
A higher value of 163.63 ± 0.0 μ was observed for blastocyst and a lower value for compact morulae 135.3 ± 2.92 μ. 8-16 cell, morulae and blastocysts were having the diameter of 147.27 μ, 140.72 ± 4.85 μ and 146.96 ± 4.55 μ.

Table.10 Developmental stage of embryos recovered from superovulated goats

Day of embryo collection	Total embryo yield	8-16 cell stage	Morula			Blastocysts		
			Early Morula	Compact Morula	Total	Early Blastocysts	Blastocysts	Total
7th day after the onset of heat	60	1 (1.67)	11 (18.33)	25 (41.66)	36 (60)	21 (35)	2 (3.33)	23 (38.33)

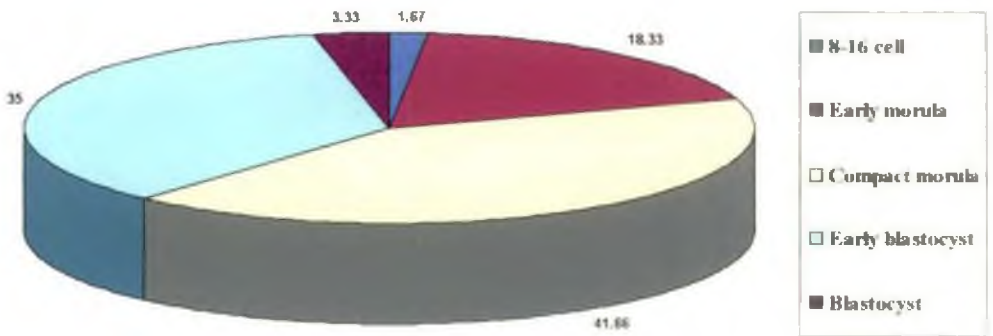
Figures in parenthesis denote percentage

Superovulatory response and embryo recovery



A: Ovulation, B: Ova and embryo recovery, B: Embryo recovery

Percentage recovery of various developmental stage embryos



4.6.2.4 Width of perivitelline space

Width of perivitelline space was higher for compact morulae (8.14 ± 1.6 μ) than morulae, blastocysts, early blastocysts and 8-16 cell stage (7.64 ± 1.7, 6.14 ± 0.0, 5.65 ± 1.28 and 5.47 μ respectively).

4.7 EMBRYO QUALITY

The details on the quality of the embryos recovered from superovulated animal are furnished in table 11. All the embryos from both fresh and repeatedly superovulated goats were pooled and then classified into Excellent, Good, Fair and Poor based on the morphological characteristics of embryo cell mass and zona pellucida. The excellent and good quality embryos were considered as transferable and the fair and poor were considered as non-transferable. All the transferable embryos were further subjected for vitrification.

4.7.1 Transferable embryos

Out of 60 embryos recovered, 55 (91.67%) were considered as transferable and its average yield per goat was 4.59, in which 14 embryos were in excellent quality and 41 embryos were good quality. Average yield of excellent and good quality embryo per goat were 1.17 and 3.42 respectively.

4.7.2 Non transferable embryos

The total number of fair quality embryos recorded was five. Since none of the goat yielded poor quality embryos, the average number of non-transferable embryos was recorded as 0.42 per goat.

4.8 EMBRYO CRYOPRESRVATION

4.8.1 Recovery rates of embryos after vitrification

The number of embryos vitrified and the number and percentage recovery of embryos after each cryopreservation method is furnished in the table 13. After CS vitrification of 17 embryos, 14 were recovered with the recovery rate of 82.35 per cent, while all the embryos vitrified by OPS could be recovered.

Table.11 Quality grade of embryos recovered from superovulated goats

Total embryo recovery	Transferable						Non transferable					
	Excellent		Good		Total (%) recovered as the percentage of fertilized ova	Average recovery per goat	Fair		Poor		Total (%) recovered as the percentage of fertilized ova	Average recovery per goat
	Total (%)	Average recovery per goat	Total (%)	Average recovery per goat			Total (%)	Average recovery per goat	Total (%)	Average recovery per goat		
60	14 (23.33)	1.17	41 (68.33)	3.42	55 (91.67)	4.59	5 (8.33)	0.42	0	0	5 (8.33)	0.42

Figures in parenthesis denote percentage

4.8.2 Viability of embryos after vitrification

After vitrification and thawing, the embryos were assessed for its morphological quality based on the intactness of zona pellucida and ooplasm. Total and percentage recovery of morphologically viable and damaged embryos are furnished in table 13 and 14 respectively. Embryos with damaged or degenerated cell mass, protrusion or lysis of the blastomeres, crack or thinning of zona pellucida were considered as non-viable. The number and percentage of embryos affected with different types of damages are shown in table 14.

4.8.2.1 Morphological viability of vitrified embryos

Out of 14 embryos recovered, seven (50 %) were retained normal morphology after CS vitrification method, while 15 embryos out of 17 (88.24 %) were retained normal morphology after OPS vitrification methods.

4.8.2.2 Non-viable vitrified embryos

Out of the 14 embryos recovered, seven (50 %) were damaged after CS vitrification and that for OPS vitrification was 11.76 per cent. Out of the 17 embryos recovered, two were lost its good-looking morphology after OPS vitrification.

4.8.2.3 Types of damages due to vitrification

Out of seven embryos subjected to CS vitrification, none showed zona pellucida damage alone; while five embryos (71.43 %) were having degenerated or broken blastomeres. Two embryos (28.57 %) showed the damage of both zona crack and embryo mass degeneration. After the OPS vitrification none of the embryos showed zona pellucida damage alone or together with the embryo cell mass damage. But two embryos (100 %) were having embryo cell mass damage alone.

4.8.2.4 FDA viability of vitrified embryos

All the morphologically viable embryos obtained after CS and OPS vitrification was stained with FDA stain to assess the membrane integrity.

Table.12 Morphometry of fresh embryos

Stages of embryo	Total diameter (μ)	Zona pellucida thickness (μ)	Diameter of inner cell mass (μ)	Width of perivitelline space (μ)
Morulae (10)	182.45±2.13	13.22±0.23	140.72±4.85	7.64±1.7
Compact morulae (14)	177.95±2.38	13.19±0.23	135.3±2.92	8.14±1.6
Early blastocysts (13)	185.41±2.73	13.58±0.05	146.96±4.55	5.65±1.28
Blastocysts (2)	196.36±0.0	10.25±0.0	163.63±0.0	6.14±0.0
8-16 cell stage (1)	180	13.63	147.27	5.47

Figures in parenthesis indicate number of observation

Table.13 Recovery rate of morphologically normal embryos after vitrification

Method of cryopreservation	No. of embryos vitrified	No. of embryos recovered after vitrification	Embryo recovery rate	Recovery rate of morphologically normal embryos	
				No.	(%)
Conventional straw vitrification	17	14	82.35%	7	50
Open pulled straw vitrification	17	17	100%	15	88.24

Any value in the same column between two groups differ non-significantly (χ^2 test)

Table.14 Type of morphological damages of vitrified embryos

Method of cryopreservation	No. of embryos recovered	Recovery rate of damaged embryos		Types of morphological damages					
				Zona pellucida alone		Embryo cell mass alone		Both zona pellucida and embryo cell mass	
		No.	(%)	No. and percentage of damaged embryo	% of total recovered embryo	No. and percentage of damaged embryo	% of total recovered embryo	No. and percentage of damaged embryo	% of total recovered embryo
Conventional straw vitrification	14	7	50	0	0	5 (71.43)	35.71	2 (28.57)	14.28
Open pulled straw vitrification	17	2	11.76	0	0	2 (100)	11.76	0	0

Any value in the same column between two groups differ non-significantly (χ^2 test)
Figures in parenthesis denote percentage

PLATE 10: VIABLE EMBRYO UNDER DIFFERENT STEPS OF CS VITRIFICATION

A. Embryo in HM before vitrification.

B. Embryo in 1 M SM after vitrification.

C. Embryo in HM after vitrification.

D. FDA stained vitrified embryo.

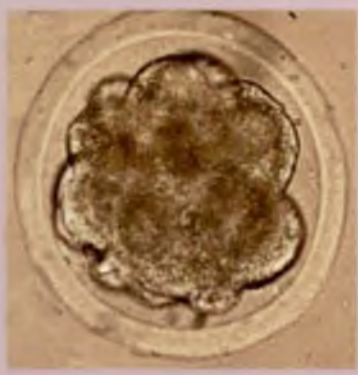
PLATE 11: NON-VIABLE EMBRYO UNDER DIFFERENT STEPS OF CS VITRIFICATION

A. Embryo in HM before vitrification.

B. Embryo in 1 M SM after vitrification.

C. Embryo in HM after vitrification.

D. FDA stained vitrified embryo (Arrow points to shadow).



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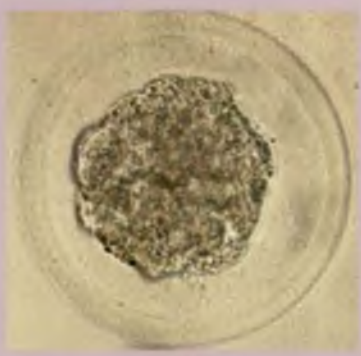

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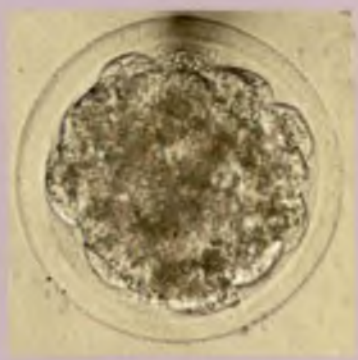

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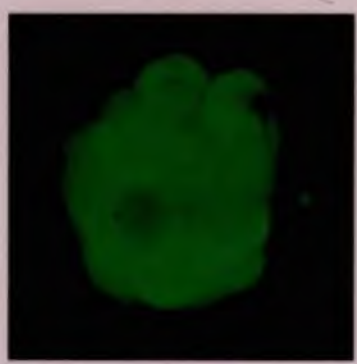

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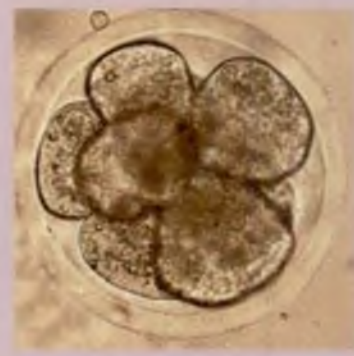

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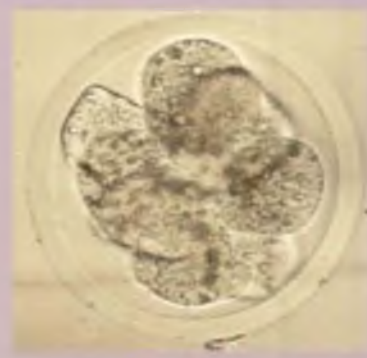

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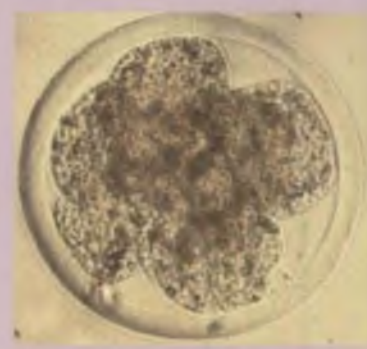

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PLATE 12: VIABLE EMBRYO UNDER DIFFERENT STEPS OF OPS VITRIFICATION

A. Embryo in HM before vitrification.

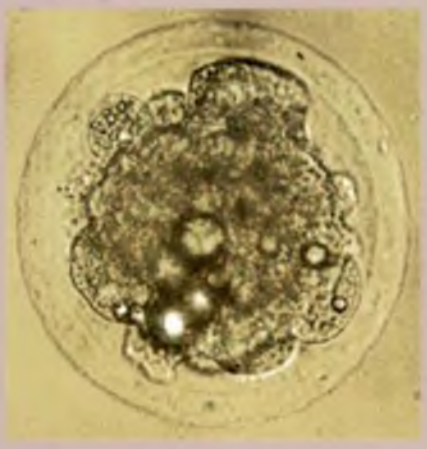
B. Embryo in equilibration medium before vitrification.

