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EVALUATION OF CRYOPRESERVATION
METHODS FOR *IN VITRO* PRODUCED
BOVINE EMBRYOS

by

Tshimangadzo Lucky Nedambale

Submitted in partial fulfillment of the requirements for the degree

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Supervisor: Prof.J.P.C. Greyling

Co-supervisor: Mr. J.M. Rust

DEDICATED TO MY FAMILY

- . *To you mom, for the love, encouragement, excellent education and guidance in my life. I could not have achieved what I achieved today without your understanding. You are a great mom.*
- . *To my uncles, for the support and encouragement they gave me when things were tough.*
- . *To my son Khathutshelo, for bringing light and direction into my life, and for understanding my absence all these years. You will always be in my heart.*
- . *To you Priscilla, for all the love, encouragement, and help in achieving this study. Above all thank you for being my best friend all these years.*
- . *To my sister Rudzani, for understanding the reason why we couldn't be together all these years.*
- . *To the rest of my family, for understanding my absence.*

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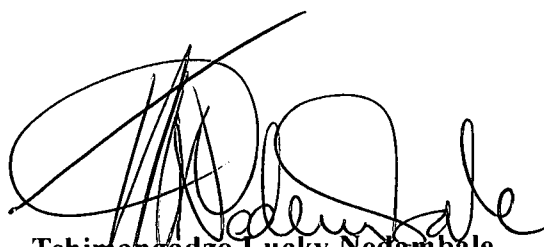
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DECLARATION

I declare that the dissertation hereby submitted by me for the **Magister Scientiae Agriculturae** degree at the University of the Orange Free State is my own independent work and has not previously been submitted by me at another University/faculty. I furthermore cede copyright of the dissertation in favour of the University of the Orange Free State.

A handwritten signature in black ink, appearing to read 'Tshimangadzo Lucky Nedambale', written in a cursive style.

Tshimangadzo Lucky Nedambale

Bloemfontein

November 1999

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	iii
DECLARATION	v
LIST OF TABLES	ix
LIST OF FIGURES	x
LIST OF ABBREVIATIONS	xii
 CHAPTER	 1
1. GENERAL INTRODUCTION	1
2. LITERATURE REVIEW	4
2.1 <i>IN VITRO</i> EMBRYO PRODUCTION (IVEP)	6
2.1.1 Sources of oocytes	6
2.1.1.1 Abattoir Material	7
2.1.1.2 Ovum pick up (OPU) by means of Ultrasound	7
2.1.2 <i>In vitro</i> maturation (IVM)	8
2.1.3 <i>In vitro</i> fertilization (IVF)	12
2.1.4 <i>In vitro</i> culture (IVC)	14
2.2 CRYOPRESERVATION OF <i>IN VITRO</i> PRODUCED BOVINE EMBRYOS	18
2.2.1 Controlled (slow) freezing and thawing	20
2.2.2 Non-permeating agents	21
2.2.3 Permeability of cryoprotectants in bovine embryos	23
2.2.4 One-Step freezing method	26
2.2.5 Vitrification	26
2.2.6 Evaluation of embryos after thawing	28
2.2.7 Survival rates of <i>in vitro</i> frozen bovine embryos	28

3.	MATERIAL AND METHODS	31
3.1	Source of embryos	31
3.2	Preparation of freezing media	33
3.2.1	Conventional slow freezing of embryos	34
3.2.2	Vitrification of Embryos	35
3.3	Thawing Procedures	36
3.4	Statistical analysis	36
4.	RESULTS	37
4.1	EVALUATION OF FOUR CRYOPRESERVATION METHODS	37
4.1.1	Survival rate of IVP bovine embryos immediately after thawing	37
4.1.2	Survival rate of IVP bovine embryos 24 hours post-thawing	39
4.1.3	Survival rate of IVP bovine embryos 48 hours post-thawing	41
4.2	COMPARISON BETWEEN THE FOUR CRYOPRESERVATION METHODS	44
4.2.1	Predicted survival rate of IVP bovine embryos immediately after thawing	44
4.2.1.1	Predicted survival rate of IVP bovine embryos vitrified by three vitrification methods immediately after thawing	45
4.2.2	Predicted survival rate of IVP bovine embryos 24 hours post-thawing	45
4.2.2.1	Predicted survival rate of IVP bovine embryos vitrified by three vitrification methods 24 hours post-thawing	46
4.2.3	Predicted survival rate of IVP bovine embryos 48 hours post-thawing	47
4.2.3.1	Predicted survival rate of IVP bovine embryos vitrified by three vitrification methods 48 hours post-thawing	48

5.	DISCUSSION	53
5.1	Conventional slow freezing of IVP bovine embryos	53
5.2.	Vitrification of IVP bovine embryos	55
5.3	Consequences of embryo cryopreservation and thawing	59
6.	GENERAL CONCLUSIONS	62
	ABSTRACT	65
	OPSOMMING	69
	REFERENCES	73

LIST OF TABLES

Tables	Page	
2.1	Young born after the freezing/thawing of mammalian embryos	19
2.2	Viability of frozen bovine embryos using various cryoprotectant solutions and rehydrated directly in holding medium	24
2.3	<i>In vitro</i> development of embryos frozen in 1.0M or 1.5M EG and rehydrated directly in holding medium	25
4.1	Embryo survival rate (%) immediately after thawing of the three vitrification methods and the slow freezing method	37
4.2	Embryo survival rate (%) 24 hours post-thawing of the three vitrification methods and the slow freezing method	39
4.3	Embryo survival rate (%) 48 hours post-thawing of the three vitrification methods and the slow freezing method	41
4.4	The predicted (\pm SE) embryo survival rate (p) immediately after thawing of three vitrification methods and the slow freezing method	44
4.5	The predicted (\pm SE) embryo survival rate (p) immediately after thawing following the three vitrification methods	45
4.6	The predicted (\pm SE) embryo survival rate (p) 24 hours post-thawing of three vitrification methods and the slow freezing method	46
4.7	The predicted (\pm SE) embryo survival rate (p) 24 hours post-thawing following the three vitrification methods	47
4.8	The predicted (\pm SE) embryo survival rate (p) 48 hours post-thawing of three vitrification methods and the slow freezing method	48
4.9	The predicted (\pm SE) embryo survival rate (p) 48 hours post-thawing following the three vitrification methods	49

LIST OF FIGURES

Figures	Page
2.1 Various stages in the development of bovine embryos (one cell to hatching blastocyst).	5
3.1 Some of the IVP bovine embryos used in the vitrification methods and the slow freezing method before being randomly assigned to four different treatment groups.	32
3.2 Freeze control (Programmable freezer) model CL 863 used in pre-programmed mode (Cryogenesis, Australia).	34
3.3 Configuration of the solutions in straw loaded with an embryo prior to being placed in liquid nitrogen (-196 °C). Vitrification solution with embryo and ViGro™ Holdingplus were sequential put into the straws to yield four liquid chambers separated by air bubbles.	35
4.1 Survival rate (%) of IVP bovine embryos immediately after thawing following vitrification in 40% EG (TMT 1); 40% EG + 0.3M trehalose (TMT 2); 40% EG + 0.3M trehalose + 20% PVP (TMT 3) and the slow freezing method in 1.5M ViGro™ EG Freezeplus (TMT 4).	38
4.2 Survival rate (%) of IVP bovine embryos 24 hours post-thawing following vitrification in 40% EG (TMT 1); 40% EG + 0.3M trehalose (TMT 2); 40% EG + 0.3M trehalose + 20% PVP (TMT 3) and the slow freezing method in 1.5M ViGro™ EG Freezeplus (TMT 4)	40
4.3 Survival rate (%) of IVP bovine embryos 48 hours post-thawing following vitrification in 40% EG (TMT 1); 40% EG + 0.3M trehalose (TMT 2); 40% EG + 0.3M trehalose + 20% PVP (TMT 3) and the slow freezing method in 1.5M ViGro™ EG Freezeplus (TMT 4)	42
4.4 IVP bovine embryos under light microscope (F10/0.25) in ViGro™ Holdingplus: Embryos were vitrified in 40% EG + 0.3M trehalose + 20% PVP (TMT 3). (A) live blastocysts immediately after thawing. (B) degenerated embryonic cells 48 hours post-thawing	43

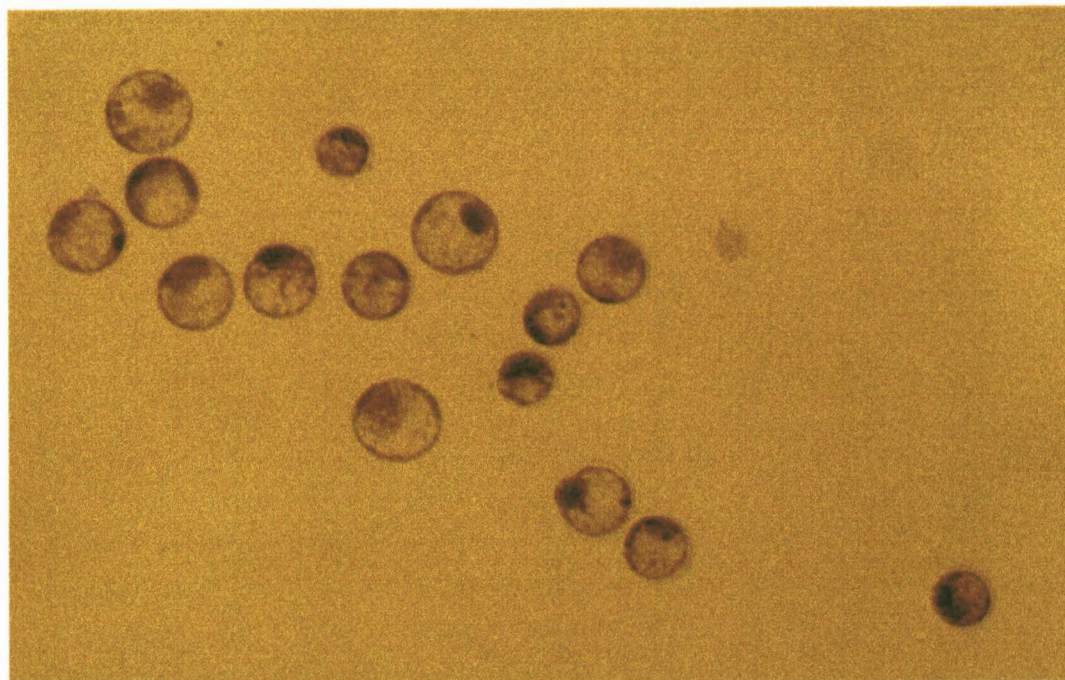
- 4.5 IVP bovine embryos under light microscope (F10/0.25) in 50
ViGro™ Holdingplus: (A) live blastocyst before being frozen by the
slow freezing method (TMT 4). (B) live blastocyst in
ViGro™ Holdingplus immediately after thawing. (C) hatched
blastocyst 48 hours post-thawing
- 4.6 IVP bovine embryos under light microscope (low magnification) in 51
ViGro™ Holdingplus: (A) two live blastocysts before being vitrified
in 40% EG + 0.3M trehalose + 20% PVP (TMT 3). (B) two live
blastocysts immediately after thawing in ViGro™ Holdingplus.
(C) two hatched blastocysts 48 hours post-thawing.
- 4.7 (A) Three live blastocysts and one degenerated blastocyst under light 52
microscope, immediately after thawing in ViGro™ Holdingplus (TMT 2).
(B) degenerated embryonic cells 24 hours post-thawing

LIST OF ABBREVIATIONS

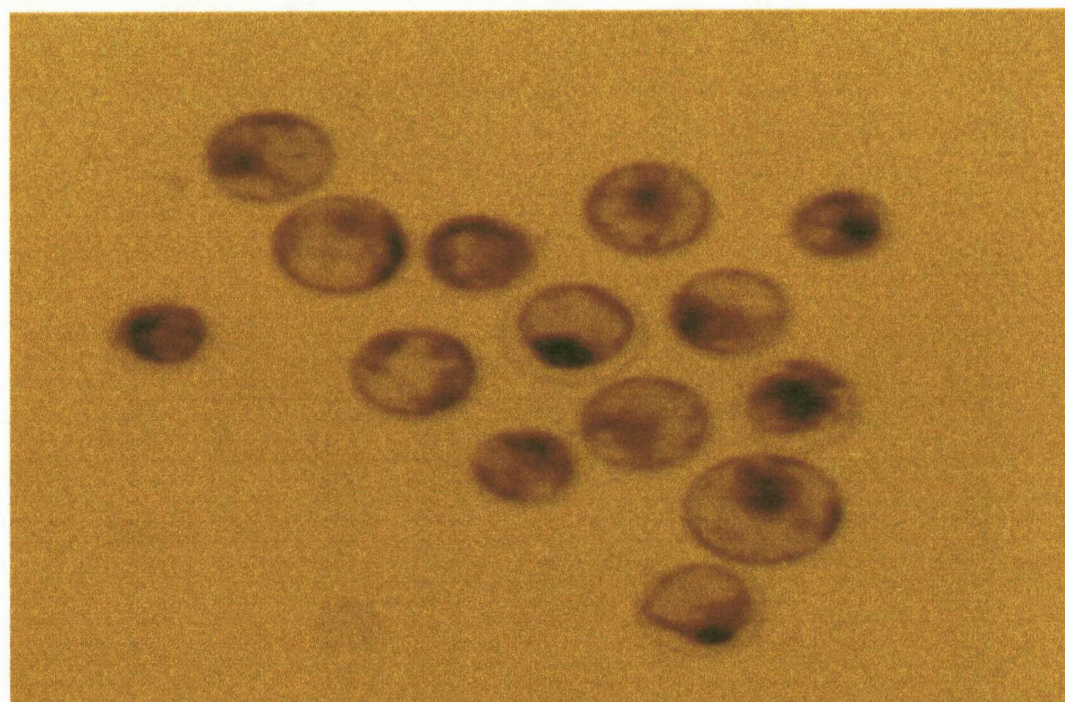
AV	Average
ARC	Agricultural Research Council
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
BO	Bovine oocyte
COCs	Cumulus oocyte complexes
CO ₂	Carbon dioxide
CR2aa	Charles Rosekrans amino acid
CP	Cryoprotectant
DMSO	Dimethyl sulfoxide
DBPS	Delbeco's buffered phosphate saline
DMPBS	Delbeco's modified phosphate buffered saline
E ₂	Estrogen
ET	Embryo transfer
EGF	Epidermal growth factor
EG	Ethylene glycol
ES	Equilibration solution
FSH	Follicle stimulating hormone
FCS	Fetal calf serum
FBS	Fetal bovine serum
GIV	Germinal vesicle
GVBD	Germinal vesicle break down
GLY	Glycerol
GLM	Generalized liner model
HCO ₃	Bicarbonate
HEPES	Hydroxyethyl piperazine ethane sulfonic acid
IVM	<i>In vitro</i> maturation

IVF	<i>In vitro</i> fertilization
IVC	<i>In vitro</i> culture
IVEP	<i>In vitro</i> embryo production
IVP	<i>In vitro</i> produced
IGF-I	Insulin like growth factor one
IGF-II	Insulin like growth factor two
KRB	Krebs-ringer-bicarbonate
LH	Luteinizing hormone
LN ₂	Liquid nitrogen
mRNA	Messenger ribonucleic acid
MPF	Maturation-promoting factor
NAHCO ₃	Sodium bicarbonate
N ₂	Nitrogen
OPU	Ovum pick up
O ₂	Oxygen
P	Prediction
PABA	Para-amino benzoic acid
PROH	Propanediol
PBS	Phosphate buffered saline
PVP	Polyvinyl pyrrolidone
PG	Propylene glycol
PO ₂	Partial pressure
RSA	Republic of South Africa
SE	Standard error
TCM	Tissue culture medium
TGF	Transforming growth factor
TGF α	Transforming growth factor alpha
TGF β	Transforming growth factor beta
TL	Tyrode's lactate
TMT	Treatment
USA	United States of America

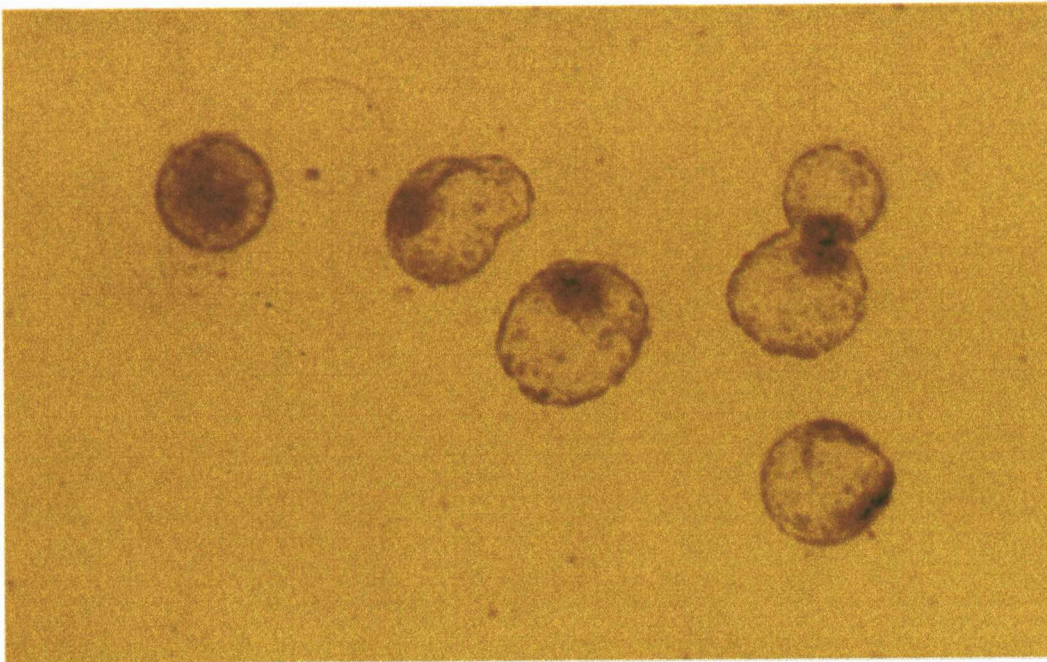
UPS Uniterruptable power supply
VS Vitrification solution



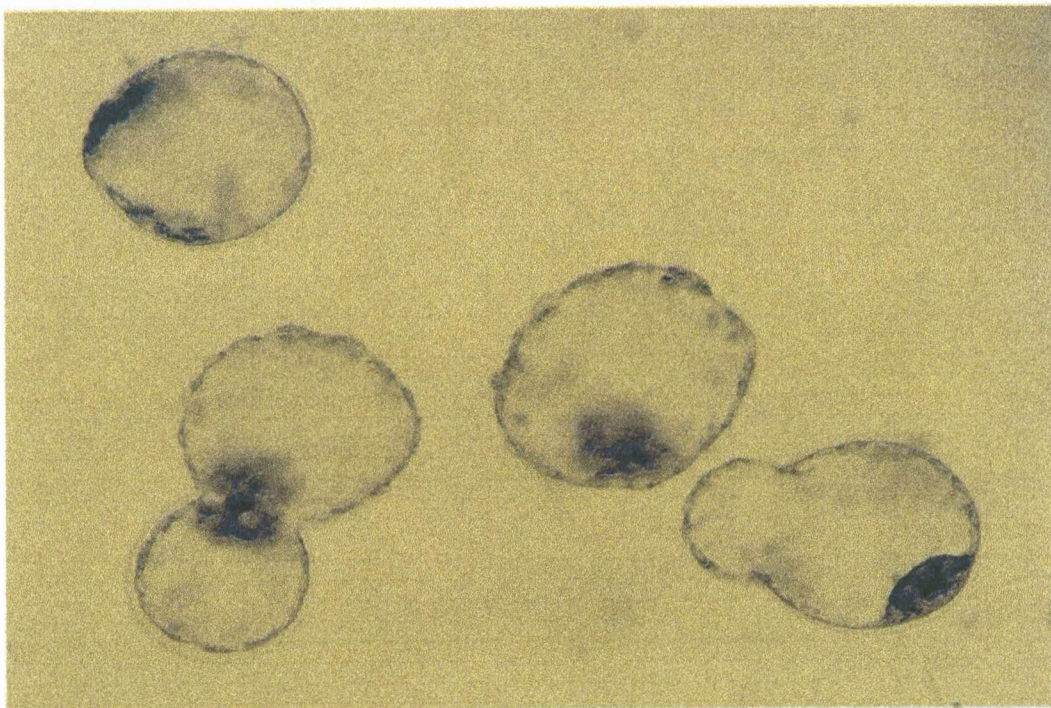
Day 7 blastocysts under light microscope (F2.5/0.08)



Day 8 blastocysts under light microscope (F2.5/0.08)



Hatched blastocysts under light microscope (F2.5/0.08)



Hatched blastocysts under light microscope (F10/0.25)

CHAPTER 1

GENERAL INTRODUCTION

The world of livestock production has undergone radical changes since researchers took steps toward the control of reproduction in farm animals. *In vitro* embryo production (IVEP) and cryopreservation is one of the assisted reproduction technology tools, which has brought radical changes to reproduction in farm animals. Furthermore, conventional superovulation and embryo collection as well as IVEP often lead to an excess of embryos that cannot all be transferred to recipients at the same time. The remaining embryos need to be stored by freezing. These embryos can then be transferred to the uterus at a later date. The embryos would be suspended in solutions designed to protect them from the effects of freezing and stored in a tank of liquid nitrogen (-196°C). When embryos are needed they are thawed, washed, and prepared for transfer. This alleviated a lot of logistical problems around an embryo transfer program.

