CRYOPRESERVATION OF BUFFALO OOCYTES BY SLOW FREEZING AND VITRIFICATION

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

DOCTOR OF PHILOSOPHY
IN
DAIRYING
(ANIMAL BIOTECHNOLOGY)

BY

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M.Sc. Dairying (Animal Biotechnology)

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KARNAL - 132001 (HARYANA), INDIA

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This is to certify that the thesis entitled, **" Cryopreservation of buffalo oocytes by slow freezing and vitrification "** submitted by **Sanjeev Kumar** towards the partial fulfilment of the award of the degree of **Doctor of Philosophy in Dairying (Animal Biotechnology)** of the **National Dairy Research Institute (Deemed University)**, Karnal (Haryana), India, is a bonafide research work carried out by him under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

Dated: 8 Feb, 2007

(R.S. Manik)
Major Advisor & Chairman
(Guide)

This thesis is dedicated to my mother.
Her constant support, encouragement and
love gave me the strength to do this work.

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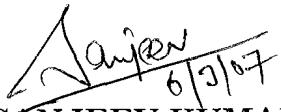

6/7/07

(SANJEEV KUMAR)

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Cryopreservation of buffalo oocytes by slow freezing and vitrification

Abstract

Immature oocytes were subjected to slow freezing in ethylene glycol (EG), dimethyl sulphoxide (DMSO) or 1,2-propanediol (PROH), each used at concentrations of 1.0 or 1.5M. The percentage of oocytes that were recovered morphologically normal was higher ($P<0.05$) for DMSO both at 1.0 and 1.5M concentrations (88 and 96%, respectively) than that for EG (44 and 76%, respectively) or PROH (57 and 75%, respectively). The nuclear maturation rate was also higher ($P<0.05$) for DMSO both at 1.0 and 1.5M concentrations (25 and 36%, respectively) than that for EG (19 and 23%, respectively) or PROH (20 and 23%, respectively). The percentage of morphologically normal oocytes and the nuclear maturation rate were higher ($P<0.05$) for 1.5 M compared to that for 1.0 M for all the 3 cryoprotectants, and highest for 1.5M DMSO among the 6 combinations compared. Following IVF, the cleavage rate and the percentage of oocytes that developed to morula and blastocyst stage were 28.8, 4.5 and 0.6%, respectively for the oocytes frozen in 1.5M DMSO compared to that of 57.0, 19.2 and 10.6%, respectively, for the controls. When in vitro matured oocytes were subjected to slow freezing, the percentage of oocytes that were recovered in a morphologically normal state was higher ($P<0.05$) for DMSO both at 1.5 and 1.0M concentrations (96 and 88%, respectively) than that for EG (78 and 68%, respectively) or PROH (81 and 68%, respectively). It was higher ($P<0.05$) for 1.5M than that for 1.0 M for all the 3 cryoprotectants, and highest for 1.5M DMSO among the 6 combinations compared. Following IVF, the cleavage rate and the percentage of oocytes that developed to morula and blastocyst stage were 36.3, 4.1 and 0.7%, respectively for oocytes frozen in 1.5M DMSO compared to that of 60.0, 19.3 and 10.6%, respectively, for the controls.

Following vitrification of immature oocytes, the percentage of oocytes recovered morphologically normal was higher ($P<0.05$) for 40%DMSO, 20%EG+20%DMSO or 20%EG+20% PROH (91, 94 and 91%, respectively) than that for 40%EG (81%). The nuclear maturation rate was higher ($P<0.05$) for EG+DMSO (53%) than that for EG+PROH (43%) which, in turn was higher than that for EG (32%) or DMSO (33%). Following IVF, the percentage of oocytes that developed to morula and blastocyst stage were higher ($P<0.01$) for EG+DMSO (14.9 and 4.0%, respectively) than those for EG+PROH (10.0 and 2.3%, respectively), EG (6.8 and 1.3%, respectively) or DMSO (7.9 and 1.8%, respectively) but lower than those for controls (20.0 and 11.5%, respectively). Following vitrification of in vitro matured oocytes, the percentage of oocytes recovered morphologically normal was higher ($P<0.01$) for EG+DMSO, EG+PROH and DMSO (96, 95 and 92% respectively) than EG (85%). Following IVF, the cleavage rate and the percentage of oocytes that developed to morula and blastocyst stage were higher ($P<0.01$) for EG+DMSO (36.2, 11.5 and 5.4%, respectively) than those for EG+PROH (26.3, 9.3 and 2.7%, respectively), EG (18.8, 7.2 and 1.6%, respectively) or DMSO (20.1, 8.0 and 2.2%, respectively) but lower than those for controls (58.0, 22.0 and 12.0%, respectively).

भैंस के अंडको का धीमा हिमन व विट्रिफिकेशन द्वारा शीत परिरक्षण

सारांश

अपरिपक्व अंडको को 1.0 एम या 1.5 एम की मात्रा वाले इथीलीन ग्लाईकोल (ई.जी.) डाईमिथाइल सल्फॉक्साइड (डी. एम.एस.ओ.) या 1,2 प्रोपेनडयाल (पी.आर.ओ.एच.) से धीमा हिमन कराया गया । सही आकारिकी वाले अंडको की संख्या डी.एम. एस.ओ. 1.0 एम या 1.5 एम में (88 एवं 96 % क्रमशः) ई.जी. (44 एवं 76 % क्रमशः) या पी.आर.ओ. एच (57 एवं 75 % क्रमशः) से सार्थक रूप से अधिक थी ($P<0.05$)। न्यूक्लीयर परिपक्वता दर भी डी.एम.एस.ओ. 1.0 एम एवं 1.5 एम मात्रा पर 25 % एवं 36 % क्रमशः थी जोकि ई.जी. द्वारा (19 एवं 23 % क्रमशः) या पी.आर.ओ.एच द्वारा (20 एवं 23 % क्रमशः) पाई गई दरो से अधिक थी। सभी परिरक्षकों द्वारा 1.5 एम मात्रा पर सही आकारिकी वाले अंडको का प्रतिशत और न्यूक्लीयर परिपक्वता दर 1.0 एम मात्रा से सार्थक रूप से ($P<0.05$) अधिक थी। 1.5 एम डी.एम.एस.ओ. द्वारा पाई गई ये दरें अन्य सभी परिरक्षकों से सर्वाधिक रही। इन विट्रो फर्टिलाइजेशन के पश्चात् क्लीवेज दर और मोरूला तथा ब्लास्टोसिस्ट की अवस्था तक विकसित होने वाले अंडको की संख्या 28.8, 4.5 एवं 0.6% क्रमशः 1.5 एम डी.एम.एस.ओ. द्वारा परिरक्षित द्वारा प्राप्त हुई जोकि मानक में 57.0, 19.2 और 10.6 % थी। जब इन विट्रो परिपक्व अंडको को धीमा हिमन में परिरक्षित किया गया तो सही आकारिकी वाले अंडको की प्राप्ति डी.एम.एस.ओ. 1.5 एम और 1.0 एम में 96 % एवं 88 % क्रमशः थी जोकि ई.जी.1.5 और 1.0 एम में 78 एवं 68% क्रमशः या पी.आर.ओ. एच. 1.5 एम या 1.0 एम में 81 एवं 68% क्रमशः थी। यह सभी परिरक्षकों की 1.5 एम मात्रा में 1.0 एम की अपेक्षाकृत सार्थक रूप से अधिक थी ($P<0.05$) और 1.5 एम डी.एम.एस.ओ. में सर्वाधिक थी। आई.वी.एफ. के उपरांत, क्लीवेज दर और मोरूला तथा ब्लास्टोसिस्ट की अवस्था तक विकसित होने वाले अंडको की दर 36.3, 4.1 एवं 0.7 % क्रमशः 1.5 एम, डी.एम.एस.ओ. द्वारा परिरक्षित अंडकों में पाई गई जिसकी अपेक्षा मानको में यह दरे 60.0, 19.3 एवं 10.6 % क्रमशः थीं।

इन विट्रो फर्टिलाइजेशन के पश्चात्, क्लीवेज दर और मोरूला तथा ब्लास्टोसिस्ट की अवस्था तक विकसित होने वाले अंडको का % 1.5 एम डी.एम.एस.ओ. द्वारा परिरक्षण में 36.3, 4.1 एवं 0.7 % था जोकि मानक परिरक्षित अंडको में 60.0, 19.3 एवं 10.6% क्रमशः था।

अपरिपक्व अंडकों के विट्रिफिकेशन के पश्चात् सही अकारिकी वाले उपलब्ध अंडको का प्रतिशत 40% डी.एम.एस.ओ., 20% ई.जी. + 20% डी.एम.एस.ओ. या 20% ई.जी. + 20% पी.आर.ओ.एच. में 91, 94 एवं 91% क्रमशः था जोकि 40% ई.जी. में पाए गए प्रतिशत (81%) से अधिक रहा ($P<0.05$) केन्द्रक परिपक्वता दर थी, ई.जी.+डी.एम.एस.ओ. में 53% थी जोकि ई.जी.+ पी.आर.ओ.एच द्वारा (43%) या ई.जी. द्वारा (32%) या केवल डी.एम.एस.ओ. द्वारा (33%) प्राप्त दरो से सार्थक रूप से अधिक रही ($P<0.05$)। इन विट्रो फर्टिलाइजेशन के पश्चात् अंडकों का प्रतिशत जोकि मोरूला तथा ब्लास्टोसिस्ट की अवस्था तक विकसित हुए, ई.जी. + डी.एम.एस.ओ. में (14.9 एवं 4.0 % क्रमशः) ई.जी.+पी.आर.ओ.एच. (10.0 एवं 2.3% क्रमशः) ई.जी. (6.8 एवं 1.3 % क्रमशः) या डी.एम.एस.ओ. में (7.9 एवं 1.8% क्रमशः) से सार्थक रूप से अधिक था ($P<0.01$) परन्तु मानको के प्रतिशत (20 एवं 11.5 % क्रमशः) से कम रहा।

इन विट्रो परिपक्व अंडको के विट्रिफिकेशन के पश्चात् सही आकारिकी के अंडको की उपलब्धता दर ई.जी.+डी.एम.एस. ओ. ई.जी.+पी.आर.ओ.एच. और डी.एम.एस.ओ. द्वारा 96, 95 एवं 92 %क्रमशः रही जोकि ई.जी. द्वारा प्राप्त दर (85 %) से सार्थक रूप से अधिक थी ($P<0.01$) इन विट्रो फर्टिलाईजेशन के क्लीवेच दर और मोरूला तथा ब्लास्टोसिस्ट तक विकसित होने वाले अंडको का प्रतिशत ई.जी.+डी.एम.एस.ओ. में (36.2, 11.5 एवं 5.4 क्रमशः था जोकि ई.जी. +पी.आर.ओ.एच. से प्राप्त दर (26.3, 9.3 एवं 2.7 प्रतिशत क्रमशः) ई.जी. से (18.8, 7.2 एवं 1.6 % क्रमशः) या डी.एम.एस.ओ. से (20.1,8.0 एवं 2.2 % क्रमशः प्राप्त दर से अधिक था। परन्तु मानकों की दर (58.0, 22.0 और 12.0 % क्रमशः) से कम था।

%	=	Per cent
μg	=	Microgram
μl	=	Microlitre
AI	=	Anaphase I
ANOVA	=	Analysis of variance
BES	=	Buffalo estrus serum
BO	=	Brackett and Oliphant
BOEC	=	Buffalo oviductal epithelial cells
BSA	=	Bovine serum albumin
buFF	=	Buffalo follicular fluid
cm	=	Centimetre
CO_2	=	Carbon dioxide
COC	=	Cumulus-oocyte-complex
CPA	=	Cryoprotective additive
CR	=	Charles – Rosenkrans
DMRT	=	Dunkan's multiple range test
DMSO	=	Di-methyl sulphoxide
DPBS	=	Dulbecco's phosphate buffered saline
EAA	=	Essential amino acids
EDTA	=	Ethylenediamine tetra-acetic acid
EG	=	Ethylene glycol
ETT	=	Embryo transfer technology
FBS	=	Fetal bovine serum
FCS	=	Fetal calf serum
FSH-P	=	Follicle stimulating Hormone Porcine
GV	=	germinal vesicle
GVBD	=	germinal vesicle Breakdown
h	=	hour(s)
Hepes	=	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
HECM	=	Hamster embryo culture medium
hpi	=	hours post insemination
hSA	=	Human serum albumin
I.U.	=	International Unit
ICM	=	Inner cell mass
IVC	=	In vitro culture
IVF	=	In vitro fertilization
IVM	=	In vitro maturation

IVMFC	=	In vitro maturation fertilization and culture
IVP	=	In vitro production
Kg	=	Kilogram
LN_2	=	Liquid nitrogen
ml, µl, l	=	Milliliter, microliter, liter
mM, µM, M	=	Millimolar, micromolar, Molar
mg, µg, ng,	=	g milligram, microgram, nanogram, gram
mCR2aa	=	Modified Charles-Rosenkrans^{+} amino acids^{-} 2
MEM	=	Minimum essential medium
mg	=	Miligram
ml	=	Mililitre
mm	=	Milimetre
mM	=	Milli mole
mosm	=	Milliosmole
MI, MII	=	metaphase I, metaphase II
mRNA	=	messenger RNA
mSOF	=	Modified synthetic oviductal fluid
mSOFaa	=	Modified synthetic oviductal fluid+ amino acids
N_2	=	Nitrogen
NEAA	=	Non-essential amino acids
O_2	=	Oxygen
^{o}C	=	Degree Celsius
OPU	=	Ovum Pick-Up
PEG	=	Polyethylene glycol
PROH	=	1,2-propanediol
PVA	=	Polyvinyl alcohol
PVP	=	Polyvinyl pyrrolidone
SBS	=	Superovulated buffalo serum
SOF	=	Synthetic oviductal fluid
SCNT	=	Somatic cell nuclear transfer
TCM-199	=	Tissue culture medium-199
TCN	=	Total cell number
TE	=	Trophectoderm
TVOR	=	Transvaginal oocyte retrieval

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CHAPTER – 1

Introduction

The superior dairy merit of buffalo as well as its contribution to dairy industry has been realized for long. Out of the current world buffalo population of around 151.6 million, nearly 97% is found in the developing nations of Southeast Asia. Buffaloes are the principal dairy animals and major contributors to meat production and generators of draught power in many of these countries. Specifically in the context of India, buffalo holds a prominent position in the dairy industry since they contribute over 55% of the total milk production. Since cattle slaughter is banned nearly all over India due to religious reasons, buffalo also contribute significantly to meat production in India.

Buffalo has also attracted considerable attention in terms of its low reproductive efficiency and scarcity of superior germplasm. Attempts are currently underway to develop several reproductive technologies, tailor-made for this species, for augmenting its reproduction and production. Among them the emphasis in 90s had been on Embryo Transfer Technology (ETT) due to its success in cattle. However, since ETT has had a very limited success in buffalo, the focus has now shifted on multiplication of superior germplasm by in vitro embryo production (IVEP). Besides IVEP, a number of other reproductive technologies, which are currently under development in this species, include cloning, transgenesis and production of embryonic stem cells. For all these reproductive technologies, availability of usable quality oocytes in large numbers is an essential prerequisite.

The abattoir ovaries are the major source of follicular oocytes. Although the oocytes obtained from abattoir ovaries cannot be used for the production of live offspring of known pedigree since the genetic background of the animal from which the ovaries were obtained is not known, even then these remain the most important source for obtaining oocytes in large numbers. Oocytes can also be obtained from live animals on a repeated basis through ultrasound-guided transvaginal oocyte retrieval (TVOR) also called Ovum Pick-Up. This technique has been successfully used for the collection of oocytes from live buffaloes. Since the pedigree of oocytes obtained through TVOR is known, such oocytes can be subjected to in vitro maturation,

fertilization and culture (IVMFC) for the production of live offspring of known pedigree. The availability of both total and usable quality oocytes is very low from slaughterhouse ovaries and from live animals. To make available usable quality oocytes in sufficient numbers, whenever required for use in any reproductive technology, necessitates their storage through cryopreservation.

Thus, cryopreservation of oocytes has the potential to be an important adjunct to reproductive technologies in domestic animals. Cryopreservation of oocytes collected from the ovaries of high genetic merit animals, which would otherwise have gone waste, can be used for the production of offspring after IVEP. Besides this, cryopreservation of oocytes offers many other advantages like creation of oocyte banks and the feasibility of salvaging genetic material from prepubertal, infertile, pregnant or even dead animals.

Cryopreservation involves arresting all biological processes and placing the cell into a suspended state of animation. Rapid scientific and technological progresses have been made in this area in the last two decades. The short fertile life of male gametes (spermatozoa) and female gametes (oocytes), which severely limits their use in assisted reproductive procedures, can be extended through cryopreservation. However, the ease and success of cryopreservation programs for spermatozoa and embryos contrast markedly with the problems associated with freezing mammalian oocytes. Using currently available protocols, the survival and fertilization rates and the developmental potential of cryopreserved oocytes are very low. Because of their high sensitivity to cooling, cryopreservation of oocytes remains difficult in most species. The results of numerous studies suggest that the survival of oocytes after cryopreservation can be affected by their stage of maturation, their quality or by biophysical factors resulting from the cryopreservation procedure used.

The procedures used for the cryopreservation of oocytes and embryos include slow freezing and vitrification. In the former, the cells are equilibrated with a cryoprotectant and seeding is done at sub-zero temperatures to minimize the formation of large ice crystals. The straws containing the oocyte are then cooled at a controlled rate to approximately -40°C before plunging them into liquid nitrogen. Although the controlled freezing method is still

widely used, its field applications are limited by complex freezing procedures and the requirement for expensive freezing machines. Vitrification, which is a relatively recent approach, is defined as a physical process by which a highly concentrated solution of cryoprotectants solidifies during cooling, without formation of ice crystals. It offers several advantages over conventional equilibrium methods e.g., faster and simplified freezing and thawing procedures and no requirement for an expensive freezing machine.

Although procedures have been developed, with varying degrees of success, for the cryopreservation of human, mouse and cattle oocytes, information on cryopreservation of buffalo oocytes using either of the two methods is very limited. Whichever be the method for cryopreservation of oocytes, the nature and concentrations of the cryoprotectants are the most important parameters, which determine its success.

Keeping these in view, the present study was conducted with the following objectives

1. To evaluate the effects of different cryoprotectants on viability of oocytes subjected to slow freezing and vitrification, before and after in vitro maturation.
2. To study the morphology of oocytes before and after cryopreservation.
3. To study the developmental competence of frozen-thawed oocytes after in vitro fertilization.

CHAPTER – 2

Review of Literature

To the scientific community, biotechnology is a field of study aimed at understanding how things work at the cellular or biochemical level, with the ultimate goal of deploying that knowledge to control or manipulate various processes that constitute life. One topic in biotechnology that swings between the practical and the conceptual is the field of cryobiology. Cryobiology comes from three Greek words: "kryos" meaning cold, "bios" meaning life and "logos" meaning discourse of study. Cryobiology is the science of studying the effects of very low temperatures on life. People long believed that very low temperatures would only exert negative effects on cells and tissues. They could not possibly imagine the advancements in cryobiology that could be achieved and possibilities of the future in this area. In the future, it may be possible to cryopreserve human cells, whole human organs, such as kidneys, hearts and livers for subsequent transplantation, preserve corneas and other delicate tissues with minimal damage long enough to allow them to be shared all over the world and protect fragile and rare plants from extinction through ice-free preservation.

2.1 HISTORY OF CRYOBIOLOGY

Cryobiology may sound like a brand new field created in the 21st century but in fact the first documented study can be traced back to Sir Robert Boyle in his 1683 monograph "New Experiments and Observations Touching Cold" in which this famous physicist documented the effects of freezing on living animals. Further research was continued through the 18th and 19th centuries, but it was not until the mid 1900's that real progress was reported. In the 1940s, Chris Polge, a scientist working at the University of Cambridge accidentally discovered the cryoprotective abilities of glycerol through the mislabeling of reagent bottles. Stories abound as to precisely how this discovery was made, at least it is clear that a mistake in the labeling of bottles of solutions in a refrigerator led to some fowl semen being frozen in a mixture of glycerol, albumin and water, rather than the intended solution of levulose. The levulose solution was ineffective but the glycerol solution was highly effective. At first, the researchers were unaware of the composition of their apparently miraculous solution but, with the help of an analytical chemist, its

true identity was revealed and a few more experiments then showed that glycerol was the active ingredient. A truly serendipitous discovery.

Discovery of the ability of glycerol to protect cells against freezing damage led to the derivation of the science of low temperature biology. In 1951, the first calf produced by artificial insemination with frozen-thawed bovine spermatozoa was born (Stewart, 1951). In the 1960s, Peter Mazur conducted extensive experiments to model the responses of microorganisms when subjected to low temperatures and freezing. These early studies resulted in the development of the discipline that is now known as cryobiology (Mazur, 1970).

2.2 ATTEMPTS TOWARDS CRYOPRESERVATION OF MAMMALIAN OOCYTES AND EMBRYOS

Cryopreservation of oocytes has become a more important technology in bioscience, agriculture and medicine. Since early 1980s, advances have been made in establishing optimal conditions for in vitro oocyte maturation (IVM), fertilization (IVF) and culture (IVC) of oocytes for in vitro embryo production (IVEP). These in vitro systems have contributed significantly to utilization of thawed oocytes and embryos and have made it possible to evaluate protocols designed to cryopreserve such biomaterials more effectively. Although cryopreservation of preimplantation embryos from various species including farm animals, human and mouse has been successfully carried out, cryopreservation of oocytes from most mammalian species has been more challenging due to their extreme sensitivity to suboptimal conditions during the cryopreservation process. Cryopreservation of mouse oocytes has been well documented and has resulted in greater success than studies with other mammalian species.

In the field of reproductive biotechnology, extensive research has been done on the cryopreservation of oocytes and embryos. Whittingham *et al.* (1972) were the first to report the successful cryopreservation of embryos of any animal species, including the births of mice from frozen-thawed embryos. They froze almost 1,000 mouse embryos in liquid nitrogen (LN_2) at -196°C and transferred hundreds of these cryopreserved embryos into recipient foster mothers, producing hundreds of normal full-term fetuses and dozens of live

mouse pups. After this first demonstration, embryo cryopreservation was applied to other species and it developed into a standard procedure for cattle producers in the 1980s. A human pregnancy was first reported by Trounson and Mohr (1983), although it aborted spontaneously. The first births in humans from frozen embryos were reported by Zeilmaker *et al.* (1984). Currently, many live births occur each year using blastocysts that had been frozen and thawed, as reported by Smith *et al.* (2005). This has allowed embryo cryopreservation to become commonplace for humans. Over the years, various protocols have been attempted for the cryopreservation of oocytes and embryos (Rall *et al.*, 2000).

Although the cryopreservation of cleavage-stage embryos is now a standard procedure, the mammalian oocyte has proven to be much more difficult to cryopreserve successfully. Early attempts with mouse oocytes used the conventional cryopreservation protocols developed for embryos, but these resulted in only 6 to 14% of the cryopreserved oocytes developing to offspring after IVF (Dulioust *et al.*, 1995). Cattle oocytes cryopreserved by slow-cooling exhibited low fertilization rates after IVF and fewer than 13% developed to 2-cell embryos (Schellander *et al.*,1994). Nevertheless, offspring have been produced from oocytes that have been successfully cryopreserved in mice (Whittingham, 1977), rabbits (Al-Hasani *et al.*, 1989), cattle (Fuku *et al.*, 1992; Otoi *et al.*, 1993) and humans (Chen-shee *et al.*, 2000). In recent years, cryopreservation of mammalian oocytes has become much more successful, most likely due to a focus on the differences between oocytes and embryos, rather than their similarities.

When fully optimized, cryopreservation of oocytes will have multiple applications for animals and human beings. Specifically in the context of farm animals, oocytes can be retrieved from live animals, even if they are prepubertal, infertile, pregnant or dead. Oocytes can also be retrieved from the ovaries of valuable dead animals. The oocytes collected from any source can be subjected to freezing for creating repositories in the form of oocyte banks. Thus, valuable genetic bloodlines from females can be preserved and oocytes, rather than embryos from a proven sire, can be marketed. Also, frozen-thawed oocytes could be later used for producing live offspring for faster multiplication of superior germplasm or for use in reproductive

technologies like production of cloned or transgenic livestock or production of embryonic stem cells.

Endangered species could also benefit from oocyte cryopreservation by conserving the female genetic material of these vulnerable animals (Holt *et al.*, 2004). Currently, there is insufficient information regarding the cryobiology of all endangered species. Cryopreservation of the oocytes of these rare species would allow one to preserve oocytes until the assisted reproductive technologies (ART) become available for use. Furthermore, oocyte cryopreservation could aid in the production and longevity of transgenic animal lines. Transgenic mice tend to have poor reproductive capabilities and production of these lines is a lengthy and costly process (Cecim *et al.*, 1995). Oocytes from laboratory animals, such as those from valuable transgenic strains of mice, could also be preserved through oocyte cryopreservation. In humans, the long-term storage of oocytes rather than embryos would help reduce legal, ethical, and moral issues associated with storing embryos (Gosden and Nagano, 2002). Storage of oocytes would also allow cancer patients without partners to preserve their genetic material for later use (Fabbri *et al.*, 2001; Porcu *et al.*, 2004).

Table 1. Applications of oocyte freezing

S. No.	Applications
1	Creation of oocyte banks
2	Alternative to embryo freezing
3	Oocyte preservation for patients with ovarian hyperstimulation syndrome
4	Oocyte donation programme
5	Prevent fertility loss through surgery
6	The treatment of congenital infertility disorders
7	Conservation of endangered species
8	Extension of fertile life of oocyte
9	Conservation of germplasm from superior genetic merit animals
10	Improve the efficiency of IVF
11	Salvaging genetic material from prepubertal, infertile, pregnant or even dead animals

2.3 FACTORS INVOLVED IN CRYOPRESERVATION

Successful cryopreservation of mammalian cells is dependent on several variables which include i) the type of cell itself, ii) the solution in which the cell is suspended and whether or not the solution contains one or more cryoprotectants, also called as cryoprotective additive (CPA), iii) the rate at which the cell is cooled to subzero temperatures, iv) the minimum subzero temperature to which the cell is cooled, v) the rate at which the cell is warmed and vi) the conditions under which the cryoprotectant is removed from the cell. Depending on the suspending solution, different types of cells exhibit different optimum cooling rates that may vary from a low rate of ~0.2°C/min to a high of 1,000°C/min. Under certain conditions, cells may even survive after being cooled at rates >100,000°C/min. For a given type of cell, identification of an appropriate CPA, an optimum cooling rate and a corresponding optimum warming rate are keys to successful cryopreservation.

A universal protocol is impractical for all types of cells due to differences among species and types of material. Although there are several protocols that have been used to successfully cryopreserve cells and tissues of many types, all of them involve the following five critical steps (Leibo and Songsasen, 2002).

1. Exposure of cells or tissues to cryoprotectants
2. Cooling of cells to temperatures below 0°C
3. Storage at the "glass" transition temperature of water, below -130°C
4. Warming and thawing, and
5. Dilution and removal of cryoprotectants prior to incubation

2.3.1 Cryoprotectants

Whatever be the method, certain compounds known as cryoprotectants or cryoprotective agents or cryoprotective additives (CPAs) are used to reduce the cellular injury when the cells are subjected to ultra low temperatures. The presence of cryoprotectants in the freezing solution is necessary to prevent cell damage during freezing and thawing of the samples.

Cryoprotectants are added to solutions used to freeze the cells to lower the freezing point. Cryoprotectants also make the cell membrane more elastic

by affecting lipids within the cell membrane. The cryoprotectants used can be broadly classified into the following three categories

i) Low molecular weight (MW) permeating cryoprotectants

These include ethylene glycol (EG, MW 62.07), 1, 2-propanediol (PROH) also called propylene glycol (PG, MW 76.1), dimethyl sulphoxide (DMSO, MW 78.13), glycerol (MW 92.1). They osmotically replace the intracellular water in the oocyte before cooling and especially during slow controlled cooling, reduce changes in the volume of the cell and prevent formation of intracellular ice. They are also thought to stabilize the intracellular proteins.

ii) Low molecular weight non-permeating cryoprotectants

These include cryoprotectants such as galactose (MW 180.2), glucose (MW 181.1), sucrose (MW 342.3) and trehalose (MW 378.3). These cryoprotectants cause dehydration of the oocyte/embryo before cooling, leading to reduced ice crystal formation during freezing.

iii) High molecular weight non-permeating cryoprotectants

Molecules with big dimension that cannot permeate the cellular membrane represent the non-permeating CPAs. They increase the concentration of extracellular solutes generating an osmotic gradient across the cell membrane, which draws water out of the cell causing the cell to dehydrate before the freezing procedure and prevent the rapid water entering into the cell after thawing during re-hydration/dilution out of the permeating CPAs.

This category includes polyvinylpyrrolidone (PVP, MW 7,34,58), polyvinyl alcohol (PVA, MW 34,109) etc. They change the ice crystal formation to an innocuous shape and size and thus protect the oocyte/embryo during freezing and thawing.

Among these cryoprotectants, the presence of low molecular weight permeating cryoprotectants is absolutely necessary. These may be combined with low molecular weight non-permeating cryoprotectants for the protection of cells during freezing. The high molecular weight cryoprotectants are not used as such. Since they are not permeable through cell membrane,

therefore, they cannot replace the water of ooplasm during dehydration, at the time of freezing.

Although cryoprotective actions of these different compounds are not entirely understood, they play different roles during cooling and thawing processes. When cells are first exposed to multimolar solutions of cryoprotectants, the cells contract due to loss of water. As the permeating cryoprotectant(s) enter the cells, water reenters and the cells return to their original volume. The cryoprotectants have different rates of diffusion into the cells. For example, the rate of diffusion for propylene glycol into oocytes is relatively fast (5-7 min) compared to the rate of diffusion for DMSO (20-30 min, Renard and Babinet, 1984). Thus the cryoprotectants osmotically replace the intracellular water in the cells before cooling besides minimizing the formation of ice crystals within the cells.

The cryoprotective actions of non-permeating cryoprotectants are based on the dehydration of cells prior to cooling which results in reduced ice crystal formation. However, low MW non- permeating cryoprotectants must be combined with low MW permeating cryoprotectants to effectively protect the cells during freezing. Cryoprotectants of high MW group protect the cells during freezing and warming by altering ice crystal formation to an innocuous size and shape.

2.3.1.1 Mechanism of cryoprotectant entry in the oocyte

When the oocyte is exposed to CPAs, it immediately contracts osmotically due to water loss because of the difference in osmotic pressure between the extracellular and the intracellular solutions. At the same time, the CPA begins to permeate the cell by simple diffusion because there is a difference in the concentration of the CPA between the extracellular and the intracellular solutions. Finally, water begins to reenter the cell to maintain osmotic equilibrium between the extracellular and the intracellular solutions. At the end, when equilibrium has been set up, the oocyte has the same concentration of CPA as that of the solution in which it is suspended, and the osmotic pressure of the cell cytoplasm is the same as that of the suspending medium. Among several variables that determine how quickly the equilibrium is established, some of the important ones are

i) Permeability property of the specific solute (e.g., ethylene glycol enters the cell faster than glycerol),

ii) Concentration of CPA (the higher the concentration, the faster the CPA will permeate cells),

iii) Temperature (the higher the temperature the faster the CPA will permeate cell) and

iv) Stage of development and species.

When aqueous/freezing solutions containing CPAs are frozen, water is removed in the form of ice, thus dissolved solutes (CPAs) become increasingly concentrated. As the temperature is decreased, more and more water leaves the solution and ice forms, increasing the concentration of the solution of the unfrozen liquid. This process continues until the sample is cooled to the eutectic point of the solution. The eutectic point is the temperature at which the entire system solidifies (Liebo, 1986).

2.3.2 Additives Used in Cryoprotectant Solutions

Compounds of various sizes that by themselves do not protect the cell from freezing damage but make the cell membrane more elastic by affecting the cell from freezing damage are used as supplements to cryoprotectant solutions. These include sugars, other large molecular weight compounds and proteins.

2.3.2.1 Sugars

Sugars are polyhydroxyl aldehydes or ketones (carbon chains with terminal aldehydes or ketones and hydroxyl side-chains). High levels of sugars and sugar alcohols are found in many polar plants, insects, fungi, etc. as non-toxic cryoprotectants. Various saccharides that have been used as supplements include mono-, di- and trisaccharides that have molecular weights ranging from ~180 to 540. The monosaccharides used as cryoprotectants include fructose, glucose, and galactose whereas the disaccharides include sucrose, trehalose and lactose, and the trisaccharides include raffinose. Trehalose has an abnormally large hydrated radius well over twice as large as other sugars and (unlike other sugars) is totally excluded from the hydration shell of proteins. Trehalose has been reported to

be very effective as a cryoprotectant supplement for vitrification of oocytes by several groups (Dinnyes *et al.*, 2000; Lj *et al.*, 2002; Begin *et al.*, 2003). Arav *et al.* (1993) showed that bovine oocytes exposed to trehalose had a higher rate of survival than oocytes exposed to sucrose and the oocytes had a very high rate of fertilization after exposure to 0.25M trehalose (70%). Sucrose is the most common sugar found in freezing-tolerant plants, some of which can increase their sucrose levels ten-fold in response to low temperatures. Sucrose and trehalose inhibit the membrane mixing associated with chilling. Both sugars fit well in the cell membranes, binding to phospholipid head groups.