C. Embryo in 0.25 M SM after vitrification.

D. Embryo in 0.15 M SM after vitrification.

E. Embryo in HM after vitrification.

F. FDA stained vitrified embryo.



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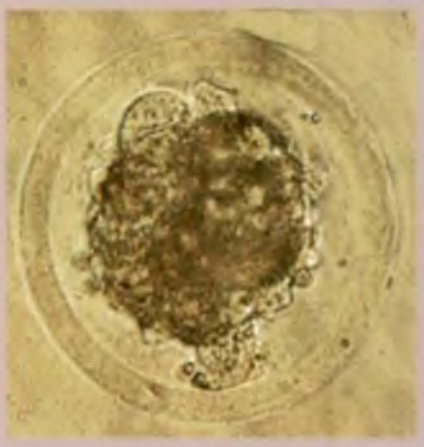

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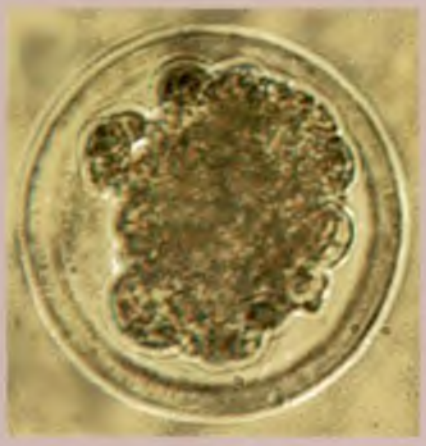

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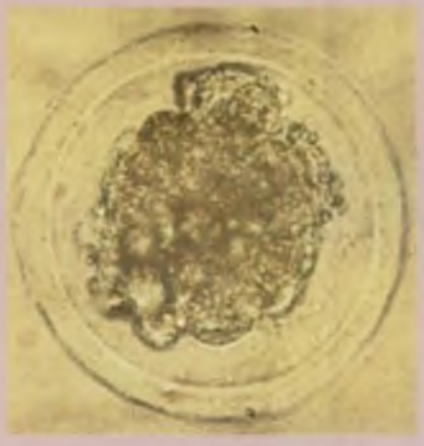

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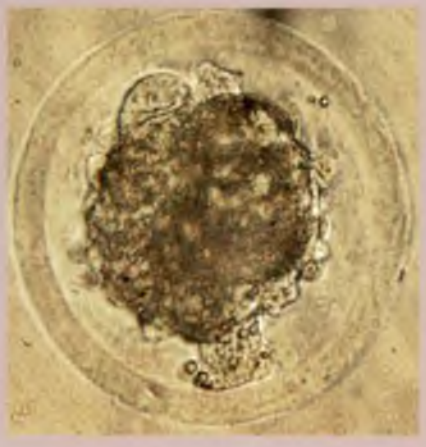

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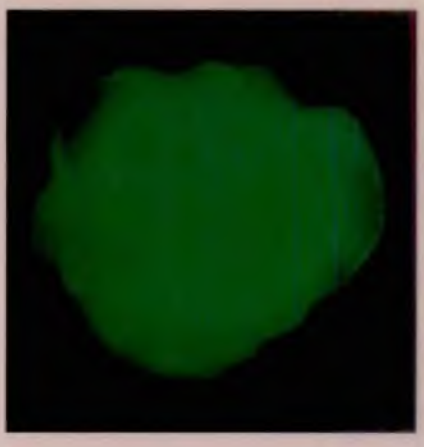


F

PLATE 13: NON-VIABLE EMBRYO UNDER DIFFERENT STEPS OF OPS VITRIFICATION

A. Embryo in HM before vitrification.

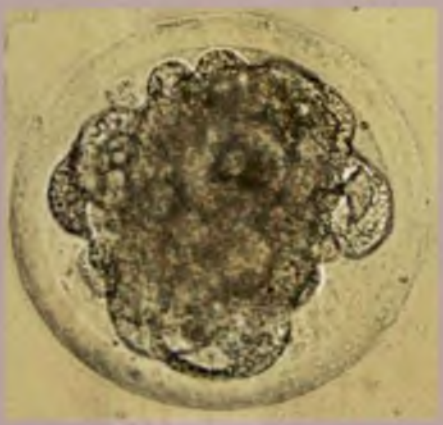
B. Embryo in equilibration medium before vitrification.

C. Embryo in 0.25 M SM after vitrification.

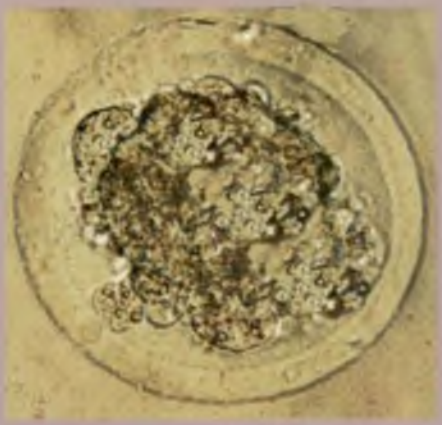
D. Embryo in 0.15 M SM after vitrification.

E. Embryo in HM after vitrification.

F. FDA stained vitrified embryo (Arrow points to shadow).



A



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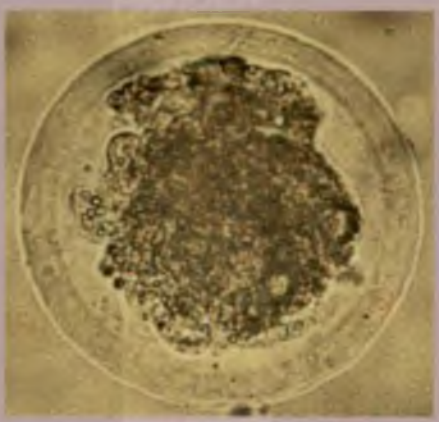

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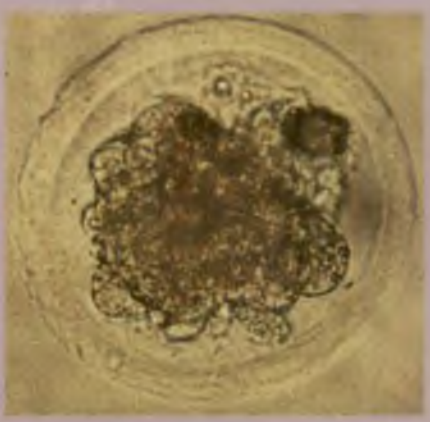
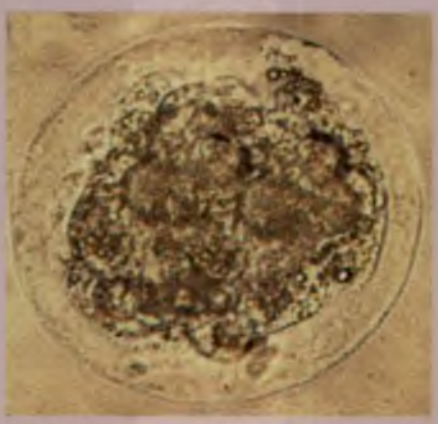


D



E

F

PLATE 13

As shown in table 15, out of seven normal good-looking embryos recovered after CS vitrification four (57.14 %) were brightly fluorescein after exposure to FDA stain. Out of 15 good quality embryos recovered after OPS vitrification, 13 (86.67 %) retained brilliant fluorescein. A higher percentage of morphologically viable embryos (76.47 %) retained the FDA viability out of the total embryo recovered (17) after OPS vitrification than after CS vitrification; 28.57 per cent retained the embryo viability out of 14 embryos recovered after CS vitrification.

Out of seven and 15 morphologically good looking embryos recovered respectively after CS and OPS vitrification method, 42.86 and 13.33 per cent respectively lost the stain completely or from some of the blastomeres and appeared as either non-fluorescein or light fluorescein.

4.8.2.5. Effect of vitrification on morphometric characteristics of embryo

Morphometry of representative number of viable embryo was taken before and after vitrification, which is presented in table 16. The total diameter, zona pellucida thickness and embryo cell mass diameter was measured.

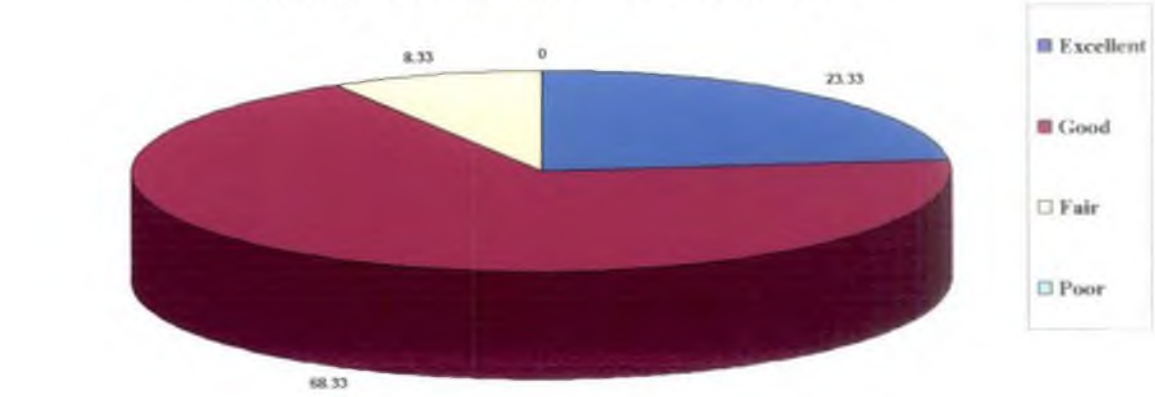
The total diameter, zona pellucida thickness and inner cell mass diameter of embryo showed an increase in the CS and OPS vitrification methods. After the CS vitrification, the total diameter of embryo was increased for the value of 1.64 ± 1.18 μ while after OPS vitrification methods was 0.05 ± 2.25 μ. Similarly the zona pellucida thickness was also showed a higher increase after CS vitrification than OPS (0.49 μ vs. 0.02 μ respectively). But inner cell mass thickness increased in similar proportion for CS and OPS vitrification (0.02 μ vs. 0.03 μ respectively).

Table.15 Viability assessment by FDA staining of vitrified morphologically normal embryos

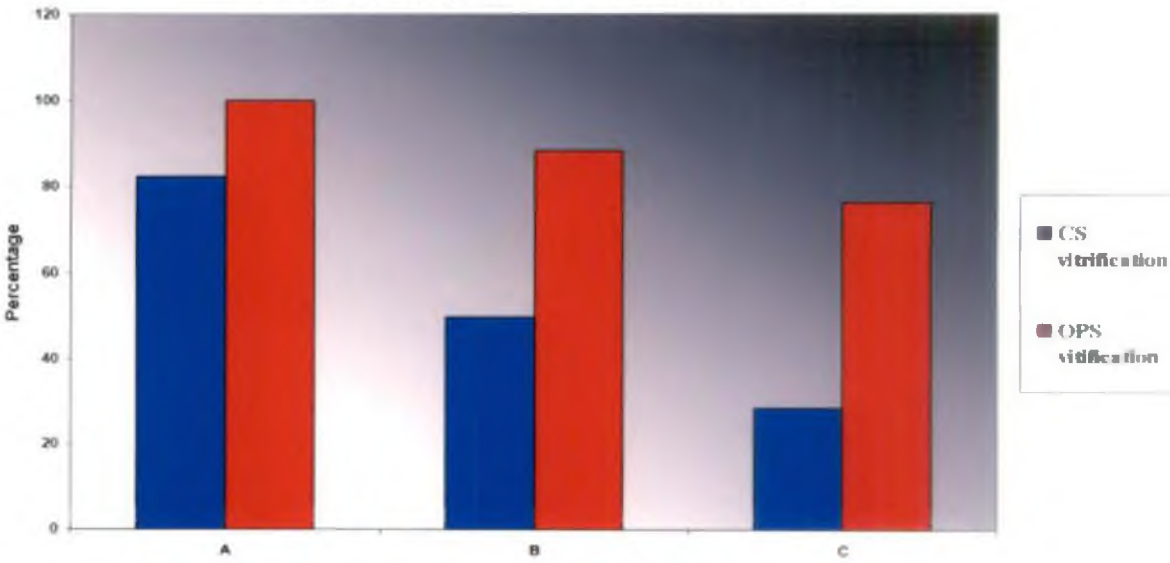
Method of cryopreservation	No. of embryos recovered after vitrification	No. of morphologically viable vitrified embryo subjected to FDA staining	FDA viable with normal morphology		FDA non-viable with normal morphology		Overall recovery of FDA viable embryo
			Total	(%)	Total	(%)	
Conventional straw vitrification	14	7	4	57.14	3	42.86	28.57
Open pulled straw vitrification	17	15	13	86.67	2	13.33	76.47