Considerable progress has been made in the improvement and simplification of cryopreservation procedures, routinely used in embryo transfer programs. Conventional slow rate, programmable freezing and vitrification of embryos have given veterinarians, scientists and animal breeders, more alternatives in their embryo transfer programs. However, pregnancy rates after cryopreservation are not comparable to that of fresh embryo transfer. No matter how embryos are cryopreserved, some embryos always lose developmental capacity after thawing. This is costly to the breeder.

The idea of cryopreserved embryos in the early 1970s emerged as a potential gene bank, when Whittingham (1971) first cryopreserved mouse embryos. Since then, embryos have successfully been preserved in other species (ranging from cow to human). However, embryo mortality is still a major problem during freezing and thawing. If the full potential value of embryo transfer in the cattle industry is to be realized, it is necessary that more emphasis should be put on developing efficient and simple methods of embryo

storage. This also entails defining and standardizing cryoprotectant solutions and improving those already in use.

The techniques of embryo storage are only now being modified to a stage where embryos are thawed and directly transferred to the recipients. Although a reasonable survival rate of frozen embryos after thawing has been achieved (50%), there is still a need to improve the existing cryopreservation methods.

Wilmut (1976) stated two advantages of embryo storage (cryopreservation); namely embryo banking and embryo export. Improvement in the freezing techniques of embryos could overcome the problems experienced with international export of live animals. Movement of animals is very expensive because of shipping, labour and quarantine costs. Furthermore, the animals may not adapt and may also succumb to local diseases, particularly when extreme environments are experienced. Many of these problems can be reduced or eliminated by embryo freezing.

Cryopreservation is an integral component of the bovine embryo transfer industry and it can be used to transport genetically superior breeds internationally. Cryopreservation of embryos is a useful tool for manipulating reproductive performances (Saha *et al.*, 1996a). Potential future applications also include the banking of embryos of genetically superior rare animals, to ensure continued availability of infrequently used strains. The aim of embryo storage *in vitro* is to preserve this genetic material in a state of suspended animation, from which it may be resuscitated after a short or longer period of storage to continue its normal development either *in vitro* or *in vivo*. It is believed that sperm and embryonic cells can probably remain viable at a temperature of -196°C , in liquid nitrogen, for perhaps a 100 000 years. The only source of damage at such low temperature is the direct ionization from background radiation (Gordon, 1994).

Up to now, research on the cryopreservation of bovine embryos has not received much attention in South Africa. Currently many aspects of cryopreservation, cryoprotectants and their potential applications are known. However there are still many aspects that are

unclear. These include how cryoprotectants act, the toxicity in embryonic cells, what events take place in an embryonic cell when it is stored in a low temperature, etc.

The main aim of this study was to compare the conventional slow freezing method and the vitrification methods (including factors involved during freezing and thawing) and find a more simple and inexpensive method of cryopreservation. The effect of these methods was tested on *in vitro* produced embryos with the aim of finding a more effective and simplified cryopreservation method. This may be a technique to increase the reproduction efficiency of bovine reproduction worldwide, and serve as an option to feed the undernourished, poor and third world communities.

CHAPTER 2

LITERATURE REVIEW

For a number of years, the development of artificial breeding technologies for use in the cattle industry, has received a great deal of attention. The expertise developed in the fields of *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) of cattle embryos are applied *in vitro* embryo production (IVEP) (Earl & Kotaras, 1997). Each cow / heifer has $\pm 150\ 000$ to $300\ 000$ potential ova in her ovaries at birth, but only 10 to 15 of those eggs develop into offspring during her life time. Therefore, the use of *in vitro* embryo production technology has the potential of maximum utilization of ova, which would otherwise have been naturally lost in a process called follicular atresia (Greve & Madison, 1991). The procedure of producing embryos from superstimulated animals and the transfer to recipient (surrogate) animals is not economical. The technique of IVEP technology is economical, however it becomes viable if many embryos are produced and successfully transferred to the recipients to produce offspring. Due to the limitations of recipients and the large number of embryos that can be obtained, it is necessary to freeze and store some these embryos, for future use.

Improved *in vitro* embryo production technologies (IVM, IVF and IVC) could facilitate basic research in the control of early blastocyst development. Increase in the implementation of embryo technology in endangered species is a reality and it also provides a source of high quality oocytes for nuclear transfer and transgenic technologies that could benefit the commercial embryo transfer industry (Watson *et al.*, 1999). Advances in embryo transfer techniques have allowed progress to be made towards increasing the number of offspring produced from genetically superior females. Even though embryo transfer programs have been proven to be quite successful, the production of embryos in large numbers for transfer remains a major problem. Transferable viable embryos remains unpredictable, because the number and quality of the oocytes collected are highly variable (Saha, 1996). The application of IVM, IVF and IVC techniques may be used to obtain large numbers of embryos, either for research purposes, for commercial fresh embryo transfer or for freezing and storage of embryos for future use.

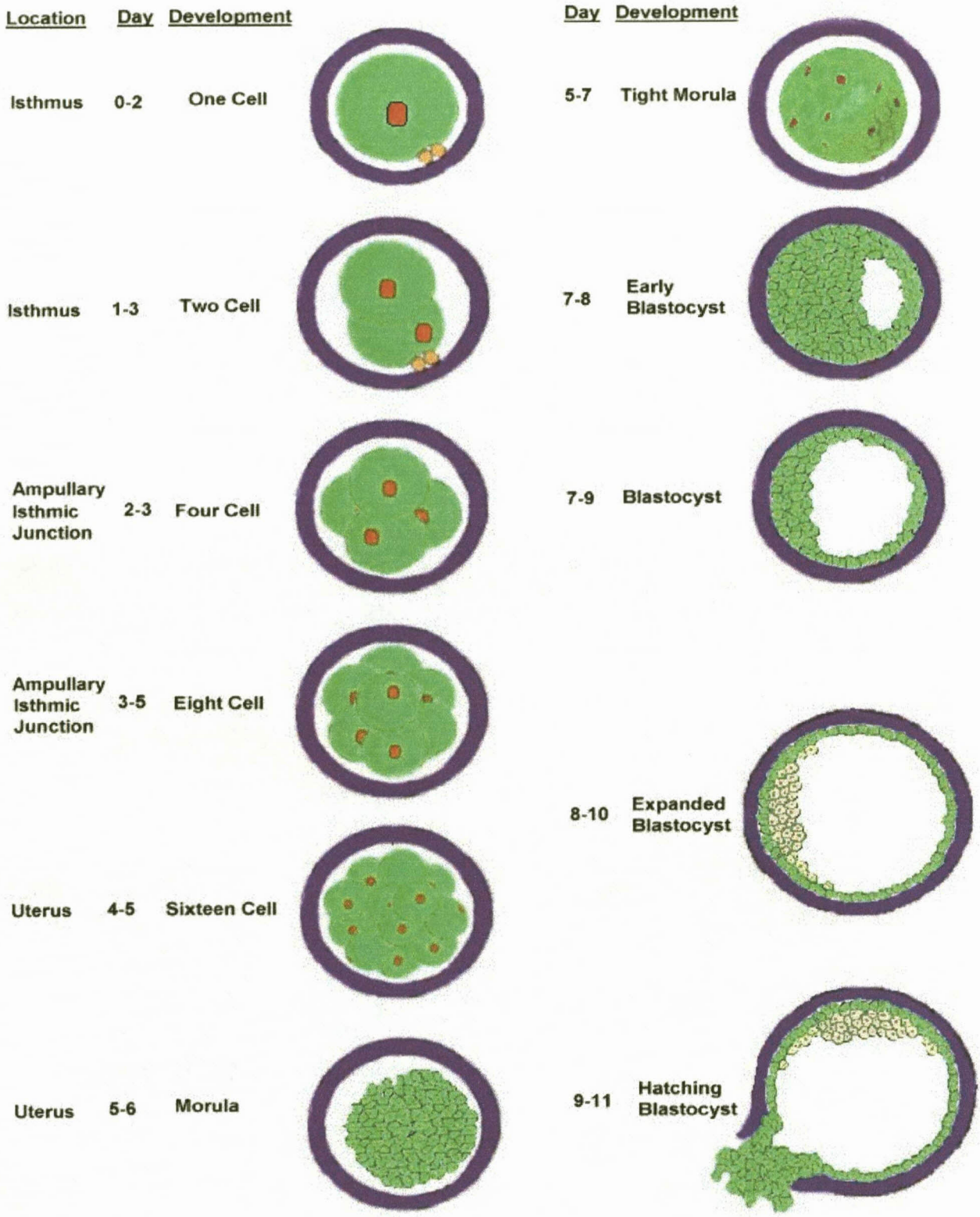


Figure 2.1 Various stages in the development of bovine embryos (one cell to hatching blastocyst)
(Geisert, 1998)

2.1 *In vitro* embryo production (IVEP)

IVEP systems allow embryos to be produced from elite donors that naturally perform poorly in normal embryo transfer (ET) programs, owing to illness, age or reproductive problems. Large numbers of embryos can be generated at a relatively low cost, with the aid of IVEP. The use of ET technology together with IVEP relies on the efficiency of the conversion of oocytes to live offspring. The efficiency depends on the effectiveness of the stages of *in vitro* maturation, *in vitro* fertilization and *in vitro* culture (Earl & Kotaras, 1997). The major difference between *in vitro* and traditional embryo transfer technologies, is that the donor female is no longer involved in the early development of embryos. The donor is only required to contribute oocytes in the same way as the male contributes sperm. This technique of IVEP raises new possibilities to maximize animal reproductive efficiency (Gordon, 1994; Petter, 1992).

The following elements of IVEP are to be addressed: Oocytes sources, *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC).

2.1.1 Sources of oocytes

Oocytes for *in vitro* embryo production (IVEP) may be obtained from different sources. The most common source is the aspiration of oocytes from the surface of ovaries collected from cows/heifers from the abattoir following slaughter. Earl and Kotaras (1997) reported that follicles may also be aspirated with the aid of an ultrasound-guided system by mid-ventral laparoscopy of live animals. The technique of laparoscopy is used more in sheep than in cows.

2.1.1.1 Abattoir Material

The traditional source of oocytes for IVEP is the abattoir (Earl & Kotaras, 1997). The collection of oocytes and production of transferable embryos from bovine ovaries obtained from abattoir materials, is described by Lu and Poldge (1992). Galli *et al.* (1994) found breed to have a significant effect on the number of oocytes collected per cow. The current success rate of recovered oocytes resulting in live born calves is approximately 10%, with embryo survival being greatly influenced by the degree of synchrony between embryo development and reproductive stage of the recipient.

Earl and Kotaras (1997) reported that embryos produced in some countries from abattoir material to have a high value. On the other hand, those produced in Australia have no economical value. Abattoir oocytes play an important role in the development of IVEP technology. Abattoir material provides a cheap source of oocytes to increase twinning in cattle. The technology of IVEP can also be utilized for oocytes obtained from the ovaries of terminally ill cows of high genetic value (Gordon, 1994).

2.1.1.2 Ovum pick up (OPU) by means of ultrasound

Oocytes can be collected from live animals in a number of ways. The most popular technique used is ultrasound guided oocytes aspiration (Fry *et al.*, 1993). This technique makes use of an ultrasound probe placed in the vagina of the animal to guide an aspiration needle to the follicles on the ovary (Rust *et al.*, 1998). The number of oocytes that can be recovered per collection from an unstimulated donor heifer varies between 2.6 and 5.4 (Fry *et al.*, 1993; 1994), with a mean of 7.4 in adult cows (Lansbergen *et al.*, 1995; Fry *et al.*, 1993). Hasler *et al.* (1995) reported a large variation in the oocyte recovery rate between 1.6 and 14.6 over the first five collections. Rust *et al.* (1998) suggests the transvaginal ultrasound guided ovum pick-up to be the least traumatic method for repeated collection of bovine oocytes.

Factors that can affect the oocyte collection rate include the experience (skill of the operator), type of needle and suction pressure used (Lansbergen *et al.*, 1995; Fry *et al.*, 1993). Even though the number of oocytes recovered per collection are relatively small with this technique, each donor animal can be aspirated twice a week. The small number of oocytes collected may present a limitation in the efficiency of IVEP (Earl & Kotaras, 1997). The development rate from oocyte to the blastocyst stage from oocytes collected from heifers older than 240 days is reported to be 18.9%, compared to 31.6% from abattoir obtained oocytes and 0% for heifers under 240 days of age (Looney *et al.*, 1995). Petter (1992) indicated that 135 oocytes could be collected per year from one donor, resulting in the production of 30 transferable embryos per donor. This technique is however, not likely to be used extensively, except in the case of genetically superior animals. This is due to the time required to process small numbers of oocytes and the relatively low embryo output once or twice a week.

2.1.2 *In vitro* Maturation (IVM)

Oocytes begin as primordial germ cells within the ovary and continue their mitotic proliferation well after the ovarian morphology has been established. At this stage of development the cells are known as **oogonia**. The mitotic phase of oogonia terminate before birth and all oogonia enter the first meiotic division. These primary oocytes progress to the stage of prophase I, before the cell cycle is interrupted. The oocytes contain a large nucleus referred to as a germinal vesicle (GIV) and enter a phase of meiotic arrest. At this stage a single layer of granulosa cells surrounds the oocytes and this unit is collectively termed the primordial follicle. The initial phase of follicular development is the resumption of growth by the primordial follicles, an event not dependent on gonadotropic hormone stimulation (Earl & Kotaras, 1997).

Follicular growth depends on an interplay between circulating gonadotropic levels and the acquisition of follicular receptor sites for the different hormones (Scaramuzzi *et al.*, 1993; Fortune & Armstrong, 1977; Dorrington *et al.*, 1975). Estradiol appears to have a

positive feedback on its own production. The next phase of growth is characterized by an increase in the number of granulosa cells and the formation of an antrum. It is at this stage that oocytes are collected from the surface of ovaries from living or recently slaughtered animals and supplied with an environment to complete *in vitro* maturation (Earl and Kotaras, 1997).

Oocyte maturation, whether *in vivo* or *in vitro*, is the most important stage of oocyte development. This stage depends on the communication between granulosa cumulus cells and oocytes (Fukui, 1990; Thibault *et al.*, 1987). Rabahi *et al.* (1993) suggested that proteins secreted by the granulosa cells play a regulatory role on the metabolism of cumulus cells and oocyte maturation. Pincus and Enzmann (1935) reported that mammalian oocytes, upon removal from ovarian follicles, could undergo spontaneous nuclear maturation in serum containing simple culture medium (Saha, 1996).

There are reports claiming that gonadotropic, steroids, buffer systems, gas composition and other physiological, physical and chemical parameters may affect the maturation process (Boone and Shapiro, 1990; Sato *et al.*, 1988). Maturation can also be achieved using a range of media, but complex media such as TCM-199 give the best results (Earl & Kotaras, 1997).

Oocytes are selected using the following criteria namely; follicle size, cytoplasmic appearance and the appearance and number of cumulus cells around the oocyte (Yang *et al.*, 1993). In cattle, immature cumulus oocyte complexes (COCs) are generally cultured for 24 to 26h in the presence of LH, FSH/ estrogen (E_2). The two principal factors known to influence the maturation process *in vitro* are proteins and hormonal supplements (Mattioli *et al.*, 1988). Maturation media are generally supplemented with a protein source such as fetal calf serum (FCS), oestrus serum and bovine serum albumin (BSA). Hormonal supplements are achieved with combinations of FSH, LH and estradiol (Mattioli, 1989; Younis *et al.*, 1989; Leibfried-Rutledge *et al.*, 1986). Protein supplement are however not essential for successful IVM (Saeki *et al.*, 1995), and not all oocytes

recovered from abattoir – derived ovaries can be matured. Oocytes are variable in quality and their developmental competence (Gordon & Lu, 1990).

Oocytes are more successfully matured *in vivo* than *in vitro* (Leibfried-Rutledge *et al.*, 1987), which might suggest that hormonal or follicular factors are required to improve maturation to obtain a normal fertilizing ability and developmental rate (Saha, 1996). Earl and Kotaras (1997) reported that some oocytes do not respond to hormonal treatments, while in others, the nucleus will mature but not the cytoplasm. For oocytes to be viable, both the nucleus and cytoplasm must mature. A number of researchers have identified possible factors which may dominate events during the late stages of follicular development and may enhance the fertilizing ability and developmental capabilities of *in vitro* matured oocytes (Saha, 1996).

The addition of gonadotropins (LH/FSH) during *in vitro* maturation has been shown to improve the developmental potential of oocytes in goats (Younis *et al.*, 1991), sheep (Staigmiller & Moor, 1984; Moor & Trounson., 1977) and cattle (Zuelke & Brackett, 1990; Brackett *et al.*, 1989; Younis *et al.*, 1989). When oocytes remain in the follicle, nuclear maturation (meiotic resumption) does not occur until after the LH surge, or in association with atresia of the follicle (Saha, 1996; Kruij *et al.*, 1983).

Lutterbach *et al.* (1987) and Critser *et al.* (1986) have shown an interaction of granulosa cells and cumulus oocyte complexes, during *in vitro* maturation, to be involved in maintaining the development of bovine oocytes. The oocytes communicate with the follicles through soluble factors, and also through gap junctions both between the oocytes and cumulus cells and between cumulus and granulosa cell (Larsen *et al.*, 1987). Cumulus cells and additional granulosa cells are needed to complete oocyte maturation (Thibault *et al.*, 1987; Xu *et al.*, 1987; Critser *et al.*, 1986). It has been clearly demonstrated that follicle cells, especially cumulus cells surrounding immature oocytes, play a central role in the developmental competence in rabbit (Saha, 1996) and bovine oocytes (Goto *et al.*, 1988).

Lonergan *et al.* (1992) recorded a 65% blastocyst rate from follicles >6mm, compared to 34%, from follicles between 2mm and 6mm in diameter. Blondin and Sirard (1995) recorded less development of blastocyst stage from follicles <3mm in diameter. The period of oocyte collection from the ovary, might influence the formation of mRNA (messenger ribonucleic acid), necessary for the production of important proteins required for later blastocyst formation. Oocyte quality and the handling therefore have a significant effect on the blastocyst production rate, making it difficult to compare results between laboratories, especially if the method of oocyte collection and selection is poorly defined (Earl & Kotaras, 1997).

Problems arising during the maturation stage, affect the fertilization quality and yield pre-implantation embryos. Various systems are quoted as achieving good maturation rates (Earl & Kotaras, 1997). Semple *et al.* (1993) found the exposure duration to maturation medium to have little effect on cleavage rate, but markedly influence the rate of development to the blastocyst stage. The temperature variations during maturation have been shown to have deleterious effects on the microtubules of the meiotic spindle, even at room temperature. This information draws the attention to the need for temperature control while collecting and handling the oocytes, if successful *in vitro* results are to be obtained (Aman & Parks, 1994).

Among the growth factors currently implicated in the modulation of oocytes maturation, are epidermal growth factor (EGF), transforming growth factor (TGF), TGF α , TGF- β , IGF-I (insulin-like growth factor I) and IGF-II. EGF is a single chain polypeptide with a molecular weight of 6045 daltons, and known to be a potent mitogen for many cells, including granulosa cells (Gordon, 1994). EGF has been shown to have the ability to stimulate the proliferation of ovarian granulosa cells (May *et al.*, 1987). EGF has also been found to have a mitogenic effect on both epidermal and non-epidermal cell types (Saha, 1996). Many researchers have indicated EGF to contribute to the promotion of oocyte maturation (Down, 1989), germinal vesicle break down (GVBD), polar body formation (Das *et al.*, 1991) and cleavage of the oocytes (Coskum *et al.*, 1991).

A maturation promoting effect of EGF was reported by Das *et al.* (1991) in mice. De Loos *et al.* (1993) recorded TGF- α and EGF to have no effect in their studies on bovine oocyte maturation. Haper and Brackett (1993), on the other hand, suggested the presence of EGF in serum to be one of the undetermined components, contributing to enhancement of oocyte maturation.

It is also well established that maturation-promoting factor (MPF) is a cytoplasmic factor that brings about germinal vesicle break down (Saha, 1996; Bavister, 1992). This factor is highly conserved among a wide variety of species, and it plays an important role in the progression of the cell cycle from interphase to metaphase, in both meiosis and mitosis. MPF is a metaphase regulating protein. The activity of MPF is controlled by the phosphorylation – dephosphorylation processes (Abey deera *et al.*, 1993). To conclude, can be said that it is essential for an appropriate chemically defined IVM system to be available to elucidate the growth factor effect.

2.1.3 *In vitro* Fertilization (IVF)

IVF is the process by which oocytes and sperm are combined in a laboratory dish in order for fertilization to take place (approximately 18h). However, the exposure of oocytes to sperm cells should not be shorter than 8h, and not longer than 18h (Visser *et al.*, 1998)

Fertilization is a complex process, which results in the union of two gametes (male and female), the restoration of the somatic chromosome number and the start of the development of a new individual (Gordon, 1994). Successful bovine IVF requires appropriate preparation of both sperm and oocyte, as well as culture conditions that are favourable for metabolic activity of the male and female gametes (Brackett, 1992; Xu & King, 1990; Sirard, 1990; Brackett, 1983; 1981).