2.3.2.2 Large molecular weight compounds

These include polyvinyl pyrrilidone (PVP), hydroxyethyl starch (HES), Ficoll and polyethylene glycol (PEG). Although these compounds will usually not protect the cells against freezing damage when used alone in solution, they will often enhance the protective effect of other low MW cryoprotectants. For example, Kuleshova and Shaw (2000) found that survival of mouse embryos after freezing could be improved by using a mixture of PVP and ethylene glycol. Several reports have shown that PVP is not successful for cryopreserving embryos and that the compound is highly toxic to embryos (Wilmut and Rowson, 1973; Fahy *et al.*, 1984). PEG has also been used to supplement CPA solutions (Rall and Fahy,1985).

2.3.2.3 Proteinacious compounds

Proteins can also be used as supplements to cryoprotectant solutions. Examples include bovine serum albumin (BSA) or fetal bovine serum (FBS). Another less common type of cryoprotectant supplement is thermal hysteresis proteins (THPs). Arctic fish live in icy polar waters that drop to temperatures of -1.8°C. DeVries and Wohlschlag (1969) isolated glycoproteins from the serum of these fish and found that the glycoproteins changed the freezing point without changing the melting point, and named these glycoproteins THPs. In another study, THPs were again isolated from fish and used for the cryopreservation of pig oocytes (Rubinsky *et al.*, 1991). These authors reported that 80% of the oocytes in the THP group were membrane-intact and 27% of these oocytes went on to mature in vitro.

2.3.3 Cooling

2.3.3.1 Cooling rates

The cooling rate is the rate of change of temperature. It is one of the principal determinants of cell survival during cryopreservation (Karow 2001). Cooling too slowly may kill the cells by exposing them to concentrated solutions; whereas cooling them too quickly can cause cell death due to ice crystal formation (Leibo and Mazur, 1971). Mazur (1970) proposed that cell survival in respect to cooling rate can be plotted as a bell-shaped curve. Essentially, cell survival is low at low cooling rates, increases to a maximum at an optimal cooling rate, and finally declines at high cooling rates. Each type of cell has its own optimal cooling rate.

When cells in suspension are cooled to subzero temperatures, ice crystals first form in the extracellular solution and the cell cytoplasm supercools. As the cell cytoplasm is cooled to lower temperatures (below -10°C or -15°C), ice crystals may form abruptly in the cytoplasm itself, a phenomenon referred to as intracellular nucleation. This is often, but not inevitably lethal to the cells. If cells that have frozen intracellularly are warmed very rapidly, the cells may be "rescued" from this damage (Mazur, 1970). In contrast, when cells are cryopreserved by vitrification, they are cooled in such high concentrations of cryoprotectant(s) solution and at such high cooling rates that intracellular ice crystals do not form. This happens when the temperature is high enough that the molecular mobility of water has increased to a point where the water molecules can move and rearrange themselves from a disorderly amorphous vitrified position to an orderly crystalline position. This occurs well below the melting point, and is therefore a potentially lethal problem (Rall *et al.*, 2000).

2.3.3.2 Supercooling

Supercooling refers to taking a liquid below its equilibrium freezing point, without freezing it. Biological solutions *in situ* can usually supercool a couple of °C or more. From the point of view of the organism, this freezing avoidance mechanism has the advantage that the solutions remain liquid and allow relatively normal, though slower, metabolism. It has the disadvantage that a supercooled solution is unstable, if an ice crystal is introduced, it tends

to freeze immediately, forming ice and a more concentrated solution. A very small volume of a pure solution may supercool as much tens of °C, but biological solutions are threatened by ice nucleators which may initiate freezing either inside or outside the cell. Supercooling is a vital strategy for some Antarctic fish that live in a solution with a higher concentration and greater freezing point depression than those of their own tissues. Their blood carries a potent protein "antifreeze". This substance, present only in tiny molar concentrations, does not depress the equilibrium freezing temperature, but works by impeding the growth of ice crystals (DeVries, 1984). The environmental temperature for such fish has a robust lower bound; they will not encounter temperatures colder than the freezing temperature of the ocean. Without such a lower bound, the supercooling strategy is a dangerous one. Plant leaves often supercool a few degrees and may thus survive mild frosts without freezing damage, whereas slightly colder temperatures can cause extensive damage (Lutze *et al.*, 1998). The importance of supercooling in cryopreservation is that it allows vitrification, discussed later in this review.

2.3.4 Storage

For the cells to be cryopreserved without a decrease in survival for the duration of storage, they must be preserved at temperatures below the glass transition temperature of the cytoplasm and the suspending solution, which is approximately -130°C. In practice, the easiest and safest way is to store cryopreserved oocytes/embryos in LN_2 at –196°C. The data of mouse model experiments indicate that cryopreserved embryos stored in LN_2 will remain "alive" for at least 2000 years (Glenister *et al.*, 1984). It was concluded that normal levels of background radiation would not be hazard to the long-term storage of mammalian embryos. Children have been born from embryos that were cryopreserved for more than 8 years (Fuller *et al.*, 2004b). Live mice and sheep have been produced from cryopreserved embryos stored for more than 15 years in LN_2 (Leibo *et al.*, 2004).

2.3.5 Thawing and Post Thawing Handling

Oocytes cryopreserved in straws are generally held in air for around 40 seconds, during which time the temperature will rise rapidly to approximately -50°C, before the straw is transferred to a water bath held at around 30°C. It also helps in preventing the bursting of the French straw.

2.3.5.1 Warming rates

The warming rate is also very important for successful cryopreservation of mammalian cells. The optimum warming rate for a given type of cell is highly dependent on the optimum cooling rate that preceded it. Early investigators assumed that rapid warming of mammalian cells after cryopreservation was always better because cells had shorter times to recrystalize and were exposed for less time to cryoprotectants. However, investigations of mouse embryo freezing by Whittingham *et al.* (1972) proved that there are exceptions to this rule. Their study showed that embryos cryopreserved by slow cooling had greater post-thaw survival when they were warmed slowly indicating that embryo survival was dependent on the warming rate. They concluded that poor survival using faster warming rates was most likely due to osmotic effects.

The most common method for warming of oocytes after they have been vitrified is a rapid and direct method. Usually, the oocytes are placed in warming solutions at 20°C to 37°C. After warming, oocytes must rehydrate and the cryoprotectants used for freezing must be removed. This is also done quickly but the debate on whether step-wise dilution is necessary still remains.

2.3.6 Removal of Cryoprotectant

The final step of the cryopreservation procedure is the removal of the cryoprotectant from the oocyte. Oocytes are cryopreserved in cryoprotectants ranging in concentration from 1–8 M (1–2 M in case of traditional slow cooling; 4–8 M in case of vitrification). In general, if oocytes frozen in permeating cryoprotectants are rapidly diluted (re-hydrated), the oocytes will be subjected to osmotic shock causing death. A common practice is to dilute the cryoprotectants in a step-wise fashion. Although this method works well in practice, it is very slow and time consuming. A much shorter method is to use a non-permeating solute such as the sucrose as an osmotic buffer to lessen the chance of an osmotic shock (Camus, 2004; Massip, 2001).

2.4 DAMAGE TO THE OOCYTES DUE TO CRYOPRESERVATION

2.4.1 Physical Damage

The causes for cellular damage and death from cryopreservation are not completely understood. During the cryopreservation process, cells

experience several changes in their milieu; water is removed from the solution in the form of ice and consequently, the solutes become more concentrated and can precipitate; the cell responds osmotically by losing water. These processes can also be caused by changes in temperature, except for the precipitation of solutes. Researchers have debated whether changes in temperature, several solution effects or both are the cause of cellular damage and death during cryopreservation (Critser and Russel, 2000; Fuller *et al.*, 2004a).

Not only during cooling but also during processing before cooling, during warming, and at recovery, cells are at risk of various types of injury. To minimize the decrease in survival, one must circumvent all of these types of injuries. Table 2 lists some of the common injuries observed after cryopreservation which may lead to cell death (Shaw *et al.*, 2000).

Table 2. Factors associated with cooling and cryopreservation that contribute to cellular injury and death in biological systems (Shaw *et al.* 2000).

System	Type/ cause of damage
All	Intracellular ice formation, extracellular ice formation, apoptosis, toxicity, calcium imbalance, free radical, ATP levels, general metabolism, fertilization failure, cleavage failure, pHi, parthenogenetic activation, cleavage
Membrane	Rupture, leakage, fusion, microvilli, phase transition
Chromosomes	Loss/gain, polyspermy, polygny (failure to extrude polar body), tetraploidy
DNA	Apoptosis, fusion, rearrangements
Cytoskeleton	Microtubules dissolve, actin
Proteins/enzymes	Dehydration, loss of function
Ultrastructure	Microvilli mitochondria, vesicles, cortical granules, zona Pellucida
Zona pellucida	Hardening, fracture
Lipids	Free radicals?

2.4.1.1 Chilling injury

Certain types of oocytes (e.g., pig and bovine oocytes, pig embryos at perihatching stage, bovine embryos at early cleavage stages) are sensitive to

cooling to below +20°C (Kasai *et al.*, 2002). Cells, having chilling sensitivity, appear dark with cytoplasmic lipid droplets, which are thought to be associated with the chilling injury. Therefore, successful cryopreservation of pig embryos has been achieved by using embryos at the perihatching stage when there are fewer droplets (Kashiwazaki *et al.*, 1991) or by removing the droplets (Nagashima *et al.*, 1995).

2.4.1.2 Intracellular ice formation

The size of mammalian oocytes is large, a large proportion of which is occupied by water. When they are cooled to low temperatures, intracellular ice is liable to form. This is fatal because even a small amount of ice is likely to recrystallize, become larger and destroy the cellular structure. To prevent this, cells must be concentrated so that vitrification i.e., solidification of a solution without crystallization occurs below the glass transition temperature for which, inclusion of a cryoprotectant is essential. In slow freezing, the cells are concentrated gradually during cooling, whereas in vitrification, the cells are concentrated upon direct suspension in a concentrated solution.

The formation of ice in the environment of the cell induces changes to which the cell must respond. When a cell suspension is cooled below its freezing point, water is removed from the solution in the form of ice, increasing the concentration of solutes which remain in the unfrozen fraction and hence increasing the osmotic pressure of the remaining solution. The resulting gradient in osmotic pressure across the plasma membrane provides the driving force for an efflux of water from cells, with the rate of efflux being limited by the permeability of the plasma membrane to water (Mazur, 1963). As the temperature is lowered, more ice is formed and the concentration of solutes in the unfrozen fraction increases. If the cooling rate is sufficiently slow to allow the cell to remain close to osmotic equilibrium, then water efflux will continue to low temperatures (Mazur, 1963). At cooling rates high enough to cause significant departure from osmotic equilibrium, where the kinetics of ice formation in the extracellular medium are much faster than the kinetics of osmotic water efflux from the cell, intracellular freezing may result (Mazur, 1963). Once ice forms within the cell, osmotic equilibrium with the extracellular solution during further cooling is maintained by *in situ* freezing of water in the cell rather than movement of water across the plasma membrane.

The mechanisms leading to intracellular freezing are unclear, as indicated by the diversity of the current hypotheses describing the genesis of intracellular ice:

a) Intracellular freezing occurs as a result of critical undercooling. As the cooling rate increases, the difference between the intracellular and extracellular osmotic pressures increases, and hence the degree of undercooling (the difference in temperature between the actual temperature of the cytoplasm and its freezing point) in the cytoplasm increases.

b) Cytoplasm is nucleated through aqueous pores in the plasma membrane. The temperature dependency of the ice nucleation rate and ice crystal growth rate results in the formation of smaller crystals at higher cooling rates (Mazur, 1961). Mazur (1963) proposed that as the cooling rate increases and the radius of the growing tip of ice crystals decreases, propagation of extracellular ice through the plasma membrane occurs when the tip radius of the crystals approximates the radius of aqueous pores in the plasma membrane.

c) Intracellular freezing occurs as a result of electrical transients at the ice interface. Electrical transients are created at a growing ice interface in aqueous solutions by the selective exclusion of charged species from ice at the interface (Workman and Reynolds, 1950). It has been suggested that this Workman-Reynolds effect may contribute to the disruption of the plasma membrane when the magnitude of the potential difference across the plasma membrane due to these electrical transients reaches a critical level (Steponkus *et al.*, 1984). Rupture of the plasma membrane would allow extracellular ice to grow rapidly into the undercooled cytoplasm. This hypothesis is consistent with observations of Dowgert and Steponkus,1983, which show that cell damage precedes the appearance of intracellular ice in isolated protoplasts (Steponkus and Dowgert, 1981) implying that intracellular ice is a result rather than the cause of injury.

The conditions leading to intracellular freezing are almost always associated with lethal injury to cells (Leibo, 1977). In the hypotheses where

intracellular freezing is the cause of cell injury, it has been proposed th injury is related to the formation (Farrant *et al.*, 1977), recrystallization (Mazu 1972), or melting (Farrant and Morris. 1973) of intracellular ice.

2.4.1.3 Extracellular ice

If mammalian oocytes are suspended in a physiological solution an cooled to low temperatures, intracellular ice forms and the oocytes will die. the sample is seeded and cooled slowly, the oocytes may be crushed by th extracellular ice, as the unfrozen fraction in which the cells are locate becomes smaller because the cells are large. (Schneider and Mazur 1987) Extracellular ice can also cause another type of injury. As the amount o extracellular water in the solution is reduced by the conversion to ice, the solution becomes concentrated, exposing the cells to high concentrations o solutes (i.e., electrolytes). However, by including a cryoprotectant in the solution, these injuries can be prevented.

2.4.1.4 Toxicity of cryoprotectants

For the cryopreservation of oocytes, the inclusion of a cryoprotectant that can permeate into the cell is essential. The mechanism of the protective action of permeating agents is considered the same, but their toxicities are different. For slow freezing, the concentration is limited to 1--2 mol/L, and the toxicity is relatively low. In vitrification, however, the concentrations can be as high as 8 mol/L, and the selection of a low toxicity agent becomes more important. In addition to permeating agents, small saccharides and macromolecules are frequently added to the solution (Kasai, 1997). However, the toxicity of these non-permeating agents is quite low.

2.4.1.5 Fracture damage

When cryopreserved oocytes are recovered, they are occasionally found to be cracked. This physical injury, called fracture damage, is thought to be caused by non-uniform changes in the volume of the liquid and solid phases of the medium during rapid phase changes. Fracture damage can be reduced by reducing the cooling and warming velocities during passage through the temperature range where the phase change would occur approximately at $-130^{\circ}C$ (Rall and Meyer 1989). Fracture damage is also related to the container used for the storage of oocytes or embryos; more

flexible ones result in less damage (Landa 1982). In vitrification using straws, fracture damage can be prevented completely by cooling and warming to the phase transition temperature in a gas phase (Kasai, 1997).

2.4.1.6 Osmotic swelling and shrinkage

Just after warming, cryopreserved oocytes contain a permeated cryoprotectant, which has to be removed. If the oocytes are directly recovered in an isotonic solution, they risk injury from osmotic swelling, because water diffuses in far more rapidly than the cryoprotectant diffuses out. The sensitivity of oocytes to osmotic swelling differs with the stage of development. The most common strategy for preventing this injury is to dilute oocytes with a hypertonic solution containing sucrose as a non-permeating solute to counteract the inflow of excess water (Kasai *et al.,* 1980; Leibo, 1980). Upon removal of the permeated cryoprotectant out of the oocytes by diffusion using a sucrose solution, they remain shrunken. This hypertonic shrinkage may also be injurious to them. As in the case of hypotonic stress, the cryopreserved cells just after warming are sensitive to this injury (Pedro *et al.,* 1997). Therefore, after diffusion of the permeated cryoprotectant out of the cell in a hypertonic solution, the oocytes should be transferred into a less hypertonic solution, and finally into an isotonic solution.

2.5 ULTRASTRUCTURAL DAMAGES

Cryopreservation mat cause damage to intracellular organelles. In mammals, the oocytes are ovulated as mature secondary oocytes at the second meiotic metaphase (MII) with the chromosomes attached to the spindle (the cytoskeletal structure involved in spatial organization of the chromosomes) and the first polar body extruded. In this condition, the chromosomes are loosely bound to the spindle, and in the absence of the enveloping nuclear membrane, they might be considered relatively unstable. The full set of maternal chromosomes is derived by normal meiotic division, yielding the normal set of haploid chromosomes on the spindle (the euploid state). Any change in the chromosomal complement (as might result from scattering or displacement from the spindle) could result in aneuploidy, with potentially severe consequences for subsequent embryonic or fetal development. For this reason, concern is frequently expressed about the

normality of the chromosome distribution in cryopreserved oocytes. Oocyte cytoskeletal organization is strongly influenced by the condensed metaphase chromosomes, with the spindle (in the case of the mouse) having a characteristic barrel shape (Johnson and Pickering, 1987; Sathananthan *et al.*, 1988).

2.5.1 Effects on Microtubules and Microfilaments

Microtubules are structures consisting of polymerized tubulin in equilibrium with the free tubulin pool in the cytoplasm, and in oocytes the major microtubular structure is the spindle. The microtubules are important for processes involving the meiotic spindle and correct distribution of chromosomes during fertilization. It has been shown for a variety of cell types that cooling can cause marked effects on microtubules, with a pronounced depolymerization and disappearance of microtubule organizing centres (Webb *et al.*, 1986). The microtubules of the meiotic spindle in unfertilized oocytes behave in this fashion, with loss of continuity in murine microtubules after 15 min at 0°C, and complete disappearance after 45–60 min (Magistrini and Szöllösi, 1980). However, progressive rewarming of the oocytes to 37°C over 15 min allowed repolymerization of the microtubules and reformation of the meiotic spindle. This ability for spindle reorganization was confirmed in mouse oocytes rewarmed from 4 or 18°C to 37°C (Johnson and Pickering, 1987), but parallel observations on the arrangement of chromosomes in the same oocytes indicated that in a significant minority of cases there was some dispersal of the chromosomes away from the reforming spindle. A similar series of events was observed in human oocytes cooled to 20°C and held for 10 or 30 min (Pickering *et al.*, 1990). Changes in spindle organization were found in all oocytes cooled and held at 20°C for 30 min, which included reduction in spindle size, disorganization of microtubules within the spindle itself, and sometimes a complete lack of microtubules.

In some oocytes, chromosome dispersal from the metaphase plate was observed, and normal spindles reformed in <50% of those oocytes after return to culture at 37°C, although in most cases chromosomal dispersal was reversed. The potential for reversal of the abnormalities resulting from cooling was confirmed in a study by Bernard *et al.*, 1992 which indicated that human

oocytes, cooled to 0°C at two different cooling rates (–3°C/min and –1000°C/min) in the absence of ice, and then returned to culture and inseminated at 37°C, were able to be fertilized and undergo subsequent development in culture at a rate similar to oocytes which had not been cooled.

The other main components of the cytoskeleton (microfilaments) are equally important for many steps, including cytokinesis. The microfilaments are composed of polymerized actin (F-actin) in dynamic equilibrium with free actin and, as with microtubules; there is a possibility that this equilibrium may be perturbed by the various stages in cryopreservation. In human oocytes, microfilaments are organized in a uniform layer enveloping the cortex of the cell (Pickering *et al.*, 1988), while in the mouse this layer appears to thicken in the region adjacent to the meiotic spindle (Longo, 1987). The activity of microfilaments is essential for spindle rotation, polar body extrusion, pronuclear migration and cytokinesis (Schatten *et al.*, 1986; Le Guen *et al.*, 1989; Vincent and Johnson, 1992).

2.5.2 Effects on the Zona Pellucida and Fertilization

An area of further concern is that the various steps in cryopreservation can induce changes in the glycoprotein coat that surrounds the mammalian oocyte. This coat (the zona pellucida) plays an important role in fertilization, since a spermatozoon must bind to the zona, undergo an acrosome reaction, penetrate the zona and fuse with specific receptors on the oocyte plasma membrane (Wassarman, 1988) to achieve normal fertilization. In particular, the fusion with the plasma membrane triggers the release of cortical granules. These are located close to the surface of the oocyte. When a single spermatozoon penetrates an oocyte, the release of cortical granules alters the glycoproteins in the zona pellucida to prevent polyspermy, the penetration of an egg by multiple spermatozoa (Webb *et al.*, 1986). Translocation and release of the cortical granules involves correct functioning of the cytoskeleton and plasma membrane organization. The zona pellucida (ZP) properties of *in vitro* produced embryos are directly related to the viability of bovine embryos after cryopreservation. In this context, Sulva *et al.* (2005) demonstrated that cryopreservation steps addresses alterations in the ZP causing irreversible damage on the further developmental competence of bovine embryos.

Recently, Larman *et al.* (2006) found that calcium-free media vitrification reduces cryoprotectant-induced ZP hardening and increased fertilization rates in mouse oocytes.

2.5.3 Cryopreservation and Chromosomal Abnormalities

Implicit in much of the previous discussion has been the possibility that, by altering the ultrastructure and integral responses of various oocyte components, successful fertilization and embryonic development will be impaired. In particular, the possibility of causing genetic abnormalities following abnormal chromosome distribution during and after fertilization is a major concern (Almeida and Bolton, 1993). Up to 15% of freshly recovered human oocytes (at metaphase II and morphologically normal by light microscopy) exhibited one or more non-spindle associated chromosomes (Van Blerkom and Henry, 1988). Studies in murine oocytes following cryopreservation during a slow cooling regime with DMSO as cryoprotectant demonstrated that in oocytes which subsequently were fertilized and reached the first cleavage stage, polyploidy was increased, but this did not appear to be linked to polyspermy (Glenister *et al.*, 1987). In comparison, Kola *et al.* (1988) reported a 3-fold an increase in aneuploidy in murine oocytes fertilized after a vitrification procedure. Bouquet *et al.* (1992) addressed this conflicting evidence in a cytogenetical study on murine oocytes using DMSO as cryoprotectant and slow cooling. These authors found an increase in polyploidy in the first and second cleavage, but this only concerned the frequency of triploidy and not monosomic or trisomic aneuploidy. Retention of the polar body seemed to be the main cause for these observations. A similar increase in polyploidy was noted in mouse oocytes cryopreserved using an ultrarapid technique (Van der Elst *et al.*, 1993); it was also defined as triploidy, although it was not possible to assign it to either diandric or digynic origins.

2.5.4 Parthenogentic Activation Associated with Cryopreservation

As outlined in the previous sections, elements of cytoskeleton are intimately involved in the various steps involving internal reorganization of oocytes prior to and after fertilization. There has been concern that cooling or cryoprotectant exposure, which may modify the cytoskeleton, could induce parthenogenetic activation in which oocyte ultrastructural reorganization and cleavage proceed without normal fertilization. (Shaw and Trounson, 1989)

Parthenogenetically activated oocytes may cleave and undergo subsequent development, with gross appearances similar to those of normally developing embryos, although the time course of development may be different from that expected for normal embryogenesis. It has been suggested that alterations in the microfilaments overlying the meiotic spindle can lead to activation (Webb *et al.*, 1986) of mouse oocytes during ageing. It has been shown in mouse oocytes exposed to either PROH or DMSO that at intermediate temperatures such as 27°C, PROH induced parthenogenesis in >50% of oocytes exposed to concentrations of 1.5 M, particularly when the oocytes were collected later than normal practice after ovulation had been induced (i.e. ageing). When the temperature of exposure was lowered to 1°C, the degree of activation was much reduced for mature ovulated oocytes, and PROH exposure gave similar results to DMSO exposure (Shaw and Trounson, 1989). Van der Elst *et al.* (1992) demonstrated that exposure to PROH could induce murine oocyte degeneration in a concentration-, temperature- and time-dependent fashion, with stronger tendency to parthenogenesis at higher temperatures and at higher concentrations. However, exposure to PROH concentrations up to 1.5 M at 0°C caused no greater incidence of parthenogenesis than recorded for the control, non-exposed population of oocytes. Studies on murine oocytes exposed to DMSO and cooled to –70°C demonstrated no parthenogenetic activation in cells recovered and cultured without insemination (Hunter *et al.*, 1991).

Pathenogenetic activation occurs due to an increase in the concentration of Ca^{2+}, which normally comes from spermatozoa. The wave of Ca^{2+} causes the release of cortical granules within the oocyte and this, in turn results in the resumption of meiosis and the formation of the second polar body (White and Yue, 1996). Furthermore, calcium fluxes are known to activate intracellular phospholipases, proteases, ATPases and endonucleases, which may result in altered plasma membrane integrity, denaturation of cytosolic proteins and chromosomal fragmentation, all of which can lead to irreversible cell injury and apoptosis. Gook *et al.* (1995) reported that human oocytes also undergo parthenogenesis and cryopreservation. They observed that 27% of fresh and 29% of aged oocytes

that had been cryopreserved were parthenogenetically activated after thawing.

Cortical granules are aligned under the oolemma of most mammalian oocytes. Zona hardening after fertilization or activation of the oocyte is caused by the release of these cortical granules (Wolf and Hamada, 1977; Gulyas and Yuan, 1985). Cortical granules are released to the outer edge of the cytoplasm during the zona reaction. This reaction is normally caused by exposure to sperm prior to fertilization and prevents polyspermy (Wassarman, 1988). Propylene glycol has been shown to cause premature cortical granule release (Schalkoff *et al.*, 1989) and disruption of cortical microfilaments (Vincent *et al.*, 1990). DMSO has been shown to cause zona hardening and a reduction of cortical granules in mouse oocytes (Vincent *et al.*, 1990). This latter study found that exposure of oocytes to DMSO between 20 and 37°C had negative effects on the zona pellucida, fertilization rate and spindle organization.

2.6 CRYOPRESERVATION OF OOCYTES AT VARIOUS DEVELOPMENTAL STAGES

Oocytes can be cryopreserved either as immature oocytes at the germinal vesicle (GV) stage or as mature oocytes at the metaphase II (MII) stage. Oocytes at the MII stage of development have undergone several developmental paths including both nuclear and cytoplasmic maturation, extrusion of the first polar body and the arranging of the chromosomes on the MII spindle whereas the immature, GV stage oocytes, come from Graafian follicles. The chromatin of these immature oocytes is still in the diplotene phase of prophase I. At this stage of development, GV oocytes do not have a spindle (Shaw *et al.*, 1999). The MII stage oocytes are vulnerable to cryoinjury due to the delicate spindle they possess which is acutely temperature sensitive. In contrast, GV oocytes seem to be less susceptible to cryoinjury than MII oocytes. The immature oocytes also have a longer period to recover from cryoinjury because they have to mature in vitro prior to insemination or other manipulations (Table 3, Shaw *et al.*, 1999).

Table 3. Factors that influence sensitivity to cryoinjury and suitability for cryostorage (Shaw *et al.*, 1999).

Material	Primordial oocyte	GV stage oocyte	MII stage oocyte
Availability	Abundant, always present	Scarce, only from antral follicles	Scarce, only at mid-cycle
Ease of collection	Easy e.g. biopsy	Oocyte retrieval	Oocyte retrieval
Size	<50 µm	80 to 300 µm	80 to 300 µm
Nuclear status	Resting prophase I, Nuclear membrane	GV, has nuclear membrane	Resting MII, temperature sensitive spindle, no nuclear membrane
Zona	No	No	No
Cortical granules	No	Central	Peripheral
Intracellular lipid	Little	May be abundant	May be abundant
Metabolic rate	Low	Low	Low
Surface: Volume ratio	High	Low	Low

Studies using MII stage oocytes suggest that oocyte survival after freezing and thawing can be affected by a number of factors. Among the morphological factors, the presence or absence of the cumulus granulosa cells during the freezing process may have a direct impact on oocyte survival, post-thaw. Furthermore, sublethal damage to the cells post-thaw may well affect their developmental capabilities. Although not proven, it is thought that the cumulus cells offer some protection against sudden osmotic changes and stresses induced by the rapid influx of the cryoprotective agents during the procedures of equilibration and removal of cryoprotectants during the prefreeze and post thaw periods (Fabbri *et al.*, 2001).

Mature human oocytes show great heterogeneity in the distribution and organization of cytoplasmic organelles which may influence the outcome of cryopreservation procedures. For example, during human oocyte maturation in vivo, the cortical granules move to the periphery and are distributed under

the oolemma. Following fertilization, the granules are exocytosed and their contents alter the biochemistry and sperm-binding characteristics of the zona pellucida, which induces zona hardening and provides the natural block to polyspermic fertilization. Premature zona hardening will undoubtedly compromise normal fertilization and may impair implantation of the embryo (Gook, 1993). In support of this hypothesis, human oocytes stored after slow cooling have lower recorded fertilization rates after standard IVF, and electron microscopy of these cells has demonstrated a reduction in the number of cortical granules (Gook, 1993) suggesting that zona hardening had occurred in response to the freeze-thaw process. However, Gook *et al.* (1995) and Al-Hasani and Diedrich (1995) found an abundance of cortical granules in the cytoplasm of cryopreserved oocytes. Where there is any potential risk of zona hardening as a result of cryopreservation, it can be bypassed by micromanipulation techniques such as intracytoplasmic sperm injection (ICSI) and assisted hatching (Nagy, 1996).

Transient cooling of human oocytes to 20°C can cause irreversible disruption of the spindle apparatus while rapid depolarization occurs when the temperature is lowered to 0°C (Pickering *et al.*, 1990; Zenzes, 2001). The appropriate organization of spindle microtubules is essential for the correct alignment and segregation of chromosomes when the spindle reassembles once the temperature returns to normal. Oocyte freezing can, therefore, increase the incidence of aneuploidies after extrusion of the second polar body through non-disjunction of sister chromatids. This disruption of the cytoskeletal architecture may also lead to abnormal cytokinesis, retention of the second polar body and alterations in the organization and trafficking of molecules and organelles (Vincent and Johnson, 1992). While deleterious effects on the cytoskeleton resulting from chilling may be avoided by cryopreservation of GV stage oocytes, the difficulties associated with IVM and extended culture appear to counteract the potential benefits of freezing oocytes at this stage. Furthermore, chilling reduces the developmental capability of GV oocytes, apart from damage to the meiotic spindle (Martino, 1996). Consequently, few pregnancies have been achieved after IVM of human oocytes (Picton and Gosden, 2000) and even fewer after the initial attempts to cryopreserve full-sized GV oocytes.

Primordial oocytes appear to be less vulnerable to cryoinjury than mature oocytes as they are smaller, lack a zona pellucida and cortical granules, and are relatively metabolically quiescent and undifferentiated. Additionally, primordial follicles are apparently more tolerant to insults such as immersion in cryoprotectant solutions and cooling to very low temperatures as their small size makes them less susceptible to damage induced by water movements into and out of the cells during freezing and thawing. Finally, primordial oocytes have more time to repair sublethal damage to organelles and other structures during their prolonged growth phase after thawing. Although the cryopreservation protocols used for the banking of human primordial oocytes have not been fully optimized, they are nonetheless effective. Using the slow freezing approach, high post-thaw follicle survival rates of 84% and 74% have been recorded for human primordial follicles stored using 1.5 M ethylene glycol and DMSO, respectively, compared with survival rates of only 44% and 10% with 1.5 M propylene glycol and glycerol, respectively (Newton, 1996).

2.7 TYPES OF CRYOPRESERVATION

There are two very different approaches to cryopreservation of mammalian oocytes, conventional slow-cooling and vitrification. Slow freezing is also known as equilibrium freezing or programmable freezing while vitrification is also known as non-equilibrium freezing. Although these methods are drastically different, both can produce successful results in mammalian oocyte cryopreservation.

2.7.1 Slow Freezing

In the slow freezing method for mammalian oocytes, samples are suspended in 1–2 mol/L solution of cryoprotectant(s) dissolved in a physiological solution, ice-seeded, and cooled very slowly (0.3– 0.5°C/min) so that the cellular contents become concentrated by gradual dehydration in response to the concentration of the extracellular unfrozen fraction during the growth of extracellular ice. In the original slow freezing process developed by Whittingham in 1972, the sample was cooled to −80°C before being preserved in liquid nitrogen. It was then found that slow cooling could be terminated at approximately −30°C before plunging the sample into liquid nitrogen; if the sample is thawed rapidly (360°C/min). In any case, such a slow cooling stage

was necessary to prevent the formation of intracellular ice. The slow freezing method proved effective for oocytes/embryos of various mammalian species, and is widely used for the cryopreservation of mammalian oocytes and embryos. However, it requires a long time for cooling and an elaborate device to control the cooling rate.

2.7.1.1 Slow freezing of bovine immature oocytes

The oocytes are at the GV stage of maturation when these are aspirated from follicles of ovaries obtained from abattoir. Since the oocyte is at the early prophase of the first meiotic division at this stage, the genetic material is contained within the nucleus and no spindle is present. Because of the presence of the nuclear membrane, it was assumed that this type of oocyte might be less prone to injuries during cryopreservation. However, there are very few reports on the cryopreservation of immature GV stage bovine oocytes.

Suzuki *et al.* (1996) compared the efficacy of 1.8 M EG, 1.3 M monomethyl ether (EME) or 1.6 M PROH and different concentrations of trehalose and PVP in terms of the post-thaw developmental competence of bovine oocytes. When bovine GV stage oocytes were frozen slowly in a mixture of EG, 5% PVP and 0.05 M trehalose, almost 80% of them developed to MII, 22% were degenerated after IVM, and none of those cryopreserved underwent parthenogenetic activation. No significant differences were observed in post fertilization development using EG, EME and PROH. In their study to compare the efficacy of PROH and DMSO for cryopreservation of immature bovine oocytes, Im *et al.* (1997) obtained nuclear maturation rates of 38 and 30%, respectively. Glycerol has also been used as a cryoprotectant in some of the studies (Yang *et al.*, 1994). The presence of cumulus cells around the oocytes is important for IVM of frozen-thawed immature bovine oocytes. The maturation rate of denuded oocytes has been reported to be significantly lower than that of cumulus oocyte complexes (COCs) after freezing (Im *et al.*, 1997).