Any value in the same column between two groups differ non-significantly (χ^2 test)

Percentage recovery of different grades of embryos



Embryo recovery after CS and OPS vitrification



A : After thawing; B : Morphologically viable; C : FDA viable

Table.16 Morphometry of vitrified morphologically normal embryos before and after vitrification

Method of cryopreservation	No. of embryos vitrified	Mean total diameter (μ)			Mean thickness of ZP (μ)			Mean diameter of embryo cell mass (μ)		
		Before freezing	After freezing	Difference	Before freezing	After freezing	Difference	Before freezing	After freezing	Difference
Conventional straw vitrification	7	178.36± 1.18	180± 0.0	1.64± 1.18	13.14± 0.32	13.63± 0.0	0.49± 0.32	135.55± 2.43	135.57± 3.02	0.02± 3.08
Open pulled straw vitrification	7	188.88± 2.52	188.23± 2.5	0.05± 2.5	13.63± 0.0	13.68± 0.02	0.02± 0.02	154.87± 2.88	154.89± 2.89	0.03± 0.02

PLATE 14: VITRIFICATION INDUCED DAMAGES OF OOCYTE

A. Oocyte having degenerated cumulus mass.

B. Oocyte showing cracked ZP.

C. Oocyte with shrunken ooplasm.

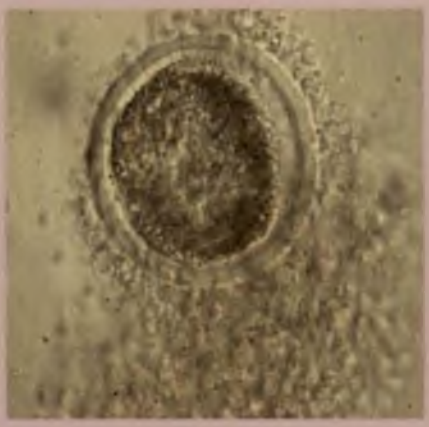
D. Oocyte with degenerated & fragmented ooplasm.

PLATE 15: VITRIFICATION INDUCED DAMAGES OF EMBRYO

A. Embryo showing lyses of blastomeres and cracked ZP.

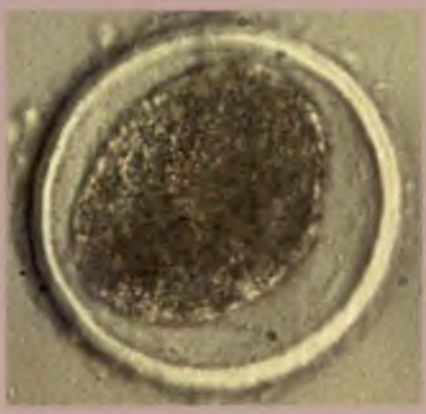
B. Embryo with leakage of blastomeres.

C. Embryo showing abnormal shape of ZP.

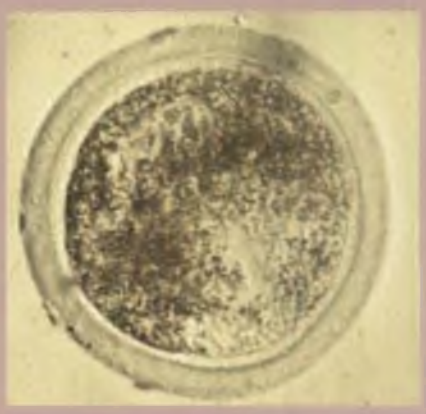


A

B

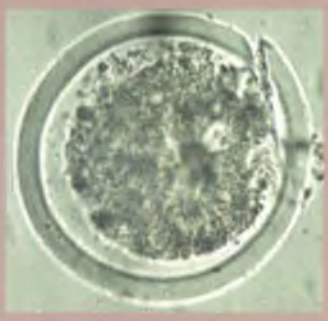


C

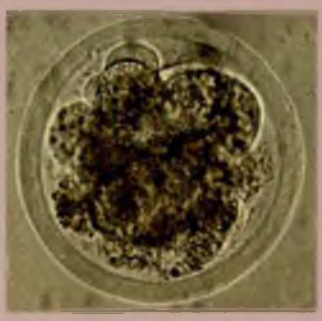


D

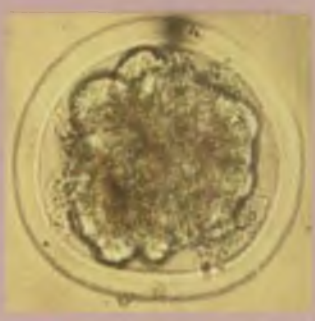
PLATE 14



A



B



C

PLATE 15

Discussion

5. DISCUSSION

5.1 RECOVERY OF OOCYTES

5.1.1 Average yield of oocytes

In the present study average yield of oocytes per ovary was 5.12 by slicing technique, which is in agreement with the findings 6.05 of Martino *et al.* (1994) and 4.99 of Vijayakumaran (1995). A lower recovery rate of 2.4 was reported by Pawshe *et al.* (1994) and higher recovery rate of 6.4 and 14.7 were observed by Das *et al.* (1996a) and Katska-Ksiazkiewicz *et al.* (2004) respectively. The high variability in the recovery rate of oocytes after slicing might be due to variation in the breed and type of animals from which ovaries were collected.

5.1.2 Morphological grading of oocytes

A higher recovery for Grade A oocytes (36.9 %) than other grades after slicing is in agreement with Vijayakumaran (1995) who recorded a higher recovery rate of 33.67 per cent for Grade A oocytes while a lowest recovery rate of 18.96 percent for grade D oocytes is in disagreement with the present study. In the present study comparatively higher recovery of 31.82 per cent was recorded for Grade D than Grade B and Grade C. The higher recovery of Grade D oocytes in the present study might be due to the fact that both denuded and degenerated oocytes were considered as Grade D.

Average yield of good quality oocytes in the present study was recorded as 2.82 per ovary, which is in partial agreement with the findings of Das (1996a) who recovered 2.29 oocytes per goat ovary after slicing. Pawshe *et al.* (1994) recorded a low recovery of 0.91 per goat ovary after slicing. This difference in recovery of good quality oocytes might be due to low recovery of total number of oocytes or difference in the quality grading. In the present study, oocytes having at least one complete layer of cumulus cells were considered as good quality. However it can be stated that slicing is simple method for oocyte recovery. Pawshe *et al.* (1994) and Das (1996a) also have made a similar observation.

5.2 OOCYTE CRYOPRESERVATION

5.2.1 Recovery rates of oocytes after vitrification

As shown in table 2 higher recovery rates of 94.94 per cent were recorded for Open pulled straw (OPS) than Conventional straw (CS) vitrification (67.06 %).

The low recovery rate (67.06 %) of oocytes after CS vitrification in the present study might be due to direct plunging of straws into liquid nitrogen as against the report of Wood *et al.* (1993) for mouse oocytes (more than 90 %) and Dhali *et al.* (2000a) and Dhali *et al.* (2000b) for buffalo oocytes (90.8 and 85 % respectively) where straws were exposed to liquid nitrogen vapour before plunging. More number of straws, which were plunged abruptly into liquid nitrogen in the present study, was showed breakage after thawing. This observation is in agreement with Dhali *et al.* (2000a) who stated that before plunging into liquid nitrogen it is necessary to precool the straw in liquid nitrogen vapor to reduce the possibility of straw fracture by rapid expansion of the crystallised sucrose column (Martino *et al.*, 1996).

The present recovery rate of OPS vitrified oocytes (94.44 %) is comparable to the report of Mavrides and Morroll (2002) who recovered 97.5 per cent of bovine oocytes after cryoloop vitrification. A slightly lower recovery rate (84 %) of OPS vitrified oocyte as compared to the present recovery rate was noted by Luna *et al.* (2001). The high recovery of OPS vitrified oocytes compared to CS vitrification in the present study might be due to loading of oocytes by capillary action of straw and avoidance of significant pressure changes of air or medium during cooling and warming. Additionally thawing and removal of oocytes to dilution medium in single step prevented unnecessary loss of oocytes.

5.2.2 Viability of oocytes after vitrification

5.2.2.1 Morphological viability of vitrified oocytes

In the present study percentage recovery of morphologically normal oocytes after CS vitrification were lower than that of OPS vitrification (42.11 vs. 70.59).

Dutta *et al.* (1998b) recorded a higher recovery rate of (73.33%) morphologically intact oocytes after CS vitrification. Lower recovery of morphologically intact oocytes in the present study of CS vitrification might be due to prolonged storage of straws in liquid nitrogen (more than 10 days vs. 24 h in the former study). Nag and Maurya (1997) reported that prolonged storage of vitrified oocytes from 10 to 30 h in liquid nitrogen resulted in non-significant reduction in maturation and fertilization. Later Dinnyes *et al.* (2000) reported that prolonged storage of vitrified oocytes in liquid nitrogen (two to three weeks) significantly reduced blastocysts development rate. Hamano *et al.* (1992) and Schmidt *et al.* (1995) also opinioned that single cell oocyte is more vulnerable to any environmental challenge than multicellular embryo. But the higher recovery rate after OPS vitrification and storage for more than 10 days in liquid nitrogen indicated the superiority of the cryopreservation by OPS.

Agrawal (1999) recovered 92.3 per cent morphologically normal caprine oocytes after CS vitrification in 25 per cent glycerol and 25 per cent Propanediol (PROH). The lower recovery rate in the present study might be due to the presence of Ethylene glycol (EG) in the vitrification medium (VM) since EG based cryoprotectant might have a toxic effect to oocyte (Chen *et al.*, 2000), due to low permeability of EG compared to PROH (Pedro *et al.*, 2005) especially for germinal vesicle stage oocytes (Hochi *et al.*, 1998). Hence high permeability enable PROH to rapidly permeate into caprine oocytes (Le Gal *et al.*, 1994) and there by reducing the intracellular ice crystal formation which is in agreement with Kharche *et al.* (2005) who recorded a high recovery rate of 81.4 per cent of morphologically intact caprine immature oocytes compared to the present study. The recovery rate of morphologically intact oocyte after CS vitrification in the present study is in partial agreement with Miyake *et al.* (1993) who could recover only 36 and 39 per cent of vitrified morphological normal oocytes after the exposure of 2 or 5 minutes respectively in EG-Ficoll-Sucrose (EFS) solution at 20°C.

In the present study glycerol was used as cryoprotectant in combination with EG for CS vitrification. Glycerol is least permeable to oocytes than EG, Dimethyl sulfoxide (DMSO) and PROH (Lim *et al.*, 1999 and Pedro *et al.*, 2005) and produces more damage to bovine oocytes (Lim *et al.*, 1999). This might be another reason for

low morphological survival of caprine oocytes after CS vitrification in the present study which is in agreement with Wani *et al.* (2004a) who observed low recovery of morphologically normal buffalo oocytes after vitrification in glycerol compared to that in DMSO, EG and PROH. In the present study of CS vitrification, oocytes were exposed to VM containing glycerol for 30-35 seconds as against 20-25 seconds for OPS vitrification in a glycerol free medium. Additionally the total cryoprotectant concentration used was 50 per cent for CS vitrification as against 33 percent for OPS. So prolonged exposure of oocyte in CS vitrification medium containing higher concentration of cryoprotectant than OPS might have resulted in cell injury due to chemical toxicity (Pedro *et al.* 2005) or osmotic over swelling due to rapid influx of water into cell against slow influx of glycerol (Wani *et al.*, 2004b).

Wood *et al.* (1993) and Bos -Mikich *et al.* (1995) observed more than 90 per cent recovery of morphologically intact hamster and mouse oocytes respectively in DMSO. Dhali *et al.* (2000a) and Dhali *et al.* (2000b) recovered 89-96 per cent and 98.4 ±1.6 per cent morphologically intact buffalo oocytes respectively after CS vitrification in DMSO - EG solution. Higher recovery rate in the former study is indicative of the superiority of DMSO alone (Massip, 2003) or in combination with EG which explain the high recovery rate (89 %) of bovine oocytes obtained by Hamano *et al.* (1992) after CS vitrification.