The first genuine IVF success of an artificially matured oocyte, is that recorded by Iritani and Niwa (1977). The first calves born following IVF of artificially matured oocytes,

were those reported by Hanada *et al.* (1986). These embryos were cultured to the blastocyst stage in rabbit oviducts and subjected to freezing and thawing prior to transfer. One of the first pregnancies produced by a total *in vitro* procedure, IVM and IVC of the early embryo was that reported by Lu *et al.* (1987).

Frozen sperm are generally used for *in vitro* embryo production. To use sperm for bovine IVF (Earl & Kotaras, 1997), special techniques of sperm preparation, e.g. concentration of motile sperm by the swim-up technique or separation on percoll gradients and *in vitro* capacitating have been devised (Parrish *et al.*, 1995). Normally spermatozoa must undergo capacitation before achieving the ability to penetrate the oocyte. The process of sperm capacitation, which normally occurs in the female reproductive tract and renders sperm cells capable of fertilization, has been a major technological barrier (Saha, 1996). In setting up an appropriate system for cattle IVF, it is clear that the medium employed must be capable of providing the secondary oocytes and capacitated sperm with the environment which readily permits sperm penetration or fertilization to occur readily (Gordon, 1994).

Media manipulation strategies for sperm preparation and washing media, include the use of high ionic strength, calcium deletion and a slightly raised pH (7.6 - 7.8). Media supplementation strategies include the use of a brief exposure of sperm to a calcium ionophore, and the addition of caffeine, heparin or serum (Earl & Kotaras, 1997). Niwa and Oghoda (1988) demonstrated the synergistic effect of heparin and caffeine on fertilization rates in bovine oocytes. The success rate of 68 to 76% when caffeine and heparin were used, was twice that obtained when caffeine and heparin were added separately in sperm capacitating medium (Maeda *et al.*, 1996).

Earl and Kotaras (1997) suggested that the success rate of IVF may also be influenced by the presence of cumulus cells around the oocytes. However, Hawk *et al.* (1992) suggested that the fertilization rate of IVF could be increased by the removal of most, but not all, cumulus cells prior to fertilization. Other researchers suggest that cumulus cells need not be present for successful IVF (Ball *et al.*, 1982), but alternative evidence supports the

presence of cumulus cells for successful IVF rates (Liu *et al.*, 1995; Chian & Sirard, 1995; Chian & Niwa, 1994). It has also been recognized that the fertilization process in cattle oocytes is temperature sensitive (Gordon, 1994) and available evidence would seem to support the view that a temperature of 39 °C is not only optimal for bovine oocyte (BO) maturation, but for sperm penetration as well. This is certainly compatible with the known events in live animals (Cheng *et al.*, 1986).

2.1.4 *In vitro* culture (IVC)

The *in vitro* culture of mammalian embryos requires a suitable environment so that the early embryo can undergo a number of cleavage divisions with the ultimate formation of the blastocyst stage of development (Petter, 1992). In recent years, the success of IVM, IVF and IVC in farm animals has been greatly improved. Pregnancies or offspring have been obtained following the transfer of embryos to recipients from *in vivo* or *in vitro* cultured oocytes (Goto *et al.*, 1988; Hanada *et al.*, 1986).

The earliest reports of a successful procedure permitting the continued cleavage of cattle embryos to the blastocyst stage outside the cow were from Ireland and it has also been confirmed that bovine embryos can be cultured to the hatching stage in rabbit oviducts (Gordon, 1994). The cells of the oviduct were used in the first successful co-culture system for sheep and cattle. It became apparent that not only oviductal cells, but also a wide variety of other somatic cells are capable of providing an environment in which farm animal embryos can develop. There is good evidence to suggest that certain current co-culture systems are highly effective in providing the needs of bovine embryos (Gordon, 1994; Gandolfi and Moor 1987).

The development of a simple and viable culture system to support embryonic development beyond the morula stage after IVM and IVF of oocytes still requires investigation (Saha, 1996). Looking toward an optimal embryo culture system, it is essential to think in terms of chemically defined media that are soundly based on the

knowledge of embryo metabolism and on known embryo preferences for energy substrates and other essential nutrients (Gordon, 1994). The components of the culture environment that are critical for embryo survival are temperature, light, pH, osmolarity of the medium, concentration of ions and energy sources, serum components (macromolecular and undefined growth factors), gas phase, quality of water and culture vessels (Petter, 1992; Bavister, 1987).

The culture of cattle embryos in conventional media developed for cell culture, has led to inconsistent results (Wright & Bondioli, 1981). Loutradis *et al.* (1987) reported the composition of many formulations used in embryo culture to be relatively simple. Many are based on a Krebs-ringer-bicarbonate (KRB) salt solution, with energy sources being pyruvate, glucose and lactate. Such media are conventionally supplemented with a protein source of fetal calf serum (FCS) or bovine serum albumin (BSA). The use of complex media such as M-199 and Ham's F-10, which contain mixtures of amino acids and other components, may not always provide the optimal conditions for development, by way of well-defined, controllable culture conditions, that could be a means of effectively formulating new media capable of supporting embryo development and viability. Some literature have demonstrated cattle embryos derived from IVM/IVF to develop *in vitro* to the morula stage in chemically defined, protein-free media, with no apparent advantage in using somatic feeder cells or serum (Pinyopummintr & Bavister, 1991).

To fully understand the needs of the early bovine embryo, it is essential to know the precise composition of the medium in which the embryos are to be cultured. It is also essential to identify important components in media e.g. serum, so that certain molecules can eventually replace substances in the IVC medium. Taken into consideration fact that conventional culture media contain many different components (Gordon, 1994).

Rosenkrans and First (1991) reported that essential and non-essential amino acids are beneficial to *in vitro* bovine embryo development in the absence of feeder cells. The role of amino acids in IVC has been the focus of recent research (Garner *et al.*, 1992). Both

essential and non-essential amino acids in the culture medium increase blastocyst development (Takahashi & First, 1992; Rosenkrans & First, 1991). Spontaneous breaking down of amino acids results in toxicity levels of ammonium in the medium. To resolve this toxicity, embryos should be transferred every 48h, into freshly prepared amino acid supplemented medium (Gardner & Lane, 1993). Moore and Bondioli (1993) used a serum-free IVC medium to culture cattle embryos from the single cell stage to the blastocyst stage. In analysis of the media, it was found that glycine and alanine are the most predominant amino acids. Glycine and alanine supplementation to the IVC medium improved development of embryos. Shamsuddin *et al.* (1993) used a cell-free serum, serum-free culture system (M-199 + BSA, insulin, transferrin and selenium), and this semi-defined medium supported the development of embryos to the blastocyst stage as readily as a complex co-culture system.

The energy substrates commonly used in embryo culture media are pyruvate, lactate and glucose (Earl & Kotaras, 1997). Gardner and Batt (1991) found that sheep embryos do not utilize any significant amounts of glucose until the 16-cell stage. This is also the case for bovine embryos (Rieger, 1992). Pyruvate utilization was substantial and remained relatively constant until the blastocyst stage development, when both pyruvate and glucose absorption increased dramatically. These findings are supported by the bovine embryo culture experiments of Takahashi and First (1992). Thompson *et al.* (1992) found that pyruvate and lactate were essential for embryonic development to the morula stage, while the addition of glucose levels above 1.5mM was detrimental to embryonic development during the first 4 or 5 cleavage divisions. The ratios of energy substrates, particularly that of pyruvate and its reduced equivalent lactate, appear to have significant impact on embryo development.

Pinyopummintr and Bavister (1991) reported a negative interaction between glucose and phosphate in bovine embryo development. In somatic cell co-culture systems, the absence of glucose during the first 36 to 48h of culture, improved the rate of normal development (Nakao & Nakatsuji, 1990) and stimulated sequential blastulations of bovine embryos (Ellington *et al.*, 1990). Much research has been done in defining the

conditions at which stage mammalian embryos are capable of developing from the one-cell to the blastocyst stage. *In vitro* experiments have suggested that glucose or phosphate is detrimental to early cleavage in some species (Chatot *et al.*, 1989).

Most laboratories perform IVM, IVF and IVC of bovine oocytes at 38 °C to 39 °C, as this temperature is close to the rectal temperature in cattle (Shi *et al.*, 1998). Temperature is of critical importance when dealing with maturation, fertilization and culturing of bovine oocytes (Gordon, 1994). It is evident that IVC at 40 °C may lead to a significantly and substantially lower yield of blastocysts or hatched blastocysts, compared to culture at 37 °C to 39 °C (Wang, 1991). Alfonso and Hunter (1992) demonstrated significantly higher cleavage rates in early bovine embryos cultured at 37 °C, compared to 39 °C.

The most environmental friendly gas phase for embryo culture systems, is the conventional 5% CO₂ in air. It is used to provide a pH of approximately 7.4 with a 26mM NaHCO₃ buffer. The same gas phase can be employed with a high a level of NaHCO₃, where a higher pH is required (Gordon, 1994). The two gas phases normally used for culture of embryos are either 5% CO₂ in air or 5% CO₂, 5% O₂ and 90% N₂. A higher pH may be achieved with higher levels of HCO₃, if higher pH values are desired. There is evidence that 5% O₂ level gives a PO₂ in the culture medium approximately that of the oviductal fluid. This gas environment is more advantageous for the culture of cattle ova than the 20% O₂ level of the air gas phase (Kane and Bavister, 1988). Numerous researchers have reported that 20% O₂ is toxic to embryos and 5% O₂ gives more satisfactory results (Wang *et al.*, 1992). Voelkel and Hu (1992a) used two O₂ concentrations within two different IVC systems. When a bovine oviductal monolayer was used, the low oxygen tension gave the highest embryo yields. Wang *et al* (1992) examined the effect of both O₂ and CO₂ on the culture of bovine embryos. It was found that 5% CO₂, 5% O₂, 90% N₂ and 10% O₂, 85% N₂, had no advantage over the conventional 5% CO₂ in air used in the IVF laboratory.

The concern has been expressed regarding the possibility that bovine zygotes and early cleavage embryos may be adversely affected by light, particularly ultraviolet light.

However, the inclusion of para-aminobenzoic acid (PABA), which is a naturally occurring compound in mammals, has been recorded as a means of providing protection (Robertson *et al.*, 1988). Severe light exposure can adversely affect the development of embryos at all stages (Gordon, 1994).

Among the factors that are of crucial importance in embryo culture is the water quality used in the preparation of the IVC media (Gordon, 1994). Keefer (1992) demonstrated that no development of IVM/IVF-derived bovine embryos (to blastocyst stage) occurred when purchased deionized distilled water was used instead of water from an ultrafiltration system.

2.2 CRYOPRESERVATION OF *IN VITRO* PRODUCED BOVINE EMBRYOS

Over the past few decades, considerable progress has been made in improving and simplifying cryopreservation procedures routinely used for embryo transfer programs (Dobrinsky, 1996). Since the first successful cryopreservation of mouse embryos (Whittingham, 1971) and cattle (Wilmut & Rowson, 1973), basic and applied research has resulted in the cryopreservation of embryos of at least 13 other mammalian species (Rall, 1992).

The success rate of freezing/thawing embryos for mammalian species is set out in Table 2.1.

Table 2.1 Young born after the freezing/thawing of mammalian embryos (Gordon, 1994).

Species	Year	Authors
Mouse	1971	Whittingham (1971)
Cow	1973	Wilmut and Rowson (1973)
Rabbit	1974	Bank and Maurer (1974)
Sheep	1974	Willadsen <i>et al.</i> (1982)
Rat	1975	Whittingham (1975)
Goat	1976	Bilton and Moore (1976)
Horse	1982	Yamamoto <i>et al.</i> (1982)
Pig	1991	Kashiwazaki <i>et al.</i> (1991)

Wilmut and Rowson (1973) were the first to record that bovine embryos survive freezing. Only one embryo out of 21 embryos transferred to recipients (< 5% survival) survived to term.

Much progress has been made in the freezing of livestock embryos, especially bovine embryos. This has led to the practical application of cryopreservation (freezing and thawing) procedures for bovine morulae and blastocysts that are nonsurgically collected and transferred (Saha *et al.*, 1996a). Cryopreservation of *in vitro* produced embryos is however becoming more important in the field of animal production, even though there is still the obstacle of large scale-commercial implementation of bovine *in vitro* produced embryos. Currently, vitrification and conventional freezing procedures are widely used for the cryopreservation of embryos and oocytes. Several researchers have tried various vitrification media to preserve embryos of different species, ranging from the mouse to the cow (Saha *et al.*, 1996b; Mahmoudzadeh *et al.*, 1995; Massip *et al.*, 1989). Successful cryopreservation of mammalian embryos can be achieved if a suitable solution could be found that will be able to promote vitrification, controlled freezing, ultra rapid freezing and thawing procedures, without harming the embryo.

2.2.1 Controlled (slow) Freezing and Thawing

The majority of bovine embryos have been cryopreserved using controlled freezing and thawing techniques (Saha, 1996). Niemann (1991) and Saha *et al.* (1996a) reported numerous modification in the following common cryopreservation stages: (i) addition of a cryoprotectant; (ii) loading of embryos into freezing vessels; (iii) transfer of embryos within straws to the cryopreservation chamber; (iv) induction of crystallization (seeding); (v) slow cooling; (vi) plunge and storage in liquid nitrogen (-196°C); (vii) thawing of the samples; and (viii) removal of the cryoprotectant.

Niemann (1991), reported that the presence of a cryoprotectant is essential to prevent damage to the embryos during freezing and thawing. Two major categories of cryoprotectants are mainly used, namely, penetrating cryoprotectants (glycerol; dimethyl sulfoxide (DMSO); 1,2 propanediol (PROH); ethanol; ethylene glycol and other alcohol) and non-penetrating cryoprotectants (sucrose; glucose; trehalose and other sugars). Saha *et al.* (1996b) indicated that the properties, which make cryoprotectants suitable for use in biological systems, is the ability to pass through cell membranes freely and the ability to dissolve electrolytes. The concentration of hydrogen-bonding substituents (providing high solubility in water) is also well correlated to cryoprotective activity. The degree of permeation of a particular embryo by a permeating cryoprotectant is dependent on the permeability co-efficient of the embryo for the respective agent and the gradient between intracellular and extra cellular concentrations of the cryoprotectant, the temperature and surface of the embryo (Niemann, 1991).

The most commonly used freezing method is that developed by Gordon (1994), this involves the placing of the bovine embryos in a concentrated glycerol solution (1.4M glycerol in PBS supplemented with BSA) at room temperature for a 20 minutes equilibration period. The straws are usually cooled from room temperature to 0 °C and seeded at -4 °C to -7 °C. Seeding is the term used to describe the controlled initiation of ice formation at slightly supercooled temperatures, generally achieved by touching the wall of the straw with very cold forceps. After seeding, ice forms quickly throughout the

entire straw; and cooling is continued at a rate of $0.3\text{ }^{\circ}\text{C min}^{-1}$ to $-30\text{ }^{\circ}\text{C}$. Then the embryo is plunged into liquid nitrogen (Gordon, 1994).

Regarding thawing, various reports have dealt with procedures for the exposure of the frozen straw to air and water. A low incidence of embryo damage when straws are warmed in air for 10 seconds prior to transfer into water has been recorded (Rall & Meyer, 1989).

2.2.2 Non-Permeating Agents

Non-permeating agents like sucrose, trehalose or other sugars do not enter the cell, but are considered to exert a significant cryoprotective effect by causing osmotic dehydration of the cell (Saha *et al.*, 1996b; Niemann, 1991). Saha *et al.* (1996a) indicated that the addition of non-permeating agents reduces cryoprotectant permeation. The high molecular weight of solutes such as polyvinyl pyrrolidone (PVP), polyethylene glycol, serum and dextran, exert a cryoprotective action. This is done by covering cell membrane defects that may arise in the course of the cryopreservation procedures, and / by helping to repair damaged cell membranes during or after embryo thawing (Saha *et al.*, 1996a; Niemann, 1991; Williams, 1983; Grill *et al.*, 1980).

When embryos are placed in a cryoprotectant (CP), the osmotic pressure rapidly forces the water out of the embryo (dehydration) in attempt to equalize the concentrations of CP on both sides of the membrane. Simultaneously the CP is forced into the cell in an attempt to achieve the same end. When the CP enters the cell it creates an osmotic pressure within the cell that causes a small amount of water to re-enter the cell. The CP will quickly equilibrate across the membrane and so bring the embryo back to 100% of its original volume. Therefore, the small amount of water that has entered the cell as a result of the CP osmotic effect is added to the volume of the cell and so makes the volume greater than 100% (possibly as high as 110%). This phenomenon causes additional stress on the cell membranes. When non-permeating agents (sucrose and trehalose) are added to the solution, the concentration of the nonpermeating agent outside

the cell maintains an osmotic pressure that counters the internal CP effect. This prevents the cells from over-expanding (Saha, 1996).

Niemann (1991) reported that the developmental stage of the embryo has its own unique permeability characteristics. The presence of a CP significantly increases the medium's osmolality, which could damage the embryo while equilibrating to the altering osmotic conditions. There are reports of one-step addition of a final concentration of CP's, with the same survival rates similar to the stepwise procedure (Saha *et al.*, 1996a; Niemann, 1985).

The procedure of using controlled freezing and thawing rates require the artificial induction of crystallization at a temperatures of approximately $-6\text{ }^{\circ}\text{C}$, to avoid excessive supercooling of the embryos. The temperature alteration due to release of heat of fusion and change in osmolality requires a holding period of 5 to 10 minutes, but not more than 10 minutes, to equilibrate temperature and cell volume. Manual seeding should be done by touching the liquid with the embryo within the straw at the opposite end to the embryo (Saha, 1996). Saha *et al.* (1996b) also reported that early cryobiological studies with bovine embryos were aimed at the approximate complete dehydration of the embryo. Embryos were cooled at a rate of 0.3 to 0.1 $^{\circ}\text{C}/\text{min}$ to -60 to $-120\text{ }^{\circ}\text{C}$ at the stage when transferred to liquid nitrogen.

Willadsen *et al.* (1978) were the first to develop a so-called fast freezing technique, in which embryos were cooled slowly to $-33\text{ }^{\circ}\text{C}$, before being plunged into liquid nitrogen ($-196\text{ }^{\circ}\text{C}$). Since glycerol is thought to be less toxic than DMSO, the majority of bovine embryos frozen until now has been frozen using glycerol as the CP and terminating the use of slow cooling, at temperatures between -30 to $-35\text{ }^{\circ}\text{C}$. Plunging at -30 to $-35\text{ }^{\circ}\text{C}$ apparently represents a good balance between hydration and extracellular ice formation and results in high survival rates (Saha, 1996).

Cryoprotectants must be removed from the thawed cells because of their toxicity at higher temperatures. However, if the embryos are placed directly into an isotonic solution

(e.g. Dulbecco's phosphate buffered saline, DPBS), intracellular hyperosmolality will cause excessive swelling of the blastomeres and subsequent death. The cryoprotectant can be removed either by a tedious stepwise procedure (5 steps with 10 in each) or the use of sucrose (Leibo & Mazur, 1978) which significantly improves embryo survival, compared to the stepwise procedure (Niemann, 1991; Niemann *et al.*, 1982).

2.2.3 Permeability of Cryoprotectants in Bovine Embryos

The first survival of cow embryos following deep freezing and thawing was obtained by the use of dimethylsulfoxide (DMSO) as a CP combined with slow freezing ($0.3 - 0.1$ °C / min) and a thawing rate of $3 - 20$ °C /min (Jensen *et al.*, 1981; Willadsen *et al.*, 1976; Wilmut & Rowson, 1973). When embryos are frozen and thawed in a conventional cryoprotectant such as glycerol or DMSO, the embryo is removed from the straw in which it was frozen. The cryoprotectant is then removed from the embryo using one of many options, each requiring 10 or more minutes for processing. The embryo is then reloaded in another straw prior to transfer. This creates a logistical problem and increases temporary *in vitro* culture of thawed embryos prior to transfer (Voelkel & Hu, 1992a).

The permeability properties of embryos, osmotic properties of the CP and the effect on the embryo's viability after thawing is a problem (Voelkel & Hu, 1992b; Leibo, 1986; Schneider & Mazur, 1984; Schneider *et al.*, 1983; Jackowski *et al.*, 1980). Conventional cryoprotectant compounds such as glycerol and DMSO do not permeate the embryonic cells as rapidly as water (Voelkel & Hu, 1992a). The embryos are placed in an isotonic medium, and then embryos are transferred to a solution of 1.5M glycerol or DMSO. The embryos respond to the high osmolality of the solution, dehydrating water to reach an osmotic equilibrium with the surrounding environment (Leibo, 1986).

Voelkel and Hu (1992a) indicated that the difference between the permeability of the embryonic cells to the CP and water, dictates the manner in which a compound must be removed from an embryo after thawing. Stepwise dilution of the cryoprotectant is widely used, with time allowed after each dilution step, for the CP to permeate the embryo.

Sucrose can be used as an osmotic buffer to maintain the osmotic equilibrium between the embryonic cell and the external environment in which embryos are suspended. The goal with each of these techniques is to minimize the degree of cellular expansion occurring in the embryo as the cryoprotectant is being removed from the cells (Voelkel & Hu, 1992b; Schneider & Mazur, 1984).