2.7.1.2 Slow freezing of bovine mature oocytes

Oocytes can also be cryopreserved at the MII stage of maturation. At this stage, the chromosomes are arranged on the metaphase plate and there

is no nuclear membrane. The cumulus mass surrounding the oocyte is expanded and cell cohesion is maintained by hyaluronic acid.

PROH has been used successfully as a cryoprotectant for the slow freezing of in vitro matured bovine oocytes (Otoi *et al.*, 1994; 1997). In one of the early studies, Xu and Betteridge (1992) subjected in vitro matured oocytes to slow freezing in 1.5M PROH. After freeze-thawing, 67.4% oocytes were found to have intact zonae pellucidae and normal looking cytoplasm indicating survival. The fertilization and cleavage rates were 22.2 and 7.9%, respectively. Sun *et al.* (1995) reported that although most (around 86%) of the bovine oocytes cryopreserved in 1.5 M PROH were recovered in a morphologically normal form, their developmental competence was severely hampered as indicated by low fertilization and cleavage rates of 36.4 and 13.5%, respectively. When PROH was used at a concentration of 1.5 M for the slow freezing of in vitro matured bovine oocytes, Mavrides and Moroll (2002) obtained the post-thaw recovery of morphologically normal oocytes to be 54.4%. After IVF, the cleavage rate was around 15%.

A number of studies have been conducted to compare the efficacy of different cryoprotectants for slow freezing of in vitro matured bovine oocytes. In one of the early studies, Otoi *et al.* (1993) compared the effectiveness of iso-molar concentrations of 1.6 M of PROH, DMSO and glycerol as cryoprotectants for slow freezing of in vitro matured bovine oocytes. The number of morphologically normal oocytes recovered after freeze-thawing was higher in PROH (49.8%) as compared to that in DMSO and glycerol (32.6 and 28.6%, respectively). After insemination, the fertilization rate of morphologically normal oocytes frozen-thawed in PROH (57.9%) was higher than that of those frozen in DMSO (38.3%) but similar to that of oocytes frozen in glycerol (50.9%). However, for PROH, glycerol and DMSO, the cleavage rate (22.4, 20.6 and 18.3%, respectively) and the proportion of surviving oocytes developing to blastocyst (1.4, 4.8 and 0.9%, respectively) were not different. In a later study by Schellander *et al.* (1994) in which the efficacy of different concentrations (1 M and 1.5 M) of various cryoprotectants (glycerol, DMSO and PROH) were compared, PROH was found to be superior to glycerol and DMSO in terms of the post fertilization cleavage rate

and the proportion of oocytes which developed to 4-cell stage. Analysis of the effects of 2 concentrations of the 3 cryoprotectants on cleavage and development to 4-cell stage indicated that glycerol 1.5 M and PROH 1.5 M were better than DMSO 1 M, but DMSO 1.5 M was better than DMSO 1 M and glycerol 1 M.

The superiority of PROH as a cryoprotectant was confirmed in a later study in which Lim *et al.* (1999) compared the efficacy of 1.0 M of PROH, DMSO and glycerol for slow freezing of in vitro matured bovine oocytes. After thawing, more oocytes were found to be morphologically normal after being frozen with DMSO (86%) or PROH (83%) than with glycerol (62%). After IVF, higher penetration rates were observed with DMSO (79%) or PROH (76%) than in glycerol (48%). The percentage of oocytes developing to 2-cell stage was also higher in DMSO (51%) and PROH (54%) than in glycerol (33%). However, a significant increase in the proportion of 8-cell embryos (46 vs. 21 to 26%) at 72 h post insemination and morulae (14 vs. 6 to 8%) were derived from oocytes frozen with PROH than with DMSO or glycerol. That the efficacy of PROH and DMSO as cryoprotectants is similar has also been reported by Im *et al.* (1997) in a study in which they found the nuclear maturation rates of oocytes to be similar after freezing them in 1.5 of PROH and DMSO (67 and 61%, respectively).

EG has also been used at a concentration of 1.8 M for the cryopreservation of in vitro matured bovine oocytes (Otoi *et al.*, 1995; Asada and Fukui 2000).

2.7.1.3 Slow freezing of oocytes of other species

In an early study, Smith compared the differences in cryopreservation survival between mammalian unfertilized oocytes and oocytes that had recently been fertilized noting that the fertilized ova had a much better chance of development in vitro than the unfertilized cells (Smith, 1961). Even though this difference between oocytes and embryos was recognized in the early days of oocyte cryopreservation research, the first mammalian oocyte cryopreservation protocols mimicked the equilibrium "slow-cooling" methods used for embryo cryopreservation (For reviews see Rall, 1992; Fabbri *et al.*, 2000; Paynter, 2000). Fabbri *et al.* (1998) described a commonly used

protocol for slow-cooling and rapid thawing of oocytes using DMSO and straws. Oocytes were frozen from 0°C or 20°C to -8°C at a rate of 2°C/min, then at a rate of 0.3°C/min to -30°C and finally at a rate of 50°C/min to -150°C. The straws were then transferred to liquid nitrogen. Thawing was performed at room temperature by washing the oocytes through a series of decreasing concentrations of cryoprotectant solutions, with the final dilution containing sucrose. Live births have been reported using similar conventional slow-cooling methods in several species including mice (Whittingham, 1977), rabbits (Al- Hasani *et al.*, 1989), cattle (Fuku *et al.*, 1992; Otoi *et al.*, 1992) and humans (Chen, 1986). A list of births resulting from oocyte cryopreservation is listed in Table 4.

2.7.2 Vitrification

An alternative approach to cryopreservation is vitrification. Conventional methods of cryopreservation have been developed to accommodate the consequences of ice formation. Vitrification is a process which, by combining the use of concentrated solutions with rapid cooling, avoids the formation of ice. Samples reach low temperatures in a glassy state, which has the molecular structure of a viscous liquid. Vitrification avoids the formation of ice crystals in the intracellular and extracellular space (Rall and Fahy, 1985). It involves the use of high concentrations (5-7 M) of cryoprotectants and ultra-rapid cooling rates (2,000-25,000°C/min). Rall (1987) noted that theoretically even water can be vitrified using a cooling rate of 107°C/sec. The cells are dehydrated by exposure to high concentrations of cryoprotectants prior to cryopreservation, which also increases the viscosity of the solution. Cells suspended in cryoprotectants when plunged directly into liquid nitrogen, form a glass-like suspension of cells. This technique completely eliminates intracellular ice formation (Porcu, 2004). However, cells may be damaged by exposure to such high concentrations of cryoprotectants. Nevertheless, vitrification has the advantage of being low-cost, since it eliminates the need for programmable freezing equipment, and it is quicker and easier to perform compared to slow-cooling.

Although most living organisms are composed of large amounts of water, it is not inevitable that freezing these organisms results in ice-

formation. Among amphibians and insects that can tolerate freezing, there is wide variation in the amount of freezing they can tolerate. According to an article on the subject in the December 1990 issue of Scientific American, woolly bear caterpillars "may spend 10 months of the year frozen solid at temperatures that descend to -50°C". Some species of frogs can spend days or weeks "with as much as 65 percent of their total body water as ice". Some amphibians achieve their protection due to the glycerol manufactured by their livers. Glycerol is "antifreeze", as it reduces ice formation and lowers the freezing point. The sugar glucose is also a cryoprotectant, and arctic frogs have a special form of insulin that accelerates glucose release and absorption into cells as temperatures approach freezing.

Insects most often use sugars as cryoprotectants. Adult arctic beetles (*Pterostichus brevicornis*) normally endure temperatures below -35°C. These beetles have been frozen in the laboratory to -87°C for 5 hours without apparent injury, i.e., they demonstrated "directed, coordinated activity such as walking, feeding, and avoidance response, and no paralysis or erratic behavior. This would seem to indicate that neurological tissue could, in principle, recover in a functional way from vitrification. However, the glycerol, sugars, and other cryoprotectants, which are produced naturally in these organisms, are not found in levels that adequately explain the remarkable freezing-tolerance.

The procedure of vitrification of embryos was first described by Rall and Fahy (1985) who showed that mouse embryos could be successfully cryopreserved by suspending them in concentrated solutions of cryoprotectants and cooling them rapidly in liquid nitrogen. Vitrified mouse embryos were subsequently shown to develop to normal offspring (Rall, 1987). This procedure has become increasingly important as an alternative method of cryopreserving oocytes, since many reports have suggested that vitrification, rather than conventional slow-cooling, might be a better method to cryopreserve mammalian oocytes. Vitrification has been used to cryopreserve oocytes of mice (Chen-shee, 2000; Lane and Gardner, 2001), rats (Nakagata, 1992), goats (Begin *et al*, 2003), horses (Maclellan *et al.*, 2002), humans (Kuleshova *et al.*, 1999; Liebermann and Tucker, 2002; Yoon *et al.*, 2000) and cattle (Dinnyes *et al.*, 2000; Le Gal and Massip; 1999; Le Gal *et al.*, 2000;

Matsumoto *et al.*, 2001; Mavrides and Morroll, 2002; Otoi *et al.*, 1998; Papis *et al.*, 2000; Vajta *et al* 1998; Vieira *et al.*, 2002). Apart from this, bovine blastocysts have been produced by transfer of nuclei from adult fibroblasts into oocytes or cytoplasts that had been vitrified (Booth *et al.*, 1999).

Table 4. First births resulting from the successful cryopreservation of mammailan oocytes

Species	Method	Author
Mouse	Slow-cooling	Parkening *et al.* (1976)
	Vitrification	Kono *et al.* (1991)
Rabbit	Slow-cooling	Al-Hasani *et al.* (1989)
	Vitrification	Vincent *et al.* (1989)
Cattle	Slow-cooling	Fuku *et al.* (1992)
	Vitrification	Vajta *et al.* (1998)
Human	Slow-cooling	Chen (1986)
	Vitrification	Kuleshova *et al.* (1999)
Horse	Vitrification, Cryoloop	Maclellan *et al.* (2002)

2.7.2.1 Vitrification of immature bovine oocytes

In one of the early studies, when immature pronuclear stage bovine oocytes were vitrified with a vitrification solution consisting of 40% EG, 0.3 M trehalose and 20% PVP in Dulbecco's phosphate buffered saline negative (DPBS-), positive (+) and NaCl (Saha *et al.*, 1996), after IVF, the cleavage rates were 23, 21 and 13%, respectively, and the blastocyst formation was 3, 3 and 1%, respectively. Vitrification of immature bovine oocytes in a mixture of 25% glycerol and 25% EG has been reported to result in a post fertilization cleavage rate of 16%, although none out of the 146 oocytes taken developed to the blastocyst stage (Le Gal and Massip 1999). In another study, Justin Tan (2004) found that bovine oocytes can be vitrified successfully with a mixture of 15% EG and 15% PROH, the survival rates were higher for oocytes vitrified without cumulus cells, and pre-incubation before insemination was not found to be necessary for the vitrification of oocytes.

2.7.2.2 Vitrification of bovine mature oocytes

In one of the earliest studies on the cryopreservation of in vitro matured bovine oocytes by vitrification, Hamano *et al.* (1992) vitrified oocytes in a mixture of 2.0 M DMSO, 1.0 M acetamide and 3.0 M PROH. After IVF and

IVC of frozen-thawed oocytes, 9 out of 88 inseminated oocytes developed to the blastocyst stage and transfer of 3 blastocysts to 3 recipients resulted in 2 pregnancies. Saha *et al.* (1996) vitrified in vitro matured oocytes with a vitrification solution consisting of 40% EG, 0.3 M trehalose and 20% PVP in Dulbecco's phosphate buffered saline negative (DPBS-), positive (+) and sodium chloride (NaCl). After IVF, the cleavage rates were 27, 18 and 14%, respectively, and the blastocyst formation was 3, 4 and 3%, respectively.

Otoi *et al.* (1998) examined the effects of stepwise addition before exposure of oocytes to vitrification solution consisting of 30% EG with 0.35 M sucrose. The rates of morphological survival and cleavage of oocytes vitrified by the three-step addition procedure were higher than those vitrified by the one-step addition procedure. In the same study, these authors reported that the survival rates of oocytes vitrified in 20 and 30% EG were lower than those in 40 and 50% EG, and that the rates of cleavage and development to blastocysts vitrified in 40% EG were the highest among the four groups. Le Gal and Massip (1999) vitrified in vitro matured bovine oocytes in a mixture of 25% glycerol and 25% EG. Although they obtained post fertilization cleavage rate of 22%, none out of the 99 oocytes taken developed to the blastocyst stage.

2.7.3 Cryopreservation of Oocytes by Ultra Rapid Methods

Injury of oocytes resulting from exposure to temperatures near 0°C is a time-dependent phenomenon (Martino, 1996). Therefore, various methods have been derived to increase cooling and warming rates to "outrace" chilling injury. These include use of electron microscope grids as a carrier of oocytes (Martino, 1996), placing oocytes into small diameter open pulled straws (Vajta *et al.*, 1998), dropping 1- to 2-µl volumes of medium containing oocytes directly onto a cold surface at -150°C (Dinnyes *et al.*, 2000), or onto films of CPA within small nylon cryoloops (Lane *et al.*, 1999). The rationale of all of these methods is that the oocytes are suspended in very small volumes of medium so that they can be cooled at extremely high rates.

2.7.3.1 Vitrification using electron microscope grids

In 1996, Martino *et al.* reported that bovine oocytes could be cryopreserved within a thin film (<1 µL) of vitrification solution placed on an

electron microscope (EM) grid. Because the grid is minute (3.05 mm in diameter and 0.037 mm thick), it is possible to cool the sample literally in an instant when it is plunged in liquid nitrogen. They compared this method to conventional straw freezing. After >20 h in culture post-warming, oocytes cryopreserved on grids had a 51% to 72% survival rate compared to 34% of the oocytes cryopreserved in straws. After IVF, 40% of the oocytes that had been cryopreserved on grids cleaved when placed into culture and 15% developed into blastocysts. The straw method resulted in a 3% cleavage rate and <1% blastocyst development. Rates of blastocyst formation of oocytes cryopreserved on grids, although significantly lower than controls, were significantly higher compared to that of oocytes cryopreserved in conventional straws. The EM grid can be stored in liquid nitrogen using a structure that is made up of cryovial cap and goblet (Parks *et al.,* 2001).

2.7.3.2 Open pulled straw vitrification

A capillary system named the open-pulled straw (OPS) was devised by Vajta *et al.* in 1997.They heat softened insemination straws (0.25 mL) and pulled them manually until the inner diameter and the wall thickness of the central part decreased from 1.7 to ~0.8 mm and from 0.15 to ~0.07 mm, respectively. Then, the straws were cut at the narrowest point with a razor blade. Embryos were loaded by means of the capillary effect by placing the narrow end in a drop of vitrification solution containing embryos, and aspirating the solution in a 2–3 cm long column (1.0–1.5 µL). Although it would be difficult to measure the precise rates of cooling and warming, it is estimated that the liquid column in OPS is cooled at 22,500°C/min (between −25 and −175°C), whereas the rate in the conventional straw is 2500°C/min. Three (3/14; 21%) normal bull calves were born after non-surgical transfer of embryos resulting from IVF of oocytes previously cryopreserved through this method (Vajta *et al.,* 1998).

Isachenko *et al.* (2001) also found that the OPS method of vitrification, when used to cryopreserve ovine GV stage oocytes, resulted in a higher number of MII stage oocytes after culture than the conventional straw freezing. Using OPS, Tecirlioglu (2003) reported for the first time, the birth of a cloned calf derived from vitrified cloned embryo produced through hand

made cloning. In another study, Lopatrova *et al.* (2002) demonstrated the survival of bovine embryos from superovulated cows vitrified by OPS. They demonstrated the same survival rates for vitrified and conventionally cryopreserved embryos of all quality grades and developmental stages during in vitro embryo culture. Expanded blastocysts survived better by vitrification than by slow freezing. Recently, the findings of Albrracin (2005) suggested that the OPS vitrification protocol has deleterious effects on organization of the meiotic spindle of calf oocytes cryopreserved at both GVBD and MII stages. OPS technique has also been used in pigs, with successful birth of piglets (Birthelot *et al.*, 2000).

Booth *et al.* (1999) first enucleated bovine oocytes and then vitrified them by the OPS method; the cryopreserved cytoplasts were then used for SCNT. Fusion rates of vitrified (83.7 ± 9.2%) and control cytoplasts (79.8 ± 4.6%) were not significantly different. However, cleavage and blastocyst development of the vitrified cytoplast group (55.7 ± 2.9% and 7.2 ± 5.0%, respectively) was significantly different than that for the control group (92.8 ± 3.9% and 32.6±7.8%, respectively). One SCNT blastocyst transferred to a recipient divided in utero, giving rise to twin calves. Unfortunately, both were dead at birth.

2.7.3.3 Solid surface vitrification

Dinnyes *et al.* (2000) developed a vitrification procedure that used a precooled metal surface (solid-surface vitrification; SSV) and small amounts of vitrification solutions dropped directly onto this metal surface. In their IVF experiment, they found a 58 to 62% cleavage rate compared to 69% for controls. Of the vitrified group of oocytes, 11 to 19% developed to blastocysts on day 9 compared with 33% for the control group. SSV and cryotube vitrification methods were compared for vitrification of pronuclear stage mouse embryos by Bagis *et al.* (2004). The results showed that both SSV and cryotube vitrification methods could result in a high rate of in vitro blastocyst development (58.3 and 68.5%, respectively).

2.7.3.4 Cryoloop vitrification

A refined system called the cryoloop, which consisted of a minute nylon loop (20 µm wide, 0.5–0.7 mm in diameter) mounted on a stainless steel pipe

inserted into the lid of a cryovial was devised by Lane et al. (1999). The cryoloop has been successfully used to cryopreserve oocytes and embryos of mouse (Lane and Gardner, 2001), rhesus monkey (Yeoman *et al.*, 2001), hamster (Lane *et al.*, 1999), cattle (Le Gal *et al.*, 2000), human (Kuleshova *et al*, 1999), horse (Maclellan *et al.*, 2002), goat (Begin *et al.*, 2003) and rabbit (Hochi *et al.*, 2004).

Lane and Gardner (2001) compared the cryoloop method of vitrification with slow cooling using sodium-free medium, replacing sodium with choline. They used the cryoloop to vitrify mouse oocytes that they fertilized after puncturing the zona pellucida with a laser. They found a 99.3% survival rate using the cryoloop for vitrification compared to 80.9% survival rate using slow-freezing. The cryoloop resulted in 69.7% fertilization and 67.1% blastocyst rates after IVF post-warming. These results were significantly higher than the rates achieved with the slow-freezing methods (39.7 and 25.9%, respectively). After transfer to foster recipient mice, the resultant embryos from IVF using vitrified-warmed oocytes developed to full-term fetuses and offspring. In the vitrified oocyte group, 52 of 92 (56.5%) blastocysts transferred resulted in viable fetuses, which was significantly higher than that for the slow-freezing group (11 of 42, 26.2%).

In an another study by Hochi *et al.* (2004), pronuclear stage rabbit zygotes were cryopreserved using cryoloop. It resulted in higher proportions of zygotes cleaving (94%) and developing to blastocysts (51%). Equine oocytes have also been cryopreserved using this method (Hochi *et al.*, 2004; Hurtt *et al.*, 2000) leading to birth of healthy foals after transfer of vitrified-warmed oocytes into inseminated mares (Maclellan *et al.* 2002). Some investigators have cryopreserved oocytes to alleviate logistical problems encountered in SCNT methods. Using equilibrium cooling, Kubota *et al.* (1998) cryopreserved bovine oocytes to serve as cytoplasts for SCNT, ultimately producing a normal calf from the 206 oocytes that had been frozen.

2.7.3.5 Vitrification using microdrops

Use of microdrops is another method used for vitrification. Bovine blastocysts have been produced by SCNT into oocytes that had been vitrified as microdrops on a metal surface at -150°C (Dinnyes *et al.*, 2000). After

SCNT, the vitrified-warmed oocyte group had an 85% cleavage rate and 27% blastocyst production rate at day 9 post insemination. This was not significantly different from a 90% cleavage rate and a 29% day 9 blastocyst rate in the control group.

2.8 STATUS IN BUFFALO

The superior dairy merit of buffalo and its contribution to the dairy industry has been realized for long. Buffalo has also attracted considerable attention in terms of its low reproductive efficiency, and attempts are currently underway to develop several new technologies for augmenting its reproduction and production. In the 80s, the major emphasis was on Embryo Transfer Technology (ETT) but due to its limited success, the focus has now shifted to In Vitro Embryo Production (IVEP, Palta and Chauhan 1998, Chauhan *et al.*, 1996, 1997;a,b,c,). IVEP has been successfully used in buffalo for the production of embryos (Chauhan *et al.*, 1998;a,b,c,d,e,f; Wani *et al.*, 2004b) and live offspring (Duran *et al.*, 2004). IVEP offers a means for the faster multiplication of superior germpasm through production of live offspring from embryos produced from buffaloes of superior genetic merit using oocytes obtained from these animas by Ovum Pick-Up (OPU), aso called Tranvaginal Oocyte retrieval (TVOR), the techniques for which are now avaiabe in buffalo (Manik *et al.*, 2002; Gupta *et al.,* 2005). Besides IVEP, a number of other reproductive technologies are currently under development for buffalo viz. transgenesis, cloning, production of embryonic stem cells etc. Availability of usable quality oocytes in suffcient numbers and at the time of choice is essential prerequisites for the development of these technologies. This can be ensured only through cryopreservation of oocytes. Although a plethora of reportes are now available on IVEP, very little work has been done in the area of cryopreservation of buffalo oocytes. The information available is discussed below.

2.8.1 Retrieval of Oocytes

Although the oocytes obtained from abattoir ovaries cannot be used for the production of live offspring of known pedigree since the genetic background of the animal from which the ovaries were obtained is not known, slaughterhouse ovaries remain the most important source for obtaining

oocytes in large numbers. Among the 3 different methods commonly employed for collecting follicular oocytes from slaughterhouse ovaries i.e., dissection, aspiration and slicing, aspiration of the visible surface follicles is the most commonly used method for recovering the oocytes. Oocytes can also be obtained from live animals on a repeated basis through ultrasound-guided TVOR also called OPU. This technique has been successfully used for the collection of oocytes from live buffaloes (Manik *et al.*, 2002; Gupta *et al.*, 2005). Since the pedigree of oocytes obtained through TVOR is known, such oocytes can be subjected to IVMFC for the production of live offspring of known pedigree.

The recovery of total as well as usable quality oocytes has been reported to be much lower in buffalo compared to that in cattle. Aspiration of more than 4600 buffalo ovaries has been reported to provide only 0.4 good quality oocytes per ovary (Madan *et al.*, 1994a). Das *et al.* (1996) reported a per ovary recovery of 0.7 ± 0.10 cumulus-oocyte complexes (COCs), with a recovery of 1.7 ± 0.18 total oocytes through aspiration of buffalo ovaries. However, Singla (1996) reported recovery of 1 good quality oocyte per ovary. Nandi *et al.* (2000) reported an average recovery of 0.6 good quality oocytes per ovary. These values are much lower than the average recovery of more than 10 good quality oocytes per ovary in cattle (Gordon 1994).

The collected oocytes are selected for IVM on the basis of their morphological appearance i.e., presence of multilayer compact cumulus oophorus and homogenous cytoplasm. A scheme for the classification of buffalo oocytes for predicting morula and blastocyst yield was reported by Chauhan *et al.* (1998b). These authors classified the aspirated COCs with homogenous cytoplasm as Grade-1 (compact COCs with an unexpanded cumulus mass having >5 layers of cumulus cells) and Grade-2 (COCs similar to those of Grade-1, but with 1-4 layers of cumulus cells). Grade-3 oocytes were without cumulus cells and/or with shrunken cytoplasm.

2.8.2 In Vitro Maturation, Fertilization and Culture of Oocytes

The technique of IVMFC of oocytes involves IVM of selected oocytes in media containing serum and hormones, IVF by frozen-thawed in vitro capacitated spermatozoa, and IVC of embryos in media either in the presence

of oviductal or cumulus cells or in specialized media like synthetic oviductal fluid (SOF) up to the morula or blastocyst stage.

2.8.2.1 In vitro maturation

Maturation of oocytes can be divided into two stages, nuclear maturation and cytoplasmic maturation. Cytoplasmic maturation encompasses all of the changes in the distribution and organization of specific organelles from the GV to the M-II stage. Cytoplasmic maturation is associated with the cellular changes that occur during nuclear maturation such as mitochondria repositioning and cortical granule migration (Mayer and Sirard, 2001). Nuclear maturation can be defined as the series of physiologic events that reflect the modification of chromatin from the diplotene phase of meiosis I to M-II phase.

The highest percentage of buffalo oocytes at the M-II stage occurs after 24 h of in vitro culture, after which the nuclear material begins to degenerate (Yadav *et al.*, 1997). For in vitro maturation of buffalo oocytes, groups of COCs of 10-15 each are cultured for 22-24 h in 50-100 µl droplets of IVM medium under sterile paraffin oil at 38.5 to 39°C in a 5% CO_2 in air atmosphere with 90-95% relative humidity (Chauhan *et al.*, 1996). The maturation process is evaluated by stripping off the cumulus mass followed by staining the oocytes with Giemsa stain (Chauhan *et al.*, 1996; Das *et al.*, 1997) or aceto-orcein (Madan *et al.*, 1994a) for observing the presence of M-II stage chromosomes. Tissue culture medium-199 (TCM 199) is the most commonly used medium for IVM of buffalo oocytes. It is generally supplemented with fetal bovine serum (FBS), luteinizing hormone (LH) and follicle stimulating hormone (FSH, Chauhan *et al.*, 1996; 1997a, b, c; 1998a, b, c, d, e, f; 1999; Das *et al.*, 1997; Nandi *et al.*, 1998).

2.8.2.2 In vitro fertilization and culture

The spermatozoa are artificially capacitated by incubation with heparin (around 10 µg/ml) for 4-6 h prior to their incubation with the oocyte for performing IVF. The post-thaw motility of spermatozoa is increased prior to IVF by pretreatment with a motility-inducing agent like caffeine (Chauhan *et al*,. 1997a, b, c, ; 1998a, b, c, d, e, f; 1999), theophylline (Chauhan *et al.*,

1998d) or a mixture of penicillamine, hypotaurine and epinephrine (PHE, Totey *et al.*, 1996).

The basic media used for IVF of buffalo oocytes include Brackett and Oliphant (BO) medium (Brackett and Oliphant, 1975) or Tyrode's modified medium (TALP, Bavister et al., 1992; Totey *et al.*, 1996). An appropriate concentration of spermatozoa is incubated with the oocyte for an optimum length of time for performing IVF. The duration of incubation is dependent on the sperm concentration used for IVF.

2.8.3 Cryopreservation of Oocytes

2.8.3.1 Slow freezing

To our knowledge, no report is available in buffalo on slow freezing of immature or mature oocytes.

2.8.3.2 Vitrification

Dhali *et al.* (2000a) reported the development of a method for the cryopreservation of buffalo oocytes by vitrification. Buffalo oocytes were vitrified in the vitrification solution consisting of 4.5 M EG and 3.4 M DMSO. Prior to this, the oocytes were exposed to the equilibration solution (50% vitrification solution) for 1 or 3 min at room temperature. The proportion of oocytes recovered in a morphologically normal form was found to be higher (98 and 88%, respectively), and the proportion of oocytes recovered in a damaged form was found to be significantly lower (2 and 12%, respectively) for the 3-min equilibration than for 1 min. The nuclear maturation rate, however, did not differ between the 1 and 3 min equilibration periods (21.5 ± 10.7 and 31.5 ± 1.5%, respectively).

In a later study Dhali *et al.* (2000b) investigated the effects of EG concentration (equilibration solution-I i.e., 4.5 M EG + 3.4 M DMSO vs equilibration solution-II i.e., 3.5 M EG + 3.4 M DMSO) and time of exposure to equilibration solution (1 min vs 3 min) on the post-thaw morphological appearance and the in vitro maturation rate of buffalo oocytes. The percentage of oocytes found to be morphologically normal varied from 89 to 96% for the two vitrification solutions and the two exposure times. Among the damaged oocytes, cracking of zona pellucida was the abnormality observed

most frequently. The nuclear maturation rate of oocytes equilibrated in equilibration solutions-I and II for 1 (28 and 24%, respectively) or 3 min (32 and 33%, respectively) did not differ significantly.

In a recent study, Wani *et al.* (2004a) compared the effects of 5 different concentrations (3.5, 4, 5, 6 and 7 M) each of 4 cryoprotectants (DMSO, EG, PROH and glycerol) on the morphological survival and in vitro maturation rate of vitrified-thawed immature buffalo oocytes. Among the different cryoprotectants, the highest maturation rate was obtained with 7 M DMSO, EG, PROH and glycerol (40.3, 42.5, 40.4 and 23.5%, respectively). Oocytes reaching to metaphase-II stage from the oocytes cryopreserved in 7 M glycerol were significantly lower than that of oocytes vitrified in DMSO, EG and PROH. In an extension of the same study, these authors studied the post fertilization developmental competence of immature buffalo oocytes vitrified using the same concentrations of different cryoprotectants as discussed above (Wani *et al.*, 2004b). The cleavage rates of oocytes vitrified in different concentrations of DMSO, EG, PROH and glycerol were lower than that of the fresh embryos. The cleavage rates were higher for the oocytes vitrified in 6 and 7 M DMSO (29.8 and 19.5%, respectively), EG (30.8 and 20.4%, respectively), PROH (27.3 and 17.7%, respectively) and glycerol (21.0 and 15.4%, respectively) compared with oocytes cryopreserved in other concentrations. However, the percentage of morula and blastocyst formation from the cleaved embryos did not vary in fresh and vitrified oocytes. In another recent study, Atabay (2004) reported for the first time, the production of Swamp buffalo somatic cell nuclear transferred embryos using vitrified bovine oocytes as recipient cytoplasts.

CHAPTER – 3

Materials and Methods

3.1 MATERIALS

3.1.1 Plasticware and Glassware

All the glassware used in the present investigation were made of high-grade pyrex glass. The glassware, wherever used, were thoroughly cleaned, rinsed with Ultrapure water and then heat sterilized at 250°C for 4 h. Disposable 35 mm x 10 mm cell culture Petri dishes, 15 and 50 ml Falcon tubes, 100 mm x 100mm square Petri dishes with 13 mm grid and Unopettes (20 µl capacity) were purchased from Becton, Dickinson and Co., Lincoln Park, NJ, USA. Disposable plastic syringes were non toxic and non-pyrogenic from Sigma Aldrich Chemicals (Norm-Ject, Henke-Sass Wolf GmbH, Germany). Disposable 19-guage hypodermic needles were from Dispovan (India) whereas the 0.22 and 0.45 µm filters were from Millipore Corporation, Bedford, MA, USA. Autoclavable disposable tips for micropipettes were obtained from Labware, USA. French straws (0.25 ml) were from IMV, L'Aigle, France.

3.1.2 Chemicals, Cell Culture Media and Supplements

The culture media used in the present study, which included tissue culture medium-199 (TCM-199), Dulbecco's modified eagle's medium (DMEM) Dulbecco's phosphate buffered saline (DPBS). The additives which included fetal bovine serum (FBS), bovine serum albumin (BSA) and antibiotics (gentamycin, penicillin and streptomycin were purchased from Sigma Chemical Co., St. Louis, MO, USA. All the cell culture media were in the form of ready-to-use liquid media. Mineral oil, the cryoprotectants used i.e., dimethyl sulphoxide (DMSO), ethylene glycol (EG) and 1,2-propanediol (PROH) and other chemicals used were also from Sigma, unless otherwise indicated. Most of the chemicals used were embryo culture tested or of cell culture grade

3.1.3 Equipment

3.1.3.1 Microscopes

The microscopes used during this study belonged to the following categories:

Low magnification zoom stereomicroscope (Nikon, Japan, Model SMZ-2T) was used for searching the aspirated oocytes, evaluating the topography and quality of oocytes, cell cultures and tissue explants.

An inverted microscope (Nikon, Japan, Model TMD) was used for examining the morphological changes before and after cryopreservation, cumulus expansion after IVM, monitoring the cell health, morphological characteristics and growth of the cultured cells. The microscope with the light source at the top and a long working distance condenser allowed cells in cell culture dishes or flasks to be viewed and photographed whenever needed. The microscope was equipped with an incubator attachment, so that optimum temperature conditions could be maintained during working and examination of oocytes and embryos. The inverted microscope was also equipped with UV fluorescence and differential interface contrast (DIC) attachment, which was used for capturing the images of the oocytes and in vitro produced embryos. The microscopes were equipped with programmable still-photography and video recording facilities.

A compound microscope (Nikon, MICROPHOT-FXA) with a movable slide holding stage, and photography facilities was used for examining the maturation status of the oocytes.

3.1.3.2 CO_2 incubator

A Thermo Forma Scientific (USA) make CO_2 incubator, with facility to maintain humidified environment, adjustable incubation temperature and CO_2 concentrations was used for the culture of cells, oocytes and embryos.

3.1.3.3 Programmable freezer

The programmable freezer used for slow freezing of oocytes was of Cryocell-AP make (Hoxan, Japan)

3.2 METHODS

3.2.1 Preparation of Different Media

For details regarding the composition of various media used in the present study, please see ANNEXURE-I.