The low recovery of morphologically intact oocytes after CS vitrification in the present study might be due to high sensitivity of GV stage oocytes to vitrification. Parks and Ruffing (1992), Taha and Schellander (1992), Yang *et al.* (1994), Agca *et al.* (2000), Dhali *et al.* (2000a) and Men *et al.* (2002) also have reported that GV stage oocytes are very sensitive to freezing. The low cryotolerance of GV stage oocytes are further supported by presence of cumulus cells (Vajta, 2000). In the present study of CS vitrification least permeable glycerol with moderately permeable EG was used for vitrification of cumulus oocyte complex. The permeability of glycerol or EG might have been further reduced by cumulus cells surrounding the oocytes which resulted in intracellular ice formation. Inadequate level of cryoprotectant reduces the viability of oocytes (Dhali *et al.*, 2000a).

Morphological survival rate of OPS vitrified oocyte in the present study is partially comparable to the report of Begin *et al.* (2003) who recovered 77 per cent of morphologically intact caprine oocytes after solid surface vitrification. Men *et al.* (2002) observed 79.6 per cent and 88.4 per cent recovery of bovine GVBD and M-II oocytes respectively after OPS vitrification in 20 per cent glycerol and 20 per cent DMSO. Compared to the present study, slightly higher recovery rate noted in the above studies might be due to stage of development (stage of maturation) of oocytes or difference in the cryoprotectant concentration and its duration.

In the present study of OPS vitrification relatively high morphological survival of oocytes when compared to CS vitrification procedure might be due to addition of 0.5 M sucrose with permeable cryoprotectant which is in agreement with the report of Chen *et al.* (1994), Hotamisligil *et al.* (1996) and O'Neil *et al.* (1997). These authors further reported that vitrification solution containing both permeating and non permeating cryoprotectant was suitable for mouse oocytes than that containing permeating cryoprotectant alone; in presence of sucrose the membrane stability of oocytes was dramatically enhanced upon prolonged exposure to cryoprotectant.

The high recovery of OPS vitrified morphologically normal oocyte as compared with CS vitrified oocyte in the present study might be due to rapid cooling rate and procedure adopted for OPS vitrification. Vajta (2000) reported that high speed vitrification method dramatically decrease the chilling injury, permit the use of less concentrated solutions and shortens the time of exposure with the final cryoprotectant both before cooling and after warming. Additionally the low volume of cryoprotectant solution prevents heterogenous ice formation (Rall, 1987). In OPS method low volume of vitrification solution is directly warmed and immediately diluted into the dilution solutions (Hurtt *et al.*, 2000), which helps to reduce exposure of oocytes to unsuitable temperature and concentrated cryoprotectant (Chen *et al.*, 2000). This might be the reason for high morphological recovery after OPS vitrification than CS vitrification.

5.2.2.2 Non-viable vitrified oocytes

In the present study 57.89 per cent damaged oocytes were recovered after CS vitrification which is in agreement with the report of Hochi *et al.* (1995) who could

recover 54.2 and 57.1 per cent damaged equine oocytes after equilibration in 20 per cent EG at 20^{0}C and 30^{0}C respectively followed by vitrification in EFS medium. A higher recovery of damaged oocytes was reported by Papis *et al.* (1995) who recovered 67, 100, 70 and 65 per cent morphologically damaged oocytes after vitrification in DMSO - Acetamide - PROH, PROH – Glycerol-Sucrose, EFS and EG - Butylene glycol – Sucrose respectively followed by direct dilution. The high recovery of damaged oocytes compared to the present study might be due to direct dilution procedure.

Morphological damage of vitrified thawed oocytes was more in CS than OPS method (Table.3). In complete disagreement of this, Chen *et al.* (2000) observed that significantly more oocytes were damaged in OPS (38 %) than in CS (19 %) after vitrification in 5.5 M EG. In another study, Chen *et al.* (2001) also recovered high percentage of morphologically damaged oocytes for OPS than CS vitrification (37 vs. 23 %) in 5.5 M EG which is contrary to the present observation. The high percentage of damaged oocyte observed after OPS than CS vitrification as against the present study might be due to difference in the cryoprotectant combination. This indicated that in addition to cooling rate, cryoprotectant combination is also important in OPS vitrification procedure to achieve high recovery rate of morphologically intact oocytes.

5.2.2.3 Types of damages due to vitrification

Perusal of table.3 showed that 57.89 per cent of the CS vitrified thawed oocytes was having ZP and ooplasm damage either alone or in combination. In contrast Agrawal (1999) recovered low percentage (7.7) of CS vitrified thawed caprine oocytes with ZP and ooplasm damage. Dhali *et al.* (2000b) also observed lower rates of damage of ZP and ooplasm (6.07 % and 1.1 % respectively) for vitrified thawed buffalo oocytes after vitrification in EG and DMSO. This difference in the degree of damage might be due to difference in the cryoprotectant combination.

Atabay *et al.* (2004) observed that most common morphological damage to *in vitro* matured oocytes after Microdroplet vitrification was the rupture of ZP with breakage/degeneration of ooplasm. But in the present study, in both OPS and CS vitrification, most common damage observed was at the level of cumulus and ooplasm.

The difference in the observation might be due to vitrification of cumulus oocytes complex in the present study as against denuded oocyte. Ooplasm of CS vitrified damaged oocytes in the present study showed uneven contraction or folding of vitellus. A similar observation was also reported by Martino *et al.* (1996). Men *et al.* (1997) reported that high concentration of cryoprotectant in the VM caused drastic shrinkage and even break down of oocytes. This observation is reinforced by the recovery of very lower number of oocytes with ooplasm shrinkage after OPS than CS vitrification in the present study. After CS vitrification of caprine oocytes in PROH, Kharche *et al.* (2005) observed 18.6 per cent oocytes with damages composed of greatly reduced cumulus mass, ooplasm rupture and abnormal shape of ZP. Compared to the present study the low recovery of damaged oocyte in the above study might be due to vitrification in highly permeable PROH.

After both methods of vitrification major damage affected the oocytes was at the level of cumulus, but higher cumulus damage was observed after CS vitrification than OPS vitrification (36.78 vs.11.7 %). Hamano *et al.* (1992) could not recover any oocytes with damaged cumulus after vitrification in DMSO-PROH-Acetamide medium. Compared to the present study the high stability of cumulus cell can be attributed to the superiority of the cryoprotectant used or the technique adopted. Fuku *et al.* (1995) and Le Gal (1996) observed that a partial loss of communication between oocytes and its cumulus layer occur as a consequence of osmotic stress generated during the cryoprotectant exposure, freezing procedure or cryoprotectant removal. Such osmotic stress can result in ice crystal formation inside the cumulus cells leading to its damage (Aono *et al.,* 2003). In the present study, OPS vitrification procedure was highly efficient than CS vitrification to reduce the osmotic damage of cumulus cells as evidenced by the low recovery rate of oocyte with damaged cumulus after OPS.

After OPS vitrification, only 3.92 per cent of the recovered oocytes showed ZP damage which was lower than the value (5.25 %) recorded after CS vitrification. This finding is in agreement with Aono *et al.* (2003) who observed 1.8-8.2 per cent of damaged mouse GV oocytes after Gel tip vitrification (Ultra rapid vitrification) in EG – DMSO medium at various concentration of cryoprotectant and equilibration step. Dinnyes *et al.* (2000) observed cracking of the vitrified sample because of temperature

difference between outer layer and inner core resulting in ZP rupture during cooling and thawing. Small volume of vitrified drops and narrowing the insulating layer between VM and cooling agent induce rapid cooling rate in OPS (Albarracin *et al.*, 2005) there by minimizing ZP damage during cooling and warming (Li *et al.*, 2002). The observation made in the present study for OPS is in agreement to this.

5.2.2.4 FDA viability of vitrified oocytes

Table. 4 of the present study showed that the percentage recovery of morphologically normal FDA viable oocytes after OPS vitrification (52.94 %) was higher than CS vitrification (31.58 %). Mohr and Trounson (1980) mentioned that FDA being non polar could readily pass into cell where it is hydrolyzed by esterase enzyme to polar substance and therefore accumulates intracellularly (Rotman and Papermaster, 1966) which yields fluorescein. Hence FDA staining can be used to assess both esterase enzyme activity and membrane integrity. Twenty five per cent of the morphologically viable vitrified oocytes by CS and OPS method did not show fluorescein which indicated that these oocytes were having damaged ooplasmic membrane which could not be assessed by the morphological examination. Didion *et al.* (1990) studied the effect of glycerol on conventional cryopreservation of porcine oocytes by Trypane blue exclusion followed by FDA staining and recorded that none of the oocytes retained fluorescein after freezing. Compared to the present study the difference in recovery of ooplasm intact oocytes based on the FDA viability in the above study might be due to type of cryoprotectant and method of freezing used.

5.2.2.5 Effect of vitrification on morphometric characteristics of oocytes

Perusal of table.5 showed that the increase in average cumulus thickness of viable oocytes was 56.69 ± 12.08 after CS vitrification and for OPS vitrification it was 17.25 ± 3.03. As this parameter was studied in a non comparable procedure no cross comparison was made between CS and OPS vitrification. Proportionate increase of total diameter of oocyte (including cumulus mass) was also noted after CS and OPS vitrification of the present study. But the extent of increase was more for CS vitrification than OPS vitrification (109.86 ± 12.77 vs. 35.07 ± 6.61). Similar observation for OPS vitrified oocytes were noted by Hurtt *et al.* (2000) who reported

that some of the cumulus mass of both bovine and equine oocytes showed expansion after OPS vitrification.

In the present study most of the vitrified damaged oocytes were devoid of cumulus cells after both methods of vitrification but the occurrence was apparently more after CS method than OPS. Hence that type of oocyte could not be accounted for morphometry studies. Le Gal (1996) mentioned that the partial loss of communication between oocyte and cumulus might be due to osmotic stress generated during the freezing procedure. Hence it can be reasonably assumed that both type of vitrification procedure generated osmotic injury to cumulus but the intensity of damage was visibly more for oocytes vitrified by CS method.

It was observed that cumulus cells of vitrified viable oocytes were easier to remove for staining and morphological studies, which is in agreement with Hurtt *et al.* (2000). Ruppert-Lingham *et al.* (2003) reported that loose attachment of cumulus with oocyte is indicative of freezing induced cumulus-oocyte junctional damage and observed reduced developmental capacity of this type of oocyte in culture. Hence it can be reasonably assumed that after *in vitro* culture, OPS vitrified viable oocyte might show a better survival rate than CS vitrified viable oocyte since compact cumulus mass around oocyte are important for the success in *in vitro* maturation of frozen thawed immature oocytes (Im *et al.*, 1997 and Papp *et al.*, 2005). However, only by *in vitro* maturation and fertilization study this fact can be convincingly established.

5.3 RESPONSE TO SYNCHRONISATION

All animals in the present study responded to synchronization after treatment with norgestomet and $PGF_{2\alpha}$ combination which is in agreement with the findings of Senn and Richardson (1992), Akinlosotu and Wilder (1993), Romano (1996), Zarkawi *et al.* (1999), Oliveira *et al.* (2001), Freitas *et al.* (2003), Joseph, (2003) and Espinosa-Marquez *et al.* (2004). Compared to the present study lower response was noted by Bretzlaff and Madrid (1985), Pendleton *et al.* (1992b) and Rowe and East (1996). This variation in response to synchronization might be attributed to the breed, type and time of administration of the drug.

In the present study prostaglandin was used as a luteolytic agent and 100 per cent response indicated that does receive the $PGF_{2\alpha}$ injection at the stage when CL was responsive (Kusina *et al.*, 2000). No animal in the present study showed oestrus with the implant *in situ*, which is in agreement with Romano (1996), Romano (2004) and Amarantidis *et al.* (2004). On contrary, Mellado and Valdez (1997) observed oestrus occurrences in goat while the implant was *in situ*. This variation might be due to exposure of doe to buck in the above study. It is well established that buck stimulus has a stimulatory effect independent of steroid feed back on hypothalamic centers (Martin *et al.*, 1983).