Embryos frozen in ethylene glycol (1.5M) were found to be tolerant to the direct rehydration in the holding medium without step-wise, or sucrose-mediated dilution of the CP. Glycerol, DMSO and propylene glycol are less efficient, when compared to ethylene glycol. Poor embryo viability was observed when propylene glycol was used as a CP. This is contrary to the findings reported by Suzuki *et al.* (1990). A high rate of *in vitro* survival was observed when bovine embryos were frozen in propylene glycol and rehydrated directly in a holding medium or transferred directly to the recipient female (Voelkel & Hu, 1992a; Suzuki *et al.*, 1991; 1990).

Voelkel and Hu (1992b) indicated that ethylene glycol can readily diffuse out of the embryonic cells, without causing gross cellular damage. However, when bovine embryos were transferred from the ethylene glycol solution into the culture medium, little osmotic response was observed, reflecting a high degree of permeability of the embryos to the CP.

Table 2.2 Viability of frozen bovine embryos using various cryoprotectant solutions and rehydrated directly in holding medium (Voelkel & Hu, 1992a)

Treatment	No. embryo frozen /No. embryo viable (%)		
	24 hours	48 hours	72 hours
1.5M EG	16/20 (80)	15/20 (75)	14/20 (70) ^a
1.5M PG	3/19 (16)	3/19 (16)	2/19 (11) ^b
1.5M DMSO	7/20 (35)	7/20 (35)	5/20 (25) ^c
1.4M GLY	6/10 (60)	3/10 (30)	3/10 (30) ^d

EG = ethylene glycol, PG = propylene glycol, DMSO = dimethyl sulfoxide, GLY = glycerol.

^{a,b} Values are different ($P < 0.0005$); ^{a,c} Values are different ($P < 0.005$); ^{a,d} Values are different ($P < 0.05$)

Table 2.2 indicates that the post-thaw viability following 72h culture was greater for embryos frozen in EG, compared to embryos frozen in other cryoprotectants. Seventy percent of the embryos frozen in 1.5M EG and rehydrated directly in holding media were viable 72h post-thawing, compared to 11% ($P<0.0005$), 25% ($P<0.005$) and 30% ($P<0.05$) for embryos frozen in 1.5M PG, 1.5M DMSO and 1.4M GLY, respectively. The loss in viability of embryos frozen in PG and DMSO was obvious after 24h in GLY and rehydrated immediately (Voelkel & Hu, 1992a; 1992b).

Table 2.3 *In vitro* development of embryos frozen in 1.0 or 1.5M EG and rehydrated directly in holding medium (Voelkel and Hu, 1992a).

No.viable embryos/No.frozen embryos (%) at different culture times			
Treatment	24 hours	48 hours	72 hours
1.0M EG	14/20 (70)	12/20 (60)	11/20 (55)a
1.5M EG	17/20 (85)	17/20 (85)	16/20 (80)b

^{a,b} Values are different ($P<0.01$)

Table 2.3 indicates the viability of embryos after a 72h-culture period and thawing of 55 and 80% ($P<0.01$) respectively for embryos frozen in 1.0 and 1.5M EG, and rehydrated directly in a holding medium. The viability was greatest for embryos frozen in 1.5M EG. This suggests that there may be advantages to this treatment, compared to a lower EG concentration. The 1.5M EG concentration was selected for use in evaluating pregnancy rates of embryos frozen in EG and then transferred directly to recipient females (Voelkel & Hu, 1992a).

EG was found to be an effective cryoprotectant for bovine embryos. High rates of post thawing survival were achieved in bovine embryos cryopreserved in EG following direct rehydration of embryos in the culture medium or after direct transfer to recipient females (Mapletoft, 1995; Voelkel & Hu, 1992a; Voelkel & Hu, 1992b).

2.2.4 One-Step Freezing Method

Several freezing methods have been previously described, one of these techniques having been referred to as the One-step Method (Voelkel & Hu, 1992a). The One-step Method is a modification of the controlled (slow) freezing and thawing technique and omits cryoprotectant dilution and microscopic evaluation of the embryos after thawing (Saha, 1996; Voelkel & Hu, 1992a; Leibo, 1984). The basic principle is to have the freezing and dilution medium in the straw separated by an air bubble. Two techniques have been described and differ mainly in the concentration of dilution medium used (Voelkel and Hu, 1992b; Leibo, 1984; Suzuki *et al.*, 1983). According to Voelkel and Hu (1992a), the French one-step freezing method involves stages with normal culture medium, freezing medium with embryos and the dilution medium (sucrose). The USA technique has two stages with sucrose and one with the freezing solution. The pregnancy rates obtained with this procedure vary between 30 and 50% (Massip *et al.*, 1987). The One-step Method offers significant advantages when embryos have to be shipped to countries where no or poor laboratory facilities for embryo handling are available (Saha *et al.*, 1996a; Voelkel & Hu, 1992b).

2.2.5 Vitrification

Vitrification is defined as the physical process by which a highly concentrated solution of cryoprotectant solidifies during cooling without the formation of ice crystals (Voelkel & Hu, 1992a). Consequently, as the temperature decreases, molecular motions in the liquid permeating the organ or cells slow down. There is a minimum amount of thermal energy required to allow molecules to move from one place to another in a liquid (translational motion). When this minimum energy becomes unavailable due to cooling, the liquid "locks up" into a solid state. This "arrested liquid" state is known as a glass, and the conversion of a liquid into glass is known as vitrification (Saha, 1996).

The procedure of successful vitrification of mammalian embryos has been the subject of many investigations, since the successful cryopreservation by vitrification of mouse

embryos (Rall & Fahy, 1985). Successful vitrification procedures have three distinctive features: (i) no ice forms in the embryo suspension during the cooling, storage or warming stages; (ii) cells are osmotically dehydrated prior to cooling by controlled equilibration in highly concentrated cryoprotectant solutions ($> 6M$); (iii) a characteristic sequence of changes occur in the osmotic volume of the embryo during the cryopreservation process (Rall, 1992).

Rall and Fahy (1985) also applied the vitrification successfully. The original vitrification solution which consisted of 20.5% DMSO, 15.5% acetamide, 10% propylene glycol and 6% ethylene glycol allowed successful cryopreservation of 8-cell mouse embryos. Scheffen *et al.* (1986) also successfully cryopreserved mouse embryos by a vitrification method with glycerol and propylene glycol. Using the same solution Massip *et al.* (1986) produced pregnancies in cattle by the transfer of embryos cryopreserved by vitrification. Since then, many researchers have successfully used vitrification to cryopreserve cleaved mouse embryos (Valdez *et al.*, 1990; Kasai *et al.*, 1990), rabbit embryos (Smorag & Gajda, 1991), sheep embryos (Szell *et al.*, 1990) and cattle embryos (Saha *et al.*, 1996; Massip *et al.*, 1986).

The solid state retains the normal molecular and ionic distributions of the liquid state and is called a glass and can be considered to be an extremely viscous supercooled liquid (Rall, 1987). A glass is microscopically a liquid that is too cold to flow. The good things about vitrification is that there is nothing about it that should be biologically damaging. A vitrified liquid is not different from the ordinary liquid except that it does not possess molecular motions and, therefore, it doesn't permit any appreciable deteriorative changes with time (Rall & Fahy, 1985). The freezing solution must contain a high concentration of one or more permeating cryoprotectants. Each solution also requires a physiological saline compound, and macromolecules have to be added to increase the ability of the solution to supercool and vitrify (Rall, 1992; 1987).

2.2.6 Evaluation of Embryos after Thawing

The grading of embryos on their morphological appearance is part of the normal routine in cattle embryo transfer (ET) operations. Frozen/thawed bovine embryos can be evaluated visually by putting them through a short-term period of IVC. This is done by assessing their progress through morphological examination, immediately after thawing, re-expansion of the blastocyst and assessing the embryos at 24h intervals for three days (Hamawaki *et al.*, 1999; Acgca *et al.*, 1998; Saha *et al.*, 1996a; Gordon, 1994; Massip *et al.*, 1993). Hamawaki *et al.* (1999) and Saha *et al.* (1996a) indicated that the development of embryos could be assessed by their ability to develop into expanded and hatched blastocysts during a 72h period of culture. Willadsen *et al.* (1978) followed the rule that no embryo can be considered to have survived freezing/thawing unless it has expanded or re-expanded into a blastocyst with a visible embryonic disc at the end of the culture period. There were also attempts to assess embryo viability after thawing by including the use of the dye-exclusion test (Dooley *et al.*, 1987), but this technique has little advantage for recommendation in routine applications (Gordon, 1994).

Hatching is one of the indicators of embryo viability as indicated in Figure 2.1. However, the fact that embryos do not hatch *in vitro*, does not necessarily mean that the embryos will not hatch within the uterus. The extended culture of the bovine blastocyst after thawing implies that hatching may be used as an indicator of its viability/normality (Gordon, 1994). Re-expansion and hatching rate is commonly used as an indicator of embryo viability (Hamawaki *et al.*, 1999; Agca *et al.*, 1998; Saha *et al.*, 1996a; Massip *et al.*, 1995a; Massip *et al.*, 1993)

2.2.7 Survival Rates of *in vitro* Frozen Bovine Embryos

Survival rates of frozen *in vitro* derived embryos, as measured by either post-thaw development in culture or by pregnancies following transfer, have been lower *in vitro* than those reported *in vivo*. Viability of *in vitro* frozen/thawed derived embryos have been reported to be affected by embryo age (Myers *et al.*, 1996; Cseh *et al.*, 1995; Massip

et al., 1995b; Takagi *et al.*, 1994a; Wurth *et al.*, 1994; Del Campo *et al.*, 1993; Suzuki, 1993), stage of embryonic development (Carvallo *et al.*, 1996; Cseh *et al.*, 1995; Dinnyes *et al.*, 1995; Del Campo *et al.*, 1993; Pavasuthipaisit *et al.*, 1993; Pollard & Leibo, 1993; Zhang *et al.*, 1993), embryo quality (Kuwayama, 1995; Han *et al.*, 1994), cryoprotectant (Ohlrichs *et al.*, 1996; Takagi *et al.*, 1994a; 1994b; Suzuki *et al.*, 1993; Takagi *et al.*, 1993; Voelkel & Hu, 1992a), pH of freezing medium (Kajihara *et al.*, 1993), freezing process (Thonon *et al.*, 1995; Wurth *et al.*, 1994; Suzuki *et al.*, 1993) and the culture system in which the embryos are produced (Pugh *et al.*, 1996; Semple *et al.*, 1995; Voelkel & Hu, 1992a; Rorie *et al.*, 1990).

Survival rates, based on re-expanded and hatching rates following cryopreservation of *in vitro* embryos, have been quoted as varying between 69.2% and 62.4% (Hasler *et al.*, 1997). Survival and pregnancy rates after transfer of frozen/thawed IVEP are significantly lower than that obtained in *in vivo* embryos, being recorded as 30 to 40% (Hasler *et al.*, 1995), 14 to 23% (Wurth *et al.*, 1994), 38% (Kajihara *et al.*, 1992) and 11% (Reichenbach *et al.*, 1991). Massip *et al.* (1995a) recorded lower success rates when expressed in terms of the number of calves born (20% compared to 59% *in vivo* frozen/thawed embryos after transfer).

A high success rate for IVEP was recorded after thawing, with a re-expansion rate of 80% and hatching rate of 65%. This was confirmed by Yang *et al.* (1992), who quoted a hatching rate of 81%. Agca *et al.* (1994) achieved a 50% pregnancy rate after bilateral twin transfer, using the vitrification procedure.

There is a discrepancy between the *in vitro* survival rate and the *in vivo* rate of development. Based on the morphology, the *in vitro* survival rate after thawing can be regarded as satisfactory. The pregnancy rate obtained after transfer is however low, a 80% survival *in vitro* frozen/thawed embryos, compared to 33% survival rate after transfer to the recipients (Massip *et al.*, 1993). Van Wagtenok-de Leeu *et al.* (1995) reported that vitrification and the one-step freezing procedure yield similar pregnancy rates a those of standard conventional (slow) freezing method. The minimum survival

rate (re-expansion within 24h) is 81% and 71% respectively for the serum free and serum treated groups on day 7 frozen-thawed embryos (Semple *et al.*, 1995). Saha *et al.* (1996a) found a significant difference between the hatching rates of day 7 (75%), day 8 (38%) and day 9 (9%) embryos.

Van Wagtenok-de Leeuw *et al.* (1997) recorded overall pregnancy rates of 44.5% following vitrification, and 45.1% following the slow freezing method. Results indicate that vitrification can be successfully used in this field without any significant reduction in the pregnancy rate. Recent reports by Vajta *et al.* (1996) indicate that vitrification in IVEP is as efficient as the conventional freezing methods. A survival rate of 73% re-expanded and 47% hatched blastocysts following vitrification and thawing was recorded. The observations in the experiment prove that the vitrification procedure is relatively harmless to the embryo and can be used for blastocysts at different developmental stages. An intact zona is not required to obtain high survival rates.

CHAPTER 3

MATERIAL AND METHODS

3.1 Source of embryos

A total of 136 early blastocysts, blastocysts and expanded blastocysts were randomly assigned to four different cryopreservation methods. The 136 blastocysts utilized for the experiment during this study were supplied by the bovine *in vitro* embryo production laboratory (ARC-Animal Improvement Institute, Irene). Embryos were obtained by IVM, IVF and IVC of bovine follicular oocytes, using the methods described by Visser *et al.* (1998) and Rosenkrans *et al.* (1993)

Collection of oocytes: Ovaries were obtained from cows directly after slaughter at a local abattoir and transported in a sterile 0.9% saline (Intra-Med, RSA) solution at a temperature of 36 °C. Ovaries were processed within 3-5h of collection. Ovaries were washed and stored in a sterile saline solution (34 °C). Oocytes were obtained from superficial follicles by slicing of the ovarian surfaces. Ovaries were rinsed in a HEPES-TL medium (Tyrode's Lactate + 5% FCS) at regular intervals during slicing. Cumulus-oocyte complexes (COC's) were identified by stereo microscopy, and rinsed in HEPES-TL medium to remove follicular fluid and cellular debris.

Oocyte maturation (IVM): COC's were matured (incubated) for 24h in a TCM199-based, IVM medium supplemented with 10% fetal bovine serum (FBS) and gonadotropic. The humidified gas atmosphere was 5% CO₂ in air and the temperature during incubation was 39 °C.

***In vitro* fertilization (IVF):** After the maturation process, the matured oocytes were transferred into 50µl droplets of modified CR2aa (IVF medium) medium supplemented with 5µg/ml heparin under oil. Frozen/thawed semen was processed by the swim-up technique (IVF media without heparin) and the selected spermatozoa washed twice by

centrifugation (250g) in the IVF media without heparin for 10 minutes. The sperm pellet was resuspended in a small volume of CR2aa medium. Ova were inseminated with a final concentration of 2×10^6 motile spermatozoa/ml IVF medium. Matured ova were co-incubated with the spermatozoa for 18h in IVF medium at 39 °C.

***In vitro* culture (IVC):** After fertilization, the presumptive zygotes were transferred to microdroplets of preconditioned IVC medium (CR2aa + FCS), containing monolayers of cumulus cells and covered with mineral oil. The presumptive zygotes were then evaluated for cleavage, 48h after insemination. On day 7, 8 and 9 post insemination, 136 early blastocysts, blastocysts and expanded blastocysts were recovered and randomly assigned to four different treatment groups.

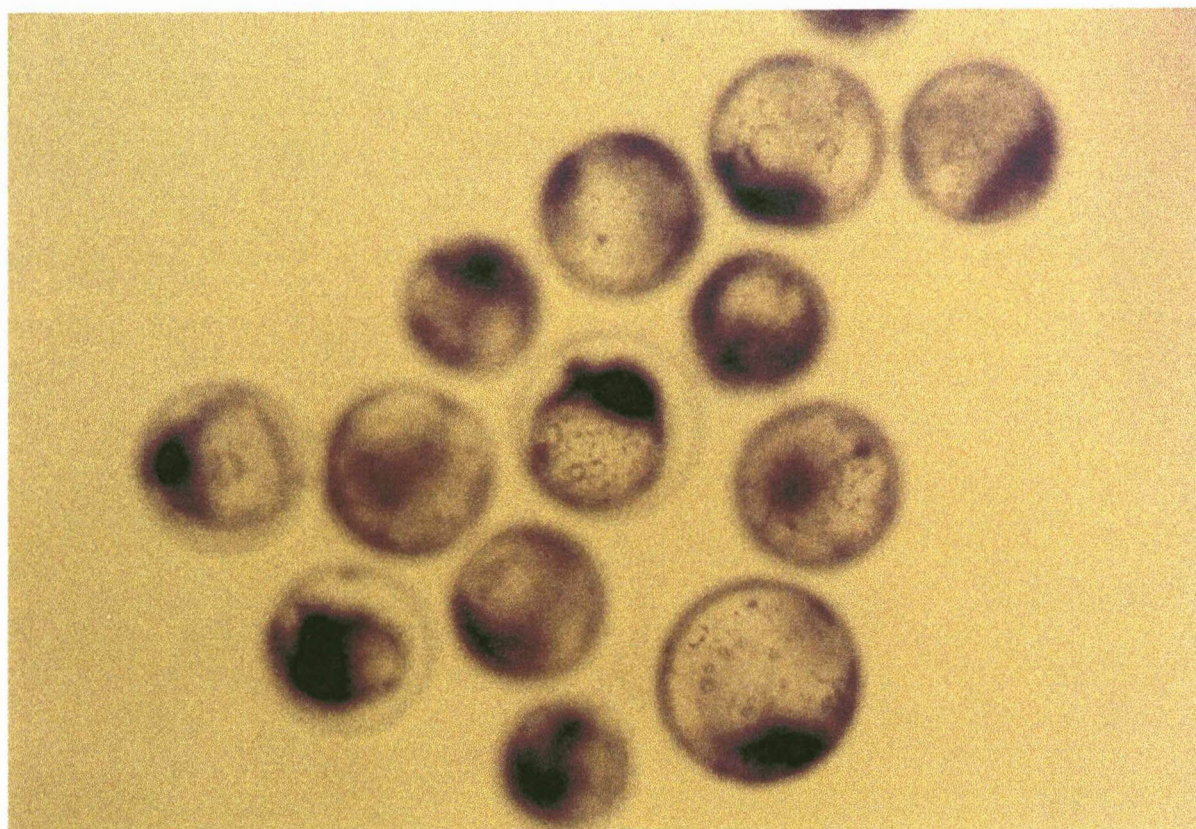


Figure 3.1 Some of the IVP bovine embryos used in vitrification methods and the slow freezing method before being randomly assigned to the four different treatment groups

3.2 Preparation of Freezing Media

Conventional (slow) freezing media

Commercial ViGro™ Holdingplus media (AB Technology, Inc. Pullman, WA, USA, 509 335-4047) was prepared for temporal storage of embryos from *in vitro* culture, before being transferred to 1.5M ViGro™ Ethylene glycol Freezeplus (AB Technology, Inc. Pullman, WA, USA). Prior to transfer, ViGro™ EG Freezeplus medium (Treatment 4) was warmed to 24 °C. (ViGro™ EG Freezeplus is a DMPBS-based solution containing 1.5M EG, 0.4% BSA and 0.1M sucrose).

Vitrification media

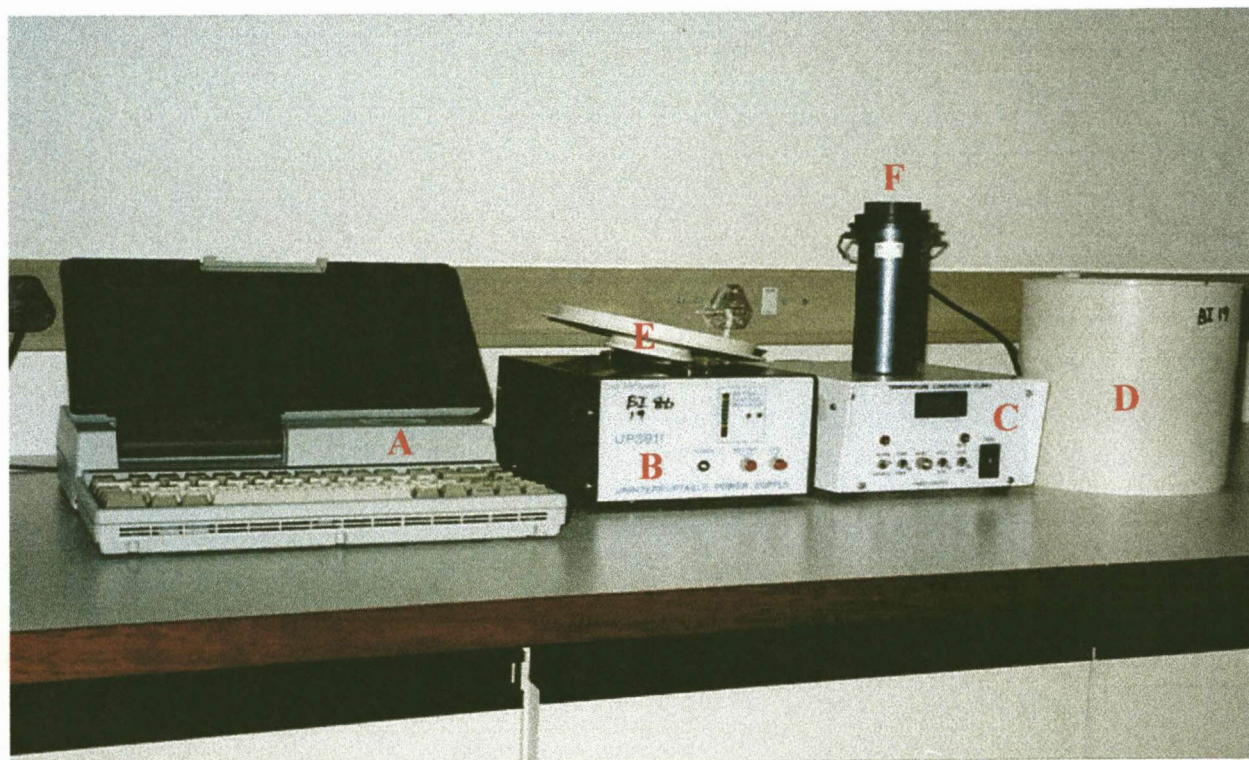
Commercial ViGro™ Holdingplus media was used for preparing the two equilibration solutions I & II and the three vitrification solutions.