3.2.2 Collection of Buffalo Serum and Follicular Fluid

Blood was collected from the jugular vein of healthy buffalo heifers and allowed to clot in slanting test tubes at ambient temperature. Care was taken to avoid the exposure of collected blood directly to bright sunlight. The serum was separated by centrifugation (12000 X g for 15 min). Serum was filtered through 0.45 µm filters, dispensed as 5 ml aliquots, and stored at -20°C till further use.

For the collection of follicular fluid, buffalo ovaries are were obtained from Delhi slaughterhouse and transported to the laboratory at 4°C within 4-5 h of slaughter. Follicular fluid was aspirated from all visible surface follicles (4 to 10 mm in diameter) with a 19-gauge needle. The cellular debris was removed by centrifugation at 3000 rpm for 30 min. The supernatant was carefully collected and filtered through 0.22 µm filter. The follicular fluid was divided into 400 µl aliquots (one aliquot required at a time) in 1.5 ml microcentrifuge tubes and stored at -20°C until further use. The same pool of buffalo follicular fluid (buFF) was used throughout each experiment.

3.2.3 In Vitro Maturation, Fertilization and Culture of Oocytes

3.2.3.1 Collection and classification of oocytes

Buffalo ovaries were collected from the slaughterhouse at New Delhi immediately after slaughter, and washed twice with sterile normal saline supplemented with antibiotics (50 µg/ml gentamycin sulphate) at 32 to 37°C. The ovaries were then transported to the laboratory in a stainless steel thermos flask maintained at 32-37°C within 4-6 h. In the laboratory, the ovaries were rinsed twice with warm saline containing antibiotics.

Oocytes were collected by aspiration of surface follicles (2-8 mm diameter) with a 19-gauge needle attached to a 10 ml syringe containing the aspiration medium (TCM-199 + 10% FBS and DPBS + 0.6% BSA in a 1:1 ratio). The contents of the syringe, which included the aspirated oocytes, follicular fluid, granulosa cells and other debris, were poured in 100 mm x 100 mm square Petri dishes with 13 mm grid (searching dishes). The oocytes were searched under a zoom stereomicroscope at around 20 x magnification. Cumulus-oocyte complexes (COCs) with an unexpanded multilayered

cumulus cells, and homogenous ooplasmic granulation were chosen. The oocytes were then shifted to 35 mm x 10 mm cell culture Petri dishes containing the washing medium (TCM-199 + 10% FBS + 0.81 mM sodium pyruvate).

The aspirated oocytes were graded according to the following criteria already in use in the laboratory:

Grade-A: Oocytes with homogeneous cytoplasmic granulation, multilayerd unexpanded cumulus cell layers associated with the oocyte.

Grade-B: Oocytes with homogeneous cytoplasmic granulation, 3-4 layers of unexpanded cumulus cell layers associated with the oocyte.

Grade-C: Oocytes with homogeneous cytoplasmic granulation, with a few or irregularly arranged unexpanded cumulus cell layers associated with the oocyte. Some of the oocytes with expanded cumulus cells were also graded as C grade oocytes.

Grade-D: deformed or irregular ooplasm without no cumulus cells associated. C and D grade oocytes were not used, though the number was recorded.

Oocytes of only grades A and B were used for further experimentation.

3.2.3.2 In vitro maturation of oocytes

For in vitro maturation, the oocytes were washed 4-6 times with the washing medium (TCM-199 supplemented with 2mM HEPES buffer, 10% FBS, 0.5 mM sodium pyruvate and 50 μg/ml gentamycin sulphate), and then twice with the IVM medium (TCM-199 + 10% FBS + 5 μg/ ml porcine FSH + 0.81 mM sodium pyruvate + 5% buffalo follicular fluid). Groups of 15-20 COCs were placed in 100 μl droplets of the IVM medium, overlaid with mineral oil in a 35 mm Petri dish, and cultured for 24 h in a humidified CO_2 incubator (5% CO_2 in air) at 38.5°C.

3.2.3.3 In vitro fertilization and culture of oocytes

The spermatozoa used for IVF throughout the study were from the same donor, which had been tested for IVF earlier. The spermatozoa were prepared for fertilization as described earlier (Chauhan et al., 1998). Briefly, two straws of frozen thawed buffalo semen were washed with BO medium

containing heparin (10 µg/ml) without BSA. The pellet was resuspended in around 0.5 ml of the washing BO medium. The spermatozoa (50 µl of the suspension) were then transferred to 50 µl droplets of capacitation and fertilization BO medium. The in vitro matured oocytes were washed twice with the washing BO medium and transferred to these 50 µl droplets of spermatozoa (15 to 20 oocytes per droplet). The droplets were overlaid with sterile mineral oil and placed in a humidified CO_2 incubator (5% CO_2 in air) for 18 h at 38.5°C for IVF. At the end of the sperm-oocyte incubation, the oocytes were separated from the sperm droplets and washed with IVC medium (mCR2aa with 0.8% BSA, fraction-V,). The medium was replaced with 50% of fresh simple culture medium (mCR2aa supplemented with 10% FBS) at 48-h intervals. The zygotes were cultured for 8-10 days and developmental competence was recorded from day 2 (48 h) of culture.

3.2.4 Slow Freezing of Immature Oocytes

3.2.4.1 Procedure for freezing and warming

COCs were washed 2 times with DPBS containing 15% FBS and 5 µg/ml gentamycin sulphate. The oocytes were exposed to the final concentration of the cryoprotectants i.e., DMSO, EG or PROH in a 2- (0.5 and 1.0 M for the final concentration of 1.0 M) or a 3-step manner (0.5, 1.0 and 1.5 M for the final concentration of 1.5 M) at 10 min intervals at room temperature (23 to 28°C). At the same time, 0.25-ml French straws were properly marked using a cryoware marker indicating the number of oocytes and date of cryopreservation. The oocytes were equilibrated in the final concentration of the cryoprotectant for 30 min at room temperature after which groups of 20-30 oocytes were loaded into 0.25-ml French straws, which were then sealed with polyvinyl alcohol powder. The straws containing oocytes were then placed in a cooling chamber in a programmable freezer and subjected to slow freezing following the cooling parameters of Im et al. (1997). Briefly the straws were cooled at a rate of -1°C /min to -6°C, automatically seeded by the freezer, held at this temperature for 7 min, cooled at a rate of -0.3°C /min to -30°C and maintained at this temperature for 10 min. The straws were then placed 1 to 2 cm above the surface of liquid nitrogen for 20 sec and plunged in liquid nitrogen. The cooling parameters used were as follows:

I.T: 25.0°C, R.1: -1.0°C, P.1: -0.6°C , K.T: 7.0 min, R.2: -0.3°C,P.2: -30.0°C, K.T: 10.0 min

Where I.T., R, P and K.T. stand for initial temperature, rate, pause and keep time, respectively.

All the cryopreserved oocytes were stored for at least 7 days in liquid nitrogen. For warming, the straws were plunged into a water bath at 37°C for 20 sec. The contents of the straws were then drained into a sterile 35 mm Petri dish. The dilution of the cryoprotectants was done in a 2- (for 1.0 M) or 3-step manner (for 1.5 M), by decreasing the concentrations of cryoprotectants by 0.5 M, at 10 min intervals at room temperature. After removal of the cryoprotectant, oocytes were washed twice with the washing medium.

3.2.4.2 Morphological evaluation of frozen-thawed oocytes

For evaluation of post-thaw morphology, the thawed oocytes were examined under an inverted microscope at 400 x. The criteria used for assessing the post-thaw morphology of frozen-thawed oocytes were as follows:

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

Abnormal: Oocytes with cracked zona pellucidae, split in two halves, leaked contents, shrunken cytoplasm, change in shape and partly or fully denuded oocytes.

3.2.4.3 Developmental potential of frozen-thawed oocytes

The in vitro developmental potential of morphologically normal frozen-thawed immature oocytes was examined by

i) Determining the nuclear maturation rate after subjecting the oocytes to IVM and

ii) Determining the proportion of oocytes which developed to morula or blastocyst stages after subjecting them to IVM, IVF and IVC

For determining the rate of nuclear maturation, the oocytes were subjected to IVM as described under 3.2.3.2. The in vitro cultured oocytes

were washed 2-3 times with DPBS containing 0.3% BSA and denuded by vortexing. For this, groups of 20-30 COCs were taken in a 1.5 ml microcentrifuge tube containing 0.5 ml of TCM-199+10% FBS and vortexed for 2 min. In addition to the frozen-thawed oocytes, a group of around 50 COCs was run as a control in each trial of IVM.

The denuded oocytes were washed 2-3 times with DPBS and transferred to 100 µl droplet of hypotonic solution and incubated at 38°C for 40 min. Chromosomal preparations were made using gradual fixation method. The oocytes were transferred to 50 µl droplets of fixative-I for 5 min. Thereafter, the oocytes were taken on a clean slide in as small a quantity of fixative –I as possible and 8-10 drops of fixative–II were added drop wise. Fixative-II was allowed to dry completely resulting in sticking of oocytes to the slide. The slides were quickly transferred to a jar containing fixative-II for 5 min. Oocytes were subsequently treated briefly (1 min) with fixative–III to soften the zona pellucida and then dried in air. For staining, the slides were put vertically in the slots of a Coplin jar containing a 2% solution of Giemsa stain in distilled water for 30 min. The slides were then washed with distilled water, air-dried and mounted with DPX-mountant. A coverslip was placed over the DPX-mountant slide and it was pressed slowly with a glass rod in such a way that no air bubbles remained between the slide and the coverslip.

The oocytes were observed for the presence of germinal vesicle (GV), germinal vesicle breakdown (GVBD), Metaphase I and II and first polar body by examination under a compound microscope. Presence of chromosomes in metaphase-II or the presence of the first polar body was taken as occurrence of nuclear maturation.

For determining the in vitro developmental competence of frozen-thawed immature oocytes subjected to slow freezing, these oocytes were subjected to IVM, IVF and IVC as described earlier under 3.2.3.2 and 3.2.3.3. The cleavage rate was recorded on day 2 post insemination and the proportion of oocytes that developed to morula and blastocyst stages was recorded at day 8 or 9 post insemination. In addition to the frozen-thawed oocytes, a group of around 50 COCs was run as a control in each trial.

3.2.4.4 Experimental design and statistical analysis

Experiment 1

The immature oocytes were divided into 6 groups (three cryoprotectants, DMSO, EG and PROH, each used at two concentrations, 1.0 and 1.5 M) and subjected to slow freezing. The percentage of oocytes, which were recovered in a morphologically normal form, was recorded. The nature of damage was recorded in the oocytes that were recovered in a damaged form. This experiment was replicated 6 times.

Experiment 2

The oocytes were divided into 7 groups, Group 1 (Control, fresh oocytes directly subjected to IVM) and Groups 2-7 (oocytes recovered morphologically normal after being subjected to slow freezing using three cryoprotectants, each used at two concentrations). The oocytes were subjected to in vitro maturation and the rate of nuclear maturation was determined. This experiment was replicated 6 times.

Experiment 3

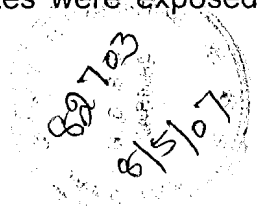
Since 1.5 M DMSO gave the best results in Experiments 1 and 2, oocytes subjected to slow freezing using this combination of cryoprotectant and concentration were subjected to IVF using non-frozen oocytes as controls in Experiment 3. This experiments was replicated 3 times.

The data of Experiments 1 and 2 were subjected to analysis of variance (ANOVA) using SYSTAT 7.0 statistical software (SPSS Inc., USA) after arc sin transformation. The pair-wise comparison of treatment means was carried out by Duncan's Multiple Range Test. Data of experiment 3 was analyzed for the test of significance by Student's t-test using SYSTAT 7.0 after arc sin transformation.

3.2.5 Slow Freezing of Mature Oocytes

3.2.5.1 Procedure for freezing and warming

Oocytes were subjected to IVM as described earlier under 3.2.3.2. Following IVM, the oocytes were washed with TCM-199 supplemented with 5% FBS, which was used as a carrier medium for different cryoprotectants. The oocytes were exposed to the final concentration of the cryoprotectants



i.e., DMSO, EG or PROH in a 2- (0.5 and 1.0 M for the final concentration of 1.0 M) or a 3-step manner (0.5, 1.0 and 1.5 M for the final concentration of 1.5 M) at 10 min intervals at room temperature (23 to 28°C). The oocytes were equilibrated in the final concentration of the cryoprotectant for 30 min at room temperature after which groups of 20-30 oocytes were loaded into 0.25-ml French straws, which were then placed in a cooling chamber in a programmable freezer and subjected to slow freezing at a cooling rate as described by Otoi et al. (1993). Briefly, the straws were cooled to -5.5°C at -1°C/min, automatically seeded by the freezer and then cooled at a rate of -0.6°C/min to -30°C. The straws were then plunged in liquid nitrogen. The cooling parameters were as follows:

I.T: 25.0°C, R.1: -1.0°C P.1: -5.5°C , K.T: 10.0 min, R.2: -0.6°C,

P.2: -30.0°C, K.T: 10.0 min

Where I.T., R, P and K.T. stand for initial temperature, rate, pause and keep time respectively.

After storage in liquid nitrogen for at least 1 week, the straws were warmed rapidly by placing plunging in a 37°C water bath for 30 sec. The contents of the straws were then drained into a sterile 35 mm Petri dish. The dilution of the cryoprotectants was done in a 2- (for 1.0 M) or 3-step manner (for 1.5 M), by decreasing the concentrations of cryoprotectants by 0.5 M, at 10 min intervals at room temperature. The oocytes were then washed twice with the washing medium.

3.2.5.2 Morphological evaluation of frozen-thawed oocytes

The evaluation of post-thaw morphology of thawed oocytes was carried out as described earlier under 3.2.4.2.

3.2.5.3 Developmental potential of frozen-thawed oocytes

The oocytes that were recovered in a morphologically normal form were subjected to IVM, IVF and IVC as described earlier. The cleavage rate was recorded on day 2 post insemination and the proportion of oocytes that developed to morula and blastocyst stages was recorded at day 8 or 9 post insemination. In addition to the frozen-thawed oocytes, a group of around 50 COCs was run as a control in each trial.

3.2.5.4 Experimental design and statistical analysis

Experiment 1

The oocytes were divided into 6 groups after IVM (3 cryoprotectants, each at 2 concentrations). The percentage of oocytes, which were recovered in a morphologically normal form, was recorded. The nature of damage was recorded in the oocytes that were recovered in a damaged form. This experiment was replicated 6 times

Experiment 2

Since 1.5 M DMSO gave the best results in terms of the oocytes recovered morphologically normal after thawing, oocytes subjected to controlled freezing using this concentration of cryoprotectant were subjected to IVF and IVC using non-frozen in vitro matured oocytes as controls. This experiment was replicated 6 times.

The data of Experiment 1 was subjected to analysis of variance (ANOVA) using SYSTAT 7.0 statistical software (SPSS Inc., USA) after arc sin transformation. The pair-wise comparison of treatment means was carried out by Duncan's Multiple Range Test. Data of Experiment 2 was analyzed for the test of significance by Student's t-test using SYSTAT 7.0.

3.2.6 Vitrification of Immature Oocytes

3.2.6.1 Preparation of vitrification solutions

The solution used as a carrier for the cryoprotectants was TCM-199 supplemented with 10% FBS, 0.35 mol/L sucrose and 5μg/ml gentamycin sulphate. This solution is hereafter mentioned as TF medium. EG was diluted to 10, 25 and 40% (v/v) concentrations in TF medium and these solutions were named E10, E25 and E40, respectively. Similarly prepared solutions for DMSO were labeled as D10, D25 and D40, respectively.

For making combinations of EG and DMSO, 10% EG and 10% DMSO were taken in TF (named as ED10) or 20% EG and 20% DMSO were taken in TF medium (named as ED20). Similarly, combinations of EG and PROH were also prepared (EP10 for 10% EG and 10% PROH and EP 20 for 20% EG and 20% PROH). All these solutions were prepared at room temperature (21–25°C).

3.2.6.2 Vitrification using EG or DMSO

For each trial, the immature oocytes were divided into 4 groups, each containing 25-30 oocytes. The oocytes of Groups 1 (E40) and 2 (D40) were exposed to the final concentration of the cryoprotectant in a 3-step manner by equilibrating the oocytes serially to 10%, 25% and 40% of the cryoprotectant. Briefly, the oocytes were placed into culture dishes containing 2 ml of medium with E10 or D10 for 1 min and were subsequently suspended to E25 or D25 for 30 sec before exposure to E40 or D40 solution. Groups of around 30 oocytes were then loaded into a 0.25 ml French straw, suspended in a 30 µl microdrop of E40 or D40 solution. The straw was then sealed with polyvinyl alcohol powder.

The oocytes were then loaded in to the straws as described below:

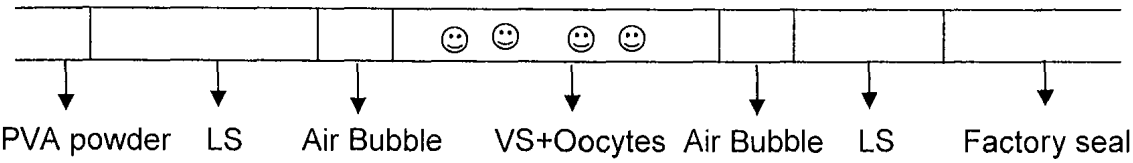


Fig.1 A 0.25 ml French straw containing oocytes. PVA stands for Polyvinyl powder; LS: loading solution; VS: Vitrification solution.

Approximately half of the straw, including the vitrification solution, was rapidly immersed in liquid nitrogen, and the rest of the straw was then immersed slowly to prevent bursting. This plunging in liquid nitrogen was done within 1 min of the final exposure of oocytes to E40 or D40 cryoprotectant solution.

3.2.6.3 Vitrification using combinations of EG+ DMSO or EG+PROH

The oocytes of Groups 3 (ED20) and 4 (EP20) were exposed to the final concentration of the cryoprotectants in a 2-step manner. The oocytes were placed in culture dishes containing 2 ml of TF medium with ED10 or EP10 for 1 min and were subsequently suspended to ED20 or EP20 for 30 sec. Groups of around 30 oocytes were then loaded into a 0.25 ml French straw, suspended in a 100 µl microdrop of ED20 or EP20 solution. The straw was then sealed with polyvinyl alcohol powder. The straws were then plunged in liquid nitrogen as mentioned above and stored for at least 7 days.

For thawing, the straws were held in air for 5-10 sec and then plunged in a water bath at 37°C for 10–15 sec. The contents of the straw were

expelled in a culture dish and the oocytes were then immediately transferred to fresh TF medium containing 0.5 M sucrose. A 3-step dilution was used for the oocytes treated with different cryoprotectants or their combinations (0.5, 0.33 and 0.17M sucrose, with I min equilibration in each solution). The oocytes were then transferred to fresh TF medium and washed several times with it.

3.2.6.4 Morphological evaluation of frozen-thawed oocytes

The evaluation of post-thaw morphology of vitrified-thawed oocytes was carried out as described earlier under 3.2.4.2.

3.2.6.5 Developmental potential of frozen-thawed oocytes

The oocytes that were recovered in a morphologically normal form were subjected to IVM, IVF and IVC as described earlier under 3.2.3.2 and 3.2.3.3. The cleavage rate was recorded on day 2 post insemination and the proportion of oocytes that developed to morula and blastocyst stages was recorded at day 8 or 9 post insemination. In addition to the frozen-thawed oocytes, a group of around 50 COCs was run as a control in each trial.

3.2.6.6 Experimental design and statistical analysis

Experiment 1

The immature oocytes were divided into 4 groups for vitrification.

Group 1 (E40): 40% EG
Group 2 (D40): 40% DMSO
Group 3 (ED20): 20% EG + 20% DMSO
Group 4 (EP20): 20% EG + 20% PROH.

The percentage of oocytes, which were recovered in a morphologically normal form, was recorded. The nature of damage was recorded in the oocytes that were recovered in a damaged form. The oocytes that were morphologically normal were subjected to IVM and their nuclear maturation rate was determined. This experiment was replicated 6 times.

Experiment 2

The immature oocytes were divided in 5 groups, Group 1-4 as for Experiment 1 and Group 5: (Control, fresh oocytes). The oocytes were subjected to IVM, IVF and IVC.

The data of Experiments 1 were subjected to analysis of variance (ANOVA) using SYSTAT 7.0 statistical software (SPSS Inc., USA) after arc sin transformation. The pair-wise comparison of treatment means was carried out by Duncan's Multiple Range Test. Data of experiment 2 was analyzed for the test of significance by Student's t-test using SYSTAT 7.0 after arc sin transformation.

3.2.7 Vitrification of Mature Oocytes

3.2.7.1 Preparation of vitrification solutions

The solutions used for the vitrification of mature oocytes were prepared as described earlier under 3.2.6.1.

3.2.7.2 Vitrification using EG or DMSO or combinations of EG+DMSO or EG+PROH

The oocytes were subjected to IVM as described earlier under 3.2.3.2. Following IVM, the oocytes were divided in 4 groups and subjected to vitrification using EG or DMSO or combinations of EG+DMSO or EG+PROH, as described earlier under 3.2.6.2 and 3.2.6.3.

3.2.7.3 Morphological evaluation of frozen-thawed oocytes

The evaluation of post-thaw morphology of vitrified-thawed oocytes was carried out as described earlier under 3.2.4.2.

3.2.7.4 Developmental potential of frozen-thawed oocytes

The oocytes that were recovered in a morphologically normal form were subjected to IVF and IVC as described earlier under 3.2.3.2 and 3.2.3.3. The cleavage rate was recorded on day 2 post insemination and the proportion of oocytes that developed to morula and blastocyst stages was recorded at day 8 or 9 post insemination. In addition to the frozen-thawed oocytes, a group of around 50 COCs was run as a control in each trial.

3.2.7.5 Experimental design and statistical analysis

Experiment 1

The in vitro cultured oocytes were divided into 4 groups for vitrification.

Group 1 (E40): 40% EG
Group 2 (D40): 40% DMSO
Group 3 (ED20): 20% EG + 20% DMSO
Group 4 (EP20): 20% EG + 20% PROH.

The percentage of oocytes, which were recovered in a morphologically normal form, was recorded. The nature of damage was recorded in the oocytes that were recovered in a damaged form. This experiment was replicated 6 times.

Experiment 2

The in vitro cultured oocytes that were recovered in a morphologically normal form were subjected to IVF and IVC, using fresh oocytes as controls. This experiment was replicated 6 times.

The data of Experiments 1 was subjected to analysis of variance (ANOVA) using SYSTAT 7.0 statistical software (SPSS Inc., USA) after arc sin transformation. The pair-wise comparison of treatment means was carried out by Duncan's Multiple Range Test. Data of experiment 2 was analyzed for the test of significance by Student's t-test using SYSTAT 7.0 after arc sin transformation.

CHAPTER – 4

Results and Discussion

4.1 SLOW FREEZING OF IMMATURE OOCYTES

4.1.1 Collection of Oocytes

A total of 2684 buffalo ovaries were collected from Delhi slaughterhouse, spread over 11 trials for this experiment, with 214 to 278 ovaries per trial. Immature oocytes were collected by aspiration of surface follicles and classified into Grades A, B, C and D. Out of the total 1703 oocytes of Grades A and B (Fig. 2,3), 340 (18%) were of Grade A whereas 1418 (82%) were of Grade B (Table 5). The mean yield of Grade A oocytes per ovary was 0.11 ± 0.02 (range 0.06 to 0.14) whereas that of Grade B oocytes was 0.52 ± 0.10 (range 0.39 to 0.63), the overall yield of Grade A and grade B oocytes per ovary being 0.63 ± 0.12 (range 0.50 to 0.75, Table 6).

Table 5. Slow freezing of immature oocytes: Number and quality of oocytes recovered through the aspiration of visible surface follicles

Trial No.	No. of ovaries aspirated	Grade of oocytes n (%)		Total (n)
		Grade A	Grade B	
1	240	32 (20)	125 (80)	157
2	270	21 (15)	117 (85)	138
3	278	30 (19)	128 (81)	158
4	276	24 (13)	156 (87)	180
5	230	33 (22)	117 (78)	150
6	214	30 (19)	132 (81)	162
7	250	34 (22)	124 (78)	158
8	238	25 (21)	95 (79)	120
9	218	14 (10)	136 (90)	150
10	250	22 (12)	158 (88)	180
11	220	20 (13)	130 (87)	150
Total	2684	340 (18)	1418 (82)	1703

Table 6. Slow freezing of immature oocytes: Oocyte recovery per ovary.

Trial No.	Grade A oocytes (n)	Grade B oocytes (n)	Total (n)
1	0.13	0.52	0.65
2	0.07	0.43	0.51
3	0.10	0.46	0.56
4	0.08	0.56	0.65
5	0.14	0.50	0.65
6	0.14	0.61	0.75
7	0.13	0.49	0.62
8	0.10	0.39	0.50
9	0.06	0.62	0.68
10	0.08	0.63	0.72
11	0.09	0.59	0.68
Mean ± SEM	0.11 ± 0.02	0.52 ± 0.10	0.63 ± 0.12

4.1.2 Post-Thaw Recovery of Morphologically Normal Oocytes

A total of 942 oocytes used in this experiment were divided into six groups of 157 each and subjected to slow freezing using three cryoprotectants i.e., EG, DMSO and PROH, each used at concentrations of 1.0 and 1.5M (Table 7). A total of 842 oocytes were recovered after thawing, with a loss of 100 oocytes during handling.

Table 7 Slow freezing of immature oocytes: Effects of different cryoprotectants on the proportion of oocytes recovered morphologically normal.

Cryo-protectant	Concen-tration (M)	Oocytes frozen (n)	Oocytes recovered (n)	Morphologically normal oocytesn (%)	Damaged oocytes n (%)
EG	1.0	157	137	61 (43.8 ± 6.3)^{c}	76 (56.1 ± 6.3)
	1.5	157	145	110 (75.5 ± 3.6)^{b}	35(24.2 ± 3.6)
DMSO	1.0	157	144	128 (88.4 ± 2.9)^{b}	16(11.5 ± 2.9)
	1.5	157	141	136 (96.2 ± 2.4)^{a}	5(3.7 ± 2.4)
PROH	1.0	157	136	76 (57.4 ± 10.4)^{c}	60(42.5 ± 10.4)
	1.5	157	139	104 (74.8 ± 4.1)^{b}	35(25.1 ± 4.1)

Data from 6 trials
Percent values are Mean ± SEM
Values with different superscripts within the same column differ significantly from each other ($P<0.05$)

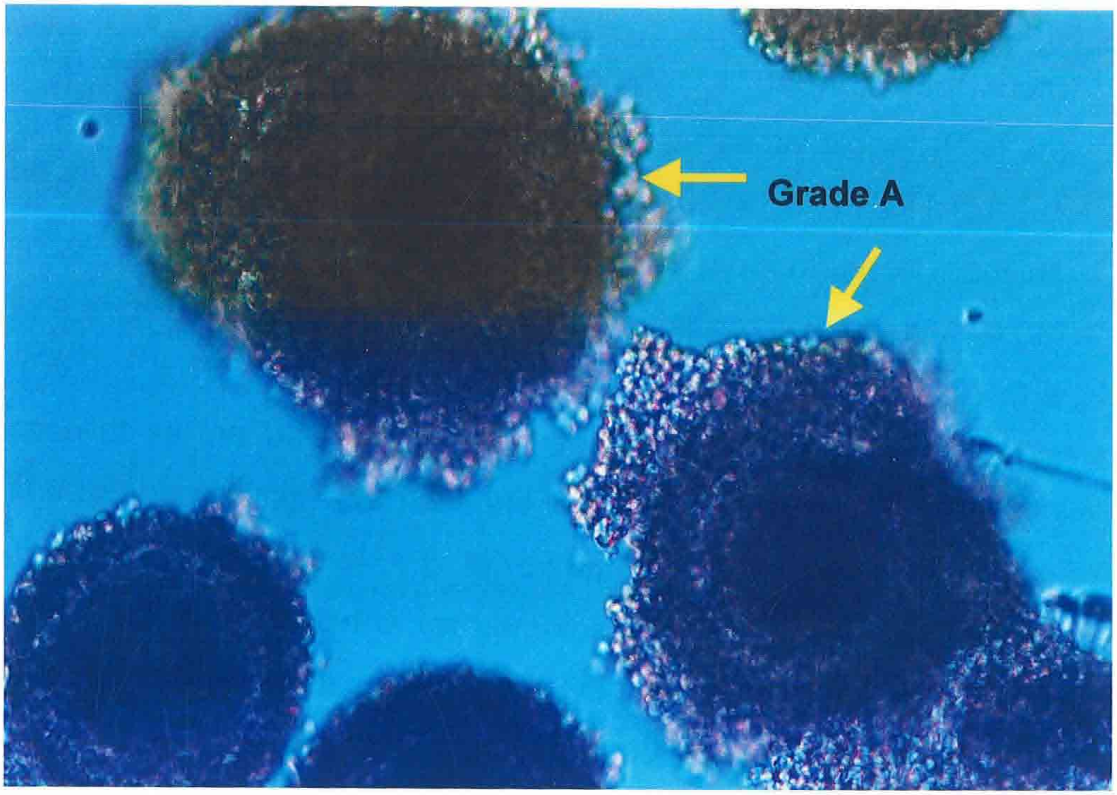


Fig.2. Immature buffalo oocytes of Grade-A

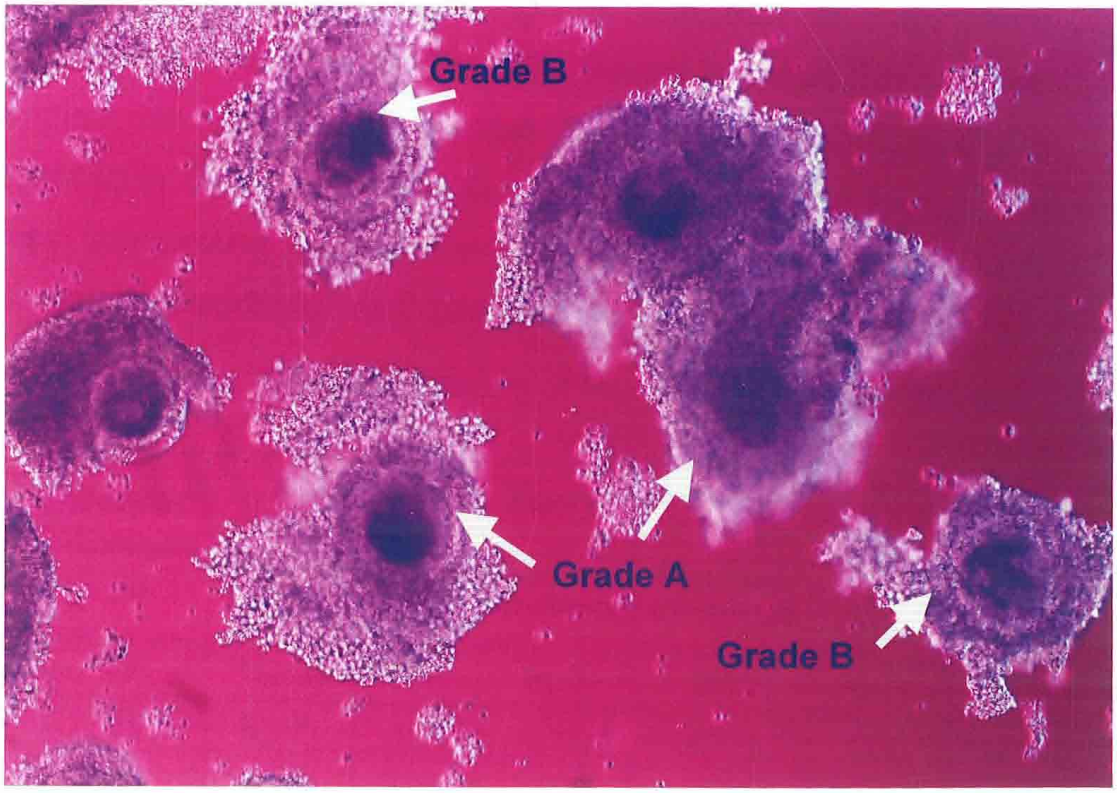


Fig.3. Immature buffalo oocytes of Grade A and B

For 1.0 M concentration, the percentage of oocytes that were recovered in a morphologically normal state was significantly higher ($P<0.05$) for DMSO (88.4 ± 2.9%) than that for EG (43.8 ± 6.3%) or PROH (57.4 ± 10.4%). Similarly, for the 1.5 M concentration, the percentage of oocytes recovered in a morphologically normal form was significantly higher ($P<0.05$) for DMSO (96.2 ± 2.4%) than that for EG (75.5 ± 3.6%) or PROH (74.8 ± 4.1%). In case of all the three cryoprotectants, the percentage of oocytes recovered in a morphologically normal state was significantly higher ($P<0.05$) for the higher concentration of 1.5 M compared to that for the lower concentration of 1.0 M. Among the six combinations of cryoprotectants and concentrations evaluated, the percentage of oocytes recovered in a morphologically normal state was the highest and that of oocytes recovered in a damaged form was the lowest ($P<0.05$) for 1.5 M DMSO.

The types of damages observed after slow freezing and thawing of immature oocytes using the six combinations of cryoprotectants and concentrations as maintained above included cracked or ruptured zona pellucida (Fig. 5-8), leaked contents (Fig. 5), shrunken or fragmented cytoplasm (Fig. 9), split in two halves (Fig. 10), change in shape (Fig. 11) and partial or complete denudation of the oocytes (Fig. 12, 13). Irrespective of the type or concentration of the cryoprotectant, partial or complete loss of the cumulus mass surrounding the oocytes was the most common damage observed followed by cracking of zona pellucida (Fig. 14,Table 8).