Both fresh and repeatedly superovulated goats in the present study exhibited 100 per cent synchronization response. While Nuti *et al.* (1987) observed that 100 per cent and 83 per cent animal responded in first and second trial of synchronization. Similarly in ewes Cordeiro *et al.* (2003) also observed that 27 and 40 per cent of animals failed to synchronise in the first and second treatment respectively. Based on the present study it can be concluded that repeat trial of synchronization procedure using norgestomet and prostaglandin did not compromise the synchronization response.

5.4 OVARIAN RESPONSE TO SUPEROVULATION

5.4.1 Anovulatory follicles

The average number of anovulatory follicle (7.17 ± 2.0) in fresh animal group is comparable to the findings of Singh *et al.* (1993) who recorded 7.25 ± 0.48 anovulatory follicle in goats treated with PMSG. The average number of anovulatory follicle in repeatedly used group was 5.17 ± 1.08, which is comparable to the report (5.8 ± 1.9) of Armstrong *et al.* (1983a). In the present study, number of anovulatory follicle in the fresh goats was higher than repeatedly used goats. Similar observation was reported by Nuti *et al.* (1987) who reported 12.83 anovulatory follicles per animal in the first trial and 4.4 anovulatory follicles per animal in the second trial. The lower number of anovulatory follicle in repeatedly used animal in the present study might be due to lowered ovarian response.

A higher number of anovulatory follicles compared to the present study were

22.1 ± 3.2 respectively. While lower number of anovulatory follicle 2.00 ± 0.09 and 0.16 respectively was observed by Tiwari *et al.* (1998) and Joseph (2003) when hCG was used with FSH. In the present study, high number of anovulatory follicle indicated the need of ovulation inducers in superovulation protocol which is in agreement with the Singh *et al.* (1993) who observed lower number of anovulatory follicle when hCG was used. Akinlosotu and Wilder (1993) reported that FSH enhanced follicular development as well as number of anovulatory follicle.

5.4.2 Superovulatory response

Superovulatory response (number of ovulation) from right and left ovaries and total response per animal was found to be higher in fresh animal than repeatedly used animal group (Table.7). In the present study, right ovary was found to be more active than left ovary among both groups of animals which is in agreement with Sarmah *et al.* (1996b). But on contrary Hasin *et al.* (2004) observed that left ovary was more active than right ovary. The role of breed as a probable cause for variability in superovulatory response is well established (Ocampo *et al.,* 1988).

A comparable number of ovulation from the fresh animal group (18.5 ± 2.19) in the present study was reported by Armstrong *et al.* (1983a), Nuti *et al.* (1987), Deshpande *et al.* (1997), Greyling *et al.* (2002) and Freitas *et al.* (2003) who recorded 17.6 ± 5.5, 17.7, 17.6, 18 and 17.4 ± 7.3 ovulation respectively in superovulated goats. Higher ovulation was reported by Senn and Richardson (1992) in Nubian goats and Senthilkumar *et al.* (2003) in Tellicherry breeds (28.7 ± 2.3 and 21.83 ± 1.99 respectively). The high variation in case of former study might be due to breed effect and later be due to comparatively lower LH content of oFSH than pFSH (McNatty *et al.,* 1989). Comparatively lower ovulation of 16.1 ± 0.8, 14.2 ± 8.11, 12.0 ± 0.5, 10.24 ±2 .75, 12.2 ± 5.3 and 11.5 ± 6.6 respectively were reported by Armstrong *et al.* (1983b), Mani *et al.* (1994), Lee *et al.* (2000) Gogai *et al.* (2001), Terblanche *et al.* (2001) and Lima-Verde *et al.* (2003). The difference compared to the present study might be due to breed (Gonzalez Bulnes *et al.,* 1999) or dose and type of hormones used (Armstrong *et al.,* 1983a and Pintado *et al.,* 1998). Using even a higher dose of 180mg Folltropin V than the dose of present study (133mg) Senthilkumar *et al.* (2003)

response might be due to season (Senn and Richardson, 1992; Gootwine *et al.*, 1997 and Pintado *et al.*, 1998) or the effect of different batches of gonadotrophin (Wollen *et al.* 1985) as commercial pFSH preparation may even vary in FSH/LH ratio with in batches (Lindsell *et al.*, 1986).

Repeatedly superovulated animal used for the present study was older than fresh animals. The overall ovulation in fresh animal was higher than that in repeatedly super ovulated animals which is in agreement with Gootwine *et al.* (1997) who reported that ovulation rate of repeated animal (15.1) was significantly less than that of freshly used animal (17.2). A similar observation was made by Baril *et al.* (1989) with pFSH. This lower superovulatory response in repeatedly superovulated animal was probably due to anti-pFSH immunological response (Remy *et al.*, 1991). But on contrary Mahmood *et al.* (1991) reported that superovulation response (19.1 ± 6.7) was significantly higher in older animals of 4-6 years than younger animals of 1.5 to three years of age (13.25 ± 3.24) in Pashmina goats treated with pFSH. The variation might be due to repeated superovulation in older animals in the present study.

5.4.3 Premature luteal regression

Percentage of goats exhibited PRCL in the fresh animal group was 66.67 and in the repeatedly superovulated animal was 50 per cent as shown in table 8 which is in agreement with Joseph (2003) who observed 51.42 per cent goats treated with FSH were having occurrence of PRCL. Compared to the present study Saharrea *et al.*(1998) reported a high percentage of animal exhibited PRCL (85.71 %) when treated with PMSG alone but a lower value of 28.57 per cent was noted in hCG treated group since hCG induce ovulation of anovulatory follicle which reduce the chance for PRCL. Anovulatory follicle produce oestradiol (Senthil kumar *et al.*, 2003) which induce endogenous secretion of $PGF_{2\alpha}$ either follicular or uterine origin (Armstrong *et al.*, 1983a). A higher percentage of PRCL noted in the present study of fresh and repeatedly superovulated animal indicated the need of ovulation inducers such as hCG (Saharrea *et al.*, 1998). Administration of prostaglandin inhibitors such as flunixine meglumine (Battye *et al.*, 1988) reduce PRCL occurrence and progesterone supplementation from later half of day four of superovulatory heat until the day of embryo collection prevent

In the present study average number of PRCL in fresh goat was 5 ± 2.45 per exhibited animal and 3.33 ± 1.87 per treated animal which is lower than repeatedly superovulated animals 7.67 ± 2.4 and 3.83 ± 2.02 respectively. Compared to the present study Pendleton *et al.* (1992a) observed a low number of 2.0 ± 1.6 PRCL for FSH treated animals. Higher number of PRCL in the present study might be due to comparatively higher number of anovulatory follicle 7.17 ± 2.0 and 5.17 ± 1.08 respectively for fresh goats and repeatedly superovulated goats as against 0.9 ± 0.8. Higher percentage of fresh animal that showed more PRCL might be due to stress, since the fresh animal used in this experiment were younger than repeatedly superovulated goat which was more susceptible to stress as reported in pigs Wan *et al.* (1994) and in goats Pintado *et al.* (1996).

5.5 EMBRYO RECOVERY RATE

5.5.1 Total ova and embryo recovery

Average and percentage recovery of ova and embryo together in the present study is 14.17 ± 2.55 and 76.57 which is significantly higher ($P<0.05$) than that in repeatedly superovulated goats (3.17 ± 0.87 and 28.79 respectively). The percentage recovery of ova and embryo from fresh animal of the present study is in agreement with the recovery rate of 76.38 per cent as noted by Pargaonkar *et al.* (1992). Chemineau *et al.* (1986) observed 78 per cent recovery rate from animals without PRCL on seventh day collection after the onset of heat which is in close proximity with the observation in fresh animal of the present study. However the slight increase compared to the present study might be due to complete flushing of the reproductive tract (Fimbria, oviduct and uterine horn). Nuti *et al.* (1987) observed low embryo and ova recovery rate of 67 per cent by uterine flushing and this low recovery rate compared to the present study of fresh goats might be due to single step flushing.

Adhesions were seen between ovary, uterus, oviduct and uterine horns (plate 5D) during the laparotomy of repeatedly used animals in the present study. This interfered with the exploration and flushing of uterus resulting in low recovery rate of ova and embryo compared to fresh animals. On contrary, Cordeiro *et al.* (2003) observed almost similar recovery rates of 78.9 and 80.4 percent respectively for ewes

after first and second collection. The similar recovery rate between first and second collection might be due to washing of genital tract with heparinised saline which might to have minimized the post operative tissue reaction.

5.5.2 Unfertilized ova recovery

In the present study average and percentage recovery of unfertilized ova from fresh animal was 4.83 ± 2.12, which is significantly higher ($P<0.05$) than that from repeatedly, superovulated animal (2.5 ± 1.05). But the percentage recovery of unfertilized ova from repeatedly superovulated goat (78.94 %) was significantly higher than that from fresh animal (34.12 %). Compared to the present study, a lower recovery rate of unfertilized ova (13 %) was reported by Tervit *et al.* (1983) and Greyling *et al.* (2002) recorded three and one percent recovery rate in Boer and indigenous African goats respectively. The low unfertilized ova recovery compared to the present study may be due to natural breeding or laparoscopic insemination unlike artificial insemination in the present study. It is demonstrated that hand mating is superior to artificial insemination in goats (Cognie *et al.*, 2003). However higher recovery rate of 7.1±1.4 and 9.67 unfertilized ova respectively were observed by Pendleton *et al.* (1992a) and Deshpande *et al.* (1997).

5.5.3 Embryo recovery

The average and percentage recovery of embryo in the present study (Table 9) for fresh animal group was respectively for 9.33 ± 3.4 and 65.89 which was significantly higher ($P<0.05$) than that in repeatedly superovulated animal (0.67 ± 0.49 and 21.05 % respectively). The percentage recovery of embryos from fresh animal in the present study is partially comparable to the recovery rate of 71.9 observed by Gonzalez – Bulnes *et al.* (2004). A comparable result of embryo recovery from fresh animal in the present study was noted by Nuti *et al.* (1987), Pendleton *et al.* (1992a), Akinlosotu and Wilder (1993), Deshpande *et al.* (1997) and Senthilkumar *et al.* (2003) who recovered 5, 7.0 ± 1.4, 10.1 8.33 and 8.33 ± 2.36 embryo respectively per superovulated goat while recovery rate from repeatedly superovulated goat in the present study is in agreement with Pendleton *et al.* (1992a) and Espinosa – Marquez *et al.* (2004) who noted 0.8 ± 0.7 and 0.1 ± 0.1 embryo per animal respectively.

Baldassarre *et al.* (2001) recovered 12.0 ± 10 embryos out of 14.1 ± 8 ova and embryo in animal treated with 160 mg Folltropin V with 5mg Lutropin. In the present study the low recovery of embryo (9.33 ± 3.4) out of 14.17 ± 2.55 ova and embryo in fresh animal might be due to absence of ovulation inducing agents in the superovulation protocol since higher number of ovulation over an extended period of time especially in FSH treated animals impede the fertilization process after programmed insemination (Selgarth *et al.*, 1990 and Cognie *et al.*, 2003).

In the present study significantly low number of embryos was recovered from repeatedly superovulated goats than fresh group. A probable reason for this low recovery is that all of the repeatedly superovulated goats showed adhesion of the reproductive tract which adversely affected the flushing efficiency. A similar observation was made by Dhandapani *et al.* (2001) who reported that goat subjected to embryo collection showed extensive adhesion involving abdominal wall, greater omentum, uterus, uterine horn, oviduct and ovaries at the time of slaughter which prevent further embryo collection procedure. Observation of lower embryo recovery from repeatedly superovulated goat in the present study is in agreement with Cordeiro *et al.* (2003) who observed a non significantly lower fertilization rate of 40 per cent in the second treatment compared to 53 per cent in the first treatment when treated with pFSH in ewes. This lowered fertility associated with repeated hormonal treatment was due to hormonal binding Baril *et al.* (1996). So in the repeatedly superovulated group of the present study, antibody might have produced against FSH, which resulted in reduced superovulatory response and lowered embryo recovery rate.