These equilibration solutions, a 10% ethylene glycol (EG, FW.62.07, Aldrich Chemical co. Inc-RSA-USA) in ViGro™ Holdingplus was prepared as equilibration solution I. 10% EG plus 0.3M trehalose (D₍₊₎Trehalose, Sigma chemical, RSA-USA) in ViGro™ Holdingplus was used for preparation of the equilibration solution II.

The three vitrification solutions (VS) were prepared as follows:

- (i) 40% EG in ViGro™ Holdingplus (Treatment 1)
- (ii) 40% EG+0.3M trehalose in ViGro™ Holdingplus (Treatment 2)
- (iii) 40% EG+ 0.3M trehalose + 20% polyvinyl pyrrolidone (Treatment 3)
in ViGro™ Holdingplus (PVP, AV.mol.wt.40 000, Sigma chemical, RSA-USA).

3.2.1 Conventional Slow Freezing of Embryos



A= Halikan Laptop, B= Uninterruptible power supply (UPS-911), C= Temperature Freeze controller CL863, D= Liquid nitrogen (LN2) bath, E= Lid of LN2 bath & F= Cryo-chamber

Figure 3.2 Freeze control (Programmable Freezer) model CL863 used in pre-programmed mode (Cryogenesis, Australia).

Day 7, 8 and 9 early blastocysts, blastocysts and expanded blastocysts were removed from IVC (39 °C) and randomly placed in holding media (ViGro™ Holdingplus) at room temperature (24 °C). Embryos were then transferred from ViGro™ Freezeplus medium to 1.5M ViGro™ EG Freezeplus (Treatment 4, n=30) at room temperature (24 °C) and immediately loaded into 0.25ml plastic straws and sealed in preparation for freezing. After sealing, embryos were immediately placed into CL 863-cryo-chamber system and the temperature maintained at -6 °C. Straws were then seeded after 5 minutes of equilibration, by touching the wall of the straws with a cold forceps at the end of the straw opposite to the embryo. Embryos were cooled at a rate of 0.3 °C/min from -6 °C to -30 °C. From -30 °C to -33 °C, the rate changed to 0.1°C/min. The programmable

freezing technique is indicated in Figure 3.2. After the targeted temperature of $-33\text{ }^{\circ}\text{C}$ was reached, embryos were transferred directly into a container with liquid nitrogen ($-196\text{ }^{\circ}\text{C}$) and straws were stored vertically in a liquid nitrogen tank.

3.2.2 Vitrification of Embryos

The day 7, 8 and 9 early blastocysts, blastocysts and expanded blastocysts were randomly assigned to vitrification solutions, using the method described by Saha *et al.* (1996). Embryos were firstly kept in 10% EG (Equilibration I, ES-I) in ViGroTMHoldingplus for 5 minutes, then in 10% EG plus 0.3M Trehalose in ViGroTMHoldingplus (Equilibration II, ES-II) for 5 minutes, both at room temperature ($24\text{ }^{\circ}\text{C}$). The embryos were then transferred to vitrification solutions containing 40% EG (Treatment 1, n=34), 40% EG + 0.3M Trehalose (Treatment 2, n=29) and 40% EG + 0.3M Trehalose plus 20% PVP (Treatment 3, n=43) in ViGroTMHoldingplus (at room temperature of $24\text{ }^{\circ}\text{C}$). Embryos were immediately loaded in 0.25ml plastic straws and plunged directly (horizontally) into liquid nitrogen and vertical stored in liquid nitrogen ($-196\text{ }^{\circ}\text{C}$).

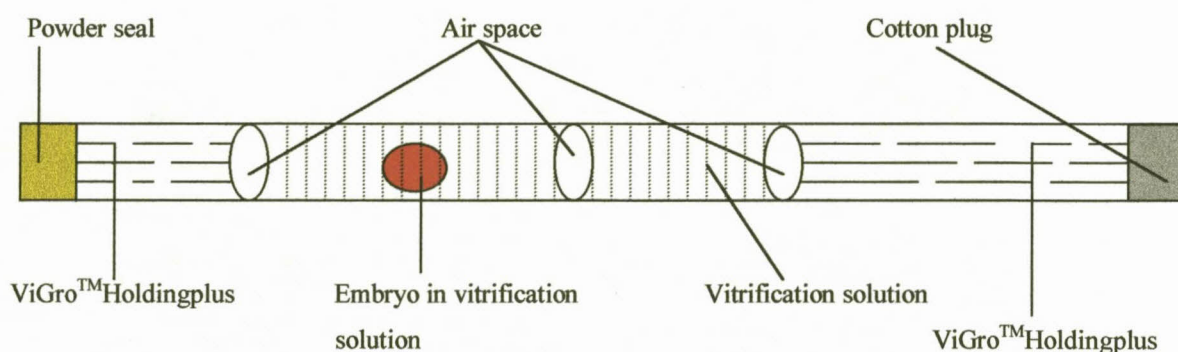


Figure 3.3 Configuration of the solutions in the straw loaded with an embryo prior to being placed in liquid nitrogen ($-196\text{ }^{\circ}\text{C}$). Vitrification solution with embryo and ViGroTMHoldingplus were sequentially put into the straw to yield four liquid chambers separated by air bubbles

3.3 Thawing Procedures

Thawing of embryos within the straw was carried out in a water bath (32 °C) at room temperature (24 °C). Each straw was placed in the water bath for 30 seconds. The straw was then dried, cut and the contents expelled into ViGro™ Holdingplus medium. Once the embryos were recovered from the straws, embryos were washed twice in fresh ViGro™ Holdingplus medium and immediately examined morphologically for survivability/re-expanded stage of the blastocyst under a stereo microscope. The survival embryos were cultured in IVC media (CR2aa + FCS) and a rate of embryo survival was recorded immediately at thawing, 24 hours and 48 hours post-thawing by monitoring the re-expansion of the blastocoel cavity and expansion of the blastocyst.

3.4 Statistical analysis

Data were analyzed using the Chi-squared test. The generalized linear modeling (GLM) was also used to test differences between fast freezing (vitrification) methods and conventional slow freezing method (Snedecor & Cochran, 1967).

CHAPTER 4

RESULTS

4.1 Evaluation of Four Cryopreservation Methods

The ratios of live : dead embryos were first evaluated using the traditional chi-squared test (Snedecor & Cochran, 1967).

4.1.1 Survival rate of IVP bovine embryos immediately after thawing

Evaluation of different cryopreservation methods for *in vitro* produced (IVP) bovine embryos was determined at thawing, 24 hours and 48 hours post-thawing. The results are presented in Table 4.1 to 4.3.

Table 4.1 Embryo survival rate (%) immediately after thawing of the three vitrification methods and the slow freezing method

Treatment Groups	Number of embryos (n)		
	Live	: Dead	Total
1 (40% EG)	18 (53%) ^{ab}	16 (47%) ^{ab}	34
2 (40% EG + 0.3M Trehalose)	12 (41%) ^a	17 (59%) ^a	29
3 (40% EG + 0.3M Trehalose + 20% PVP)	33 (77%) ^b	10 (23%) ^b	43
4 (1.5 ViGro TM EG Freezeplus)	15 (50%) ^{ab}	15 (50%) ^{ab}	30

^{ab}Values within the same column with different superscripts, differ significantly ($P < 0.05$)

There was a significant ($P < 0.05$) difference between the survival rate of blastocysts frozen/thawed in Treatment (TMT) 3 and Treatment (TMT) 2 (77% vs. 41% respectively). There was no significant difference in survival rate between blastocyst frozen/thawed by vitrification methods (TMT 1 vs. TMT 2 & TMT 1 vs. TMT 3) and the conventional slow freezing method (TMT 1 vs. TMT 4; TMT 2 vs TMT 4; TMT 3 vs. TMT 4).

The graphic presentation of the survival rate (%) of IVP bovine embryos cryopreserved by vitrification methods (40% EG; 40% EG + 0.3M trehalose; 40% EG + 0.3M trehalose + 20% PVP) and the slow freezing method (1.5M ViGroTMEG Freezeplus), immediately after thawing are presented in Figure 4.1

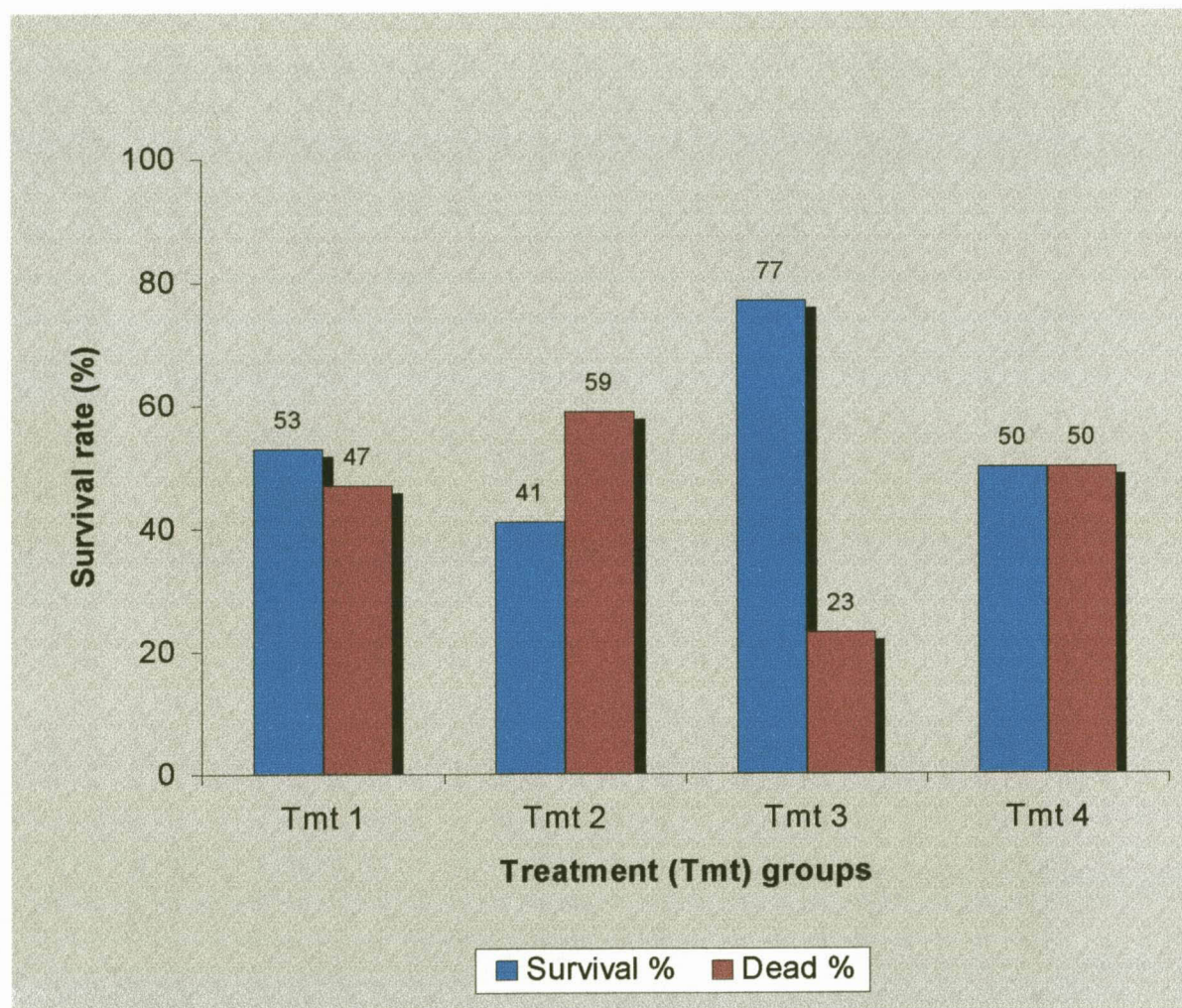


Figure 4.1. Survival rate (%) of IVP bovine embryos immediately after thawing following vitrification in 40% EG (TMT 1), 40% EG + 0.3M trehalose (TMT 2); 40% EG + 0.3M trehalose + 20% PVP (TMT 3) and the slow freezing method in 1.5M ViGroTMEG Freezeplus (TMT 4).

4.1.2 Survival rate of IVP bovine embryos 24 hours post-thawing

Table 4.2 Embryo survival rate (%) 24 hours post-thawing of the three vitrification methods and the slow freezing method

Treatment groups	Number of embryos (n)		
	Live	: Dead	Total
1 (40% EG)	9 (26%) ^a	25 (74%) ^a	34
2 (40% EG + 0.3M Trehalose)	6 (21%) ^a	23 (79%) ^a	29
3 (40% EG + 0.3M Trehalose + 20% PVP)	26 (60%) ^b	17 (40%) ^b	43
4 (1.5 ViGro TM EG Freezeplus)	12 (40%) ^{ab}	18 (60%) ^{ab}	30

^{ab}Values within the same column with different superscripts, differ significantly ($P < 0.05$)

There was a significant ($P < 0.05$) difference in survival rate between TMT 2 and TMT 3 (21% vs 60% respectively). There was also a significant ($P < 0.05$) difference between TMT 1 and TMT 3 (26% vs 60% respectively), with TMT 3 having the highest survival rate. There was no significant difference in survival rate between the other vitrification methods (TMT 1 vs. TMT 2), and vitrification methods and the slow freezing method (TMT 1 vs TMT 4; TMT 2 vs. TMT 4; TMT 3 vs. TMT 4), 24 hours post-thawing.

The graphic presentation of the survival rate (%) of *in vitro* produced bovine embryos following the slow freezing method (TMT 4) and vitrification methods (40% EG (TMT 1), 40% EG + 0.3M trehalose (TMT 2) & 40% EG + 0.3M trehalose + 20% PVP (TMT 3), 24 hours post-thawing is indicated in Figure 4.2.

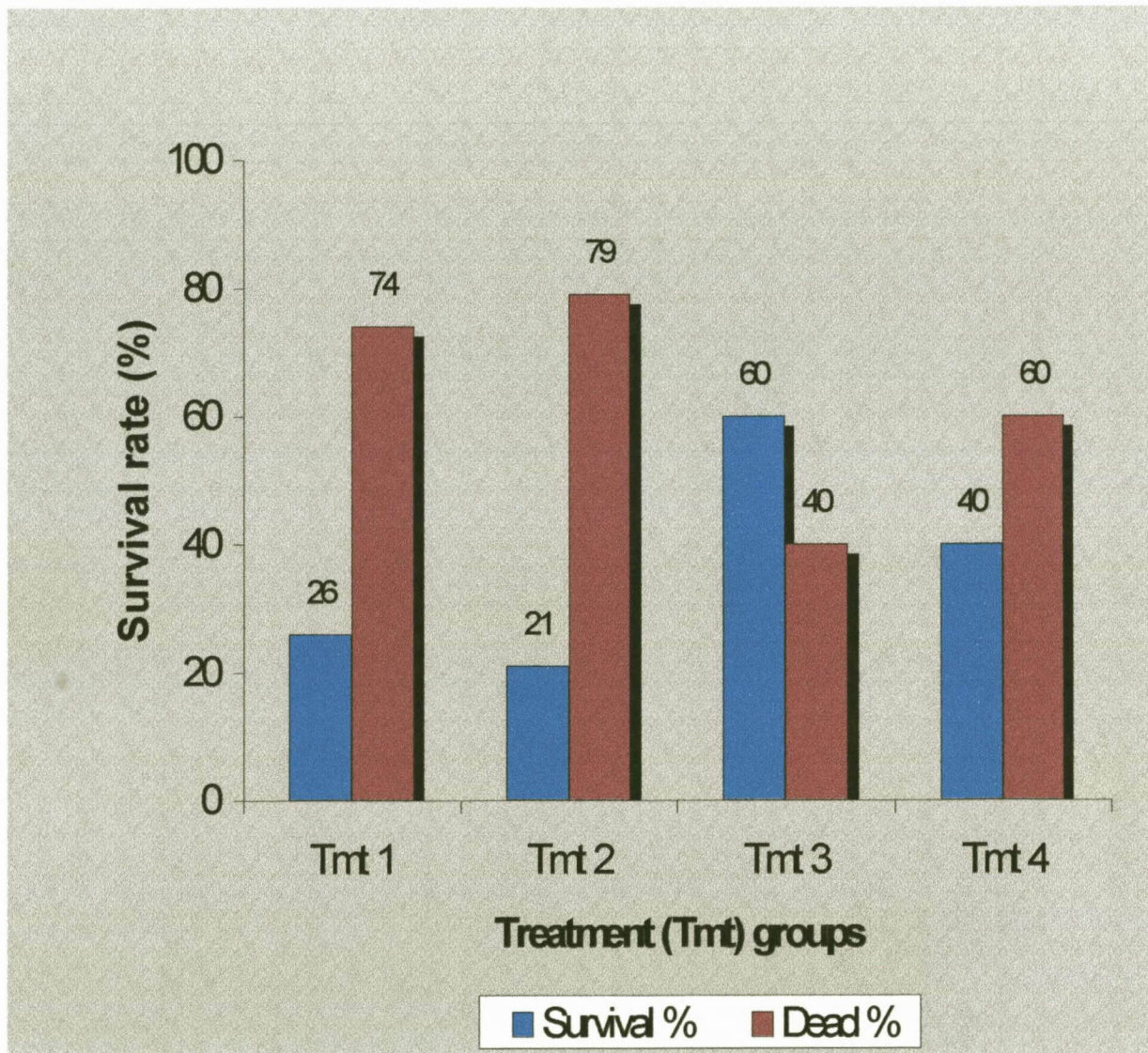


Figure 4.2 Survival rate (%) of IVP bovine embryos 24 hours post-thawing following vitrification in 40% EG (TMT 1); 40% EG + 0.3M trehalose (TMT 2); 40% EG + 0.3M trehalose + 20% PVP (TMT 3) and slow freezing method in 1.5M ViGroTMEG Freezeplus (TMT 4)

4.1.3 Survival of IVP bovine embryos 48 hours post-thawing

Table 4.3 Embryo survival rate (%) 48 hours post-thawing of the three vitrification methods and the slow freezing method

Treatment groups	Number of embryos (n)		
	Live	: dead	Total
1 (40% EG)	4 (12%) ^a	30 (98%) ^a	34
2 (40% EG + 0.3M Tre.)	3 (10%) ^a	26 (90%) ^a	29
3 (40% EG + 0.3M Trehalose. + 20% PVP)	16 (37%) ^a	27 (63%) ^a	43
4 (1.5M ViGro TM EG Freezeplus)	9 (30%) ^a	21 (70%) ^a	30

^aValue within the same column with different superscript do not differ significantly

There were no significant differences in the survival rate among embryos frozen/thawed by three vitrification methods (TMT 1 vs TMT 2; TMT 1 vs TMT 3; TMT 2 vs TMT 3), and in the conventional slow freezing method (TMT 1 vs TMT 4; TMT 2 vs TMT 4; TMT 3 vs TMT 4).

The graphic presentation of the survival rate (%) of *in vitro* produced bovine embryos following the slow freezing method (TMT 4) and vitrification methods 40% EG (TMT 1), 40% EG + 0.3M trehalose (TMT 2) and 40% EG + 0.3M trehalose + 20% PVP (TMT 3), 48 hours post-thawing is presented in Figure 4.3.