Table 8. Types of damages after slow freezing of immature oocytes in different cryoprotectants.

Cryopro-tectant	Concen-tration (M)	Oocytes damaged (n)	Type of damage n (%)					
			Cracked ZP	Split In two halves	Leaked contents	Shrunken cytoplasm	Change in Shape	Partly/ Fully denuded
EG	1.0	76	11(14)	7(9)	5(7)	6(8)	5(7)	42(55)
	1.5	35	6(17)	1(3)	4(11)	4(11)	2(6)	18(52)
DMSO	1.0	16	4(25)	1(6)	2(13)	2(13)	0(0)	7(43)
	1.5	5	1(20)	1(20)	1(20)	0(0)	0(0)	2(40)
PROH	1.0	60	11(18)	3(5)	7(12)	7(12)	3(5)	29(48)
	1.5	35	6(17)	2(6)	5(14)	4(11)	2(6)	16(46)

Data from 6 trials

4.1.3 Nuclear Maturation Rate

A total of 754 oocytes were used in this experiment (Table 9). The oocytes were divided into 7 groups, 6 out of which were the three cryoprotectants, each used at two concentrations, as mentioned above. The 7th group was that of fresh non-frozen, immature oocytes which served as controls. The immature oocytes were subjected to slow freezing after which the nuclear maturation was determined. The nuclear maturation rate of frozen-thawed oocytes which varied from 18.6 ± 3.3% to 35.9 ± 1.6% for the 6 combinations of cryoprotectants and concentrations used was significantly lower ($P<0.05$) than that for the non-frozen control oocytes (71.0 ± 1.7%). In case of all the three cryoprotectants, the nuclear maturation rate of frozen-thawed oocytes was significantly higher ($P<0.05$) for the higher concentration of 1.5 M compared to that for the lower concentration of 1.0 M. For 1.0 M concentration, the nuclear maturation rate was significantly ($P<0.05$) higher for DMSO (25.2 ± 1.0%) than that for EG (18.6 ± 3.3%) or PROH (20.2 ± 1.43%). Similarly, for 1.5 M concentration, the nuclear maturation rate for DMSO (35.9 ± 1.6%) was significantly higher ($P<0.05$) than that for EG (22.8 ± 0.9%) or PROH (23.2 ± 2.0%). Among the six combinations of cryoprotectants and concentrations evaluated, the nuclear maturation rate was found to be the highest ($P<0.05$) for 1.5 M DMSO.

Table 9. Slow freezing of immature oocytes: Effects of different cryoprotectants on the post-thaw nuclear maturation rate of oocytes recovered morphologically normal.

Cryoprotectant	Concentration (M)	Oocytes taken (n)	Matured oocytes n(%)
EG	1	61	10 (18.6 ± 3.3)^{c}
	1.5	110	25 (22.8 ± 0.9)^{bc}
DMSO	1	128	32 (25.2 ± 1.0)^{b}
	1.5	136	48 (35.9 ± 1.6)^{a}
PROH	1	66	15 (20.2 ± 1.43)^{c}
	1.5	104	24 (23.2 ± 2.0)^{bc}
Control		149	106 (71.0 ± 1.7)^{d}

Data from 6 trials
Percent values are Mean ± SEM
Values with different superscripts within the same column differ significantly from each other ($P<0.05$)

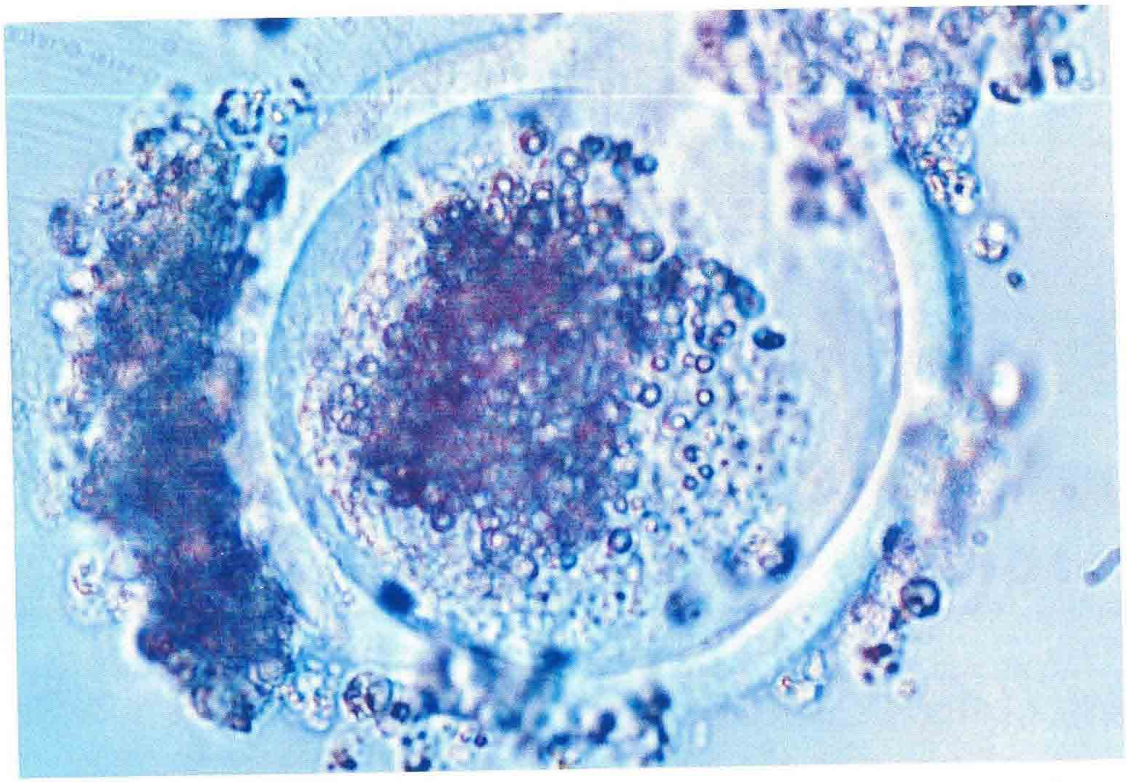


Fig.6. Zona rupturing

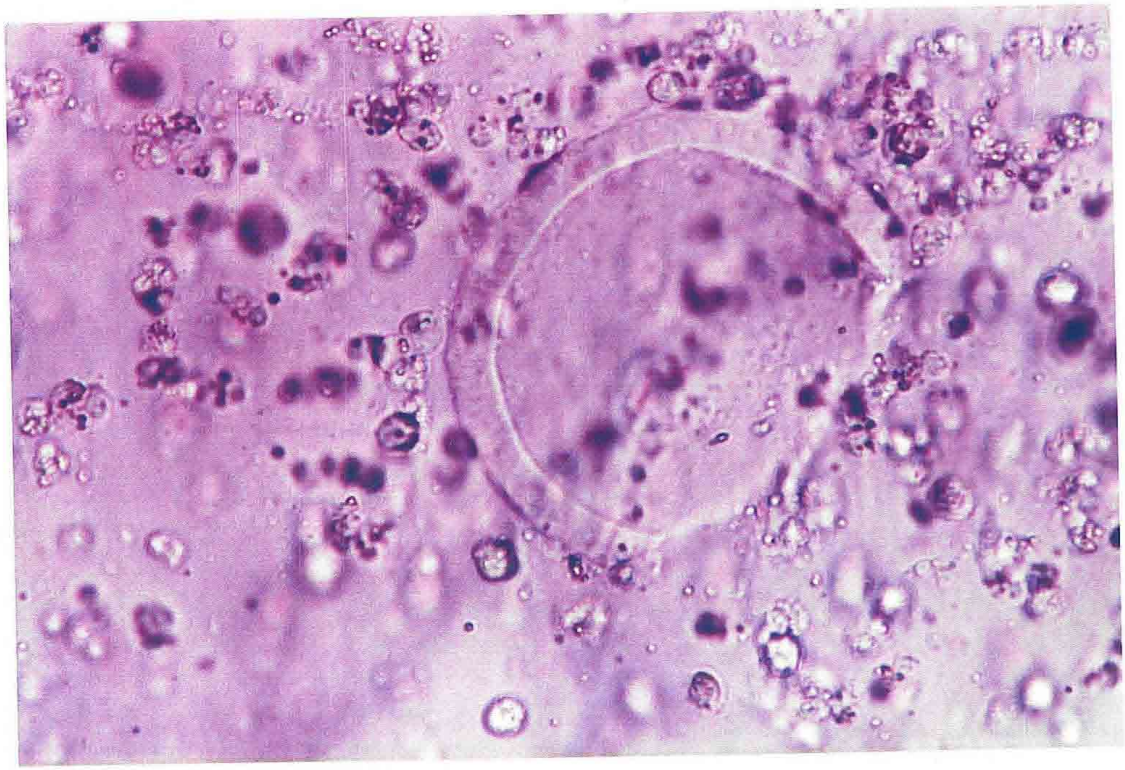


Fig.7. Excessively damaged zona

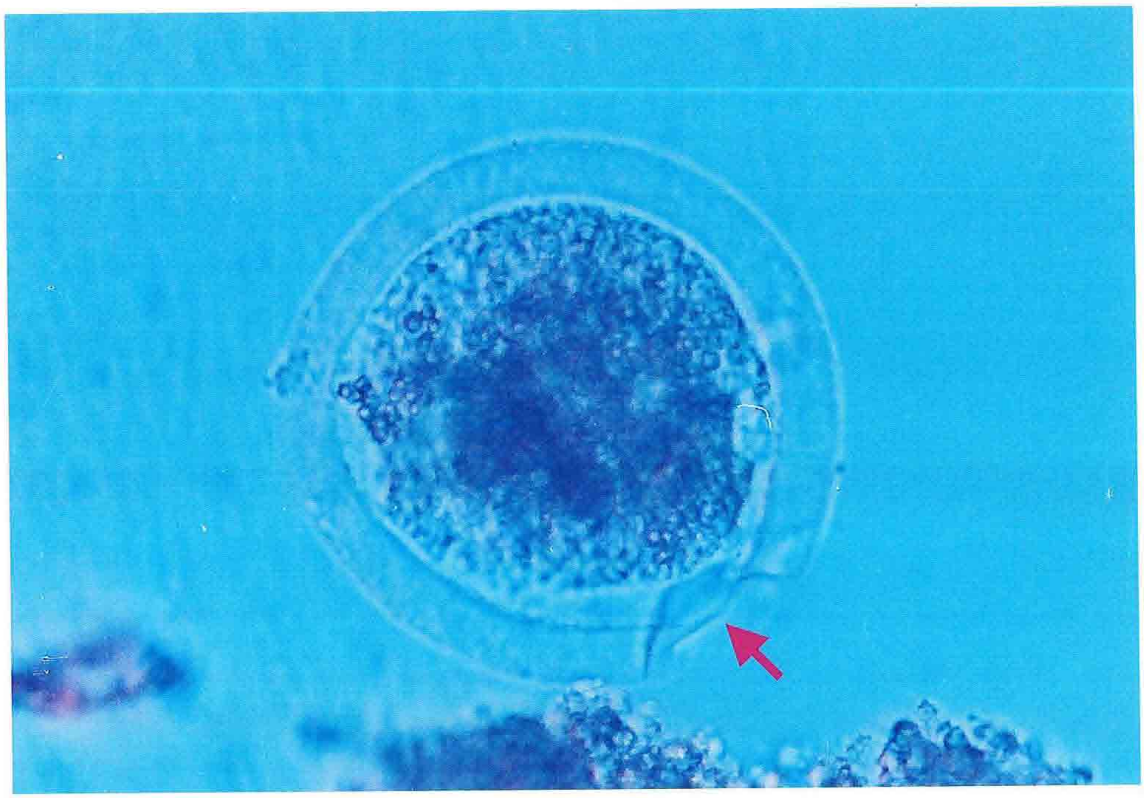


Fig.8. Damaged zona

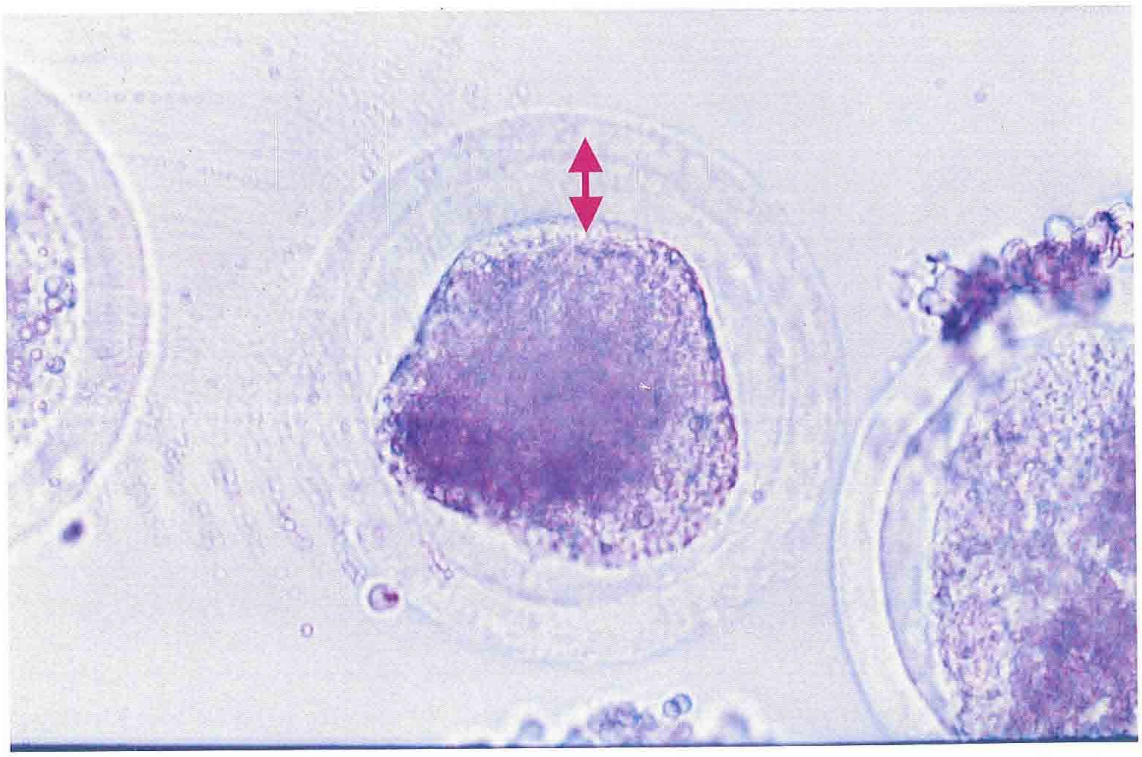


Fig.9. Shrinkage of ooplasm

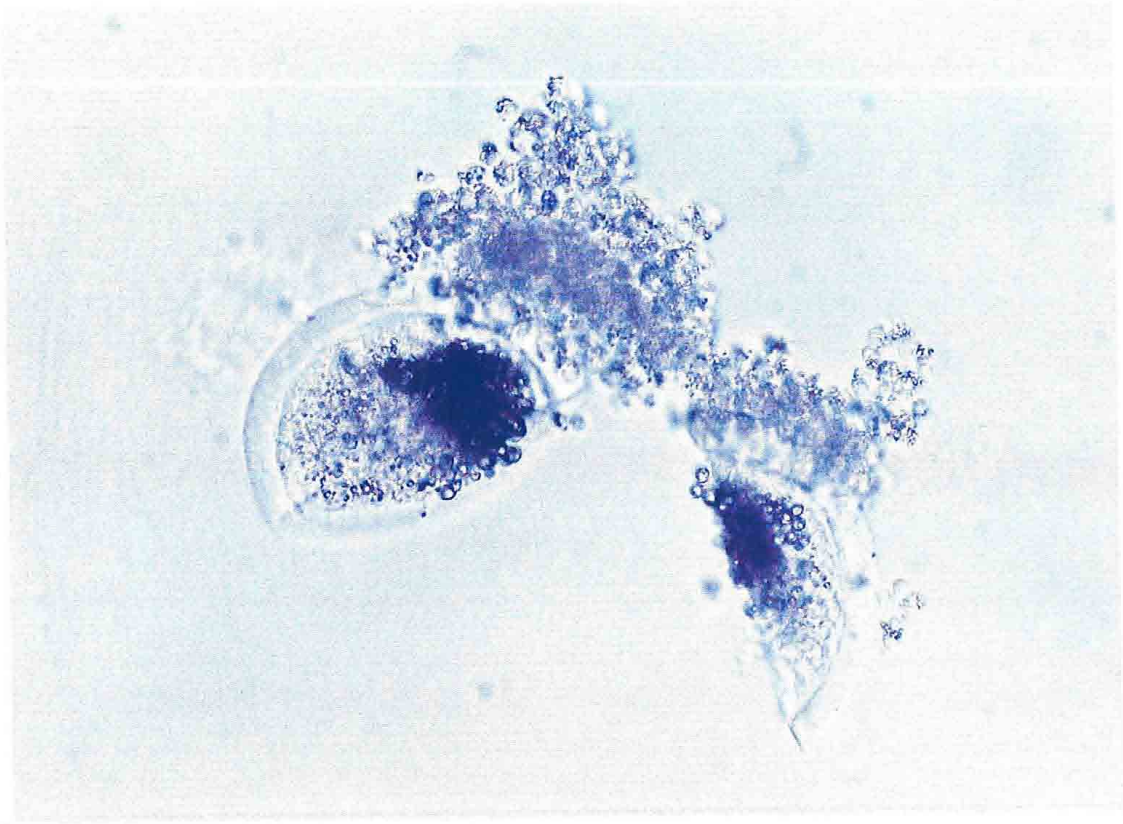


Fig.10. Splitting of oocyte in two halves

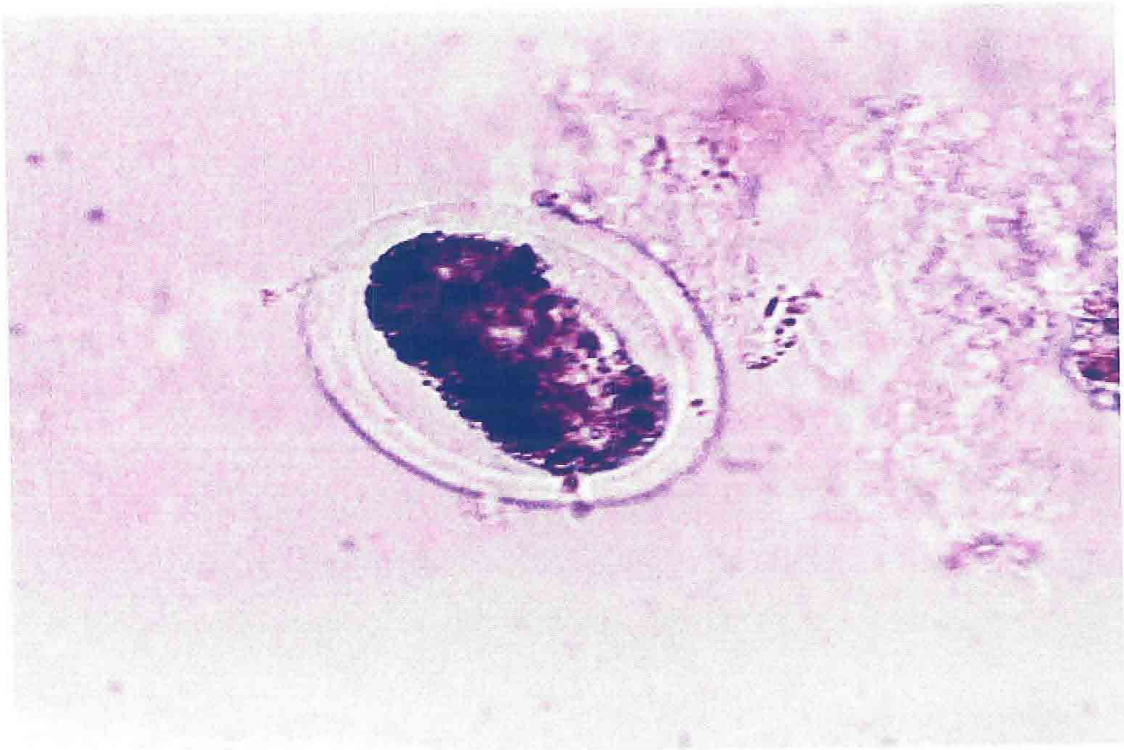


Fig.11. Change in shape of oocyte

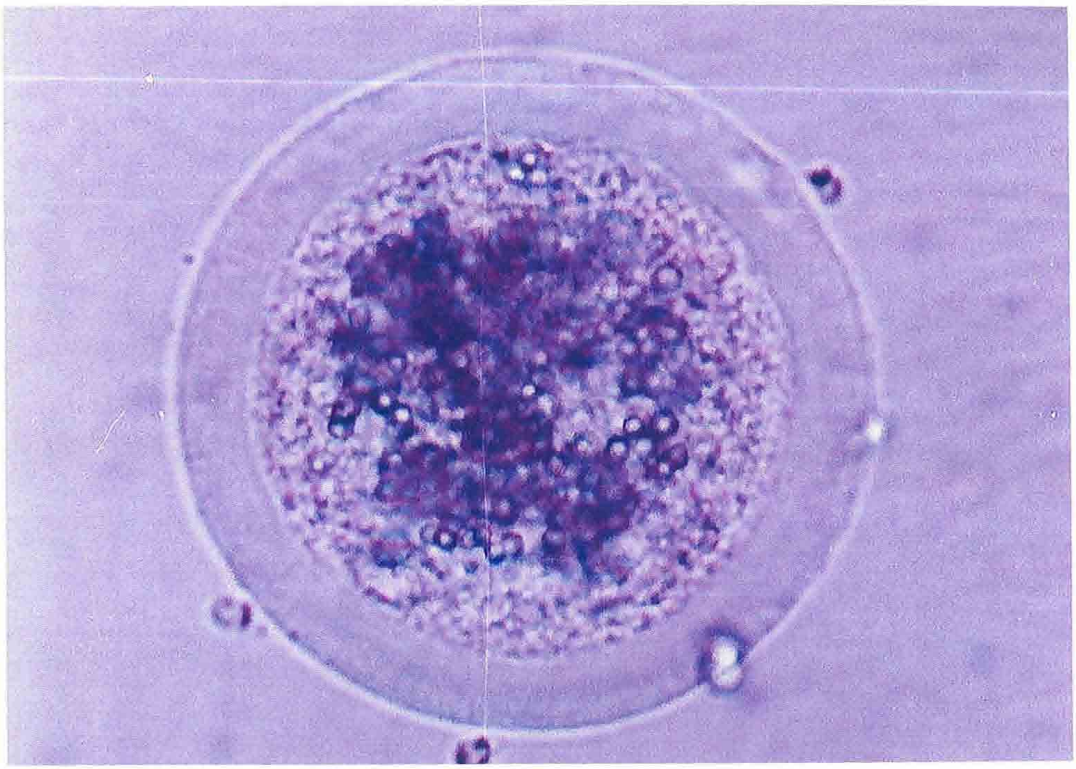


Fig.12. Fully denuded oocyte

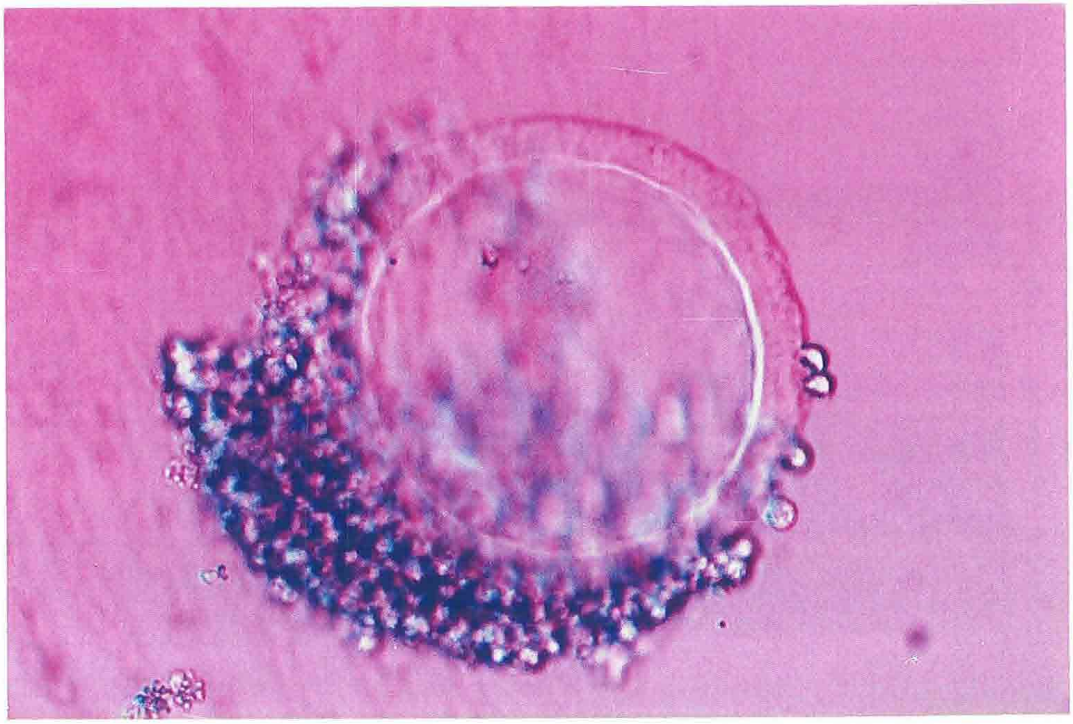
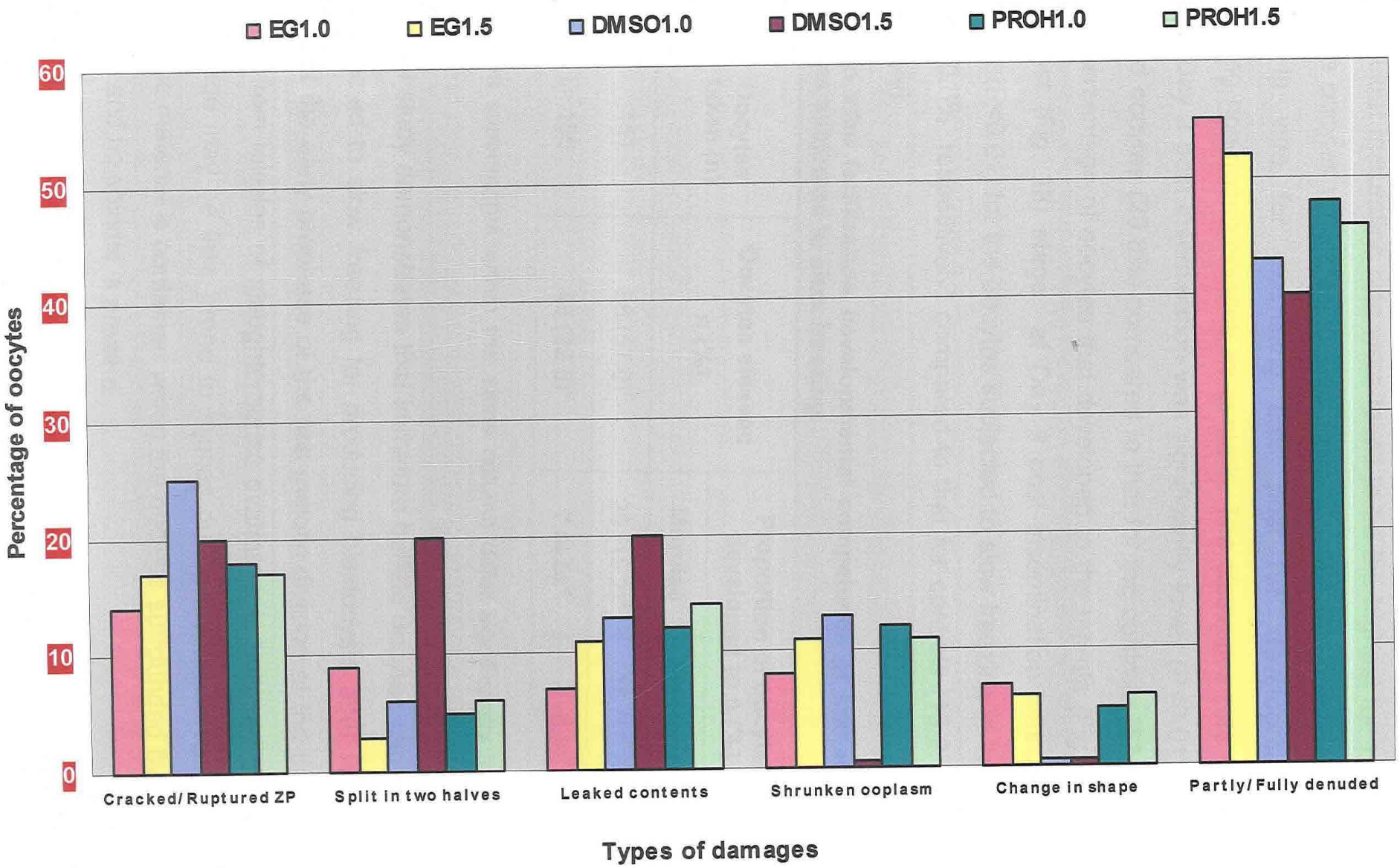


Fig.13. Partially denuded oocyte

Fig. 14 Types of damages after slow freezing of immature oocytes



4.1.4 Post IVF Developmental Competence

Since the proportion of oocytes recovered in a morphologically normal state and the nuclear maturation rate were found to be the highest for 1.5 M DMSO, immature oocytes subjected to slow freezing using this combination were fertilized in vitro for examining their post IVF developmental competence, using non-frozen oocytes as controls. The cleavages rate (Fig. 15) recorded at Day 2 post insemination was significantly lower ($P<0.01$) for the frozen-thawed oocytes (28.8%) compared to that for the controls (57.0%, Table 10). The percentage of oocytes that developed to the morula (Fig. 17) and the blastocyst (Fig. 18) stage at Day 9 post insemination was also significantly lower ($P<0.01$) for the oocytes subjected to slow freezing in 1.5 M DMSO (4.5 and 0.6%, respectively) compared to that for controls (19.2 and 10.6%, respectively).

Table 10. Post in vitro fertilization developmental competence of immature oocytes subjected to slow freezing.

Cryoprotectant	Oocytes taken (n)	Oocytes cleaved n (%)	Proportion of oocytes developed to n (%)	
			Morulae	Blastocysts
Control	151	86 (57.0)[a]	29 (19.2)[a]	16 (10.6)[a]
DMSO (1.5 M)	156	45 (28.8)[b]	7 (4.5)[b]	1 (0.6)[b]

Data from 3 trials
Values with different superscripts within the same column differ significantly from each other ($P<0.01$)

The present study demonstrates that immature buffalo oocytes can be successfully subjected to slow freezing for producing blastocysts after IVF. The oocytes are at the early prophase of the first meiotic division at the time of their aspiration from follicles of slaughterhouse ovaries. It is believed that oocytes at this stage may be less prone to injuries during cryopreservation because the genetic material is contained within the nucleus surrounded by a nuclear membrane and no spindle is present.

Only those oocytes, which were surrounded by a cumulus mass all around were taken for this study since the post-thaw developmental competence of immature cattle oocytes subjected to slow freezing has been reported to be lower for partly or wholly denuded oocytes than that for those

which had a dense cumulus mass around them (Im *et al.*, 1997). The choice of cryoprotectants i.e., DMSO, PROH and EG, and the concentrations at which these were used was based on the results of earlier reports on the slow freezing of immature cattle oocytes (Otoi *et al.*, 1995; Im *et al.*, 1997; Suzuki *et al.*, 1996). Post-thaw morphological examination of the oocytes revealed that the incidence of damage was lower with the higher concentration of 1.5 M than that with 1.0 M for all the three cryoprotectants evaluated. DMSO was more effective than PROH or EG at both the concentrations tested. This indicates that the type of the cryoprotectant as well as its concentration were important factors affecting the post-thaw survival of immature oocytes subjected to slow freezing. However, irrespective of the type or concentration of the cryoprotectant, partial or complete loss of surrounding cumulus mass resulting in their denudation was the most common damage.