Compared to the percentage recovery of embryos from fresh animal group, a higher recovery rate of 78.6 per cent was noted by Gonzalez - Bulnes *et al.* (2003). The low recovery from fresh animal group of the present study may be due to seventh day embryo collection in presence of PRCL and high superovulatory response (18.5 ± 2.19) as against 14.3 ± 0.5. Armstrong *et al.* (1983b) reported that in PRCL condition, the recovery rate of embryos were significantly decreased as the interval from oestrus to flushing increased and Cognie *et al.* (2003) indicated that as the superovulatory response increased (>15CL), the fertilization rate decreased followed by vaginal or cervical insemination which may be attributed to a disturbance in sperm transport

(Evans and Armstrong, 1984). In the present study the embryo collection was carried out 7 days after the onset of heat and the superovulatory response was moderately high.

5.6. DEVELOPMENTAL STAGES AND MORPHOMETRY OF EMBRYOS

5.6.1 Developmental stages

Perusal of table 10 reveals that 60 per cent of embryos collected were morula (early and compact morula), 38.33 per cent were blastocysts (early and blastocysts) and 1.67 per cent were 8-16 cell stage upon embryo collection on seventh day after the onset of heat (ie. sixth day after AI). Compared to the present study Joseph (2003) recovered high percentage of morula (86.67 %) from adult goats and a lower recovery of 44.44 per cent from peripubertal goats. This difference might be due to difference in the protocol and type of animal used for superovulation. Dutta *et al.* (1998a) recovered higher number of blastocyst than morula during the uterine flushing on day 6 of post oestrus. In the present study high recovery of morulae on 6th day after AI indicates that delayed ovulation might have occurred and hence the need of ovulation inducers in the present superovulation protocol is reinforced.

5.6.2 Morphometry of embryos

In the present study total diameter of embryos ranged from 177.95 ± 2.38 to 185.41 ± 2.73 except for blastocysts, which was having the diameter of 196.36 ± 0.0. Diameter of all the stages of embryo in the present study was slightly higher than that reported by Agrawal and Bhattacharyya (1984) and Bonia *et al.* (1998) for goat embryo who reported the diameter ranging from 167.38 to 173.87 for 32 cells to late blastocysts of Barbai and Assam goat. All stages of embryo in the present study except blastocysts showed almost similar ZP thickness approximately 13 μ. But in contrast Bonia *et al.* (1998) recorded similar thickness of 10.38 μ for morula to blastocysts stage. In the present study Inner Cell Mass (ICM) diameter was ranging from 135.3 ± 2.92 to 163.63 ± 0.0; lower for compact morula and higher for blastocysts. In comparison with the present study Bonia *et al.* (1998) noted lower diameter for ICM in all stages of caprine embryos except morula, where a higher diameter of 145.51 ± 3.77 was noted. But with the report of blastocysts and 8-16 cell stage embryos the number of observation are not enough for making any genuine conclusion. Perusal of literature showed only very

limited works on morphometry of embryos. Hence the discussion on this aspect is limited to that extent.

5.7 EMBRYO QUALITY

5.7.1 Transferable embryos

In the present study the embryos from both fresh and repeatedly superovulated group were pooled and the average number of transferable embryos per goat and its percentage yield were estimated as 4.59 and 91.67 respectively. The percentage recovery rate of transferable embryos in the present study is in exact agreement with Tervit *et al.* (1983) who reported 91.67 per cent recovery rate in Angora goats when treated with PMSG. Selvaraju *et al.* (2003) also recorded similar recovery rate of transferable quality embryo (92.06 %) as the present study despite the difference in the quality assessment.

Pendleton *et al.* (1992a) and Deshpande *et al.* (1997) recovered higher number of transferable quality embryo from superovulated goat with pFSH (6.7 ± 1.5 and 8.33 respectively). Following the similar quality grading assessment as in the present study, Senthilkumar *et al.* (2003) recovered 7.16 ± 1.96 transferable embryos per goat treated with pFSH (Folltropin-V) in Tellicherry breed. The low recovery in the present study may be due to pooling of embryos from both fresh and repeatedly superovulated groups and increased number of anovulatory follicle in the present study, since oestradiol producing capacity of anovulatory follicle have more impact on embryo recovery rate and embryo quality (Armstrong *et al.*, 1983b; Tervit *et al.*, 1983 and Senthilkumar *et al.*, 2003).

5.7.2 Non-transferable embryos

In the present study percentage recovery and average number of non-transferable embryo was 8.33 percent and 0.42 respectively. A comparable number of 0.83 and 0.33 non transferable embryos per treated animal with pFSH and PMSG respectively were observed by Senthilkumar *et al.* (2003) in Tellicherry breed of goat. Compared to the present study a higher non-transferable embryo was recovered by Biswas *et al.* (2001) using PMSG in Black Bengal goats (0.80 ± 0.13 and 1.60 ± 0.17

fair and poor quality embryo respectively). This high variation compared to the present study might be due to difference in the protocol used.

5.8 EMBRYO CRYOPRESERVATION

5.8.1 Recovery rates of embryo after vitrification

Perusal of table 13 revealed that the embryo recovery rate after OPS was higher than CS vitrification (100% vs. 82.35%). Similar values were recorded by Silvestre *et al.* (2003) for CS vitrification and El-Gayar and Holtz (2001) and Naik *et al.* (2005) for OPS vitrification. Silvestre *et al.* (2003) recovered 82 per cent of rabbit embryos after one step vitrification in Ethylene Glycol (EG), Dimethyl Sulfoxide (DMSO) and Sucrose solution followed by direct plunging of straws into liquid nitrogen. El-Gayar and Holtz (2001) and Naik *et al.* (2005) reported 100 per cent recovery rate for goat and rabbit embryos respectively after OPS vitrification.

Yuswiati and Holtz (1990), Mc Ginnis and Youngs (1990), Yang *et al.* (1992) and Silvestre *et al.* (2003) recovered 95.65, 94.7, 94.9 and 82 - 100 per cent of embryo respectively after direct plunging of straws into liquid nitrogen, which are higher than the present value for CS vitrification. But Szell and Windsor (1994), Agrawal *et al.* (1994), Saito *et al.* (1994) and Han *et al.* (2003) reported 96 -100 per cent recovery rate of embryos after exposure to liquid nitrogen vapour followed by plunging of straws into liquid nitrogen which are comparable to the present value for OPS.

When straws were cooled in liquid nitrogen vapour prior to plunging into liquid nitrogen the incidence of fractures of straws were less frequent than when plunged directly into liquid nitrogen (-2500^{0}C/minute) (Rall, 1987). The low recovery rate observed in CS vitrification in the present study might be due to direct plunging of straws into liquid nitrogen with associated higher rate of breakage of straws. Ishimori *et al.* (1993), Szell and Windsor (1994), Bautista *et al.* (1997), Naitana *et al.* (1997), Bautista *et al.* (1998a), Kaidi *et al.* (1998) and Markkula *et al.* (2001) also reported about the possibility of such type of damage to straws in their studies.

5.8.2 Viability of embryos after vitrification

5.8.2.1 Morphological viability of vitrified embryos

Recovery rate of morphologically viable embryos in this study was higher after OPS vitrification (88.24 %) than CS vitrification (50 %) (Table13). Perusal of literature shows the involvement of a series of factors in deciding the success rate of vitrification. The type of cryoprotectant used and their concentration, toxicity of individual cryoprotectant and their combination effect, exposure time, cooling rate, thawing procedure and molarity of sucrose for dilution etc are the major among them.

Agrawal (1992) did not observe any apparent morphological damage in any of the thawed goat embryos after CS vitrification in 25 per cent glycerol and 25 per cent Propanediol (PROH). The low percentage of good quality embryos after CS vitrification in the present study might be due to low percentage of EG (25 %) since Szell and Windsor (1994) mentioned that the optimal cryoprotectant concentration varies with the type of cryoprotectant so that the EG has to be used in a high concentration than PROH when combined with glycerol to provide a comparable level of cryoprotection.

Presence of highly toxic glycerol in the CS vitrification medium might be another reason for low survival rate of early developmental stages of embryo after CS vitrification in the present study since the cryoprotectant toxicity increases in the order of EG, Methanol, DMSO, glycerol, PROH and Butylene glycol (Ali and Shelton, 1993c). High concentration of cryoprotectant (50 %) containing 25 per cent glycerol makes CS vitrification medium more toxic than OPS vitrification medium containing lower cryoprotectant concentration (33 %) without glycerol. Miyake *et al.* (1993) and Han *et al.* (2003) recovered 83.3 and 100 per cent respectively morphologically intact morula - blastocyst stage embryo after vitrification in Ethylene Glycol-Ficoll-Sucrose (EFS) medium, which is superior to PROH and glycerol combination for ovine morula (Martinez and Matkovic, 1998). Traldi *et al.* (2000) observed that slow freezing in EG is better than vitrification in 3.4 M glycerol and 4.4 M EG for goat embryos and Le Gal *et al.* (1993) observed that EG is a better cryoprotectant than glycerol for goat embryos at both developmental stages (morula and blastocyst stages).

In the present study 88.24 percent embryo with intact morphology could be recovered after OPS vitrification method using 16.5 per cent EG and 16.5 per cent DMSO which is in agreement with Naik *et al.* (2005) who recovered 91 per cent morphologically intact rabbit morulae after OPS vitrification in EG and DMSO combination. A lower recovery rate was reported by Okada *et al.* (2002b) who could recover only 53.52 per cent of excellent quality ovine morula to blastocyst stage after closed OPS vitrification with 25 per cent EG and 25 per cent glycerol medium. Compared to the present study lower recovery rate observed may be due to difference in the cryoprotectant combination, which indicated the superiority of EG and DMSO combination. EG and DMSO are highly permeable agents into the cell (Ishimori *et al.,* 1992c) or the combination has high permeability than EG alone (Vicente and Garcia-Ximenez, 1994).

In the present study most of the embryos assigned for vitrification were at morula and compact morula stage, which might be a reason for low morphological viability in the CS vitrification protocol. This observation is in agreement with Yuswiati and Holtz (1990), Saito *et al.* (1994), Mahmoudzadeh *et al.* (1994), Szell and windsor (1994), Dinnyes *et al.* (1995), Dinnyes *et al.* (1999), Naitana *et al.* (1997), Martinez *et al.* (1998), Baril *et al.* (2001) and Okada *et al.* (2002a) who mentioned low survival rate of early developmental stages of embryo and indicated that cryotolerance increased as the developmental stages advanced. The major difference between the morula and blastocyst stage is that cells of morula are larger than that of blastocyst which render morula to more osmotic stress induced by the removal of the permeated cryoprotectant (Naitana *et al.,* 1996) and also the water content of morula is present inside the cells while in blastocyst and expanded blastocyst, blastocoel contains largest amount of water. So in presence of cryoprotectant solution blastocoel dehydrate faster owing to a lower membrane barrier against the water. Sufficient dehydration of morula can be achieved by increasing the exposure time to cryoprotectant but it produces more toxic effect due to prolonged exposure to cryoprotectant toxicity Mahmoudzadeh *et al.* (1995) and Valdez *et al.* (1990). But the higher recovery rate for early development stages after OPS vitrification might be due to the specific combination of cryoprotectant used in the present study. Ishimori *et al.* (1992a) and Ishimori *et al.*

(1992b) also observed that EG and DMSO combination is equally suitable for CS vitrification of *in vivo* derived morula and blastocyst.

Agrawal *et al.* (1994) observed 78.57 per cent recovery rate of morphologically normal goat embryos (Morula - blastocyst) after CS vitrification in 25 per cent glycerol and 25 per cent PROH. Compared to the present study the high recovery of morphologically intact embryos was due to vitrification in vapour prior to plunging into liquid nitrogen. Vitrification in vapour is thought to prevent sample fracture which can damage ZP or embryo (Rall and Meyer, 1989; Kasai, 1996 and Kasai *et al.*, 1996). Fractures are known to occur with solution of high concentration cooled ultra rapidly by plunging abruptly into liquid nitrogen. This might be a reason for the low recovery of normal embryos after CS vitrification in the present study. Minimization of the volume of the cryoprotectant solution in the narrower part of OPS down to 0.5 µL, low heat insulation characteristics of straw due to thinning of wall thickness and ten fold acceleration of freezing (16,700^{0}C/minute) (Vajta *et al.*, 1998) when the straws are immersed into liquid nitrogen prevents the formation of crystalline ice and zona fracturing (Vajta *et al.*, 1998; Vajta *et al.*, 1999c and Lazar *et al.*, 2000) and also toxic and osmotic effects at thawing are minimized by immersion of the embryo containing capillary into a thawing solution in OPS vitrification methods makes it more superior than CS vitrification.