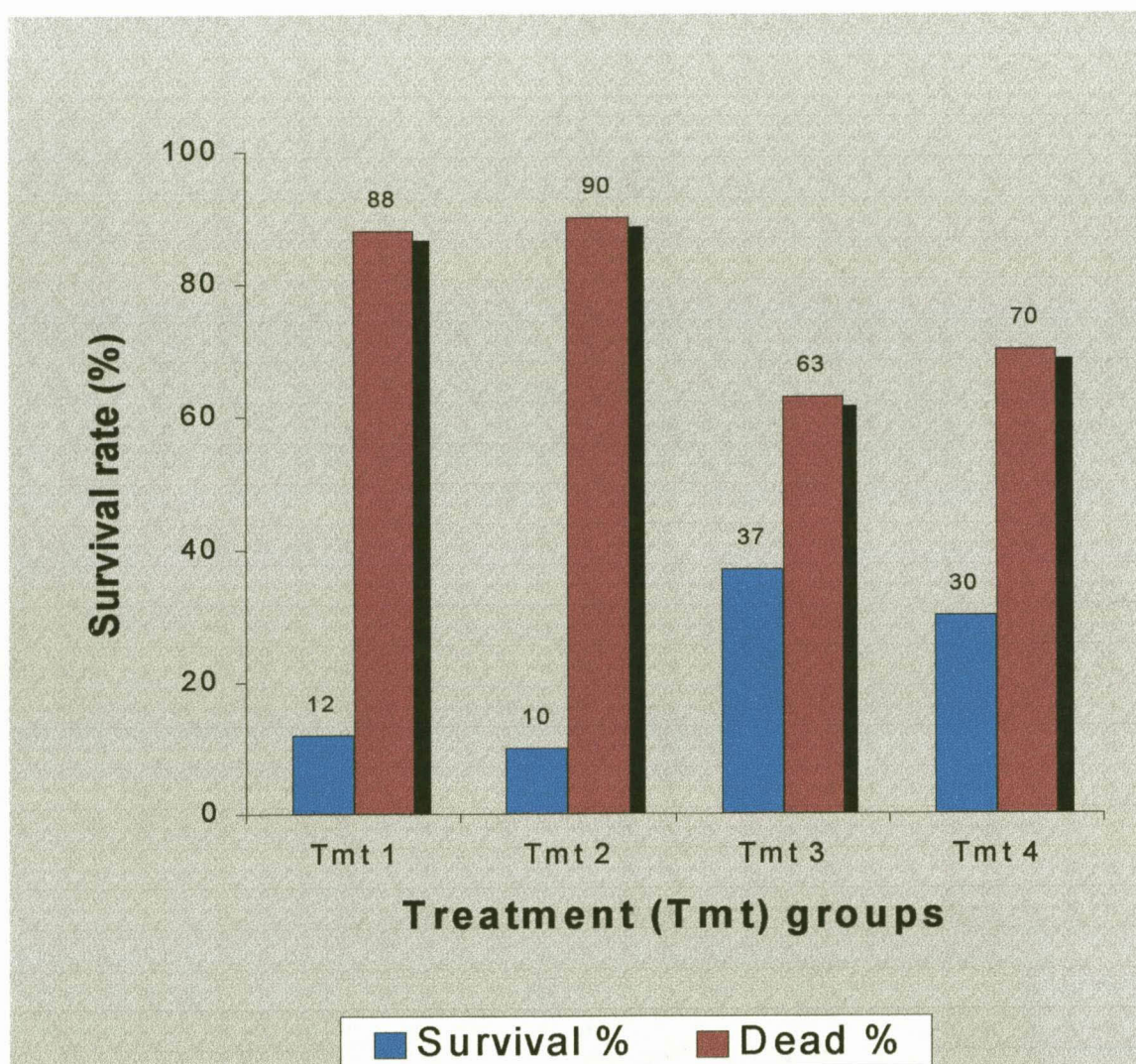


Figure 4.3 Survival rate (%) of IVP bovine embryos 48 hours post-thawing following vitrification in 40% EG (TMT 1); 40% EG + 0.3M trehalose (TMT 2); 40% EG + 0.3M trehalose + 20% PVP (TMT 3) and the slow freezing method in 1.5M ViGroTMEG Freezeplus (TMT 4)

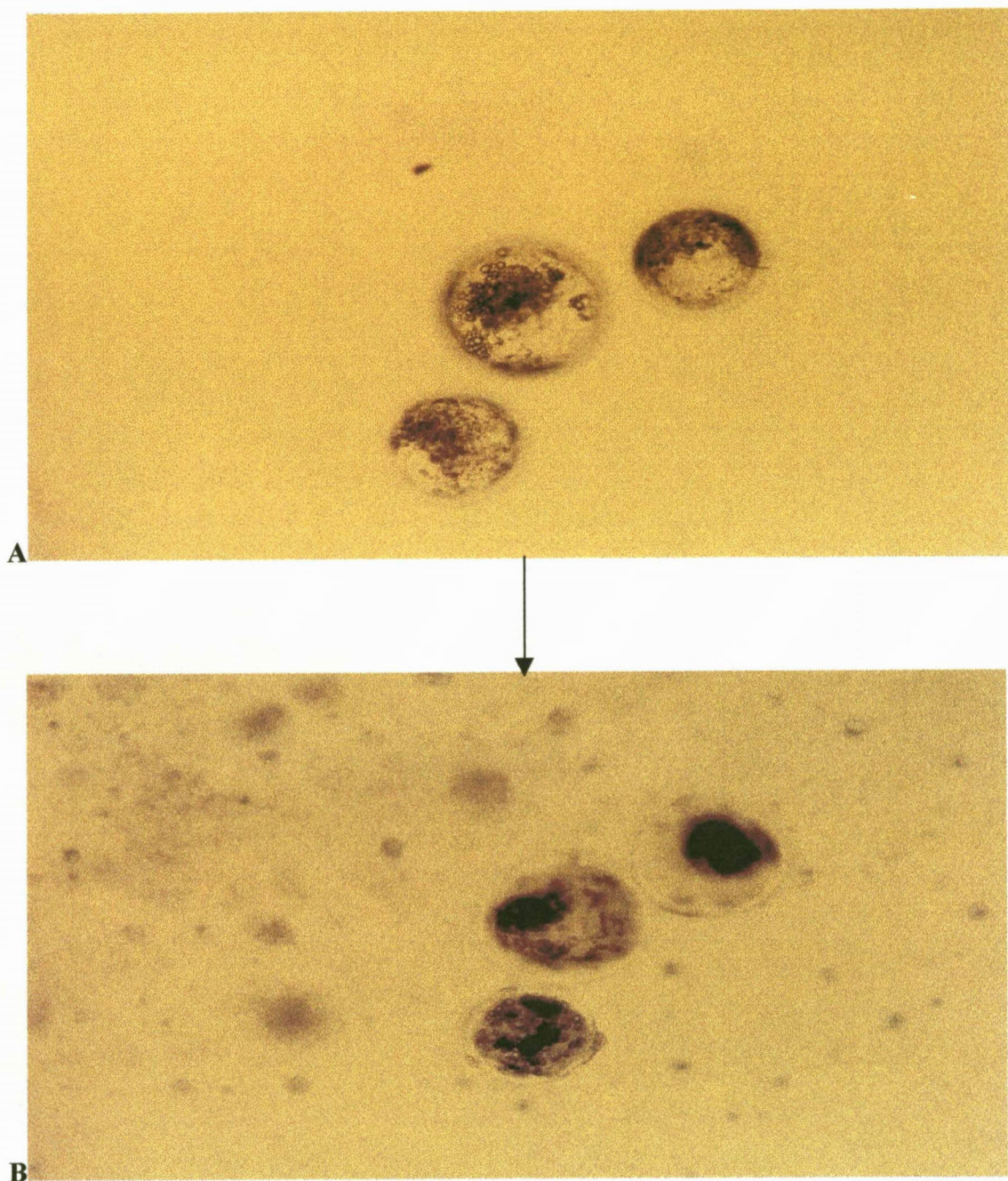


Figure 4.4 IVP bovine embryos under light microscope (F10/0.25) in ViGroTM Holdingplus: Embryos were vitrified in 40% EG plus 0.3M trehalose plus 20% PVP (TMT 3). (A) Live blastocysts immediately after thawing. (B) degenerated embryonic cells 48 hours post-thawing.

4.2 Comparison Between the Four Cryopreservation Methods (Bonferroni multiple comparison test)

Since the survival rate of embryos was binomially distributed and not normally distributed, the technique known as generalized linear modelling (GLM; Bonferroni multiple comparison test) was used to test the difference between the vitrification methods and the conventional slow freezing method. This modern statistical technique is valuable to compare the proportion (number of embryos surviving out of the total embryos frozen) and also compute likelihood ratios between treatments.

4.2.1 Predicted survival rate of IVP bovine embryos immediately after thawing

Table 4.4 The predicted (\pm SE) embryo survival rate (p) immediately after thawing of three vitrification methods and the slow freezing method

Treatment groups	p	SE
1 (40% EG)	52.9 ^{ab}	8.56
2 (40% EG+0.3M Trehalose 2)	41.4 ^a	9.15
3 (40% EG+0.3M Trehalose+20%PVP)	76.7 ^b	6.44
4 (1.5M Vigro TM EGFreezeplus)	50.0 ^{ab}	9.13

^{ab}Column with different superscripts differ significantly ($P < 0.05$)

The Bonferroni multiple comparison test was used to test the differences in the predictions for the three fast freezing methods (vitrification methods) and the slow freezing method. There was a significant ($P < 0.05$) difference in the survival rate between embryos frozen/thawed with TMT 2 (41% \pm 9.15) and TMT 3 (77% \pm 6.44).

The embryos frozen/thawed in TMT 1 (53%) are predicted to be 1.1 times more likely to survive, compared to those frozen in TMT 4 (50%). The embryos frozen/thawed in TMT 2 (41%) are predicted to be 1.4 times less likely to survive compared to those in TMT 4 (50%). Embryos frozen/thawed in TMT 3 (77%) are predicted to be 3.3 times more likely to survive, compared to those frozen in TMT 4 (50%).

4.2.1.1 Predicted survival rate of IVP bovine embryos vitrified by three vitrification methods immediately after thawing

To compare only the vitrification methods, the Bonferroni multiple comparison test was repeated, excluding the conventional slow freezing method.

The predicted embryo survival rate (p) and standard errors (se) for each prediction is indicated in Table 4.5.

Table 4.5 The predicted (\pm SE) embryo survival rate(p) immediately after thawing following the three-vitrification methods

Treatments	p	SE
1 (40% EG 1)	52.9 ^{ab}	8.56
2 (40% EG + 0.3M trehalose)	41.3 ^a	9.15
3 (40% EG + 0.3M trehalose + 20% PVP)	76.7 ^b	6.44

^{ab}Column with different superscripts differ significantly ($P < 0.05$)

There was a significant ($P < 0.05$) lower predicted survival rate for embryos frozen in TMT 2 (41%), compared to TMT 3 (77%).

Embryos frozen/thawed in 40% EG (TMT 1) are predicted to be 2.9 times less likely to survive compared to embryos frozen/thawed in 40% EG + 0.3M trehalose + 20% PVP (TMT 3). Embryos frozen/thawed in 40% EG + 0.3M trehalose (TMT 2) are predicted to be 4.7 times less likely to survive compared to those frozen/thawed in 40% EG + 0.3M trehalose + 20% PVP (TMT 3)

4.2.2 Predicted survival rate of IVP bovine embryos 24 hours post-thawing

The predicted embryo survival rate (p) and standard errors (se) for each prediction are indicated in Table 4.6.

Table 4.6 The predicted (\pm SE) embryo survival rate (p) 24 hours post-thawing of three vitrification methods and the slow freezing method

Treatment groups	p	SE
1 (40% EG)	26.5 ^a	7.56
2 (40% EG + 0.3M trehalose)	20.5 ^a	7.52
3 (40% EG + 0.3M Trehalose + 20% PVP)	60.5 ^b	7.46
4 (1.5M ViGro TM EG Freezeplus)	40.0 ^{ab}	8.94

^{ab}Column with different superscripts differ significantly ($P < 0.05$)

There was a significant ($P < 0.05$) lower predicted survival rate for embryos frozen/thawed in 40% EG (TMT 1; 26.5%), compared to 40% EG + 0.3M trehalose + 20% PVP (TMT 3; 60.5%). There was also a significant ($p < 0.05$) lower predicted survival rate for embryos frozen/thawed in 40% EG + 0.3M trehalose (TMT 2; 20.5%), compared to 40% EG + 0.3M Trehalose + 20% PVP (TMT 3; 60.5%).

Embryos frozen/thawed in TMT 1 (27%) are predicted to be 1.9 times less likely to survive, compared to those frozen in TMT 4 (40%). Embryos frozen/thawed in TMT 2 (21%) are predicted to be 2.6 times less likely to survive, compared to those in TMT 4 (40%). Embryos frozen/thawed in TMT 3 (61%) are predicted to be 2.3 times more likely to survive, compared to those frozen/thawed in TMT 4 (40%).

4.2.2.1 Predicted survival rate of IVP bovine embryos vitrified by three vitrification methods, 24 hours post-thawing

The predicted embryo survival rate (p) and standard error (se) for each prediction are indicated in Table 4.7.

Table 4.7 The predicted (\pm SE) embryo survival rate (p) 24 hours post-thawing of the three vitrification methods

Treatments	P	SE
1 (40% EG)	26.47 ^a	7.56
2 (40% EG + 0.3M Trehalose)	20.69 ^a	7.52
3 (40% EG + 0.3M Trehalose + 20% PVP)	60.47 ^b	7.46

^{ab}Column with different superscripts differ significantly ($P < 0.05$)

There was a significant ($P < 0.05$) higher predicted survival rate for embryos vitrified in 40% EG (TMT 1; 26%), compared to those frozen in 40% EG + 40% EG 0.3M trehalose + 20% PVP (TMT 3; 60%). Similarly, a significant ($P < 0.05$) difference was predicted in survival rate for embryos vitrified in 40% EG + 0.3M trehalose (TMT 2; 21%), compared to those frozen/thawed in 40% EG + 0.3M trehalose + 20% PVP (TMT 3; 60%).

Embryos vitrified in 40% EG (TMT 1; 26%) are predicted to be 4.3 times less likely to survive, compared to those vitrified in 40% EG + 0.3M trehalose + 20% PVP (TMT 3; 60%). Embryos vitrified in 40% EG + 0.3M trehalose (TMT 2; 21%) are predicted to be 5.9 times less likely to survive, compared to those vitrified in 40% EG + 0.3M trehalose + 20% PVP (TMT 3; 60%).

4.2.3 Predicted survival rate of IVP bovine embryos 48 hours post-thawing

The predicted embryo survival rate (p) and standard error (se) of each prediction is set out in Table 4.8.

Table 4.8 The predicted (\pm SE) embryo survival rate (p) 48 hours post-thawing of three vitrification methods and the slow freezing method

Treatment groups	p	SE
1 (40% EG)	11.7 ^a	5.53
2 (40% EG + 0.3M Tre.)	10.3 ^a	5.66
3 (40% EG + 0.3M Tre. + 20% PVP)	37.2 ^a	7.37
4 (1.5M ViGro TM EG Freezeplus)	30.0 ^a	8.37

^aColumn with the same superscript do not differ significantly

No statistical difference in the predicted embryo survival rate was found among the four treatment groups.

Embryos frozen/thawed in TMT 1 (12%) are predicted to be 3.2 times less likely to survive, compared to those frozen/thawed in TMT 4 (30%). Embryos frozen/thawed in embryos frozen/thawed in TMT 2 (10%) are predicted to be 3.7 times less likely to survive, compared to those frozen-thawed in TMT 4 (30%). Embryos frozen/thawed in TMT 3 (37%) are predicted to be 1.4 times more likely to survive, compared to those in TMT 4 (30%).

4.2.3.1 Predicted survival rate of IVP bovine embryos vitrified by three vitrification methods 48 hours Post-thawing

The predicted embryo survival rate (p) and standard error for each prediction (se) are set out in Table 4.9.

Table 4.9 The predicted (\pm SE) embryo survival rate (p) 48 hours post-thawing of the three vitrification methods

Treatments	P	SE
1 (40% EG)	11.7 ^a	5.53
2 (40% EG + 0.3M Trehalose)	10.3 ^a	5.66
3 (40% EG + 0.3M Trehalose + 20% PVP)	37.2 ^a	7.37

^aColumn with the same superscript do not differ significantly

No significant differences are predicted in the embryo survival rate between the vitrification methods (Table 4.9).

Embryos vitrified in 40% EG (TMT 1; 12%) are predicted to be 4.4 times less likely to survive, compared to those vitrified in 40% EG + 0.3M trehalose + 20% PVP (TMT 3; 37%). Embryos vitrified in 40% EG + 0.3M trehalose (TMT 2; 10%) are predicted to be 5.1 times less likely to survive, compared to those vitrified in 40% EG + 0.3M trehalose + 20% PVP (TMT 3; 37%), at 48 hours post-thawing.

Figure 4.5 to 4.7 present IVP bovine embryos under light microscope (F2.5/0.08, low magnification and F10/.25, second magnification) before being frozen or after thawing in ViGroTM Holdingplus.

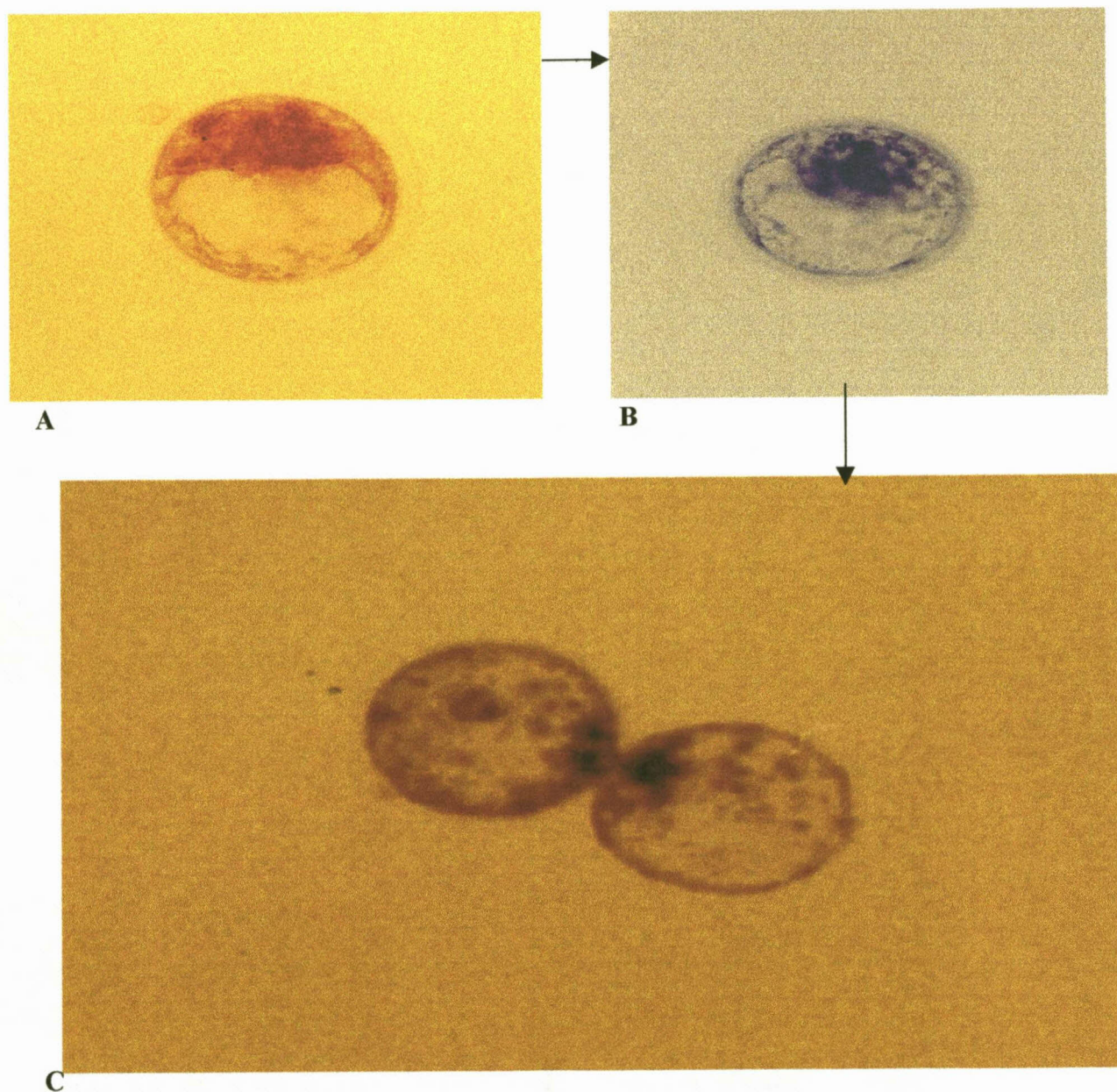


Figure 4.5 IVP bovine embryos under light microscope (F10/.25) in ViGro™ Holdingplus: (A) live blastocyst in ViGro™ Holdingplus before frozen by slow freezing method (TMT 4). (B) live blastocyst in ViGro™ Holdingplus immediately after thawing. (C) hatched blastocyst 48 hours post-thawing.