DMSO was found to be to be more effective than either PROH or EG in terms of the nuclear maturation rate also. One possible reason for this could be the higher permeability of bovine oocytes to DMSO than that to EG (Agca *et al.*, 1998). Others have found both DMSO and PROH to be equally effective for the slow freezing of immature cattle oocytes (Im *et al.*, 1997). Similarly, no significant differences were observed in post fertilization development using 1.8 M EG, 1.3 M monomethyl ether (EME) or 1.6 M PROH EG, EME and PROH when bovine GV stage oocytes were subjected to slow freezing (Suzuki *et al.*, 1996). The nuclear maturation rate of 35.9% obtained with 1.5 M DMSO in the present study is similar to that of 30-38% reported for cattle oocytes with 1.5 M PROH or DMSO (Im *et al.*, 1997). The post-thaw recovery of morphologically normal oocytes and the cleavage rate obtained with 1.5 M DMSO in the present study (96.2% and 28.8%, respectively) are higher than 29-32% and 9.1%, respectively, obtained when immature cattle oocytes were directly immersed into 2.0 M PROH (Fuku *et al.*, 1992) or 8.2-9.2% and 2.9-7.0%, respectively, when cattle oocytes were immersed directly in 1.8 M EG for slow freezing (Otoi *et al.*, 1995). Higher post-thaw survival, nuclear maturation rate and cleavage rate obtained in the present study could be not only due to use of cryoprotectant concentrations optimal for immature buffalo oocytes but also because of a three step addition and dilution of the

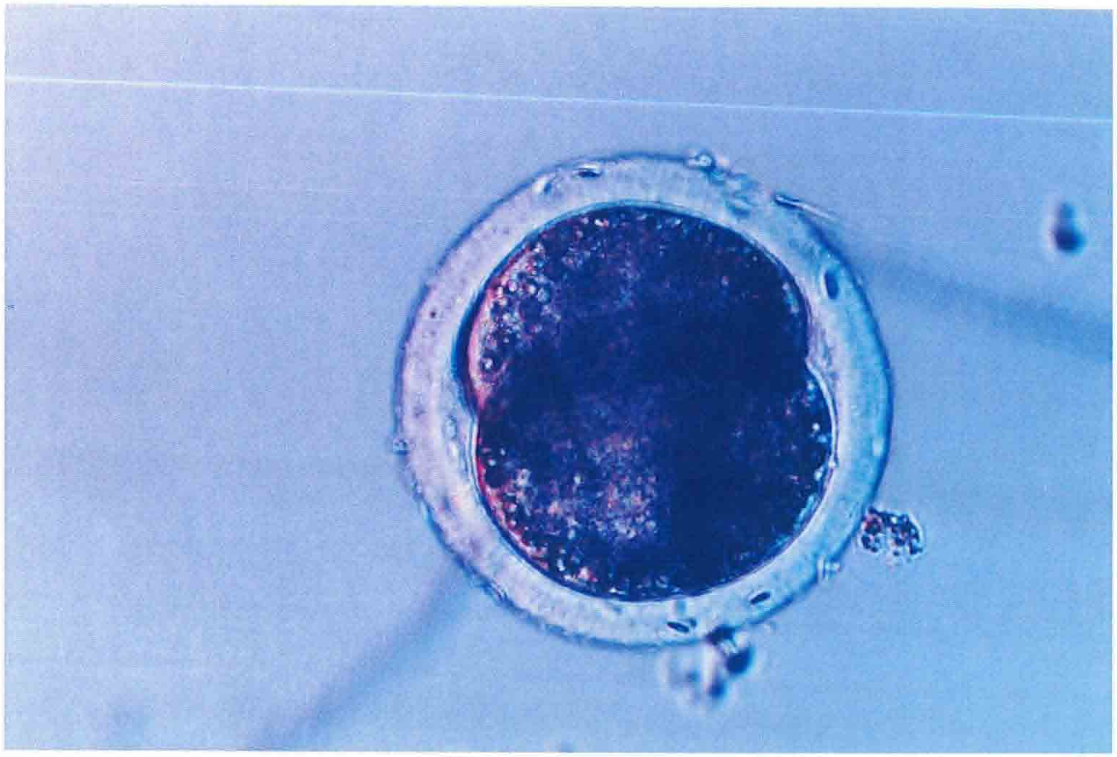


Fig.15. 2-cell stage embryo formed after cryopreservation

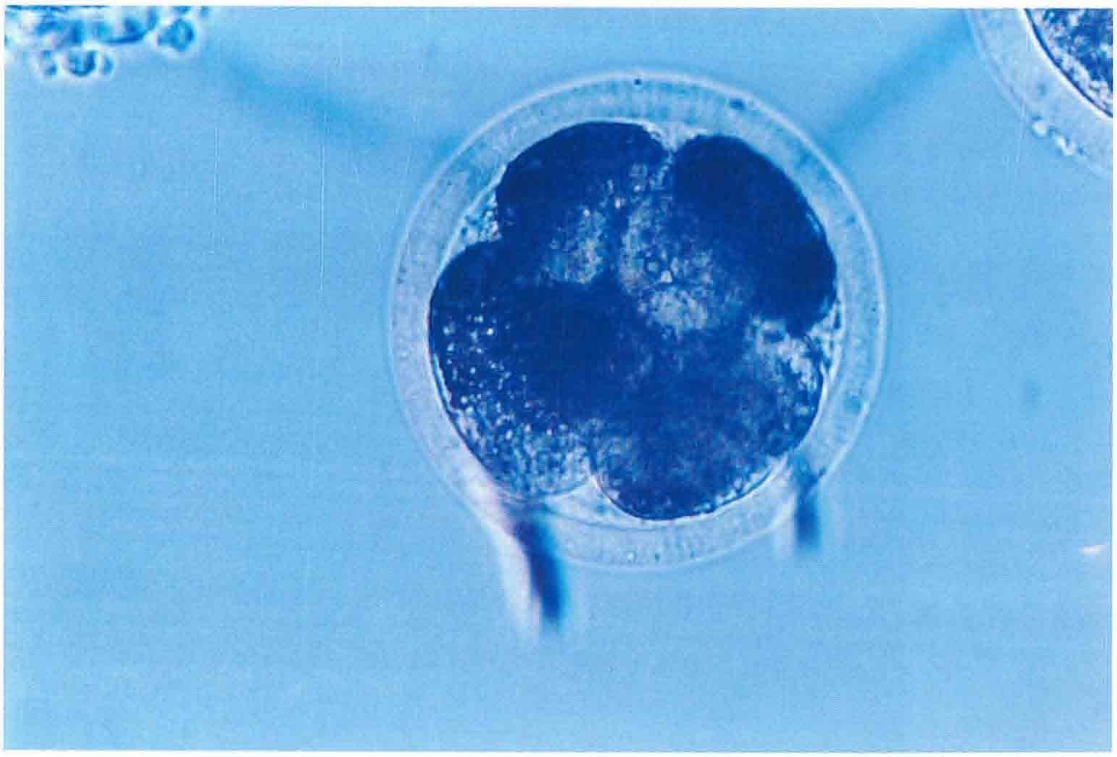


Fig.16. 4-cell stage embryo produced after cryopreservation

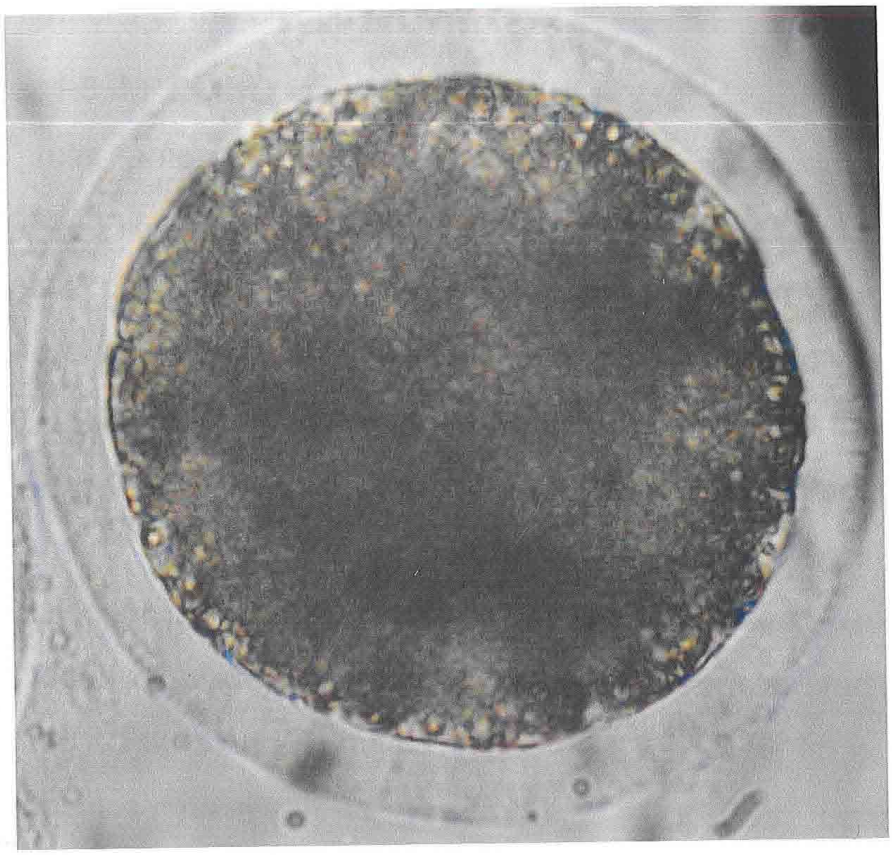


Fig.17. Morula produced from frozen-thawed oocyte

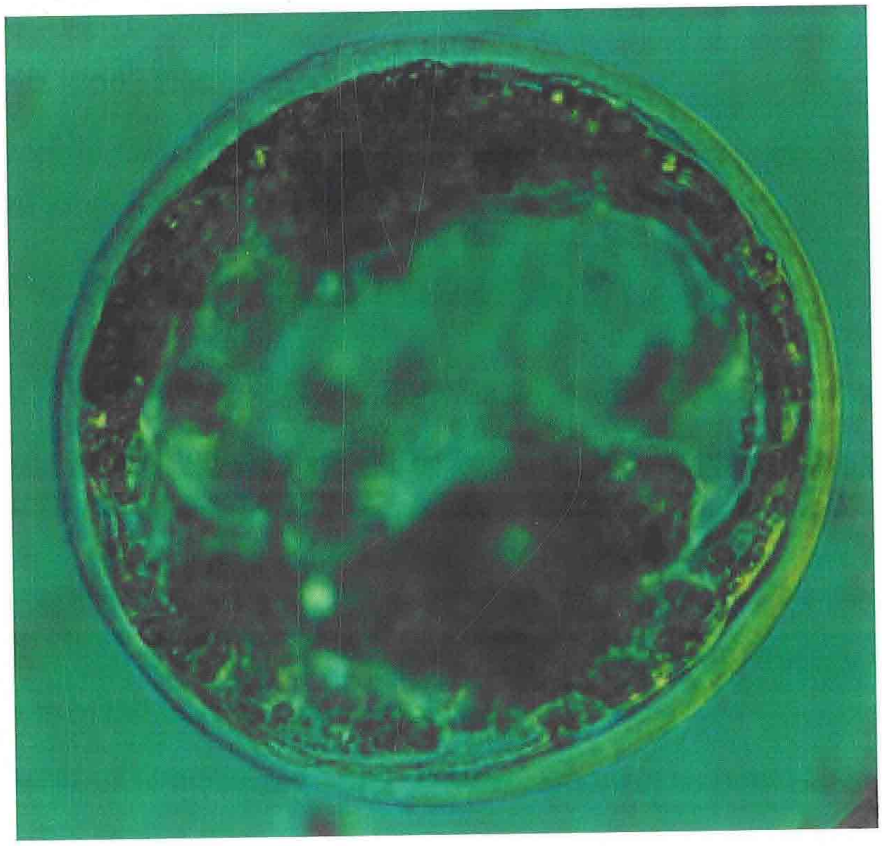


Fig.18. Blastocyst produced from frozen-thawed oocyte

cryoprotectant in the present study, unlike a direct exposure of oocytes to a high concentrations of the cryoprotectant in other studies.

Since 1.5 M DMSO gave the best post-thaw survival and nuclear maturation rate among the six combinations of cryoprotectants and concentrations, this combination was selected for studying the post IVF developmental competence of frozen-thawed immature oocytes. The cleavage rate and the proportion of oocytes that developed to the blastocyst stage at day 9 post insemination was significantly lower for the frozen-thawed compared to the control oocytes. It is believed that it is much more difficult to cryopreserve immature than mature oocytes. Both fertilizability and post IVF developmental competence of immature oocytes has been reported to be lower than those for in vitro matured oocytes in all the earlier studies in cattle (Lim *et al.*, 1991; Parks and Ruffing 1992; Otoi *et al.*, 1995; Im *et al.*, 1997). Immature oocytes have been reported to be less permeable to cryoprotectants than mature oocytes in bovine (Agca *et al.*, 1998) and goat (Le Gal *et al.*, 1999). Also, immature bovine oocytes are more sensitive to anisotonic stress than mature oocytes (Agca *et al.*, 2000).

In conclusion, DMSO was more effective than EG or PROH for the slow freezing of immature buffalo oocytes and blastocysts could be produced from immature buffalo oocytes subjected to slow freezing in 1.5 M DMSO.

4.2 SLOW FREEZING OF IN VITRO MATURED OOCYTES

4.2.1 Collection of Oocytes

A total of 2333 buffalo ovaries were collected, spread over 9 trials for this experiment (Table 11). Out of the total 1387 oocytes of Grades A and B (Fig. 2,3) collected, 311 (22%) were of Grade A whereas 1076 (78%) were of Grade B. The yield of Grade A oocytes per ovary varied from 0.08 to 0.2 among different trials, with a mean yield of 0.12 ± 0.04 per ovary (Table 12) whereas the yield of Grade B oocytes varied from 0.33 to 0.58 per ovary, with a mean yield of 0.45 ± 0.09 oocytes per ovary. The cumulative yield of Grade A and B oocytes recovered per ovary was 0.57 ± 0.13.

Table 11. Slow freezing of in vitro matured oocytes: Number and quality of oocytes recovered through the aspiration of visible surface follicles.

Trial No.	No. of ovaries aspirated	Grade of oocytes n (%)		Total (n)
		Grade A	Grade B	
1	230	32 (25)	98 (75)	130
2	250	25 (21)	95 (79)	120
3	268	32 (19)	136 (81)	168
4	290	42 (23)	139 (77)	181
5	240	48 (27)	132 (73)	180
6	280	38 (29)	94 (71)	132
7	274	41 (25)	122 (75)	163
8	223	30 (19)	130 (81)	160
9	278	23 (15)	130 (85)	153
Total	2333	311 (22)	1076 (78)	1387

Table 12. Slow freezing of in vitro matured oocytes: Oocyte recovery per ovary.

Trial No	Grade A oocytes (n)	Grade B oocytes (n)	Total (n)
1	0.13	0.42	0.55
2	0.10	0.38	0.48
3	0.11	0.50	0.61
4	0.14	0.47	0.61
5	0.20	0.55	0.75
6	0.13	0.33	0.46
7	0.14	0.44	0.58
8	0.13	0.58	0.71
9	0.08	0.46	0.54
Mean ± SEM	0.12 ± 0.04	0.45 ± 0.09	0.57 ± 0.13

4.2.2 Post-Thaw Recovery of Morphologically Normal Oocytes

Immature oocytes were subjected to IVM for 24h after which the in vitro matured oocytes (Fig. 4) were divided into 6 groups (three cryoprotectants i.e., EG, DMSO and PROH, each used at 1.0 and 1.5 M concentrations). A total of 911 in vitro matured oocytes were subjected to slow freezing, out of which 878 were recovered after thawing, thus resulting in a loss of 33 oocytes during handling (Table 13). Morphological examination of the oocytes revealed that for 1.0 M concentration, the percentage of oocytes recovered in a morphologically normal form after freezing-thawing was significantly higher ($P<0.05$) for DMSO (88.3 ± 1.9%) compared to that for EG (67.6 ± 2.4%) or PROH (67.5 ± 5.0%). Similarly, for 1.5 M concentration, the percentage of oocytes recovered in a morphologically normal form was significantly higher ($P<0.05$) for DMSO (96.0 ± 1.6%) compared to that for EG (78.4 ± 3.1%) or PROH (80.6 ± 4.6%).

Table 13. Slow freezing of in vitro matured oocytes: Effects of different cryoprotectants on the proportion of oocytes recovered morphologically normal.

Cryoprotectant	Concentration (M)	Oocytes frozen (n)	Oocytes recovered (n)	Morphologically normal oocytes n (%)	Damaged oocytes n (%)
EG	1.0	149	142	97(67.6 ± 2.4)^{c}	45(32.4 ± 2.4)^{c}
	1.5	153	145	114(78.4 ± 3.1)^{b}	31(22.4 ± 2.6)^{b}
DMSO	1.0	148	144	127(88.3 ± 1.9)^{b}	17(11.6 ± 1.9)^{b}
	1.5	159	153	147(96.0 ± 1.6)^{a}	6(3.9 ± 1.6)^{a}
PROH	1.0	150	146	101(67.5 ± 5.0)^{c}	45(32.5 ± 5.0)^{c}
	1.5	152	148	120(80.6 ± 4.6)^{b}	28(17.8 ± 3.4)^{b}

Data from 6 trials
Percent values are Mean ± SEM
Values with different superscripts within the same column differ significantly from each other ($P<0.05$)

Among the types of damages observed after slow freezing and thawing of in vitro matured oocytes, which included cracked zone pellucida, shrunken or fragmented cytoplasm, leaked contents, oocytes split in two halves, change in shape and partial or complete denudation of the oocytes (Fig. 5 to Fig. 13), the last one was the most common damage observed followed by cracking of zona pellucida, irrespective of the type or concentration of the cryoprotectant (Fig. 19, Table 14).

Table 14. Types of damages after slow freezing of in vitro matured oocytes in different cryoprotectants

Cryoprotectant	Concentration (M)	Oocytes damaged (n)	Type of damage n (%)					
			Cracked ZP	Split In two halves	Leaked contents	Shrunken cytoplasm	Change in Shape	Partly/ Fully denuded
EG	1.0	45	7(16)	4(9)	3(7)	4(9)	3(7)	24(53)
	1.5	31	5(16)	1(3)	4(13)	4(13)	2(6)	15(48)
DMSO	1.0	17	4(23)	1(6)	1(6)	2(12)	1(6)	8(47)
	1.5	6	1(17)	1(17)	1(17)	1(17)	0(0)	2(33)
PROH	1.0	45	8(18)	2(4)	5(11)	5(11)	2(4)	23(51)
	1.5	28	5(17)	2(7)	5(17)	3(11)	1(4)	12(43)

Data from 6 trials

4.2.3 Post IVF Developmental Competence

Among the 6 combinations of cryoprotectants and concentrations evaluated for the slow freezing of in vitro matured oocytes, the proportion of oocytes recovered in a morphologically normal state and the nuclear maturation rate were found to be the highest for 1.5 M DMSO. Therefore, in vitro matured oocytes subjected to slow freezing using this combination were fertilized in vitro for examining their post IVF developmental competence, using non-frozen oocytes as controls. A total of 445 oocytes were used for this experiment. The cleavages rate recorded at Day 2 post insemination was significantly lower ($P<0.01$) for the frozen-thawed oocytes (36.3%) compared to that for the controls (60.0%) as shown in Table 15. The percentage of oocytes that developed to the morula and the blastocyst stage at Day 9 post insemination was also significantly lower ($P<0.01$) for the oocytes subjected to slow freezing in 1.5 M DMSO (4.1 and 0.7%, respectively) compared to that for controls (19.3 and 10.6%, respectively).

Table 15. Post in vitro fertilization developmental competence of in vitro matured oocytes subjected to slow freezing.

Cryoprotectant	Oocytes taken (n)	Oocytes cleaved n (%)	Proportion of oocytes developed to n (%)	
			Morulae	Blastocysts
Control	150	90 (60.0)[a]	29 (19.3)[a]	16 (10.6)[a]
DMSO (1.5 M)	295	107 (36.3)[b]	12 (4.1)[b]	2 (0.7)[b]

Data from 6 trials

Values with different superscripts within the same column differ significantly from each other ($P<0.01$)

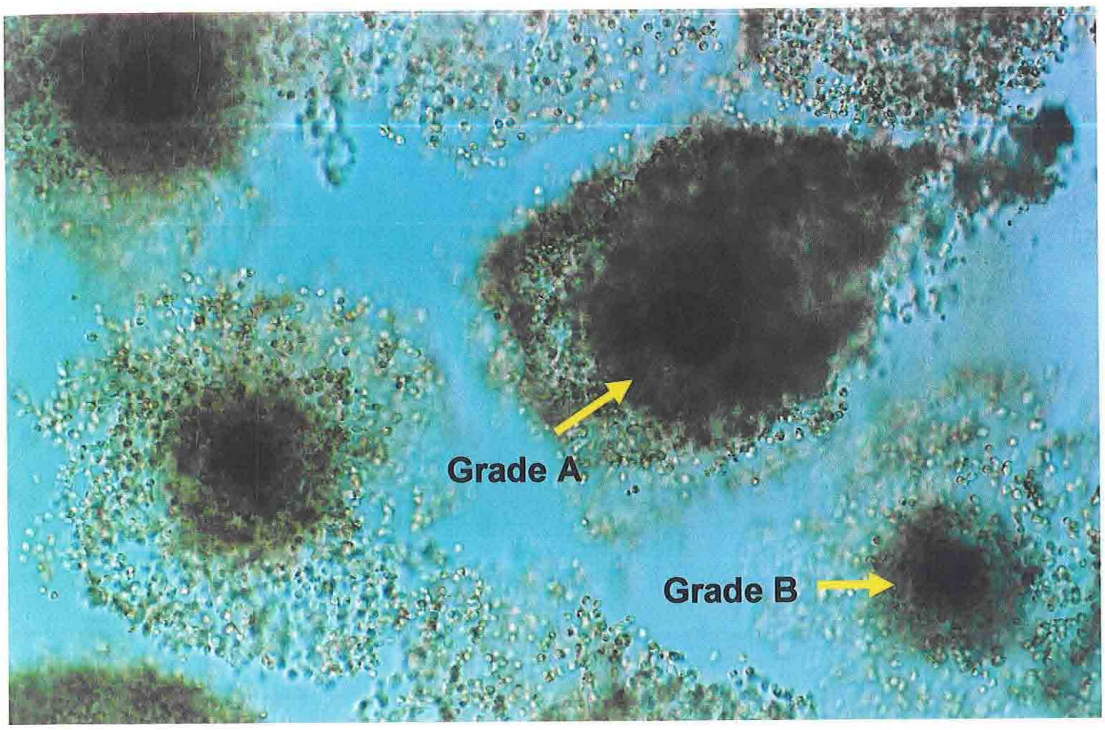


Fig.4. In vitro matured oocytes of Grade A and B

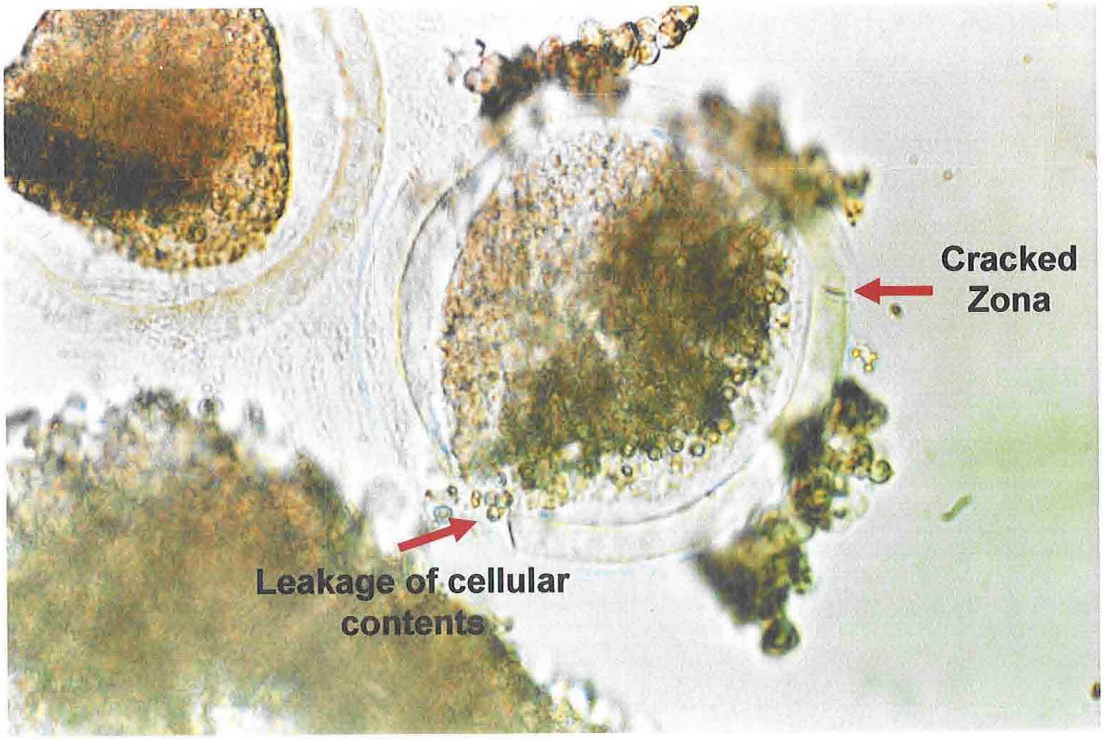
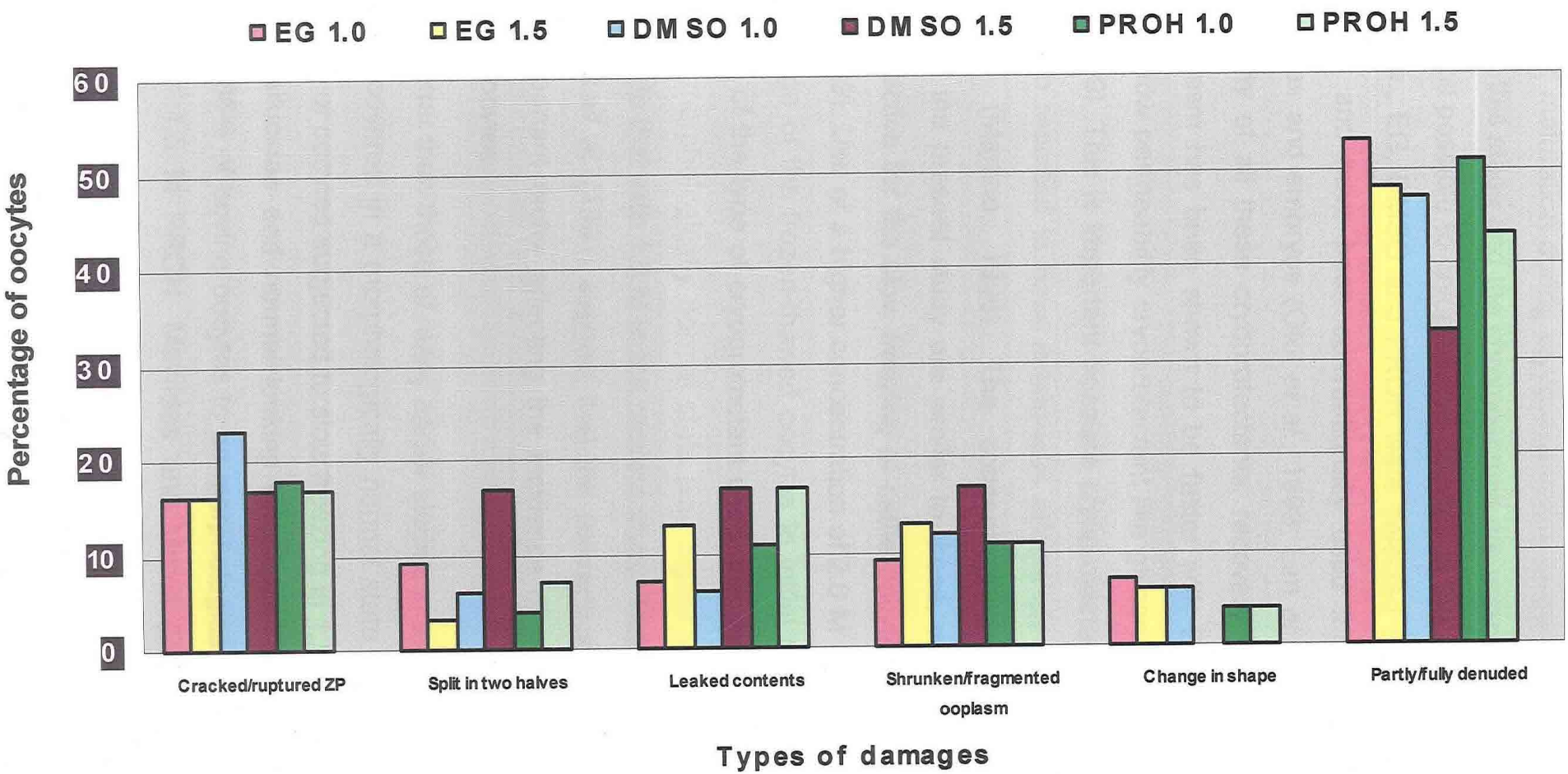


Fig.5. Zona cracking and leakage of cellular contents

Fig. 19 Types of damages after slow freezing of in vitro matured oocytes



Although it is believed that the oocytes are more prone to injury at the mature i.e., MII stage of maturation during cryopreservation since no nuclear membrane is present at this stage and the chromosomes are arranged on the metaphase plate, it is still possible to cryopreserve oocytes at this stage. The three cryoprotectants i.e., EG, DMSO and PROH were selected since these have high permeability and have been conventionally used for the slow freezing of cattle oocytes and embryos (Otoi *et al.*, 1993; Lim *et al.*, 1999). Due to high permeability of all these cryoprotectants, recovery from initial shrinkage of the cytoplasm has been shown to be faster with DMSO and PROH than that with a low permeability cryoprotectant like glycerol for cattle oocytes (Lim *et al.*, 1999). This is important because cryoprotectant-induced osmotic stress has been reported to have deleterious effects on survival of mature cattle oocytes (Martino, 1996). The concentrations of these cryoprotectants used in the present study are similar to those, which have been reported to be effective for the slow freezing of cattle oocytes (Otoi *et al.*, 1993; Lim *et al.*, 1999). Use of a higher concentration of 2.0 M was found to lead to severe damage of the frozen-thawed oocytes in initial trials (data not shown). Irrespective of the type of cryoprotectant used, the proportion of oocytes recovered in a morphologically normal state was higher with 1.5 M concentration compared to that with 1.0 M in the present study. These results, as also reported by Lim *et al.* (1991) suggest that the concentration of the cryoprotectant is an important factor affecting the incidence of cryoinjuries during slow freezing of oocytes.

Our results are better than those of many earlier studies in terms of the proportion of oocytes recovered in a morphologically normal state. Xu *et al.* (1992) found only 67.4% of oocytes subjected to slow freezing in 1.5M PROH to have intact zonae pellucidae and normal looking cytoplasm. Sun *et al.* (1995) obtained around 86% of bovine oocytes to be morphologically normal after cryopreservation in 1.5 M PROH. Mavrides and Moroll (2002) who obtained the post-thaw recovery of morphologically normal oocytes to be 54.4%, and post IVF cleavage rate to be around 15% when in vitro matured bovine oocytes were subjected to slow freezing in 1.5 M PROH. The number of morphologically normal oocytes recovered after freeze-thawing was reported to be 49.8% for PROH and 32.6% for DMSO when in vitro matured

bovine oocytes were subjected to slow freezing in 1.6 M of PROH or DMSO (Otoi *et al.*, 1993).

A comparison of the three cryoprotectants in the present study revealed that DMSO was better than PROH and EG in terms of recovery of morphologically normal oocytes at both 1.0 and 1.5 M concentrations. In a study similar to ours, Schellander *et al.* (1994) compared the efficacy of different concentrations (1 M and 1.5 M) of various cryoprotectants (glycerol, DMSO and PROH) and found that PROH was superior to glycerol and DMSO in terms of the post fertilization cleavage rate and the proportion of oocytes which developed to 4-cell stage. In terms of the cleavage and development to 4-cell stage indicated that glycerol 1.5 M and PROH 1.5 M were better than DMSO 1 M, but DMSO 1.5 M was better than DMSO 1 M and glycerol 1 M. In a similar earlier study in which Otoi *et al.* (1993) compared the effectiveness of iso-molar concentrations of 1.6 M of PROH, DMSO and glycerol as cryoprotectants for slow freezing of in vitro matured bovine oocytes, post IVF fertilization rate of morphologically normal oocytes frozen-thawed in PROH (57.9%) was found to be higher than that of those frozen in DMSO (38.3%) but similar to that of oocytes frozen in glycerol (50.9%). However, for PROH, glycerol and DMSO, the cleavage rate (22.4, 20.6 and 18.3%, respectively) and the proportion of surviving oocytes developing to blastocyst (1.4, 4.8 and 0.9%, respectively) were not different. In another study in which Lim *et al.* (1999) compared the efficacy of 1.0 M of PROH, DMSO and glycerol for slow freezing of in vitro matured bovine oocytes, higher post IVF penetration rates were observed with DMSO (79%) or PROH (76%) than in glycerol (48%). The percentage of oocytes developing to 2-cell stage was also higher in DMSO (51%) and PROH (54%) than in glycerol (33%). However, a significant increase in the proportion of 8-cell embryos (46 vs. 21 to 26%) at 72 h post insemination and morulae (14 vs. 6 to 8%) were derived from oocytes frozen with PROH than with DMSO or glycerol.

Whereas some authors have reported DMSO and PROH to be equally effective for the slow freezing of cattle oocytes (Lim *et al.*, 1999), others have found PROH to be superior to DMSO (Otoi *et al.*, 1993). However, it is not possible to compare the results of this study with those of others in which the efficacy of different cryoprotectants has been compared because besides the

type of cryoprotectant employed, a number of other factors viz. the concentration of the cryoprotectant, protocols used for slow freezing, thawing procedures, species and the developmental state of the oocytes significantly affect the effectiveness of the cryoprotectant. Irrespective of the type of cryoprotectant, the loss of cumulus cells resulting in partial or complete denudation of the oocytes was the most commonly encountered damage. Since presence of an intact cumulus mass around the oocyte is necessary for fertilization and development to the blastocyst stage (Nandi *et al.*, 1998), the oocytes which get denuded during freezing-thawing cannot be used for IVF.