However, when embryos permeated by a cryoprotectant are directly recovered into an isotonic solution they are threatened by injury from osmotic swelling because water permeated more rapidly than the cryoprotectant diffuse out. To counteract excess water inflow, embryos are often suspended in a sucrose solution (Rall, 1987; Mahmoudzadeh *et al.*, 1995 and Kasai, 1996). In the present study of CS vitrification, vitrified embryos were directly diluted in higher concentrations of 1 M sucrose. Dobrinsky *et al.* (1992) showed that one step dilution with 1 M sucrose was detrimental to embryos exposed to VM and the embryo survival after vitrification was improved by decreasing the concentration of sucrose to 0.3 M and time of exposure to 10 minutes. For the mouse morula sucrose had harmful effects at 30^0 C or at high concentration level of 1 M (Kasai *et al.*, 1992b). Mahmoudzadeh *et al.* (1993) found that 0.5 M sucrose was also efficient for dilution of EG. Naitana *et al.* (1997) observed 100 per

cent morphologically intact ovine embryos after CS vitrification in 3.4 M glycerol and 4.6 M EG when 0.5 M sucrose solution was used for initial dilution. The high sucrose concentration of 1 M in CS vitrification in comparison with 0.25 M used in OPS vitrification might be a reason for low recovery rate of morphologically viable embryos after CS vitrification in the present study.

Another advantage of OPS vitrification method over CS vitrification is the reduction in osmotic injury to OPS vitrified embryo due to the combination effect of non-permeating cryoprotectant (sucrose) with permeating cryoprotectant (EG and DMSO). According to Culleo *et al.* (2004a) the use of decreasing concentration of sucrose (0.4 M) in OPS methods avoids sudden osmolarity changes in the embryos. Szell and Shelton (1986) reported that non permeating compound contribute the dehydration of the cells prior to permeation by the cryoprotectant which lessened the exposure time of the cells to the toxic effects of the cryoprotectants (Liebermann *et al.*, 2002). The non permeating compounds also acted as an osmotic buffer preventing the entry of water inside the embryo and osmotic injury (Szell and Shelton, 1986 and Rall, 1987). Non addition of sucrose with permeable cryoprotectant in the present protocol for conventional vitrification might be another reason for low survival recovery.

The embryos are exposed to large amount of VM and longer duration of inappropriate condition during the process of thawing under the CS vitrification method. This might have caused higher incidence of damage to embryos in the present study whereas the efficacy of the OPS vitrification is enhanced by the direct contact of embryos with the thawing solution from the very beginning of the thawing procedure itself and minimization of duration of exposure to it's toxic and other adverse effects (Lazar *et al.*, 2000).

Ishimori *et al.* (1993) recovered 100 per cent vitrified morphologically normal bovine embryo (Morale-Blastocyst) after CS vitrification in 25 per cent EG and 25 per cent DMSO. The high survival rates compared to present study of CS vitrification might be due to the difference in cryoprotectant combination. DMSO is usually regarded as less permeable (Gordon, 1994) but in combination with EG the permeability of the two cryoprotectants seems to be even higher than that of EG alone

(Vicente and Garcia Ximenez, 1994). The combination was successfully adopted by Vicente and Garcia Ximenez (1996), Vajta *et al.* (1996a), Vajta *et al.* (1996b), and Vajta *et al.* (1997a) and Ito *et al.* (1999) for CS vitrification of embryo. Later Naik *et al.* (2005) suggested that the high recovery of morphologically intact embryos in the OPS vitrification method need not be entirely due to the OPS itself but it might be at least partly due to the composition of medium in the OPS vitrification and the cryoprotectant dilution. If it is so then it can be reasonably assumed that by the use of EG-DMSO combination instead of glycerol-EG in the CS vitrification in the present study, the survivability of embryos could have been substantially increased.

5.8.2.2 Non-viable vitrified embryos

Recovery rate of morphologically non-viable vitrified embryos after CS vitrification in the present study (50 %) is in partial agreement with Yuswiati and Holtz (1990) who obtained 43 per cent non-transferable goat morula after CS vitrification in 25 per cent glycerol and 25 per cent PROH. A comparable level of recovery (50 %) is noted in the present study after CS vitrification of embryo though the cryoprotectant used were glycerol and EG. High recovery rate of damaged embryo even after using highly permeable (Szell *et al.*, 1989; Ali and Shelton, 1993b; Songsasen *et al.*, 1995 and Bautista and Kanagawa, 1998) and least toxic (Ali and Shelton, 1993c) EG might be due to the presence of highly toxic glycerol in the medium. However Agrawal and Polge (1989) recovered mouse embryos with damages as low as 11.6 and 3.57 per cent respectively in one step and two step dilution after vitrification in glycerol and PROH. Compared to the present study low percentage of damaged embryo recovered by various others (Han *et al.*, 2003; Kasai *et al.*, 1992a; Miyake *et al.*, 1993 and Nguyen *et al.*, 2000) when glycerol free EFS medium was used for cryopreservation, the probability of glycerol toxicity is reinforced.

The damaged embryos recovery rate after OPS vitrification in present study (11.76 %) is comparable to the recovery rate of 18 per cent and 12.5 per cent respectively for equine embryos after OPS and Cryoloop vitrification (Oberstein *et al.*, 2001). A similar observation was made by Lopez-Bejar and Lopez-Gatius (2002) who recovered 10.5 and 13.2 per cent of damaged rabbit embryos after vitrification in 25 per cent glycerol-25 per cent EG medium and 25 per cent EG-SM respectively in modified

OPS which was less than what they had obtained after CS vitrification (18.8 vs. 23.5 %). Later Naik *et al.* (2005) made a similar observation of low recovery rate of damaged rabbit morula after OPS than CS vitrification (9 % vs. 35.15 %).

5.8.2.3 Types of damages due to vitrification

In the present study after CS vitrification, none of the embryos showed any single type damage of ZP. Out of the total embryos recovered after CS vitrification 71.43 and 28.57 per cent of damaged embryos respectively had damage to ICM alone or combined damage of both ZP and ICM. But all the damaged embryos recovered after OPS vitrification showed only single type damage of ICM, not any damage of ZP alone or ZP-ICM combined (Table 14).

In the present study 14.28 per cent of conventionally vitrified embryos were having ZP damage as against four and 3.6 per cent respectively obtained by Schiewe *et al.* (1991) and Kasai *et al.* (1992a). Rapid plunging of straws into liquid nitrogen in the present study might have caused breakage of straw or the fracture damage of embryo suspension as against slow plunging of straws adopted by Schiewe *et al.* (1991) and Kasai *et al.* (1992a) which might have helped in reducing the fracturing of embryo suspension (Rall and Polge, 1984; Rall, 1987 and Kasai, 1996). However Bautista and Kanagawa *et al.* (1998) reported that rapid warming was essential to prevent the devitrification (crystallization) of cryoprotectant. So it can be suggested that optimum level of cooling and warming rate is essential for survival of embryos. Ali and Shelton (1993c) also have made a similar observation.

Compared to the present study higher percentage of ZP damage was noted for goat embryo (21.43) by Agrawal *et al.* (1994) and for ovine early morula, morula and blastocysts (17, 33 and 29 respectively) by de Paz *et al.* (1994) after CS vitrification in 25 per cent glycerol with 25 per cent PROH. This higher incidence of damage in the above study might be due to the presence of PROH in the VM since PROH is less permeable than EG (Szell *et al.*, 1989 and Mahmoudzadeh *et al.*, 1993) used in the present study. Less permeable cryoprotectant leads to inadequate dehydration and resulted in devitrification during warming (de Paz *et al.*, 1994). Based on the present study it can be suggested that the low recovery of ZP damaged embryos (0 %) after

OPS vitrification may be due to glycerol free VM in addition to the advantage of cooling rate. In the present study of CS vitrification most of the blastomeres of the damaged embryos were appeared as broken due to osmotic swelling, which indicated that intracellular ice might have been formed in the embryo (Kasai *et al.*, 2002) which is in agreement with Bautista and Kanagawa (1998) who reported that inadequate level of cryoprotectant in the cell before cooling resulted in excessive swelling of blastomeres.

Absence of embryos having ZP damage after OPS vitrification in the present study indicated the superiority of OPS procedure for cryopreservation of the caprine embryos which is in agreement with Lopatarova *et al.* (2002) who mentioned that ZP damage of OPS vitrified embryos did not exceed one per cent. As compared to conventional straw there is no air column adjacent to the VM in the open pulled straw. So the pressure change of the air column in the conventional straw during rapid cooling as reported by Vajta *et al.* (1998) could be avoided in OPS vitrification and there by the occurrence of ZP damage. Another advantage of OPS vitrification is the speed with which the embryos can be loaded and preserved there by reducing the time of exposures to the embryos to high concentration of VM and the high speed of cooling and warming rates resulted in rare occurrence of ZP damage (Lazar *et al.*, 2000) in comparison with CS vitrification.

5.8.2.4 FDA viability of vitrified embryos

Morphologically intact embryos recovered after CS and OPS vitrification were further assessed for its membrane integrity by FDA staining method. Table 15 revealed that a high percentage (76.47 %) of morphologically intact embryos recovered after OPS retained membrane integrity of ICM than CS vitrified embryos (28.57 %). The survival rate of CS vitrified embryos after the FDA staining in the present study is comparable with the report of Wang *et al.* (1988) who observed fluorescence in 31.25 per cent of the frozen goat embryos. Noto *et al.* (1991) observed the survival rate of 60 per cent for frozen human multi pronucleate cleaved embryo (derived from polyspermic zygote) after FDA staining. This high survival rate compared to the CS vitrification of the present study might be because embryo, which showed fluorescein for at least 50 per cent of the blastomeres, was also counted positively for assessing

survival rate. But in the present study embryos with all blastomeres showing brilliant fluorescence only were counted for the viability assessment. No published work on FDA staining of vitrified embryos could be located in the available literature.

Presence of morphologically viable non – fluorescence embryos in the present study indicated that it is not possible to clearly assess the membrane intactness of blastomeres by routine morphological evaluation. Minor membrane damage which cannot be assessed by the morphological examination can be expressed by FDA staining. However Butler and Biggers (1989) indicated that FDA measures only basic cell function and not the developmental potential.

5.8.2.5 Effect of vitrification on morphometric characteristics of embryos

In the present study, table 16 revealed that there is not much increase in the ICM diameter, ZP thickness and total diameter of morphologically viable embryo before and after vitrification by both methods. Compared to the present study Saha *et al.* (1996) reported that the shape and cell to cell contact of ICM from vitrified blastocysts were distorted (Iwasaki *et al.*, 1994) as a result of freezing and thawing. In the present study ICM diameter of vitrified viable embryo showed only a slight increase after CS and OPS vitrification method (0.02 ± 3.08 vs.0.03 ± 0.02) which further indicated that ICM of morphologically viable embryos were intact without any distortion or lysis. However Oberstein *et al.* (2001) noted a decrease in the total diameter of vitrified equine embryos, after OPS vitrification.

Based on the present study it can be stated that OPS vitrification is superior to CS vitrification to get more viable early stage caprine embryos. Routine morphological evaluation of embryo coupled with FDA staining can be adopted as an accurate criterion to assess the viability of embryos. However considering the fact that FDA measuring only the basic cell function and not the developmental potential, production of viable young one's can have only the real viability status.

Summary

6. SUMMARY

The study was designed and conducted with the objective of evaluating the cryopreservability of caprine oocytes and embryos by two vitrification methods namely Conventional Straw (CS) and Open Pulled Straw (OPS) vitrification.

Ovaries were collected from recently slaughtered Malabari/ its cross bred goats and oocytes were retrieved by slicing method. After grading, a total of 139 oocytes having at least one complete layer of cumulus cells were randomly assigned to CS (85) and OPS (54) vitrification procedures. After a storage period of minimum 10 days, the vitrified oocytes were examined for morphological viability. The membrane integrity of morphologically viable oocytes was assessed subsequently by FDA staining technique.

Average yield and percentage recovery of grade A, B, C and D oocytes were 1.89 and 36.9 per cent, 0.93 and 18.18 per cent, 0.67 and 13.1 per cent and 1.63 and 31.82 per cent respectively. The total recovery of good quality oocytes (Grade A and B) was recorded as 206 and its average yield per ovary was 2.82.