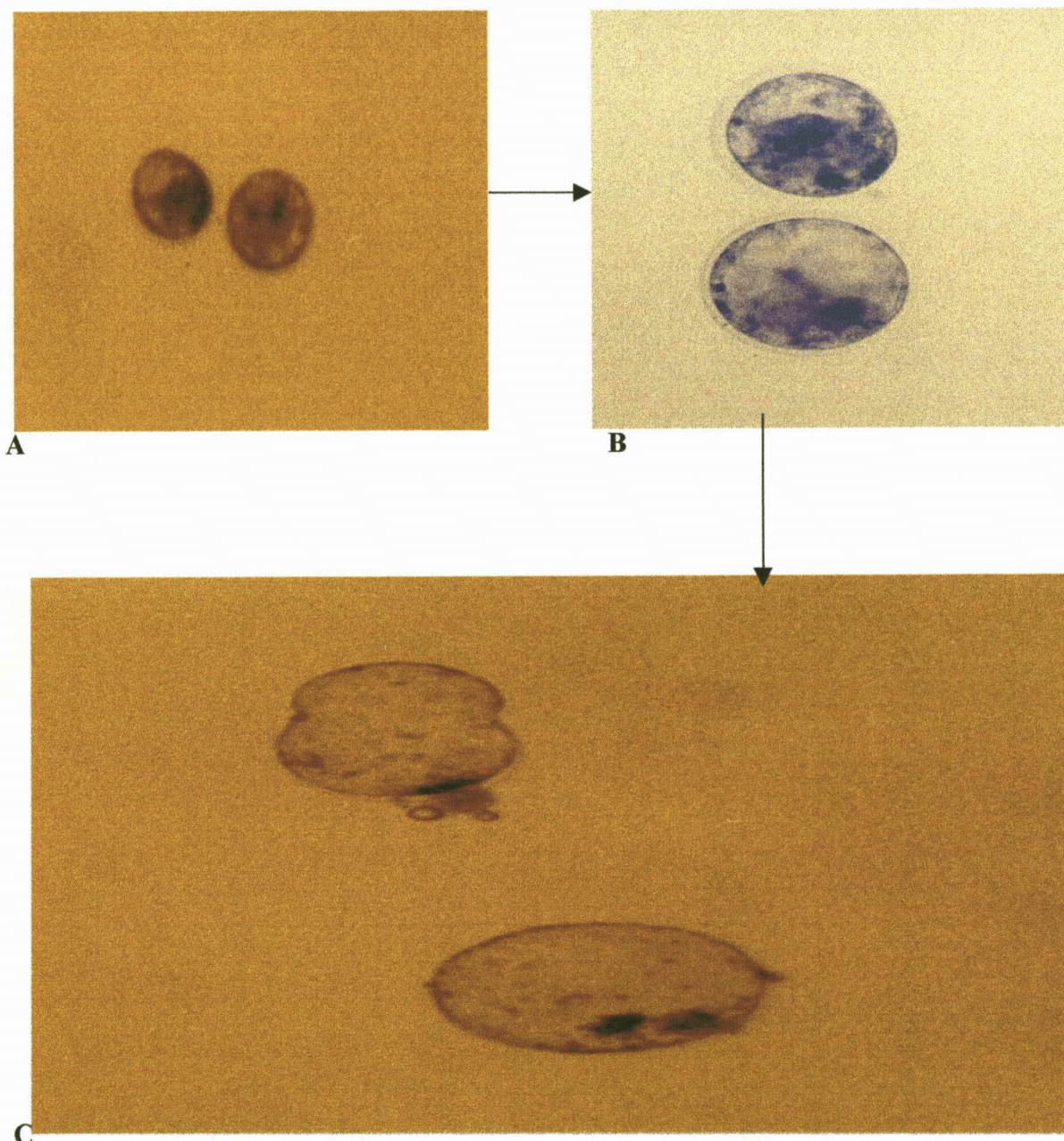


Figure 4.6 IVP bovine embryos under light microscope in ViGro™ Holdingplus: (A) two live blastocysts under light microscope (low magnification) before being vitrified in 40% EG + 0.3M trehalose + 20% PVP (TMT 3). (B) two live blastocysts immediately after thawing in ViGro™ Holdingplus. (C) two hatched blastocysts 48 hours post-thawing.

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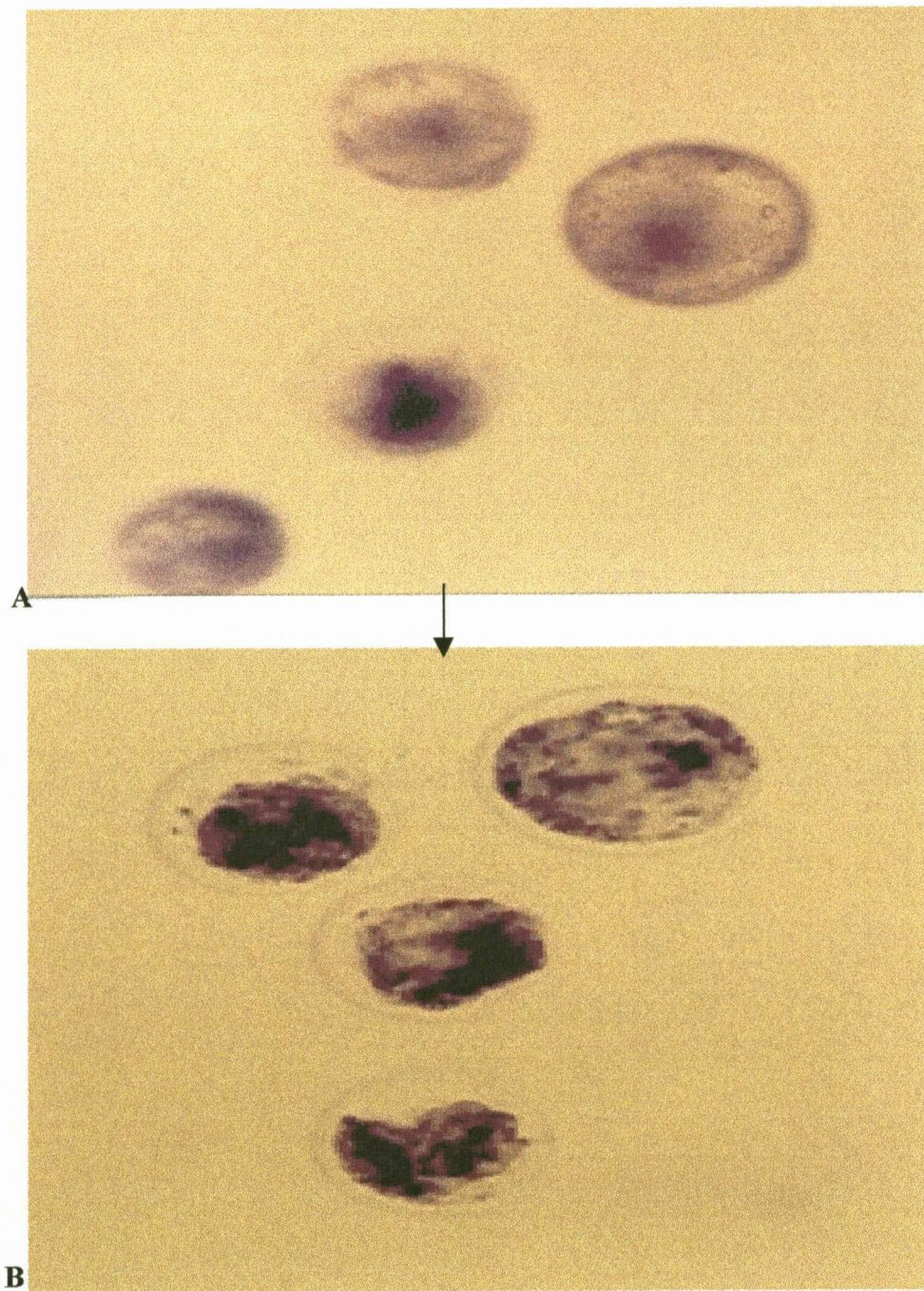


Figure 4.7 (A) Three live blastocysts and one degenerated blastocyst under the light microscope, immediately after thawing in ViGro™ Holdingplus (TMT 2). (B) degenerated embryonic cells 24 hours post-thawing.

CHAPTER 5

DISCUSSION

5.1 Conventional slow freezing of IVP bovine embryos

In the absence of a cryoprotectant, mammalian embryos do not survive slow cooling to temperatures below -20°C , at a rate low enough to allow osmotic dehydration (Schneider & Mazur, 1984). The cryoprotectant used in this study was ethylene glycol (EG), the cryoprotectant first used as a cryoprotective agent by Miyamoto and Ishibashi (1977; 1978) for the freezing of mouse embryos using the conventional slow freezing method. High survival rates were obtained with mouse frozen/thawed embryos from the 8-cell to the mid blastocyst stage, using 1.5M EG compared to other cryoprotectants (Urano *et al.*, 1986). Since then, the majority of *in vivo* produced bovine embryos have been successfully cryopreserved by controlled slow freezing, using the same concentration of ethylene glycol, such as 1.5M EG. Normal offspring have been obtained, although there are still major obstacles in the cryopreservation of *in vitro* produced bovine embryos (Pollard and Leibo, 1994). *In vitro* produced bovine embryos, especially those at an earlier stage of development have been considered to more sensitive to cryopreservation than their *in vivo* counterparts (Hochi *et al.*, 1996). *In vitro*-produced bovine embryos are seriously damaged when cryopreserved by controlled slow freezing techniques (Pollard & Leibo, 1994). The sensitivity of IVP bovine embryos to freezing can be prevented by appropriate rapid cooling techniques (Mahmoudzadeh *et al.*, 1994).

In this study, commercial 1.5M ViGroTMEG Freezeplus was used for slow freezing (TMT 4) and ViGroTMHoldingplus for direct thawing. Evaluation of IVP bovine embryos was done immediately after thawing in ViGroTMHoldingplus, then cultured *in vitro* (5% CO₂ at 39°C) for further evaluation at 24 hours and 48 hours post-thawing. After an initial slow cooling rate of $0.3^{\circ}\text{C}/\text{minute}$ (from -6°C to -30°C), the rate was changed to $0.1^{\circ}\text{C}/\text{minute}$ (from -30°C to -33°C). This is normally performed during controlled freezing, and it was compared with vitrification methods (TMT 1, TMT 2 and TMT 3).

The results obtained clearly indicate a possibility of a beneficial effect of the vitrification method on the survival rate of cryopreserved IVP bovine embryos. The survival rate of IVP bovine embryos was lower for the conventional slow freezing method (TMT 4), compared to the vitrification method using 40% EG + 0.3M trehalose + 20% PVP (TMT 3). Although the survival rate of IVP bovine embryos cryopreserved by TMT 4 (40%) was higher than TMT 1 (26%) and TMT 2 (21%) 24 hours post-thawing, and also higher (TMT 4; 30%) than TMT 1 (12%) and TMT 2 (10%) 48 hours post thawing (Table 4.1-4.8), TMT 3 was the method that gave the highest survival rate following thawing (77%), 24 hours post-thawing (60%) and 48 hours post-thawing (37%). There was no statistical differences between the conventional slow freezing method (TMT 4) and the vitrification methods (TMT 1 vs. TMT 4, TMT 2 vs. TMT 4 & TMT 3 vs. TMT 4) from immediately after thawing to 48 hours post-thawing. TMT 4 (slow freezing method) was the next best method in terms of the survival rate of IVP bovine embryos.

Mahmoudzadeh *et al.* (1994) reported that the low survival rate of the embryos after controlled-rate freezing was not due to the freezing protocol, but due to the higher sensitivity of the *in vitro* produced embryos to slow cooling. The survival rate differed between 0% and 46% for embryos at the morula stage (Pollard & Leibo, 1994; Leibo & Loskutoff, 1993) and between 43% and 80% for embryos at the blastocyst stage (Mahmoudzadeh *et al.*, 1994; Pollard & Leibo, 1994; Takagi *et al.*, 1994a; Iwasaki *et al.*, 1994; Zhang *et al.*, 1993). Mahmoudzadeh *et al.* (1994) suggested that the extreme sensitivity of *in vitro* produced embryos to freezing injury could possibly be prevented by a adequate rate of cooling.

Hochi *et al.* (1996) reported that the use of ethylene glycol (EG) as a cryoprotective additive allows for direct transfer without dilution after warming. It was also reported that embryos frozen in EG seem to exhibit a higher survival rate when the embryos are warmed in a water bath compared to those warmed in air, even though embryos have been cooled as slowly as 0.3 °C/minute (Massip *et al.*, 1995b). Voelkel and Hu (1992a) recorded a higher rate of viability of embryos frozen in EG and rehydrated directly in a culture medium, compared to embryos frozen in other cryoprotectants. It was indicated

that EG can readily diffuse out of embryonic cell, without causing gross cellular damage. When bovine embryos are placed into 1.5M EG prior to freezing and thawed embryos are transferred from EG solution into culture medium, little osmotic response is observed. This reflects the high degree of permeability of the embryo to the cryoprotectant.

5.2 Vitrification of IVP bovine embryos

In this study, vitrification of IVP bovine embryos was performed in a simple way, with a single type of cryoprotectant. The cryoprotectant used was ethylene glycol (EG), which has the advantage (compared to other cryoprotectants) of having a higher permeability and lower toxicity to embryos due to its lower molecular weight (Saha *et al.*, 1996a; Kobayashi *et al.*, 1993). Sufficient permeation of EG into embryos during vitrification takes places quicker than that of other cryoprotectants. Removal of EG from embryos during thawing is faster than other cryoprotectants, resulting in a decrease in time of exposure to the cryoprotectant, which may lead to decreased embryo toxicity (Mahmoudzadeh *et al.*, 1993).

Trehalose was chosen as a non-permeating carbohydrate/agent because of its reported improved effectiveness (Sampedro *et al.*, 1998; Saha *et al.*, 1996b; Smorag *et al.*, 1990; Robertson *et al.*, 1989; Krag *et al.*, 1985). The concentration of trehalose used, was 0.3M (11.3%), a concentration reported to yield better results compared to other concentrations (Saha *et al.*, 1996a). It has also been reported that the presence of trehalose in the cryoprotectant solution prevents osmotic shock due to diffusion of the cryoprotectant out of the embryo after thawing. Its presence in the cryoprotectant solutions restricts water movement across the membranes, preventing lysis of embryonic cells during diffusion of the cryoprotectant out of the embryo (Shaw *et al.*, 1997; Saha *et al.*, 1996b). In this study, the presence of trehalose, and 40% EG in ViGroTMHoldingplus (TMT 2) decreased the survival rates of IVP bovine embryos. This may be due to the presence of sucrose in the ViGroTMHoldingplus media. *In vitro* produced bovine embryos frozen in 40% EG (TMT 1) and thawed in ViGroTMHoldingplus had higher survival rates than those frozen in 40%

EG + 0.3M trehalose (TMT 2). Perhaps the addition of trehalose in a solution (ViGro™ Holdingplus) already having a non-permeating agent (sucrose), creates a detrimental/toxic effect on IVP bovine embryos. Suzuki *et al.* (1995) suggested that it may be necessary to include a low concentration of sugar in the holding medium to protect the embryos from osmotic shock.

The addition of 0.3M trehalose and 20% PVP with 40% EG (TMT 3) in ViGro™ Holdingplus, increased the survival rates of IVP bovine embryos. However, the addition of 0.3M trehalose alone with 40% EG (TMT 2) in ViGro™ Holdingplus, produced a lower survival rate. In this study, it was observed that immediately after thawing, 24 hours post-thawing and 48 hours post-thawing, embryos frozen in 40% EG + 0.3M trehalose in ViGro™ Holdingplus (TMT 2) had the lowest survival rates, compared to other treatment groups. This may be caused by the presence of sucrose in ViGro™ Holdingplus. Sucrose is a non-permeating agent, like trehalose. It may be that the presence of sucrose may have increased the concentration of non-permeating agent (sugar) in the solutions. High non-permeating agent concentrations may be detrimental/toxic to embryos. Saha *et al.* (1996a) reported an increase in the survival rate of embryos cryopreserved with 40% EG + 0.3M trehalose in mPBS and 0.3% BSA. The exclusion of mPBS and BSA in this study, may have decreased the survival rate of the embryos vitrified by 40% EG + 0.3M trehalose (TMT 2) in ViGro™ Holdingplus.

Smorag *et al.* (1990) reported a significant ($P < 0.01$) decrease in the viability of one-cell and two-cell rabbit embryos when exposed to high concentrations of sucrose (2.5M sucrose) or when exposed to 1.45M trehalose. These embryos did not maintain their ability to develop further, nor do they return to their original physiological shape and volume. This might be the consequence of irreversible changes in the permeability of the cell membrane.

Dobrinsky *et al.* (1992) recorded a significant beneficial effect of sucrose concentration ($P < 0.01$) on the proportion of embryos developing to the blastocyst stage (0.1M=56%; 0.3M=54%; 1.0M=28%). Embryos not exposed to sucrose, only developed to blastocysts

(68%). It was also found that 1-step dilution with 1.0M sucrose is detrimental, if embryos are exposed to vitrification media. Embryo survival rate after vitrification, was improved by decreasing the concentration of sucrose to 0.3M and the time of exposure to 10 minutes. Sampedro *et al.* (1998) reported that non-permeating carbohydrates or agents protect cytosolic proteins against adverse conditions such as cold, desiccation and heat. Among the carbohydrates, trehalose was found to be the most effective protective agent.

The macromolecule polyvinyl pyrrolidone (PVP), along with EG (cryoprotectant) and trehalose (sugar) in ViGro™ Holdingplus were also used in this study. The addition of 20% PVP with 40% EG and 0.3M trehalose dramatically increased the survival rates of IVP bovine embryos. The PVP used in this study has an average molecular weight of 40 000mw, compared to the average of 30 000 mw, used by Saha *et al.* (1996b). It has been reported that PVP is a large, interface-seeking molecule. It has also been suggested that PVP coats the cells immediately after thawing, providing a protection mechanism against osmotic stress. Although the mechanism of protection by large polymer PVP is not yet known, it may prevent osmotic injury in embryos during thawing or protect the embryo membranes from denaturation. The concentration of PVP used was 20%, this concentration (20% PVP) was found to give better embryo survival rate, when used with 40% EG, 0.3M trehalose and 0.3% BSA in mPBS (Saha, 1996). It has also been shown that the inclusions of macromolecule (PVP) in solutions facilitate vitrification by increasing the tendency of the solutions to supercool (Fahy *et al.*, 1984). The presence of a high concentration of endogenous macromolecules in the dehydrated cytoplasm of embryos ought to facilitate intracellular glass (Rall, 1987). The large polymer PVP (macromolecules) may also prevent devitrification during thawing (Kasai *et al.*, 1990).

The presence of trehalose (non-permeating agent), may reduce the toxicity that is associated with the PVP and EG, by causing the embryo to shrink rapidly. This reduces the amount of cryoprotectant (EG) in the cells (Kasai *et al.*, 1990). When the embryos are exposed to the cryoprotectant plus non-permeating agent solution, only the cryoprotectant itself permeates the cell. The extra osmolarity created by the non-permeating agent cause dehydration, which reduces the formation of intracellular ice (Szell & Shelton, 1986).

The presence of a non-permeating agent in the medium of 40% EG + 0.3M trehalose + 20% PVP restricts water movement across the membranes, thus preventing cell lysis during diffusion of the cryoprotectant out of the embryo. Non-permeating agents are also involved in active ion transport through the trophoctoderm. The active transport is controlled by the Na⁺ K⁺ ATP-ase system, which is inhibited by some cryoprotectants (Saha *et al.*, 1996a)

Vitrification of embryos was performed in three types of vitrification solutions, namely 40% EG (TMT 1), 40% EG + 0.3M trehalose (TMT 2) and 40% EG + 0.3M trehalose + 20% PVP (TMT 3) in ViGroTM Holdingplus. The fourth treatment group was the conventional slow freezing solution (1.5M ViGroTM EG Freezeplus, TMT 4). Thawing was done directly in ViGroTM Holdingplus. Embryos vitrified in 40% EG + 0.3M trehalose + 20% PVP (TMT 3) exhibited higher survival rates (Table 4.1-4.9). Saha (1996) recorded a survival rate of 52.9% for embryos frozen in 40% EG; 75% for embryos frozen in 40% EG + 0.3M trehalose and 84% for embryos frozen in 40% EG + 0.3M trehalose + 20% PVP in mPBS. Vajta *et al.* (1996) recorded survival rates of 79% following vitrification in 12.5% EG and 12.5% DMSO in TCM-Hepes and 7.5% FCS. The vitrification methods used in this study differed slightly from those previously described by Saha *et al.* (1996b). In this study ViGroTM Holdingplus was used in place of mPBS and BSA. It also included fewer media changes, but the basic cryoprotectant was the same. Embryos were directly thawed in ViGroTM Holdingplus and cultured *in vitro* for further morphological evaluation at 24 hours and 48 hours post-thawing. The purpose was to further evaluate the development rate of IVP bovine embryos cultured in the incubator (humidity, air, 5% CO₂ at 39 °C).

The predicted percentage survival of IVP bovine embryos achieved by this experimental study were 53% (TMT 1); 41% (TMT 2); 77% (TMT 3) immediately after thawing (Table 4.4 to 4.9). The predicted survival rates were highest in embryos vitrified in 40% EG + 0.3M trehalose +20% PVP (TMT 3). The actual survival rate of embryos vitrified in TMT 3, was significantly (P<0.05) higher compared to TMT 2 immediately after thawing. The actual survival rate in TMT 3 (60%) was significantly (P<0.05) higher

compared to TMT 1 (26%) and significantly higher compared to TMT 2 (21%) during 24 hours post thawing. They were no significant differences between the treatments at 48 hours post-thawing (Table 4.9). However, TMT 3 (37%) gave the highest actual survival rates compared to the other three treatment groups (TMT 1; TMT 2; TMT 4). This study found TMT 3 to be the most effective method of cryopreservation for IVP bovine embryos. These results are in accordance with those of Saha *et al.* (1996a), who recorded high survival (84.1%) and hatching rates (68.2%) for embryos cryopreserved with 40% EG + 0.3M trehalose + 20% PVP in mPBS and 0.3% BSA.

Ishimori *et al.* (1993) recorded a 20%, 73% and 85% development rate 24h post-thawing, using day 7 *in vivo* bovine blastocysts that were vitrified after exposure in mixture of EG + DMSO in PBS. Yang *et al.* (1992) vitrified day 7 bovine blastocysts using EG/propylene glycol in combination with glycerol. 90.4% to 97% of the blastocysts survived thawing. Hatching rates ranging from 51% to 80% were also recorded. In this study, very few hatched blastocysts were recorded, but hatching depends on the quality of embryos frozen (Gordon, 1994).