The cleavage rate on Day 2 post insemination and the proportion of cleaved oocytes that developed to the blastocyst stage on Day 9 post insemination after IVF of the oocytes in the control group was similar to that reported earlier from our laboratory (Chauhan *et al.*, 1998a,) and significantly higher than that for the frozen-thawed oocytes indicating that both fertilizability and the post-fertilization developmental competence was compromised due to adverse effects of exposure to a cryoprotectant and low temperatures. Earlier studies have suggested that exposure to cryoprotectants like PROH or DMSO at ambient temperatures can lead to premature cortical granule release in human oocytes (Schalkoff *et al.*, 1989), zona reaction, zona hardening and reduced fertilization in mouse (carroll *et al.*, 1990; Johnson and Pickering, 1987). Chromosomal abnormalities in form of polyploidy in mouse (Bouquet *et al.*, 1992) or aneuploidy in cattle oocytes (Asada and Fukui, 2000) have also been believed to be associated with decreased fertilizability of frozen-thawed oocytes. However, the primary reason for the loss of developmental competence during freezing has been reported to be the loss of metaphase spindles. Less than 10% of metaphase-II spindles have been reported to be in their normal formation after exposure to a cryoprotectant for 1 min (Aman and Parks, 1994). In other studies, spindles have been reported to completely disappear in more than 70% of frozen-thawed bovine oocytes (Saunders and Parks, 1999) and repeat culture failed to enable the bovine oocytes to recover normal appearance of meiotic spindle (Saunders and Parks, 1999; Asada and Fukui, 2000). It has been shown that in human oocytes, not only are meiotic spindles very sensitive to simple cooling, effective cryoprotectants like DMSO do not provide substantial stabilization of the spindles even at 0°C (Sathananthan *et al.*, 1988).

In conclusion, the type and the concentration of cryoprotectants are important factors affecting the post thaw survival of in vitro matured oocytes subjected to slow freezing. DMSO was more effective than EG or PROH for the slow freezing of in vitro matured buffalo oocytes.

4.3 VITRIFICATION OF IMMATURE OOCYTES

4.3.1 Collection of Oocytes

A total of 3097 buffalo ovaries were collected from slaughterhouse, spread over 12 trials for this experiment (Table 16). Out of the total 1552 oocytes of Grades A and B (Fig. 2,3) collected, 610 (39%) were of Grade A whereas 942 (61%) were of Grade B. The yield of Grade A oocytes per ovary varied from 0.18 to 0.22 among different trials, with a mean yield of 0.20 ± 0.05 per ovary (Table 17) whereas the yield of Grade B oocytes varied from 0.28 to 0.34 per ovary, with a mean yield of 0.29 ± 0.06 oocytes per ovary. The cumulative yield of Grade A and B oocytes recovered per ovary was 0.49 ± 0.11.

Table 16. Vitrification of immature oocytes: Number and quality of oocytes recovered through the aspiration of visible surface follicles.

Trial No.	No. of ovaries aspirated	Grade of oocytes n (%)		Total (n)
		Grade A	Grade B	
1	242	48 (41)	69 (59)	117
2	256	49 (38)	81 (62)	130
3	248	48 (39)	76 (61)	124
4	258	58 (43)	78 (57)	136
5	242	52 (43)	68 (57)	120
6	268	52 (40)	78 (60)	130
7	251	46 (39)	72 (61)	118
8	278	55 (40)	84 (60)	139
9	269	50 (37)	84 (63)	134
10	262	52 (39)	80 (61)	132
11	265	52 (38)	86 (62)	138
12	258	46 (35)	88 (65)	134
Total	3097	610 (39)	942 (61)	1552

Table 17. Vitrification of immature oocytes: Oocyte recovery per ovary.

Trial No.	Grade A oocytes (n)	Grade B oocytes (n)	Total (n)
1	0.19	0.29	0.48
2	0.19	0.32	0.51
3	0.19	0.31	0.50
4	0.22	0.31	0.53
5	0.22	0.28	0.50
6	0.19	0.29	0.48
7	0.18	0.29	0.47
8	0.20	0.30	0.50
9	0.19	0.31	0.50
10	0.20	0.30	0.50
11	0.20	0.32	0.52
12	0.18	0.34	0.52
Mean ± SEM	0.20± 0.05	0.29± 0.06	0.49 ± 0.11

4.3.2 Post-Thaw Recovery of Morphologically Normal Oocytes

A total of 1321 oocytes used in this experiment were divided into 4 groups for vitrification using 40% EG or 40% DMSO or 20% EG+20% DMSO or 20% EG+20% PROH. After thawing, a total of 1292 oocytes were recovered, resulting in a loss of 29 oocytes during handling (Table 18). The morphological examination of the oocytes revealed that the percentage of oocytes recovered in a morphologically normal state was similar for 40% DMSO (91.1 ± 1.5%), 20% EG+20% DMSO (93.7 ± 1.1%) and 20% EG+20% PROH (90.8 ± 1.2%). The proportion of oocytes recovered in a morphologically normal state was significantly lower ($P<0.01$) for 40% EG (80.6 ± 2.3%) compared to that for these groups.

Table 18. Vitrification of immature oocytes: Effects of different cryoprotectants on the proportion of oocytes recovered morphologically normal.

Cryopro-tectant/ Combi-nation	Cryopro-tectant	Concen-tration (%)	Oocytes frozen (n)	Oocytes recovered (n)	Morphologically normal oocytes n (%)	Damaged oocytes n (%)
EG	EG	40	322	315	259 (80.6±2.3) [a]	56 (16.1±2.3)[a]
DMSO	DMSO	40	328	322	298 (91.1±1.5) [b]	24 (7.2±1.5)[b]
EG +DMSO	EG	20	335	327	316 (93.7±1.1) [b]	11(3.0±1.1)[b]
	DMSO	20				
EG +PROH	EG	20	336	328	304 (90.8±1.2) [b]	24 (7.3±1.2)[b]
	PROH	20				

Data from 10 trials
Percent values are Mean ± SEM
Values with different superscripts within the same column differ significantly from each other (P<0.01)

The different types of damages observed included cracked ZP, split into two halves, leaked contents, shrunken/fragmented ooplasm, change in shape and partial/complete denudation of the oocyte (Fig. 5 to Fig. 13). Among these, partial or complete loss of the cumulus mass surrounding the oocytes was the most prevalent damage followed by cracked ZP, irrespective of the type of the cryoprotectant/ combination used (Fig. 20, Table 19).

Table 19. Types of damages after vitrification of immature oocytes in different cryoprotectants.

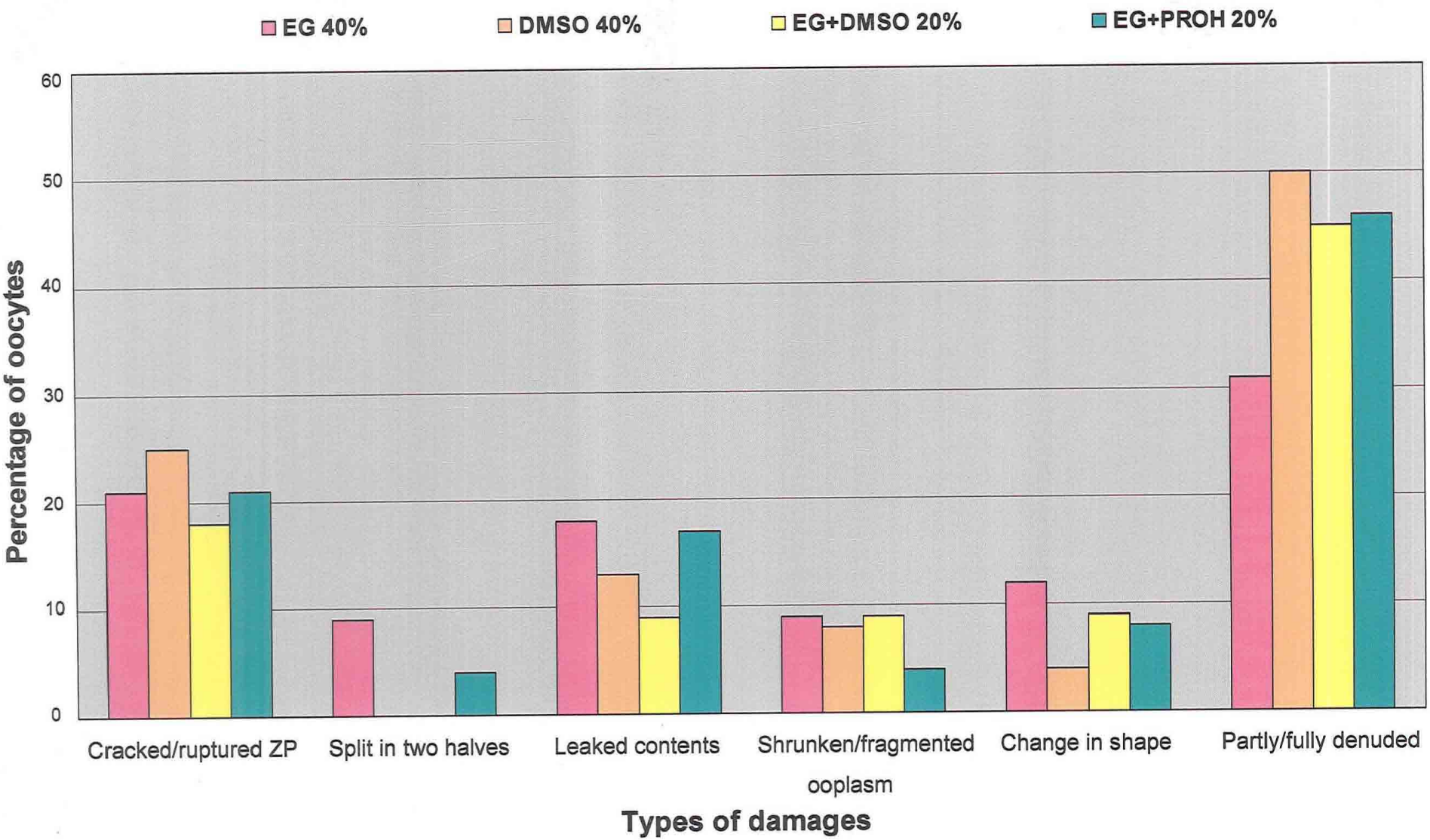
Cryopro-tectant	Concen-tration (M)	Oocytes damaged (n)	Type of damage n (%)					
			Cracked ZP	Split In two halves	Leaked contents	Shrunken cytoplasm	Change in Shape	Partly/ Fully denuded
EG	40	56	12 (21)	5 (9)	10 (18)	5 (9)	7 (12)	17 (31)
DMSO	40	24	6 (25)	0 (0)	3 (13)	2 (8)	1 (4)	12 (50)
EG +DMSO	20+20	11	2 (18)	0 (0)	1 (9)	1 (9)	1 (9)	6 (45)
EG +PROH	20+20	24	5 (21)	1 (4)	4 (17)	1 (4)	2 (8)	11 (46)

Data from 10 trials

4.3.3 Nuclear Maturation Rate

A total of 588 immature oocytes were divided into 4 groups and subjected to vitrification using the cryoprotectant/combination mentioned above. After thawing 579 oocytes were recovered, with a loss of 9 oocytes

Fig. 20 Types of damages after vitrification of immature oocytes



during handling (Table 20). The nuclear maturation rate of the vitrified-thawed oocytes was significantly higher (P<0.05) for 20% EG+20% DMSO (53.2 ± 1.8%) than that for 20% EG+20% PROH (42.8 ± 1.9) which, in turn, was significantly higher (P<0.05) than that for 40% EG (31.7 ± 2.4%) or 40% DMSO (32.9 ± 1.2%). However, the nuclear maturation rate obtained with all the cryoprotectants/ combinations was significantly lower (P<0.05) than that for controls (70.0 ± 1.0%).

Table 20. Vitrification of immature oocytes: Effects of different cryoprotectants or their combinations on the post-thaw nuclear maturation rate of oocytes recovered morphologically normal.

Cryopro-tectant/ combination	Concen-tration (%)	Oocytes frozen (n)	Recovered (n)	Morpholo-gically normal oocytes taken for staining (n)	Matured oocytes n (%)
EG	40	142	140	112	38 (31.7 ± 2.4)[a]
DMSO	40	148	146	135	47 (32.9 ± 1.2)[a]
EG +DMSO	20+20	150	147	142	77 (53.2 ± 1.8)[b]
EG +PROH	20+20	148	146	148	67 (42.8 ± 1.9)[c]
Control				200	140 (70.0 ± 1.0)[d]

Data from 4 trials
Percent values are Mean ± SEM
Values with different superscripts within the same column differ significantly from each other (P<0.05)

4.3.4 Post IVF Developmental Competence

Immature oocytes were divided in 4 groups and subjected to vitrification using cryoprotectants/combinations mentioned above. The frozen-thawed oocytes were subjected to IVM with a group of non-frozen oocytes run as controls. Following IVF, the cleavage rate recorded at Day 2 post insemination was found to be significantly higher (P<0.01) for the controls (57.0 ± 0.3%) compared to that for the vitrified-thawed oocytes of different groups (Table 21). The cleavage rate was found to be significantly higher (P<0.01) for the combinations of cryoprotectants i.e., 20% EG+20% DMSO (33.9 ± 0.13%) and 20% EG+20% PROH (27.8 ± 0.32%) compared to that when a single cryoprotectant was used i.e., 40% EG (16.3 ± 0.2%) or 40% DMSO (17.8± 21%). The percentage of oocytes which developed to the morula stage at Day 9 post insemination was significantly higher (P<0.01) for 20% EG+20% DMSO (14.9 ± 0.13%) compared to that for 20% EG+20%

PROH (10.0 ± 0.4%) or 40% EG (6.8 ± 0.32%) or 40% DMSO (7.9 ± 0.15%). It was, however, significantly lower (P<0.01) than the corresponding value for controls (20.0 ± 0.4%). Similarly, the proportion of oocytes which developed to the blastocyst stage at Day 9 post insemination was also significantly higher (P<0.01) for 20% EG+20% DMSO (4.0 ± 0.03%) compared to that for 20% EG+20% PROH (2.3 ± 0.02%) or 40% EG (1.3 ± 0.02%) or 40% DMSO (1.8 ± 0.02%). It was, however, significantly lower (P<0.01) than the corresponding values for controls (11.5 ± 0.02%).

Table 21. Post in vitro fertilization developmental competence of immature oocytes subjected to vitrification.

Group	Oocytes taken for IVF (n)	No of cleaved oocytes n (%)	Proportion of oocytes developed to n (%)	
			Morulae	Blastocysts
Control	200	114(57.0 ± 0.30)[a]	40(20.0 ± 0.40)[a]	23(11.5 ± 0.02)[a]
EG (40%)	147	24(16.3 ± 0.20)[b]	10(6.8 ± 0.32)[b]	2(1.3 ± 0.02)[b]
DMSO (40%)	163	29(17.8 ± 0.21)[b]	13(7.9 ± 0.15)[b]	3(1.8 ± 0.02)[b]
EG+DMSO (20%+20%)	174	59(33.9 ± 0.13)[c]	26(14.9 ± 0.13)[c]	7(4.0 ± 0.03)[c]
EG+PROH (20%+20%)	169	47(27.8 ± 0.32)[c]	17(10.0 ± 0.04)[b]	4(2.3 ± 0.02)[b]

Data from 6 trials
Percent values are Mean ± SEM
Values with different superscripts within the same column differ significantly from each other (P<0.01)

A comparison of the results of the present study with those of earlier studies on the vitrification of bovine immature oocytes indicates that the proportion of oocytes recovered in a morphologically normal form and the post IVF cleavage rate and the proportion of oocytes which developed to the blastocyst stage are higher with all the cryoprotectants and combinations used in the present study than those reported earlier in cattle. In one of the early studies, when immature pronuclear stage bovine oocytes were vitrified with a vitrification solution consisting of 40% EG, 0.3 M trehalose and 20% PVP in Dulbecco's phosphate buffered saline negative (DPBS-), positive (+) and NaCl, the cleavage rates were found to be 23, 21 and 13%, respectively, and the blastocyst formation was 3, 3 and 1%, respectively (Saha *et al.*, 1996). In a later study by Le Gal and Massip (1999), vitrification of immature

bovine oocytes in a mixture of 25% glycerol and 25% EG was reported to result in a post fertilization cleavage rate of 16%, although none out of the 146 oocytes taken developed to the blastocyst stage. Successful vitrification of bovine immature oocytes with a mixture of 15% EG and 15% PROH was reported recently by Justin Tan (2004) in a recent study.

The nuclear maturation rate obtained with 40% EG or 40% DMSO (31.7 and 32.9%, respectively) obtained in the present study is lower than those reported by Hochi *et al.* (1998) after vitrification of immature bovine oocytes in a mixture of 40% EG, Ficoll and sucrose as a vitrification medium (47.5%). However, the nuclear maturation rate obtained after vitrification of immature buffalo oocytes in combinations of cryoprotectants i.e., 20% EG+ 20% DMSO or 20% EG+ 20% PROH (53.2 and 42.8%) are similar to the nuclear maturation rate reported by these authors. In contrast to the results of the present study, Cetin and Bastan (2006) reported better nuclear maturation with 40% EG (34.1%) than with 20% EG+ 20% DMSO (20.7%).

There are very few reports on the vitrification of buffalo immature oocytes. In one of the early reports on vitrification of buffalo immature oocytes, Dhali *et al.* (2000a) used a vitrification solution consisting of 4.5 M EG and 3.4 M DMSO. Prior to exposure to this solution, the oocytes were exposed to the equilibration solution (50% vitrification solution) for 1 or 3 min at room temperature. The proportion of oocytes recovered in a morphologically normal form was found to be higher (98 and 88%, respectively) for the 3-min equilibration than for 1 min. The nuclear maturation rate, however, did not differ between the 1 and 3 min equilibration periods (21.5 ± 10.7 and 31.5 ± 1.5%, respectively). In a later study Dhali *et al.* (2000b) investigated the effects of EG concentration (equilibration solution-I i.e., 4.5 M EG + 3.4 M DMSO vs equilibration solution-II i.e.,3.5 M EG + 3.4 M DMSO) and time of exposure to equilibration solution (1 min vs 3 min) on the post-thaw morphological appearance and the in vitro maturation rate of buffalo oocytes. The percentage of oocytes found to be morphologically normal varied from 89 to 96% for the two vitrification solutions and the two exposure times. The nuclear maturation rate of oocytes equilibrated in equilibration solutions-I and II for 1 (28 and 24%, respectively) or 3 min (32

and 33%, respectively) did not differ significantly. Although the proportion of oocytes recovered in a morphologically normal form reported by these authors is similar to that obtained in the present study with combinations of cryoprotectants i.e., 20%EG+20%DMSO or 20%EG+20%PROH, the nuclear maturation rates obtained in the present study (53.2 ± 1.8 and 42.8 ± 1.9%, respectively) are much higher than those reported by these authors.

The nuclear maturation rates obtained in the present study are similar to those reported by Wani *et al.* (2004a) in a recent study. These authors compared the effects of 5 different concentrations (3.5, 4, 5, 6 and 7 M) each of 4 cryoprotectants (DMSO, EG, PROH and glycerol) on the morphological survival and in vitro maturation rate of vitrified-thawed immature buffalo oocytes. Among the different cryoprotectants, the highest maturation rate was obtained with 7 M DMSO, EG, PROH and glycerol (40.3, 42.5, 40.4 and 23.5%, respectively). Oocytes reaching to metaphase-II stage from the oocytes cryopreserved in 7 M glycerol were significantly lower than that of oocytes vitrified in DMSO, EG and PROH.

To our knowledge, there is only one earlier report on the post fertilization developmental competence of immature buffalo oocytes subjected to vitrification. Using the same concentrations of different cryoprotectants as discussed above Wani *et al.*, 2004b reported that the cleavage rates of oocytes vitrified in different concentrations of DMSO, EG, PROH and glycerol were lower than that of the fresh embryos, as also observed in the present study. The cleavage rates were higher for the oocytes vitrified in 6 and 7 M DMSO (29.8 and 19.5%, respectively), EG (30.8 and 20.4%, respectively), PROH (27.3 and 17.7%, respectively) and glycerol (21.0 and 15.4%, respectively) compared with oocytes cryopreserved in other concentrations. The percentage of oocytes inseminated that developed to the morula stage varied from 10 to 11% and those which developed to the blastocyst stage varied from 3 to 4% for the 7 M concentration of DMSO, EG or PROH. One possible reason for the lower nuclear maturation rates and the proportion of inseminated oocytes that developed to the morula and blastocyst stages obtained by these authors compared to the corresponding values obtained in the present study could be the use of a single cryoprotectant by these authors

unlike the use of combinations of cryoprotectants used in the present study. A comparison between single cryoprotectants i.e., 40% EG or 40% DMSO with a combination of cryoprotectants i.e., 20% EG+ 20% DMSO or 20% EG+20% PROH in the present study indicates that the nuclear maturation and cleavage rates, and the proportion of oocytes that developed to the morula and the blastocyst stage are significantly higher when a combination of cryoprotectants is used.

It can be concluded that among the 4 cryoprotectants/combinations (40%EG, 40% DMSO, 20%EG + 20%DMSO and 20%EG + 20% PROH) examined for the vitrification of immature oocytes, 20%EG + 20%DMSO gave the best results in terms of nuclear maturation rates and morula and blastocyst yields.

4.4 VITRIFICATION OF IN VITRO MATURED OOCYTES

4.4.1 Collection of Oocytes

A total of 2253 buffalo ovaries were used for this experiment, spread over 9 trials (Table 22). Out of the total 1159 oocytes of Grades A and B (Fig. 2, 3) collected, 464 (40%) were of Grade A whereas 695 (60%) were of Grade B. The yield of Grade A oocytes per ovary varied from 0.19 to 0.23 among different trials, with a mean yield of 0.20 ± 0.02 per ovary (Table 23) whereas the yield of Grade B oocytes varied from 0.29 to 0.32 per ovary, with a mean yield of 0.31 ± 0.10 oocytes per ovary. The cumulative yield of Grade A and B oocytes recovered per ovary was 0.51 ± 0.12.

Table 22. Vitrification of in vitro matured oocytes: Number and quality of oocytes recovered through the aspiration of visible surface follicles.

Trial No.	No. of ovaries aspirated	Oocytes n (%)		Total (n)
		Grade A	Grade B	
1	248	48 (39)	76 (61)	124
2	220	56 (42)	76 (58)	132
3	256	58 (43)	78 (57)	136
4	272	60 (41)	88 (59)	148
5	240	48 (40)	72 (60)	120
6	270	52 (39)	80 (61)	132
7	247	46 (39)	72 (61)	118
8	258	48 (37)	80 (61)	128
9	242	48 (40)	73 (60)	121
Total	2253	464 (40)	695 (60)	1159

Table 23. Vitrification of in vitro matured oocytes: Oocytes recovery per ovary.

Trial No	A grade oocytes (n)	B grade oocytes (n)	Total (n)
1	0.19	0.31	0.50
2	0.22	0.29	0.51
3	0.23	0.30	0.53
4	0.22	0.32	0.54
5	0.20	0.30	0.50
6	0.19	0.30	0.49
7	0.19	0.29	0.48
8	0.19	0.31	0.50
9	0.19	0.31	0.50
Mean ± SEM	0.20 ± 0.02	0.31 ± 0.10	0.51 ± 0.12

4.4.2 Post-Thaw Recovery of Morphologically Normal Oocytes

Following IVM, the oocytes were subjected to vitrification using either 40% EG or 40% DMSO or 20% EG+20% DMSO or 20% EG+20% PROH. After vitrification of a total of 811 oocytes, 782 oocytes were recovered resulting in a loss of 29 oocytes during handling (Table 24). The percentage of oocytes recovered in a morphologically normal form after vitrification-thawing of in vitro matured oocytes was not significantly different ($P<0.01$) for 20% EG+20% DMSO (95.6 ± 1.4%), 20% EG+20% PROH (85.2 ± 2.1%) and 40% DMSO (91.6 ± 2.5%), while the percentage of oocytes recovered in a morphologically normal form for 40% EG (85.2 ± 2.1) was significantly lower ($P<0.01$) than the above three groups.

Table 24. Vitrification of in vitro matured oocytes: Effects of different cryoprotectants on the proportion of oocytes recovered morphologically normal.

Cryopro-tectant/ Combi-nation	Cryopro-tectant	Concen-tration (%)	Oocytes frozen (n)	Oocytes recovered (n)	Morphologically normal oocytes n (%)	Damaged oocytes n (%)
EG	EG	40	214	206	180(85.2 ± 2.1)^a	26(14.8 ± 2.1)^a
DMSO	DMSO	40	194	185	174(91.6 ± 2.5)^b	11(8.4 ± 2.5)^b
EG +DMSO	EG	20	189	185	182(95.6 ± 1.4)^b	3(4.4 ± 1.4)^b
	DMSO	20				
EG +PROH	EG	20	214	206	196(95.1 ± 2.1)^b	10(4.9 ± 2.1)^b
	PROH	20				

Data from 10 trials
Percent values are Mean ± SEM
Values with different superscripts within the same column differ significantly from each other ($P<0.01$)

Among the different type of damages observed i.e., cracked ZP, split into halves, leaked contents, shrunken/ fragmented ooplasm, change in shape and partial /full denudation of the oocytes (Fig. 5 to Fig. 13), the last one was found to be the most prevalent, irrespective of the cryoprotectant/combination used (Fig. 21, Table 25).

Table 25. Types of damages after vitrification of in vitro matured oocytes in different cryoprotectants.

Cryopro-tectant	Concen-tration (M)	Oocytes damaged (n)	Type of damage n (%)					
			Cracked ZP	Split In two halves	Leaked contents	Shrunken cytoplasm	Change in Shape	Partly/ Fully denuded
EG	40	26	4 (15)	1 (3)	3 (12)	0 (0)	3 (12)	15 (58)
DMSO	40	11	1 (9)	0 (0)	0 (0)	1 (9)	0 (0)	9 (82)
EG +DMSO	20+20	3	1 (33)	0 (0)	0 (0)	0 (0)	1 (33)	1 (33)
EG +PROH	20+20	10	1 (10)	1 (10)	1 (10)	2 (20)	2 (20)	3(30)

Data from 10 trials

4.4.3 Post IVF Developmental Competence

After IVM, the oocytes were divided in 4 groups and subjected to vitrification using cryoprotectants/combinations mentioned above. The frozen-thawed oocytes were subjected to IVF with a group of non-frozen in vitro matured oocytes run as controls. Following IVF, the cleavage rate recorded at Day 2 post insemination was found to be significantly higher ($P<0.01$) for the controls (58.0 ± 0.4%) compared to that for the vitrified-thawed oocytes of different groups (Table 26). The cleavage rate was found to be significantly higher ($P<0.01$) for 20% EG+20% DMSO (36.2 ± 0.13%) than that for 20% EG+20% PROH (26.3 ± 0.34%) or 40% EG (18.8 ± 0.3%) or 40% DMSO (20.1± 0.21%). The percentage of oocytes which developed to the morula stage at Day 9 post insemination was also significantly higher ($P<0.01$) for 20% EG+20% DMSO (11.5 ± 0.08%) compared to that for 20% EG+20% PROH (9.3 ± 0.07%) or 40% EG (7.2 ± 0.14%) or 40% DMSO (8.0 ± 0.06%). It was, however, significantly lower ($P<0.01$) than the corresponding value for controls (22.0 ± 0.22%). Similarly, the proportion of oocytes which developed to the blastocyst stage at Day 9 post insemination was also significantly higher ($P<0.01$) for 20% EG+20% DMSO (5.4 ± 0.02%) compared to that for

20% EG+20% PROH (2.7 ± 0.01%) or 40% EG (1.6 ± 0.01%) or 40% DMSO (2.2 ± 0.02%). It was, however, significantly lower (P<0.01) than the corresponding values for controls (12.0 ± 0.09%).

Table 26. Post in vitro fertilization developmental competence of in vitro matured oocytes subjected to vitrification.

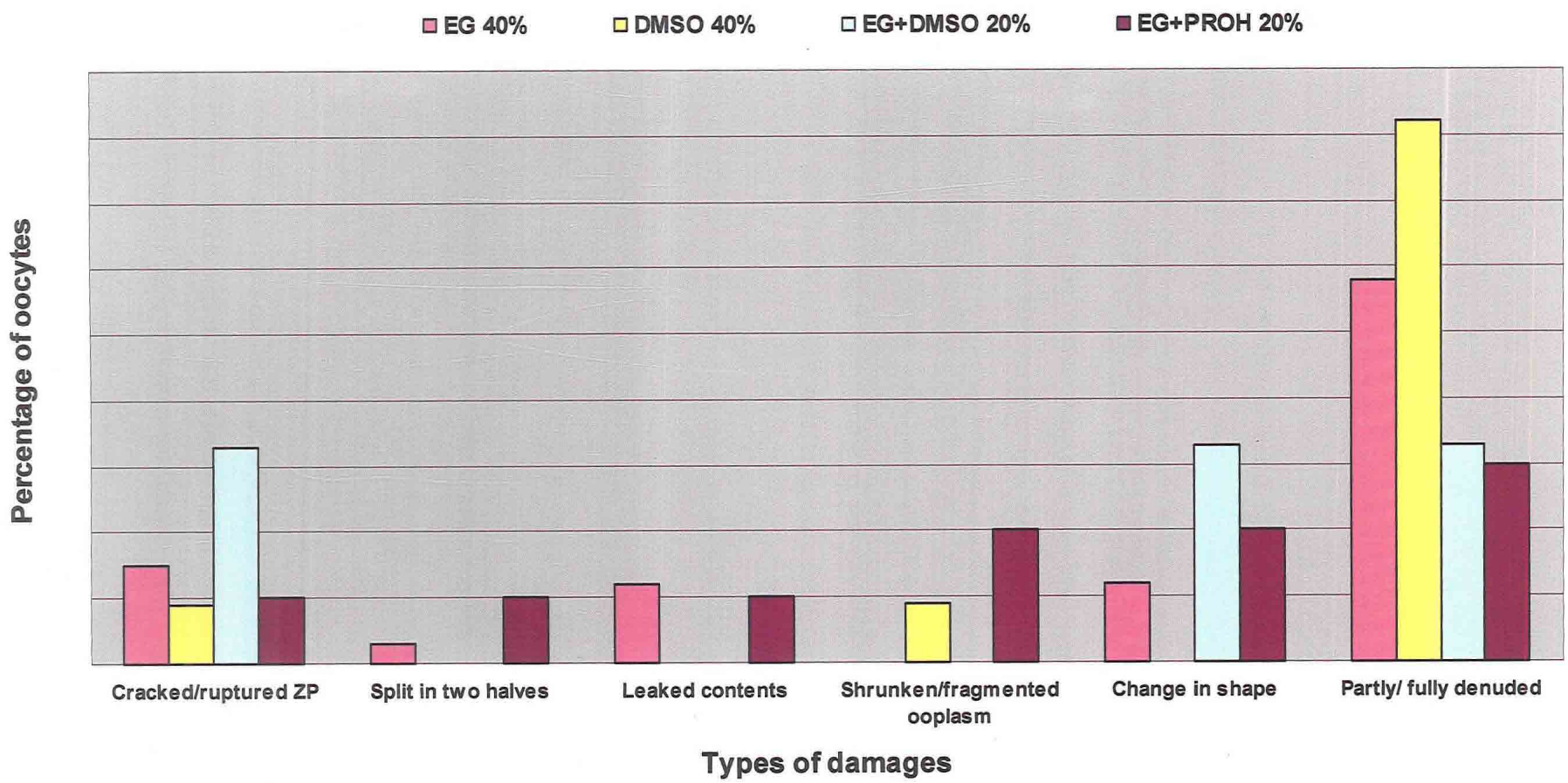
Group	Oocytes taken for IVF (n)	No of cleaved oocytes n (%)	Proportion of oocytes developed to n (%)	
			Morulae	Blastocysts
Control	150	87 (58.0 ± 0.4)[a]	33(22.0 ± 0.22)[a]	18 (12.0 ± 0.09)[a]
EG (40%)	180	34(18.8 ± 0.3)[b]	13(7.2 ± 0.14)[b]	3 (1.6 ± 0.01)[b]
DMSO (40%)	174	35(20.1 ± 0.21)[b]	14(8.0 ± 0.06)[b]	4 (2.2 ± 0.02)[b]
EG+DMSO (20%+20%)	182	66(36.2 ± 0.13)[c]	21(11.5 ± 0.08)[c]	10 (5.4 ± 0.02)[c]
EG+PROH (20%+20%)	182	48(26.3 ± 0.34)[b]	17(9.3 ± 0.07)[b]	5 (2.7 ± 0.01)[b]

Data from 6 trials
Percent values are Mean ± SEM
Values with different superscripts within the same column differ significantly from each other (P<0.01)

The proportion of oocytes that developed to the blastocyst stage, as observed in the present study is lower than that obtained by Hamano *et al.* (1992) who reported that 9 out of 88 inseminated oocytes developed to the blastocyst stage after vitrification of in vitro matured bovine oocytes in a mixture of 2.0 M DMSO, 1.0 M acetamide and 3.0 M PROH. However, the cleavage rate and the blastocyst formation rate in the present study is similar to that reported by Saha *et al.* (1996) after vitrification of in vitro matured oocytes in a vitrification solution consisting of 40% EG, 0.3 M trehalose and 20% PVP. Our cleavage rates and blastocyst formation rates are also similar to those obtained after vitrification of bovine oocytes in 40% EG reported by Otoi *et al.* (1998). To our knowledge, there is no report available on the vitrification of in vitro matured buffalo oocytes.

In conclusion, among the 4 cryoprotectants/combinations (40%EG, 40% DMSO, 20%EG + 20%DMSO and 20%EG + 20% PROH) examined for the vitrification of in vitro matured oocytes, 20%EG + 20%DMSO gave the best results in terms of morula and blastocyst yields.