A higher recovery of 94.94 per cent was noted for OPS than 67.06 per cent for CS vitrification. The percentage recovery of morphologically viable oocytes after vitrification was higher for OPS than CS (70.59 vs. 42.11). The high recovery rate of OPS vitrified oocytes compared to CS vitrification was due to superiority of the OPS technique and its protocol.

The percentage damage after CS and OPS vitrification for Cumulus (36.83 vs. 11.76), ZP (5.25 vs. 3.92) and ooplasm (57.83 vs. 29.4) indicated that rapid cooling rate of OPS reduced osmotic injury to oocytes.

After CS and OPS vitrification, 24 and 36 morphologically viable oocytes respectively were recovered and subjected to FDA staining. Out of the vitrified oocytes a higher percentage were found to be FDA viable after OPS than after CS vitrification (52.9 vs.31.58). Twenty five per cent of the morphologically viable vitrified oocytes in both methods failed to retain fluorescein, which indicated the weakness in viability assessment by routine morphological method to identify minor membrane damage.

The vitrification efficiency of caprine embryos were studied using embryos obtained from Fresh and repeatedly superovulated adult Malabari goats. Superovulation was achieved with 133 mg pFSH after synchronization using 1.5 mg norgestomet and 10 mg of prostaglandin. All the responded animals were inseminated at 12 h interval till the end of the oestrus and subjected to surgical embryo collection by uterine flushing on day seven after the onset of heat. Thirty four transferable quality embryos equally divided and subjected to vitrification by CS and OPS methods. After storage period of minimum 10 days the vitrified embryos were examined for morphological characteristics and membrane integrity was assessed by FDA staining.

Response to synchronization was hundred per cent in fresh and repeatedly superovulated groups. The average number of anovulatory follicles of > 5 mm diameter observed in fresh goat (7.17 ± 2.0) was higher than that in repeatedly superovulated goat (5.17 ± 1.08). Average number of ovulation in fresh goat was higher (18.5 ± 2.19) than repeatedly superovulated goats (11 ± 2.74).

Average and percentage recovery of ova and embryo together in fresh goats (14.17 ± 2.55 and 76.57 %) were significantly higher ($P<0.05$) than that in repeatedly superovulated group (3.17 ± 0.87 and 28.79 %). A similar trend of significantly higher ($P<0.05$) average and percentage recovery of embryos were observed in fresh (9.33 ± 3.4 and 65.89 %) than in repeatedly superovulated group (0.67 ± 0.49 and 21.05 %). Adhesion of reproductive tract could be attributed to the low recovery rate of embryos from repeatedly superovulated goats.

Embryo recovery rate after OPS vitrification was higher than CS vitrification to the extent of 100 and 82.35 per cent respectively. This low recovery rate observed after CS vitrification can be attributed to direct plunging of straws into liquid nitrogen leading to its breakage. Higher recovery noted in OPS method indicated the superiority in loading and warming procedures. Recovery rate of morphologically viable embryos was higher after OPS (88.24 %) than CS vitrification (50 %). High concentration of cryoprotectant (50 %) containing 25 per cent glycerol in CS medium might have adversely affected the survival rate of embryos. Ethylene glycol- DMSO combination

with high cooling rate in OPS method is highly efficient for retaining the morphological viability of vitrified early stage caprine embryos.

While 14.28 per cent of the CS vitrified thawed embryos showed ZP damage, none in OPS vitrified group showed similar damage. A higher per cent of ICM mass damage was observed in CS than OPS vitrified embryos (35.71 vs. 11.76). In the CS vitrification most of the blastomeres of damaged embryos appeared as broken.

After FDA staining a good per cent (76.47) of OPS vitrified embryos retained membrane integrity of blastomeres than CS vitrified embryos (28.57). Presence of morphologically viable non fluorescein embryos in the present study indicated the weakness in viability assessment by routine morphological methods to identify minor membrane damages.

Morphological and FDA viability assessment of vitrified oocytes and embryos revealed that OPS vitrification is far superior to CS for obtaining high percentage of viable oocytes and embryos. However a normal morphological appearance never prove that a cell will function, nor can appearance be quantified in the manner required of a viability assay. Hence vitrified oocytes and embryos have to be subjected to *in vitro* fertilization/ transfer studies, as the birth of a normal young one is the only final indicator of viability.

ANNEXURE

Details of Hormones, Media, Drugs, Chemicals and Equipments

Sl.No	Name of items	Patent name and / catalogue No.	Manufacturer
I	**HORMONES AND DRUGS**		
1	pFSH (FSH from porcine pituitary)	Folltropin-V	Vetrepharm Canada Inc
2	Norgestomet ear implant with injection	Crestar	Intervet international, Boxmeer, Holland
3	Prostaglandin analogue (Dinoprost)	Lutalyse	Pharmacia and Upjohn, Belgium
4	Xylazine	Xylaxin	Indian Immunologicals, India
5	Ketamine	Ketmin - 50	Themsis, Hyderabad
6	Amoxycillin and Cloxacillin	Intamox-0.5	Intas Pharmaceuticals, India
II	**MEDIA AND CHEMICALS**		
1	mDPBS	Modified DPBS D-6650	Sigma Chemicals, St. Louis, USA
2	mDPBS	Modified DPBS D-4031	"
3	TCM-199 10X	M-0650	"
4	BSA fraction-V	A-9418	"
5	Ethylene glycol	E-9129	"
6	Glycerol	G-7893	"
7	Dimethyl Sulphoxide	DMSO Hybri-Max D-2650	"

8	Sucrose	S-1888	Sigma Chemicals, St. Louis, USA
9	Sodium Pyruvate	S-8636	„
10	Sodium Bicarbonate	S-5761	„
11	D-(+) Glucose	G-7021	„
12	Potassium chloride	P-5405	„
13	Sodium phosphate mono basic	S-5011	„
14	Phenol red	P-0290	„
15	Magnesium chloride	M-2393	„
16	$CaCl_2$	C-7902	„
17	Fluorescein Diacetate	F-1397	„
18	Heparin sodium salt	H-3149	„
19	Hepes	H-3784	Sigma Aldrich, USA
20	Acetic Acid	Acetic acid	BDH laboratories, England
21	Sodium lactate	Sodium lactate (60%)	Central drug house (P) Ltd.
22	Gentamycin sulphate	RM-461	Hi- Media Laboratories Ltd. Mumbai
23	Acetone	A-10529	Nice Laboratory, India

III	**DISPOSABLE WARES**		
1	Foley's catheter No.8	Foley's catheter No.8	Rusch, Kernen, Germany/ Teleflex Medical Private Ltd. Pondichery
2	100 X 15 mm square style petridishes	Falcon, 35 1112	Becton Dickinson,USA
3	Petridishes 35mm 60mm 90mm	Tarsons, 460035 Tarsons, 460060 Tarsons, 460090	Tarsons Products Pharma Trust, Laboratory Instruments,Mumbai
4	Syringe filters	Millex Cat.No. SLGS 025 0S	Millipore Corporation Bedford, MA 01730 USA
5	0.25 and 0.5 ml plastic straw	0.25 and 0.5 ml cassou straw	IMV France
6	Graduated plastic tubes 15 ml 50 ml	Falcon Polystyrene conical tube No. 352095 No.352073	Becton Dickinson,USA
7	Capillary pipettes	Unopette - 5888	Becton Dickinson,USA
IV	**EQUIPMENTS**		
1	Stereo zoom microscope	MZ6	Leica Microsystems Wetzlar,GmbH, Germany
2	Fluorescent-Phase contrast microscope	DMIL	Leica Microsystems Wetzlar,GmbH, Germany
3	Millipore ultra filtration water purification system	Milli 2 UF Plus	Millipore Corporation USA
4	Stage Warmer	Linkam MC 60	Linkam, England
5	Digital Camera	Leica DC 300	Leica Microsystems Wetzlar,GmbH, Germany
6	Electronic analytical Precision Balance	Sartorius CP 224 S	Sartorius, Germany
7	Digital pH Meter	Cyber scan 2500	Eutech Instruments, Singapore

8	Water bath for thawing	Cito Warm Water Thaw Model No. CT 12-2	Cito Products, USA
9	Straw sealer	Quick Sealer	Sevana, India
10	Stream line vertical Laminar flow Cabinet	Stream line vertical Laminar flow Cabinet	Esco, India
11	Micropipetter	Labopette 0.5 – 10 µl 100 – 1000 µl	Hirschmann Laborgerate, Germany
12	Micropipetter	Transferpette 10- 100 µl	Brand, Germany

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CRYOPRESERVABILITY OF CAPRINE OOCYTES AND EMBRYOS BY CONVENTIONAL STRAW AND OPEN PULLED STRAW VITRIFICATION

RATHEESH BABU. M.

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Faculty of Veterinary and Animal Sciences
Kerala Agricultural University, Thrissur

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Department of Animal Reproduction, Gynaecology and Obstetrics
COLLEGE OF VETERINARY AND ANIMAL SCIENCES
MANNUTHY, THRISSUR-680651
KERALA, INDIA

ABSTRACT

Objective of the study was to compare the cryopreservability of caprine oocytes and embryos by Conventional Straw (CS) and Open Pulled Straw (OPS) vitrification.

Oocytes retrieved by slicing the abattoir origin ovaries of Malabari goats/ its cross bred, having at least one complete layer of cumulus cells were randomly assigned for CS and OPS vitrification procedure. After thawing and cryoprotectant removal oocytes were examined again for morphological viability and membrane integrity by FDA staining.

The total recovery of good quality oocytes from 73 ovaries and its average yield per ovary were 206 and 2.82 respectively. Out of this 85 and 54 oocytes respectively were subjected to CS and OPS vitrification.

The percentage recovery of morphologically viable oocytes after thawing was higher for OPS than CS vitrification (70.59 vs. 42.11). The higher recovery rate of oocytes for OPS over CS vitrification indicated the superiority of the technique in maintaining the morphological viability of oocytes. Only 3.92 per cent of the OPS vitrified thawed oocytes showed ZP damage, which was lower than that after CS vitrification (5.25). Higher cumulus damage observed after CS than OPS vitrification (36.8 vs. 11.7 %) indicated the higher efficiency of OPS in reducing the osmotic damage of cumulus cells. A higher percentage of oocytes (52.9) were recorded as FDA viable after OPS than CS vitrification (31.58).

The vitrification efficiency of caprine embryos were studied using embryos obtained from fresh and repeatedly superovulated adult Malabari goats. Superovulation was achieved with 133 mg pFSH after synchronization using 1.5 mg norgestomet and 10 mg of prostaglandin. Thirty four transferable quality embryos were equally divided and subjected to vitrification by CS and OPS methods. After a storage period of minimum 10 days the vitrified embryos were examined for morphological characteristics and membrane integrity by FDA staining.

The response to synchronization was hundred per cent in fresh and repeatedly superovulated groups. The average number of anovulatory follicles observed in fresh and repeatedly superovulated goat was 7.17 ± 2.0 and 5.17 ± 1.08 respectively. Average number of ovulation in fresh goat (18.5 ± 2.19) was higher than that of repeatedly superovulated goat (11 ± 2.74). Significantly higher ($P<0.05$) average recovery of embryos was noted in fresh animal (9.33 ± 3.4) than in repeatedly superovulated animal (0.67 ± 0.49). Adhesion of reproductive tract due to earlier surgical handling led to lower recovery of embryos from repeatedly superovulated goats.

Embryo recovery rate after OPS was higher than CS vitrification (100 % vs. 82.35 %). Similarly the recovery rate of morphologically viable embryos was also higher for OPS (88.24 %) than CS vitrification (50 %). After CS vitrification, 14.28 per cent of the vitrified embryos showed ZP damage while none of the embryos subjected to OPS vitrification showed similar damage. After FDA staining a higher percentage (76.47) of OPS vitrified embryos retained membrane integrity of blastomeres than CS vitrified embryos (28.57).

Present study indicated that OPS is a far superior technique than CS vitrification for cryopreservation of caprine oocytes and embryos. Presence of morphologically viable non-fluorescein oocytes and embryos with FDA staining inferred that viability assessment of oocyte and embryo based on morphological characteristics alone is not enough, but it should be coupled with other non-invasive technique such as FDA staining. The study further revealed that the embryo yielding efficiency of fresh animal was significantly higher than repeatedly superovulated animals.