Fig. 21 Types of damages after vitrification of in vitro matured oocytes



4.5 GENERAL DISCUSSION

The yield of usable quality oocytes of grades A + B was found to vary between 0.48 and 0.75 per ovary in the different experiments on slow freezing and vitrification of oocytes. These values fall within the range of recovery of usable quality oocytes reported in various earlier studies in which these have been found to be only 0.4 (Totey *et al.*, 1992; Madan *et al.*, 1994a), 0.9 (Das *et al.*, 1996). A comparison with the corresponding figure in cattle which is around 8-12 acceptable quality oocytes per ovary (Gordon, 1994) indicated that the recovery of total and usable quality oocytes per ovary is much lower in buffalo compared to that in cattle. Cryopreservation could be a means of preserving buffalo oocytes for making them available as and when required for various reproductive technologies like transgenesis, cloning, production of embryonic stem cells or multiplication or conservation of superior germplasm.

The present study was divided into 4 parts. Two methods of cryopreservation i.e., slow freezing and vitrification, each for immature and in vitro matured oocytes. The choice of cryoprotectants i.e., DMSO, PROH and EG, and the concentrations at which these were used was based on the results of earlier reports on the slow freezing of immature (Otoi *et al.*, 1995; Im *et al.*, 1997; Suzuki *et al.*, 1996) or mature cattle oocytes (Otoi *et al.*, 1993; Lim *et al.*, 1999) or the vitrification of oocytes of immature or mature cattle or buffalo (Hamano *et al.*, 1992; Hochi *et al.*, 1998; Cetin and Bastan 2006; Wani *et al.*, 2004a,b). All these cryoprotectants are low molecular weight permeating cryoprotectants which replace the water inside the oocyte osmotically. The molecular weights of the 3 cryoprotectants used were EG: 62.07; PROH: 76.1and DMSO: 78.13.

Only oocytes of Grades A and B i.e., oocytes surrounded by a cumulus mass all around were used since the post-thaw developmental competence of partly or wholly denuded oocytes subjected to cryopreservation has been reported to be lower than for those which had a dense cumulus mass around them in cattle (Im *et al.*, 1997). It is generally believed that the mature oocytes are more difficult to cryopreserve than GV-stage immature oocytes because the former are more prone to ultrastuctural damages. However, in the present study, the cleavage rates (28.8 vs. 36.3%) or the proportion of cleaved embryos that developed to the blastocyst stage at Day 9 post insemination

(0.6 vs, 0.7%) were not very different between immature and in vitro matured oocytes when these were subjected to slow freezing in 1.5 M DMSO. Similarly, the cleavage rates (33.9 vs. 36.2%) or the proportion of cleaved embryos that developed to the blastocyst stage at Day 9 post insemination (4.0 vs, 5.4%) were not different between immature and in vitro matured oocytes subjected to vitrification in 20% EG+ 20% DMSO. These results indicate that the lack of a nuclear membrane, the presence of chromosomes in metaphase-II or the presence of cumulus mass in an expanded state in the in vitro matured oocytes do not make them more prone to cryoinjury.

Irrespective of the techniques employed, or the type of oocytes used, the cleavage rate recorded at Day 2 post insemination and the proportion of cleaved embryos that developed to morula or blastocyst stages at Day 9 post insemination was much lower for the frozen-thawed oocytes compared to that for non-frozen controls. Two things, common to the cryopreservation techniques examined in the present study are – exposure of oocytes to a cryoprotectant solution and a drastic reduction in temperature. Both have been shown to have detrimental effects on the developmental competence of the oocytes in many species due to cryoinjuries which may me morphological or more importantly, ultrastructural.

Cryoprotectants which are used to permeate cells in molar concentrations are likely to alter structures which are stabilized by hydrophobic interactions and depend upon the equilibrium between polymerized and depolymerized components, including the microtubular system of the spindle (Webb *et al.*, 1986; Vincent *et al.*, 1990). Addition of DMSO to mouse oocytes at 37°C has been found to produce an early increase in the formation of cytoplasmic microtubule asters, as a result of microtubule elongation, towards the centre of the cell (Johnson and Pickering, 1987). More prolonged exposure at 37°C was found to cause spindle disruption and dispersal of chromosomes from the equatorial plate. When the temperature of exposure to DMSO was lowered to 20°C (Van der Elst *et al.*, 1988) and the mouse oocytes were diluted back into normal culture medium and maintained thereafter at 37°C, normal spindle morphology was observed in 60% of the cells. This was not different from oocytes cooled to 20°C without DMSO and returned to 37°C, where only 77% of cells exhibited normal

spindles. Reducing the temperature of exposure to DMSO to 4°C has been shown to reduce both spindle depolymerization and chromosomal scattering in mouse oocytes (Johnson and Pickering, 1987). These observations would be consistent with cryopreservation studies, where in both mouse (Whittingham, 1977) and human (Hunter *et al.*, 1991) oocytes, DMSO exposure and equilibrium were performed at 0°C before freezing, and in both cases good fertilization and embryonic growth in culture were observed (George and Johnson, 1993).

Another commonly used cryoprotectant, 1,2-propanediol, has also been studied in relation to effects on spindle morphology (Van der Elst *et al.*, 1988). Exposure of mouse oocytes to 1,2-propanediol at 20°C showed there was a protection of spindle morphology compared to oocytes maintained at that temperature without cryoprotectant. However, when these murine oocytes were used for fertilization and embryo culture studies, it was found that the fertilization rate was not significantly lower than that of untreated cells. When the temperature of 1,2-propanediol exposure was raised to 37°C, low concentrations of 1,2-propanediol (<1.0 M) caused spindle depolymerization, while higher concentrations of 1.5–2.0 M appeared to stabilize spindle morphology (Joly *et al.*, 1992). The percentage of spindle abnormalities in human oocytes exposed to 1,2-propanediol at 20°C and then cryopreserved was not significantly greater than in untreated oocytes (Gook *et al.*, 1993). Lowering of temperature of the oocytes in the absence of a cryoprotectant can also have adverse effects on their ultrastructure. When human oocytes were cooled to 20°C in the absence of a cryoprotectant, a higher degree of change in spindle morphology was noted (Vincent and Johnson, 1992).

Zona hardening is also believed to be one of the possible reasons for the lower cleavage rates. Following fertilization, the cortical granules are exocytosed and their contents alter the biochemistry and sperm-binding characteristics of the zona pellucida, which induces zona hardening and provides the natural block to polyspermic fertilization. Premature zona hardening will undoubtedly compromise normal fertilization and may impair implantation of the embryo (Gook *et al.*, 1993). In support of this hypothesis, human oocytes stored after slow cooling have lower fertilization rates after standard IVF, and electron microscopy of these cells has demonstrated a

reduction in the number of cortical granules (Gook *et al.*, 1993) suggesting that zona hardening had occurred in response to the freeze-thaw process. However, Gook *et al.* (1995) and Al-Hasani and Diedrich (1995) found an abundance of cortical granules in the cytoplasm of cryopreserved oocytes.

In the present study, vitrification was found to offer higher yields of Day 9 morulae and blastocysts compared to slow freezing. Whereas the proportion of cleaved embryos that developed to the blastocyst stage after slow freezing of immature and in vitro matured oocytes was only 0.6 and 0.7%, the corresponding values for vitrification were 4.0 and 5.4%, respectively. A comparison of the cleavage rates obtained after slow freezing of immature or in vitro matured oocytes (28.8 and 36.3%, respectively) or their vitrification in 20% EG+ 20% DMSO (33.9 and 36.2%, respectively) reveals that these were not very different whereas the proportion of cleaved embryos that developed to the blastocyst stage at Day 9 post insemination was over 6-fold higher for vitrification compared to that for slow freezing in case of immature as well as mature oocytes. These results indicate that the beneficial effects of vitrification are manifested at the post cleavage stages of embryonic development rather than at early embryonic divisions. Most of the publications comparing traditional freezing and vitrification of transferable stage domestic animal embryos report either equal in vitro or in vivo survival rates or improved results after vitrification (Mahmoudzadeh *et al.*, 1994; Dinnyes *et al.*, 1995; Hasler *et al.*, 1995; Reinders *et al.*, 1995; Agca *et al.*, 1996; Lane *et al.*, 1999).

The strategy of vitrification is basically different from that of slow cooling. A slow rate of cooling attempts to maintain a delicate balance between the various factors, which may result in damage, such as ice crystal formation, osmotic injury, toxic effect of cryoprotectants, concentrated intracellular electrolytes, chilling injury, zona fracture, and alterations of intracellular organelles, cytoskeleton and cell-to-cell contacts (Massip *et al.*, 1995; Dobrinsky, 1996; Martino *et al.*, 1996; Saha *et al.*, 1996), whereas vitrification totally eliminates ice crystal formation. A negative consequence of this strategy is the increased probability of nearly all forms of injury except for those caused by ice crystal formation. Different approaches are used to minimize toxic, osmotic and other injuries; application of less toxic chemicals, combination of two or three cryoprotectants including at least one permeable

cryoprotectant, stepwise addition and/or exposure of cells to precooled concentrated solutions (Fahy *et al.*, 1984; Rall, 1987; Kasai *et. al.*, 1990; Smorag and Gajda, 1994; Palasz and Mapletoft, 1996). In the past 15 years, at least 20 different combinations of cryoprotectants have been reported for vitrification of mammalian oocytes and embryos. The number of variations regarding the concentrations, incubation times and other conditions is almost infinite.

The cooling rates are much higher in vitrification than in slow freezing. Achievement of these higher cooling rates (2500°C/sec) in vitrification requires the use of approximately 5 to 7 M concentration of cryoprotectants, which is several orders higher than that needed for traditional equilibrium freezing approximately 1 to 2 M (Rall, 1987; Massip et al., 1989). Although this high concentration of cryoprotectants is always harmful to the cells, it helps to avoid formation of ice crystals. Vitrification has been reported to result in some positive consequences apart from the total elimination of ice crystal formation. The increased cooling rate decreases chilling injury, i.e. damage of the intracellular lipid droplets, lipid-containing membranes and the cytoskeleton, passing rapidly through the dangerous +15° to -5°C zone (Dobrinsky, 1996; Martino *et al.*, 1996; Isachenko *et al.*, 1998; Zeron *et al.*, 1999). In addition, vitrification does not require expensive programmable cooling machines or special skill and can be performed very quickly. Injury of oocytes resulting from exposure to temperatures near 0°C is a time-dependent phenomenon (Martino *et al.*, 1996). Therefore, various methods have been derived to increase cooling and warming rates to "outrace" chilling injury. These include use of electron microscope grids as a carrier of oocytes (Martino *et al.*, 1996), placing oocytes into small diameter open pulled straws (Vajta *et al.*, 1998), dropping 1- to 2-µl volumes of medium containing oocytes directly onto a cold surface (solid surface vitrification) at -150°C (Dinnyes *et al.*, 2000), or onto films of CPA within small nylon cryoloops (Lane *et al.*, 1999). The rationale of all of these methods is that the oocytes are suspended in very small volumes of medium so that they can be cooled at extremely high rates.

In conclusion, the results of the present study show that immature and in vitro matured oocytes can be successfully cryopreserved to obtain blastocysts after in vitro fertilization.

CHAPTER – 5

Summary and Conclusions

5. SUMMARY AND CONCLUSIONS

Availability of usable quality oocytes in large numbers, as and when required, is an essential prerequisite for the development of various reproductive technologies like in vitro embryo production, cloning, transgenesis and production of embryonic stem cells, for buffalo. Poor availability of total and usable quality oocytes from slaughterhouse ovaries and from live buffaloes through Ovum pick-Up necessitates their storage through cryopreservation. It also offers many other advantages like creation of oocyte banks and the feasibility of salvaging genetic material from prepubertal, infertile, pregnant or even dead animals. Cryopreservation, which involves arresting all biological processes and placing the cell into a suspended state of animation, can be carried out through slow freezing or vitrification. In the former, the oocytes cells are equilibrated with a cryoprotectant and cooled at a controlled rate. In vitrification, the oocytes present in a highly concentrated solution of cryoprotectants solidify during cooling, without formation of ice crystals. It offers faster and simplified freezing and thawing procedures and no requirement for an expensive freezing machine. Very little information is available in buffalo on the cryopreservation of oocytes. The present study was, therefore, conducted to i) evaluate the effects of different cryoprotectants on viability of oocytes subjected to slow freezing and vitrification, before and after in vitro maturation, ii) study the morphology of oocytes before and after cryopreservation and iii) study the developmental competence of frozen-thawed oocytes after in vitro fertilization.

Oocytes obtained by aspiration of surface follicles (2-8 mm diameter) from slaughterhouse buffalo ovaries were classified into Grades A, B, C and D based on the appearance and thickness of the cumulus mass surrounding them and the appearance of ooplasm. Oocytes with homogeneous cytoplasmic granulation and ≥3 layers of unexpanded cumulus layers were subjected to in vitro maturation (IVM) by placing groups of 15-20 oocytes in 100 µl droplets of the IVM medium (TCM-199+10% FBS+5 µg/ ml pFSH+0.81 mM sodium pyruvate+5% buffalo follicular fluid) and culturing for 24 h in a CO_2 incubator (5% CO_2 in air) at 38.5°C. For in vitro fertilization (IVF), the oocytes were incubated with processed spermatozoa for 18 h in fertilization

medium, after which these were cultured for up to 8-10 days post insemination in modified Charles Rosenkrans medium with amino acids (mCR2aa). The cleavage rate was recorded on Day 2 and the proportion of oocytes that developed to the morula and the blastocyst stage was recorded on Day 9 post insemination.

The percentage of oocytes that were recovered in a morphologically normal form and the nuclear maturation rates were compared among different groups by ANOVA with DMRT after arc sin transformation of data. The cleavage rate and the proportion of oocytes that developed to the morula and the blastocyst stage were compared among different groups by Student's 't' test in slow freezing experiments and by DMRT in vitrification experiments.

For slow freezing of immature oocytes, these were exposed to the final concentration of the cryoprotectants i.e., dimethyl suphoxide (DMSO), ethylene glycol (EG) or 1,2-propanediol (PROH) in a 2- or 3-step manner at room temperature (23 to 28°C). Groups of 20-30 oocytes were loaded into 0.25-ml French straws and subjected to slow freezing (-1°C/min to -6°C, automatically seeded by the freezer, held at this temperature for 7 min, cooled at a rate of -0.3°C /min to -30°C and maintained at this temperature for 10 min). The straws were then placed 1 to 2 cm above the surface of liquid nitrogen for 20 sec, plunged and stored for at least 7 days in liquid nitrogen. For warming, the straws were plunged into a water bath at 37°C for 20 sec. The dilution of the cryoprotectants was also done in a 2- or 3-step manner. The oocytes with spherical and symmetrical shape with no visual signs of lysis, membrane damage, swelling, vacuolization, degeneration or leakage of the cellular content were considered morphologically normal whereas those with cracked zona pellucidae, split in two halves, leaked contents, shrunken cytoplasm, change in shape and partly or fully denuded oocytes were considered morphologically abnormal after examination under an inverted microscope.

When immature oocytes were subjected to slow freezing using three cryoprotectants i.e., EG, DMSO and PROH, each used at concentrations of 1.0 and 1.5M,

i) The percentage of oocytes recovered in a morphologically normal state was significantly higher ($P<0.05$) for 1.5 M compared to that

for 1.0 M. For 1.0 M concentration, the percentage of oocytes that were recovered in a morphologically normal state was significantly higher ($P<0.05$) for DMSO (88.4 ± 2.9%) than that for EG (43.8 ± 6.3%) or PROH (57.4 ± 10.4%). Similarly, for the 1.5 M concentration, the percentage of oocytes recovered in a morphologically normal form was significantly higher ($P<0.05$) for DMSO (96.2 ± 2.4%) than that for EG (75.5 ± 3.6%) or PROH (74.8 ± 4.1%).

ii) Among the 6 combinations of cryoprotectants and concentrations evaluated, the percentage of oocytes recovered in a morphologically normal state was the highest and that of oocytes recovered in a damaged form was the lowest ($P<0.05$) for 1.5 M DMSO.

iii) The nuclear maturation rate of frozen-thawed oocytes which varied from 18.6 ± 3.3% to 35.9 ± 1.6% for the 6 combinations of cryoprotectants and concentrations used was significantly lower ($P<0.05$) than that for the non-frozen control oocytes (71.0 ± 1.7%).

iv) In case of all the 3 cryoprotectants, the nuclear maturation rate of frozen-thawed oocytes was significantly higher ($P<0.05$) for the higher concentration of 1.5 M compared to that for the lower concentration of 1.0 M. For 1.0 M concentration, the nuclear maturation rate was significantly ($P<0.05$) higher for DMSO (25.2 ± 1.0%) than that for EG (18.6 ± 3.3%) or PROH (20.2 ± 1.44%). Similarly, for 1.5 M concentration, the nuclear maturation rate for DMSO was significantly higher ($P<0.05$) than that for EG (22.8 ± 0.9%) or PROH (23.2 ± 2.0%).

v) Among the 6 combinations of cryoprotectants and concentrations evaluated, the nuclear maturation rate was found to be the highest ($P<0.05$) for 1.5 M DMSO. When this combination was used for the slow freezing of immature oocytes, the cleavages rate recorded at Day 2 post insemination was significantly lower ($P<0.01$) for the frozen-thawed oocytes (28.8%) compared to that for the controls (57.0%). The percentage of oocytes that developed to the morula

and the blastocyst stage at Day 9 post insemination was also significantly lower (P<0.01) for the oocytes subjected to slow freezing in 1.5 M DMSO (4.5 and 0.6%, respectively) compared to that for controls (19.2 and 10.6%, respectively).

For slow freezing of in vitro matured oocytes, these were exposed to the final concentration of the cryoprotectants i.e., DMSO, EG or PROH in a 2- or 3-step manner as for immature oocytes. Groups of 20-30 in vitro matured oocytes were then loaded into 0.25-ml French straws and subjected to slow freezing (-5.5°C at -1°C/min, automatically seeded by the freezer and then cooled at a rate of -0.6°C/min to -30°C). The straws were then plunged in liquid nitrogen and stored for at least a week. The straws were warmed in a 2- or 3-step manner as for immature oocytes. When in vitro matured oocytes were subjected to slow freezing using three cryoprotectants i.e., EG, DMSO and PROH, each used at concentrations of 1.0 and 1.5M,

i) For 1.0 M concentration, the percentage of oocytes recovered in a morphologically normal form after freezing-thawing was significantly higher (P<0.05) for DMSO (88.3 ± 1.9%) compared to that for EG (67.6 ± 2.4%) or PROH (67.5 ± 5.0%). Similarly, for 1.5 M concentration, the percentage of oocytes recovered in a morphologically normal form was significantly higher (P<0.05) for DMSO (96.0 ± 1.6%) compared to that for EG (78.4 ± 3.1%) or PROH (80.6 ± 4.6%).

ii) When in vitro matured oocytes subjected to slow freezing using this combination were fertilized in vitro, the cleavages rate recorded at Day 2 post insemination was significantly lower (P<0.01) for the frozen-thawed oocytes (36.3%) compared to that for the controls (60.0%). The percentage of oocytes that developed to the morula and the blastocyst stage at Day 9 post insemination was also significantly lower (P<0.01) for the oocytes subjected to slow freezing (4.1 and 0.7%, respectively) compared to that for controls (19.3 and 10.6%, respectively).

For vitrification, the immature and in vitro matured oocytes were divided into 4 groups, 40% EG, 40% DMSO, 20%EG+20%DMSO and 20%EG+20%PROH. The oocytes of groups 40% EG and 40% DMSO were exposed to the final concentration of the cryoprotectant in a 3-step manner

whereas those of the other 2 groups were exposed to the final concentration in a 2-step manner. Groups of around 30 oocytes were then loaded into 0.25 ml French straws, plunged and stored in liquid nitrogen for at least a week. The straws were thawed and the cryoprotectant solution was diluted in a 3-step manner. For the immature oocytes subjected to vitrification-thawing

i) The percentage of oocytes recovered in a morphologically normal state was similar for 40% DMSO (91.1 ± 1.5%), 20% EG+20% DMSO (93.7 ± 1.1%) and 20% EG+20% PROH (90.8 ± 1.2%). The proportion of oocytes recovered in a morphologically normal state was significantly lower ($P<0.01$) for 40% EG (80.6 ± 2.3%) compared to that for these groups.

ii) The nuclear maturation rate of the vitrified-thawed oocytes was significantly higher ($P<0.05$) for 20% EG+20% DMSO (53.2 ± 1.8%) than that for 20% EG+20% PROH (42.8 ± 1.9) which, in turn, was significantly higher ($P<0.05$) than that for 40% EG (31.7 ± 2.4%) or 40% DMSO (32.9 ± 1.2%).

iii) Following IVF, the cleavage rate recorded at Day 2 post insemination was significantly higher ($P<0.01$) for the controls (57.0 ± 0.3%) compared to that for the vitrified-thawed oocytes of different groups. The cleavage rate was significantly higher ($P<0.01$) for the combinations of cryoprotectants i.e., 20% EG+20% DMSO (33.9 ± 0.13%) and 20% EG+20% PROH (27.8 ± 0.32%) compared to that when a single cryoprotectant was used i.e., 40% EG (16.3 ± 0.2%) or 40% DMSO (17.8± 21%).

iv) The percentage of oocytes which developed to the morula stage at Day 9 post insemination was significantly higher ($P<0.01$) for 20% EG+20% DMSO (14.9 ± 0.13%) compared to that for 20% EG+20% PROH (10.0 ± 0.4%) or 40% EG (6.8 ± 0.32%) or 40% DMSO (7.9 ± 0.15%). It was, however, significantly lower ($P<0.01$) than the corresponding value for controls (20.0 ± 0.4%).

v) The proportion of oocytes which developed to the blastocyst stage at Day 9 post insemination was also significantly higher ($P<0.01$)

for 20% EG+20% DMSO (4.0 ± 0.03%) compared to that for 20% EG+20% PROH (2.3 ± 0.02%) or 40% EG (1.3 ± 0.02%) or 40% DMSO (1.8 ± 0.02%). It was, however, significantly lower ($P<0.01$) than the corresponding values for controls (11.5 ± 0.02%).

For the vitrification of in vitro matured oocytes, immature oocytes were first subjected to IVM and then vitrified. For these oocytes

i) The percentage of oocytes recovered in a morphologically normal form after vitrification-thawing was not significantly different ($P<0.01$) for 20% EG+20% DMSO (95.6 ± 1.4%), 20% EG+20% PROH (95.1 ± 2.1%) and 40% EG (91.6 ± 2.5%) while for 40% EG (85.2 ± 2.1) was significantly different ($P<0.01$) than the above three groups.

ii) Following IVF, the cleavage rate recorded at Day 2 post insemination was significantly higher ($P<0.01$) for the controls (58.0 ± 0.4%) compared to that for the vitrified-thawed oocytes of different groups. The cleavage rate was significantly higher ($P<0.01$) for 20% EG+20% DMSO (36.2 ± 0.13%) than that for 20% EG+20% PROH (26.3 ± 0.34%) or 40% EG (18.8 ± 0.3%) or 40% DMSO (20.1± 0.21%).

iii) The percentage of oocytes which developed to the morula stage at Day 9 post insemination was also significantly higher ($P<0.01$) for 20% EG+20% DMSO (11.5 ± 0.08%) compared to that for 20% EG+20% PROH (9.3 ± 0.07%) or 40% EG (7.2 ± 0.14%) or 40% DMSO (8.0 ± 0.06%). It was, however, significantly lower ($P<0.01$) than the corresponding value for controls (22.0 ± 0.22%).

iv) The proportion of oocytes which developed to the blastocyst stage at Day 9 post insemination was also significantly higher ($P<0.01$) for 20% EG+20% DMSO (5.4 ± 0.02%) compared to that for 20% EG+20% PROH (2.7 ± 0.01%) or 40% EG (1.6 ± 0.01%) or 40% DMSO (2.2 ± 0.02%). It was, however, significantly lower ($P<0.01$) than the corresponding values for controls (12.0 ± 0.09%).

It can be concluded from the results of the present study that

i) Blastocysts can be produced from immature and in vitro matured oocytes after slow freezing or vitrification.

ii) Among the different type of damages observed i.e., cracked ZP, split into halves, leaked contents, shrunken/fragmented ooplasm, change in shape and partial/full denudation of the oocytes, the last one was the most common, irrespective of the type of oocyte, type or concentration of the cryoprotectant/combination used or cryopreservation method employed.

iii) The proportion of oocytes recovered in a normal form was higher for the higher concentration of 1.5 M compared to that for the lower concentration of 1.0 M after slow freezing of immature or mature oocytes in EG, DMSO or PROH.

iv) For the slow freezing of immature oocytes in EG, DMSO or PROH, the nuclear maturation rate was higher for the higher concentration of 1.5 M compared to that for the lower concentration of 1.0 M.

v) Among the 6 combinations of cryoprotectants (EG, DMSO and PROH) and concentrations (1.0 and 1.5 M) examined for the slow freezing of immature and in vitro matured oocytes, DMSO at 1.5 M concentration gave the best results.

vi) Among the 4 cryoprotectants/combinations (40%EG, 40% DMSO, 20%EG + 20%DMSO and 20%EG + 20% PROH) examined for the vitrification of immature and in vitro matured oocytes, 20%EG + 20%DMSO gave the best results in terms of morula and blastocyst yields.

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ANNEXURE

ANNEXURE

Solutions for oocyte collection

Normal saline containing antibiotics

Composition	-	**Volume (1000ml)**
Sodium chloride	-	9.0 gm
Penicillin G	-	0.12 gm
Distilled water	-	1000 ml

Aspiration medium (for about 250-300 ovaries)

Composition	-	**Volume (100 ml)**
TCM-199 (Hepes modified)	-	50 ml
DPBS	-	50 ml
BSA	-	0.3 gm
Gentamycin	-	50μg/ml
L-glutamine	-	As recommended by the manufacturer

In vitro maturation media

Washing medium

Composition	-	**Volume (40 ml)**
TCM-199 (Hepes modified)	-	36 ml
FBS	-	4 ml
Sodium pyruvate	-	0.0036 gm
Gentamycin	-	50μg/ml
L-glutamine	-	As recommended by the manufacturer

Maturation medium

Composition	-	**Volume (10 ml)**
Washing medium	-	10 ml
Porcine FSH	-	5 μg/ml
Follicular Fluid	-	500μl

In vitro fertilization media

Brackett and Oliphant (BO) medium

Solution A (Stock)

Sodium chloride	-	4.3092 gm
Potassium chloride	-	0.1974 gm
Calcium chloride dihydrate	-	0.2171 gm
Magnesium chloride-hexahydrate	-	0.0697 gm
Sodium hydrogen phosphate-dihydrate	-	0.0840 gm

Dissolve the above in 500 ml of distilled water. Mix 0.1 ml of 0.5% phenol red for coloring and indication of pH of the solution. A yellowish color will appear after addition of phenol red. Add penicillin (50 I.U./ ml) and streptomycin (5 µg/ml) for preservation of longer periods.

Solution B (Stock)

Sodium bicarbonate	-	2.5873 gm

Dissolve it in 200 ml of distilled water. Mix 0.1 ml of 0.5% phenol red for indication of pH of the solution. Pink color will appear. Add penicillin/ streptomycin as given above.

Working BO medium

Composition	-	**Volume (50 ml)**
Solution A	-	38 ml
Solution B	-	12 ml
Heparin	-	10 µg/ml
Sodium pyruvate	-	0.0068 gm
Caffeine sodium benzoate	-	0.0971 gm

(Heparin can be used 50-100 µg/ml solution)

BO medium for capacitation and fertilization

Working BO media	-	10 ml
BSA (Fatty acid free)	-	0.1 gm

In vitro culture media

Washing medium for presumed zygotes

mCR2aa	-	20 ml
BSA (Fraction V) @ 0.8%	-	0.16 gm
Gentamycin	-	50μg/ml

In vitro culture medium

mCR2aa	-	9.0 ml
FBS	-	1.0 ml
Gentamycin	-	50 μg/ml

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

Modified Charles Rosenkrans-2 medium with amino acids (mCR2aa)

The mCR2aa medium was prepared in 100 ml aliquots as per the compositions given below. The pH and the osmolarity of the media were checked each time a fresh lot of the medium was prepared.

Composition of mCR2aa

S. No.	Component	Molarity (mM)
1	Water	-
2	NaCl	108.3
3	$NaHCO_3$	24.9
4	NEAA*	1.0 ml/100 ml
5	EAA**	2.0 ml/100 ml
6	Glutamine	1
7	KCl	2.9
8	Hemicalcium lactate	2.5
9	Na Pyruvate	0.5
10	Glycine	0.5
11	Alanine	0.5
12	Glucose	1
13	Phenol red	5 µg/ml
14	Gentamycin	50µg/ml
15	BSA	0.6%

*MEM Non-essential amino acids (Gibco, Catalog No. 11140-050)

*BME Essential amino acids (Sigma, catalog No. B6766)

Solutions for slow freezing of immature oocytes

Amount of various components for making solution for slow freezing of immature oocytes

Cryoprotectant	Amount of Cryoprotectant /DPBS/FBS (µl)			
	0.5 M	1.0 M	1.5 M	2.0 M
EG	55/1,635/300	110/1,580/300	165/1,525/300	220/1,470/300
DMSO	71/1,619/300	142/1,548/300	213/1,477/300	284/1,406/300
PROH	73/1,617/300	146/1,544/300	219/1,471/300	292/1,.398/300

The total volume in each case was 2 ml. The concentration of FBS in this was 15%. DPBS taken already contained 5 µg/ml gentamycin sulphate. During preparation of the solutions, first the required volume of DPBS was taken in a 35 mm Petri dish. The required amounts of FBS and respective cryoprotectants were added and the mixture was kept at 4°C. The solutions were filtered through a 0.22 µm filter just before use. Fresh solutions were prepared during every trial.

Solutions for slow freezing of in vitro matured oocytes

Amount of various components for making solutions for slow freezing of mature oocytes

Cryoprotectant	Amount of Cryoprotectant /TCM-199/FBS (µl)			
	0.5 M	1.0 M	1.5 M	2.0 M
EG	55/1,835/100	110/1,780/100	165/1,725/100	220/1,670/100
DMSO	71/1,819/100	142/1,748/100	213/1,677/100	284/1,606/100
PROH	73/1,817/100	146/1,744/100	219/1,671/100	292/1,598/100

The total volume in each case was 2 ml. The concentration of FBS in this was 5%. TCM-199 taken already contained 50µg/ml gentamycin sulphate. During preparation of the solutions, first the required volume of TCM-199 was taken in a 35 mm Petri dish. The required amounts of FBS and respective cryoprotectants were added and the mixture was kept at 4°C. The solutions were filtered through a 0.22 µm filter just before use. Fresh solutions were prepared during every trial.

Solutions for vitrification of immature and in vitro matured oocytes

Composition - 4.0 ml

Amount of various components for preparation of vitrification solutions

Cryoprotectant/ combination	Cryopro-tectant	Concen-tration (%)	Molarity	Amount (µl)	TCM-199 (ml)	Sucrose (gms)	FBS (µl)
EG	EG	10	1.5	345	3.25	0.47	400
		25	3.8	862	2.73		
		40	6.2	1380	2.22		
DMSO	DMSO	10	1.4	400	3.20		
		25	3.5	1000	2.60		
		40	5.6	1600	2.00		
EF + DMSO	EG	10	1.5	345	2.85		
	DMSO	10	1.4	400			
	EG	20	3.1	690	2.11		
	DMSO	20	2.8	800			
EG + PROH	EG	10	1.5	345	2.86		
	PROH	10	1.3	395			
	EG	20	3.1	690	2.12		
	PROH	20	2.7	790			

Solution for loading of vitrified oocytes

Volume: 2 ml

Sucrose (M)	Amount (gm)	TCM-199 (ml)	FBS (µl)
0.5	0.34	1.8	200

Solution for thawing of vitrified oocytes

Volume: 2 ml

Sucrose (M)	Amount (gm)	TCM-199 (ml)
0.5	0.34	2.0
0.33	0.22	2.0
0.17	0.11	2.0
0.00	0.00	2.0

Solutions for oocyte fixation and staining

Hypotonic solution

Composition	-	**Volume (10.0 ml)**
Distilled water	-	10.0 ml
Tri-sodiumcitrate	-	0.09 gm

First fixative

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

Composition	-	**Volume (10.0 ml)**
Methanol	-	5.0 ml
Glacial acetic acid	-	1.0 ml
Distilled water	-	4.0 ml

Second fixative

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

Composition	-	**Volume (4.0 ml)**
Methanol	-	3.0 ml
Glacial acetic acid	-	1.0 ml

Third fixative

Third fixative contained methanol, glacial acetic acid and distilled water in the ratio of 3:3:1.

Composition	-	**Volume (7.0 ml)**
Methanol	-	3.0 ml
Glacial acetic acid	-	3.0 ml
Distilled water	-	1.0 ml

Giemsa stain

Composition	-	**Volume (50.0 ml)**
Giemsa	-	2.0 ml
Double distilled water	-	48.0 ml

Aceto-orcein stain

Stock solution (A)

Composition	-	**Volume (45.0 ml)**
Orcein	-	1.0 gm
Acetic acid	-	45.0 ml

The solution was boiled, filtered and then preserved at room temperature.

Working Solution

Composition	-	**Volume (10.0 ml)**
Solution A	-	5.5 ml
Double distilled water	-	4.5 ml

The working solution was prepared just before use.

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