5.3 Consequences of embryo cryopreservation and thawing

During the initial pre-freeze phase, the embryos are exposed and equilibrated to a cryoprotectant. Embryos exposed to permeating cryoprotectants, shrink by losing water until the equilibrium stage is reached. Fahning and Garcia (1992) reported shrinkage is to be due to the initial hyperosmoticity of the extracellular solution. Embryos are much more permeable to water than to cryoprotectants. Shrinkage is stopped when equilibrium is reached between the efflux of water and the influx of the cryoprotectant. As the additive permeates the embryos, it gradually re-expands because of the re-entry of water to maintain osmotic equilibrium. Leibo (1989) suggested that the rate at which re-expansion occurs reflect the following:

- (i) the specie of the embryo
- (ii) the stage of embryonic development

- (iii) the embryo surface to volume ratio
- (iv) the cryoprotectant itself
- (v) the temperature of exposure

In addition to the choice of cryoprotectant (due to its permeability), and its technique of addition to the blastocysts, the rate at which embryos are cooled and rewarmed (thawed) determines the survival rate. The cryoprotectants function is to drive as much water from the embryos as possible and thus prevent intracellular ice formation, while keeping the cytoplasm supercooled until freezing. Seeding is also done in the slow freezing method in order to induce a phase of change from water to ice that allows an increase in the concentration of salts in a suspending solution (Fahning & Garcia, 1992). Once the ice is formed, the embryos are cooled slowly, allowing them to respond osmotically to concentration changes.

It was observed that embryos initially lose water and shrink immediately after transfer into vitrification solutions, especially the vitrification solution containing PVP (40% EG + 0.3M trehalose + 20% PVP, TMT 3). Initial loss of water and shrinkage of IVP bovine embryos when transferred into the vitrification solutions, was to compensate for the high extracellular osmolarity caused by EG, trehalose, sucrose and PVP. When embryos were exposed to vitrification solutions, only EG permeates the embryo cells. The extra osmolarity created by a non-permeating agent (trehalose and sucrose) causes dehydration, which reduces the occurrence of intracellular ice formation. Embryos re-expand again and return to their original normal shape after equilibrium stage is reached in the solution. Szell and Shelton (1986) observed the same effects when embryos were exposed to glycerol-sucrose solutions. Glycerol permeates the cells, not as fast as the EG. The occurrence of glycerol permeation in glycerol-sucrose solutions is time consuming compared to EG.

The two main causes of cellular death during freezing and thawing are intracellular ice formation and solution effects (Mazur, 1970). Intracellular ice formation occurs when the cells are not sufficiently dehydrated at a given sub-zero temperature. Dehydration by

slow freezing rates or by exposure to a non-permeable solute, protects the cells against intracellular ice formation (Nguyen *et al.*, 1983; Whittingham *et al.*, 1979; Leibo, 1978). The lethal event in a rapidly cooled cell is the growth of intracellular ice crystals, rather than their initial formation. The ice formed in cells as a result of rapid cooling is likely to grow by re-crystallization during thawing, especially if thawing is slow. The damage as a result of slow thawing could not be a manifestation of recrystallization. It occurs when cooling too slowly. Slow cooling is also damaging to slowly cooled nucleated cells under certain conditions (Mazur, 1970).

Thawing of embryos in air, followed by immersion in water produces minimal damage to the zona pellucidae (Shea *et al.*, 1993). The incidence of zona damage to bovine embryos frozen in straws is dependant on the thawing conditions, with no damage observed when thawing is conducted at 20 °C in air. However, thawing in water baths at 20 °C and 36 °C caused zona damage in 17% and 24% of the embryos, respectively (Rall & Meyer, 1989). In this study, there were few incidences of zona pellucida damage (2.94%), especially in the frozen expanded blastocysts. There is a difference between *in vitro* survival rate and *in vivo* development. Although based on morphology, *in vitro* survival rate after thawing may be satisfactory. The pregnancy rate after transfer can be low, e.g. 80% survival rate *in vitro* versus 33% pregnancy rate after transfer to recipients (Massip *et al.*, 1993).

In view of the above mentioned, it is suggested that further studies be launched regarding transfer of embryos to recipient cows in order to verify *in vitro* results obtained in this study.

CHAPTER 6

GENERAL CONCLUSIONS

The best overall embryo survival rate were obtained by the vitrification method (TMT 3), while the conventional slow freezing method (TMT 4) resulted in the second best survival rate. The embryo survival rate in TMT 3 was 77% (immediately after thawing), compared to the other cryopreservation survival rates of 53% (TMT 1); 41% (TMT 2) and 50% (TMT 4). The addition of PVP to the solution with 40% EG and 0.3M trehalose (TMT 3) significantly ($P < 0.05$) increased the survival rate of IVP bovine embryos. It seems as if PVP plays a highly significant role of protecting embryo during freezing and thawing.

Embryos frozen in 40% EG + 0.3M trehalose (TMT 2), gave the lowest survival rate. The addition of trehalose may have been responsible for this decrease in survival rate. Embryos frozen only in 40% EG (TMT 1) in ViGro™ Holdingplus had a slightly higher survival rate, compared to embryos frozen in TMT 2. ViGro™ Holdingplus medium contains sucrose (non-permeating agent). Perhaps the addition of trehalose (non-permeating agent) in a solution already containing sucrose (ViGro™ Holdingplus), which is also a non-permeating agent, increases the concentration of sugar in the solution. This may have led to embryo toxicity during the freezing and thawing procedures. It seems as if higher concentrations of the non-permeating agent during freezing and thawing could stress and damage the embryo.

The survival rate of frozen embryos can be predicted, depending on the type of cryoprotectants and quality of embryos used. This means that survival rate of embryos can be predicted from the total number of embryos frozen. In this study, IVP bovine embryos frozen in 40% EG + 0.3M trehalose + 20% PVP (TMT 3) were predicted to be more likely to survive, compared to other three treatment groups (Table 4.4 to 4.9). The application of this prediction model in cryopreservation has different concepts and

different approaches that have to be taken into account for an accurate prediction of the embryo survival rate. These factors are, the cryoprotectant (EG) used, which can easily permeate the embryos; the quality and stage of the blastocysts, low concentration of non-permeating agent (0.3M) used and inclusion of the macromolecules PVP. The way in which embryos are handled during the cryopreservation process should also be taken into account. It can be a factor effecting embryo survival rate after thawing. All factors mentioned above, if taken into account, may increase the accuracy of the survival rate prediction, derived from various cryopreservation techniques.

Despite the radical cryopreservation procedures used in the vitrification methods and the slow freezing methods, both seek a common goal, the highest possible survival rates of embryos after thawing. This includes the prevention of toxicity and osmotic stress generally associated with the freezing and thawing of embryos. Early developmental stage is critical and good quality embryos have a better chance of surviving the freezing and thawing procedures.

Vitrification could greatly improve cryopreservation in the near future. It could become the accepted method in cryopreservation programmes, especially in IVP bovine embryos. The method of vitrification is simple, reliable and relatively easy to use. Embryos frozen by vitrification can be thawed directly in holding medium and immediately transferred to recipients. It does not require a step-wise removal procedure of the cryoprotectants. This method offers new opportunities in transfer of embryos immediately after thawing, and saves time, compared to the conventional slow freezing method. The most effective vitrification method for long term cryopreservation of IVP bovine embryos will depend on the use of less toxic media, and less harmful steps introduced during the freezing and thawing procedures. These include cryopreservation procedures that would minimize osmotic stress in the re-culturing of the embryos after thawing.

It is concluded that the combination of 40 % EG + 0.3M trehalose + 20% PVP (TMT 3), used as a vitrification solution, be recommended as a suitable method for cryopreservation of IVP bovine embryos. It gave the highest embryo survival rate from

immediately after thawing to 48 hours post-thawing. The advantage of this vitrification technique being that it is simple, quick and inexpensive.

Additional research is needed to develop an effective cryopreservation method that will reduce the sensitivity problem of *in vitro* produced embryos. *In vitro* produced embryos contain lipids that cause them to be more sensitive to the freezing process, compared to those produced *in vivo*. The ability of vitrified *in vitro* produced bovine embryos further needs to be evaluated for their development *in utero*, in controlled embryo transfer programmes.

ABSTRACT

EVALUATION OF CRYOPRESERVATION METHODS FOR IN VITRO PRODUCED BOVINE EMBRYOS

by

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Key words: IVEP, bovine embryos, vitrification, conventional slow freezing, IVM, IVF, PVP, EG, trehalose, survival rate

The objective of this study was to evaluate four cryopreservation techniques for *in vitro* produced bovine embryos, and to select the best method for practical application. The cryopreservation methods investigated were three vitrification methods and a slow freezing method. This study was done at the ARC-Animal Improvement Institute in conjunction with the University of the Orange Free State (Department of Animal Science).

Embryos were obtained by the IVM, IVF and IVC of bovine follicular oocytes. A total of 136 early blastocysts, blastocysts and expanded blastocysts were randomly assigned to four different treatment groups. In the conventional slow freezing method, the IVP bovine embryos were first held in ViGro™ Holdingplus medium before being transferred to 1.5M ViGro™ EG Freezeplus medium (TMT 4). In this technique, the IVP embryos were loaded into 0.25ml straws. The straws containing the embryos were immediately placed into a programmable freezer (CL-863 cryo-chamber) at -6 °C. Straws were seeded

after a 5 minutes equilibration period. Embryos were initially cooled from -6°C to -30°C at a rate of $0.3^{\circ}\text{C}/\text{min}$. Thereafter, from -30°C to -33°C , the rate was changed to $0.1^{\circ}\text{C}/\text{min}$. After the target temperature was reached, straws were immediately transferred to liquid nitrogen.

Vitrification of IVP bovine embryos was performed according to the following procedures: Embryos were initially placed in 10% EG in ViGroTM Holdingplus medium for 5 minutes (Equilibration I), thereafter in 40% EG + 0.3M trehalose in ViGroTM Holdingplus medium for 5 minutes (Equilibration II), both at room temperature. Embryos were then transferred to vitrification solutions, containing 40% EG (TMT 1); 40% EG + 0.3M trehalose (TMT 2); 40% EG + 0.3M trehalose + 20% PVP (TMT 3) in ViGroTM Holdingplus. Embryos were then loaded into 0.25ml straws, and plunged directly into liquid nitrogen (LN_2). The straws were vertically stored in liquid nitrogen (-196°C) until thawing and evaluation took place.

Thawing of embryos within the straws was carried out in a water bath (32°C). Each straw was placed in a water bath for 30 seconds. The straws were dried, cut and the contents transferred to ViGroTM Holdingplus medium. Recovered embryos were washed twice in fresh ViGroTM Holdingplus, and embryos were morphological examined for their viability under a stereo microscope. The viable embryos were cultured in IVC media. Embryo survival was recorded immediately after thawing, 24 hours and 48 hours post-thawing by monitoring the re-expansion of the blastocoel and expansion of the blastocyst.

Statistically, there was a significant ($P < 0.05$) difference in survival rate between embryos frozen in TMT 3 (77%), compared to those frozen in TMT 2 (41%), immediately after thawing. There was no significant difference in embryo survival rate for the other treatment groups. At 24 hours post-thawing, there was a significant ($P < 0.05$) difference in survival rate between embryos frozen in TMT 3 (60%), compared to those frozen in TMT 1 (26%). There was also a significant ($P < 0.05$) higher survival rate for embryos frozen in TMT 3 (60%), compared to those frozen in TMT 2 (21%). At 48 hours post-thawing, however, there was no significant difference in survival rate for embryos frozen

in all the treatment groups. TMT 3 had the highest survival rates of embryos (37%). The generalized linear model (Bonferroni multiple comparison test) was used to test and predict the embryo survival rate between the treatment groups. The predicted (theoretical) embryo survival rate correlated highly and significantly ($P < 0.05$) higher with the survival rate of embryos frozen in TMT 3. Embryos frozen in TMT 3 were also predicted to be more likely to survive, compared to the other treatment groups. The results clearly indicate the beneficiary effect of this vitrification method (TMT 3). Vitrification is simple and more cost effective, compared to the slow freezing method (TMT 4), which is time consuming and expensive. Although there was no significant difference 48 hours post-thawing, TMT 3 could be recommended as the method for cryopreservation of IVP bovine embryos.

The addition of 0.3M trehalose with 40% EG in the ViGro™ Holdingplus medium decreased the survival rates of the IVP bovine embryos. Embryos frozen and thawed in 40% EG in ViGro™ Holdingplus had higher survival rates, compared to those frozen/thawed in TMT 2, from immediately after thawing, to 48 hours post-thawing. Perhaps the addition of trehalose in the solution (ViGro™ Holdingplus), already containing non-permeating agent (sucrose), increased the concentration of non-permeating agent in the freezing solution. High concentrations of non-permeating agent may be detrimental or toxic to the embryos.

The presence of 20% PVP with 0.3M trehalose and 40% EG dramatically increased the survival rate of IVP bovine embryos. The PVP plays some kind of protective role during the freezing and thawing processes. Although the mechanism of protection is not clear, it may be that it prevents water from entering the cells during vitrification and thawing, which in turn prevents intracellular ice formation. Intracellular ice formation is lethal to embryos during thawing.

It can be concluded that the combination of 40 % EG + 0.3M trehalose + 20% PVP (TMT 3), used as a vitrification solution, be recommended as suitable method for cryopreservation of IVP bovine embryos. It gave the highest embryo survival rate from

immediately after thawing to 48 hours post-thawing. The advantage of this vitrification technique is that it is simple, quick and inexpensive.

Additional research is needed to develop an effective cryopreservation method that will reduce the sensitivity problem of *in vitro* produced embryos. *In vitro* produced embryos contain lipids that cause them to be more sensitive to freezing, compared to those produced *in vivo*. The ability of vitrified *in vitro* produced bovine embryos still needs to be evaluated for their development *in utero*, in controlled embryo transfer programs.

OPSOMMING

DIE EVALUASIE VAN KRIOPRESERVERINGSTEGNIEKE VIR *IN VITRO* GEPRODUSEERDE BEESEMBRIOS

deur

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Sleutel Woorde: IVEP, beeseembrios, vitrifikasie, konvensionele stadige bevriesing, IVM, IVF, PVP, EG, trehalose, oorlewingstempo

Die doel van die studie was om vier kriopreserverings tegnieke vir *in vitro* geproduseerde beeseembrio's te evalueer en die beste metode te selekteer vir praktiese toepassing. Die kriopreserveringsmetodes ondersoek, het bestaan drie vitrifikasiemetodes en 'n stadige stapsgewyse metode. Die studie is uitgevoer by die LNR Diereverbeteringsinstituut in samewerking met die Universiteit van die Oranje Vrystaat (Departement Veekunde).

Embrios is verkry deur IVM, IVB en IVK van follikulêre beesoosiete. 'n Totaal van 136 vroeë blastosiste, blastosiste en laat blastosiste was ewekansig toegedeel tot die vier behandelingsgroepe. In die konvensionele stapsgewyse bevriesingsmetode is die embrios in 'n Vigro TM houmedium geplaas en daarna in 1.5 M Vigro TM EG freezeplus medium oorgeplaas – behandeling 4 (TMT4). Met die tegniek is die embrios in 0.25 ml strooitjies gelaai en direk in 'n programmeerbare bevriesingsapparaat (CL 863 cryochamber) by 'n

temperatuur van -6°C . geplaas Na 'n ekwilibrasieperiode van 5 minute is die strooitjies geïnduseer vir vinnige ysvorming. Embrios is inisieel afgekoel teen 'n tempo van $0.3^{\circ}\text{C}/\text{minuut}$ tot 'n temperatuur van -30°C , daarna van -30°C tot en met -33°C teen 'n tempo van $0.1^{\circ}\text{C}/\text{minuut}$. Nadat die teikentemperatuur bereik is, is die strooitjies direk in vloeibare stikstof gedompel. Strooitjies is vertikaal gestoor in vloeibare stikstof (-196°C) tot en met ontdooiing en evaluasie.

Vitrifasie van embrios is gedoen volgens die volgende prosedures. Embrios is aanvanklik geplaas in 'n 10% EG in Vigro™ houmedium vir 'n periode van 5 minute (ekwilibrasie I), daarna in 40% EG + 0.3 M trehalose in Vigro™ houmedium vir 5 minute (ekwilibrasie II). Beide stappe is by kamertemperatuur uitgevoer. Embrios is daarna oorgeplaas na vitrifikasie oplossings bevattende 40% EG (TMT1); 40% EG + 0.3 M trehalose (TMT2); 40% EG + 0.3 M trehalose + 20% PVP (TMT3) in ViGro™ Houmedium. Embrios is gelaai in 0.25 strooitjies en direk in vloeibare stikstof geplaas. Embrios is vertikaal in vloeibare stikstof (-196°C) geberg, totdat hul ontdooi en geëvalueer is.

Ontdooiing van embrios is uitgevoer in 'n waterbad (32°C). Elke strooitjie is in die bad water geplaas vir 30 sekondes waarna dit afgedroog, geknip en die inhoud oorgeplaas is na 2 ml ViGro™ Houmedium. Herwonne embrios is twee maal gewas in vars ViGro™ Houmedium, waarna hul morfologies ondersoek is vir lewensvatbaarheid onder 'n stereomikroskoop. Die lewendige embrios is gekweek in IVK media en die embrio oorlewings tempo is vasgestel net na ontdooiing, 24 uur en 48 uur na ontdooiing deur die monitering van die her-uitsetting van die blastosol en uitsetting van die blastosist.

Statisties was daar 'n betekenisvolle ($P < 0.05$) verskil, (onmiddellik na ontdooiing), in oorlewings tussen embrios gevries in TMT3 (77%), vergeleke met die gevries in TMT2 (41%). Geen betekenisvolle verskil in oorlewings is verkry tussen die ander groepe nie. By 24 uur na ontdooiing, was daar 'n betekenisvolle ($P < 0.05$) verskil in oorlewings tempo tussen embrios in TMT3 (60%) en TMT 1 (26%). Daar was ook 'n betekenisvolle ($P < 0.05$) hoër oorlewings tempo vir embrios na TMT3 behandeling (60%), vergeleke met

daardie gevries in TMT2 (21%). By 48 uur na ontdooiing is daar egter geen betekenisvolle verskil in oorlewingstempo tussen die behandelingsgroepe gekry nie. TMT3 het die hoogste oorlewing vir die IVP beesembrios getoon (37%).

Die liniêre model (Bonferroni meervoudige vergelykingstoets) is gebruik om die oorlewing tussen behandelingsgroepe te evalueer en te voorspel. Die voorspelde (teoretiese) embryo oorlewingstempo het 'n hoë oorlewing vir embrios in TMT3 getoon. Embrios gevries in TMT3 behandeling, is ook voorspel om meer waarskynlik te oorleef, vergeleke met die ander behandelingsgroepe. Resultate toon 'n definitiewe voordeel ten opsigte van hierdie vitrifikasietegniek (TMT3). Vitrifikasie is 'n eenvoudige tegniek en nie so duur, vergeleke met die stapsgewyse bevringsmetode (TMT4), wat tydrowend en duur is. Alhoewel daar geen betekenisvolle verskil na 48 uur na-ontdooiing was nie, kan TMT3 aanbeveel word as die beste metode vir die kriebewaring van IVP beesembrios. Die byvoeging van 0.3M trehalose met 40% EG in die ViGro™ Houmedium medium veroorsaak 'n afname in die oorlewing van IVP beesembrios. Embrios gevries en ontdooi in 40% EG in ViGro™ Houmedium, het hoër oorlewing getoon, vergeleke met embrios gevries in TMT2, onmiddellik na ontdooiing tot 48 uur na ontdooiing. Waarskynlik het die byvoeging van trehalose in die oplossing, wat reeds 'n nie-deurlaatbare middel (sukrose) bevat, die konsentrasie van nie-deurlaatbare middels in die bevringsmedium verhoog. Hoë konsentrasies nie-deurlaatbare middels kan nadelig of toksies wees vir die embrios.

Die teenwoordigheid van 20% PVP met 0,3M trehalose en 40% EG het die oorlewing van die IVP beesembrios dramaties verbeter. PVP speel 'n beskermende rol tydens bevrings en ontdooiing. Alhoewel die meganisme van beskerming nie duidelik is nie, mag dit wees dat dit die invloed van water in die sel verhoed tydens vitrifikasie en ontdooiing. So doendeword die vorming van intrasellulêre ys verhoed. Intrasellulêre ysvorming is fataal vir die oorlewing tot gevolg gehad van embrios tydens ontdooiing.

Samevattend kan gesê word dat 40% EG + 0.3 M trehalose + 20% PVP (TMT 3) die aanbevole metode van kriebewaring van IVP beesembrios is. Dit het die hoogste

oorlewing gegee van ontdooiing tot 48 uur na ontdooiing. Die voordeel van hierdie tegniek vitrifikasie is dat dit eenvoudig, vinnig en goedkoop is.

Addisionele navorsing is egter nog nodig om 'n effektiewe kriopreserveringstegniek te ontwikkel wat die sensitiwiteit van *in vitro* geproduseerde embrios sal verlaag. *In vitro* geproduseerde embrios bevat meer lipiede as *in vivo* embrios wat 'n verhoogde sensitiwiteit vir bevriesing veroorsaak. Die vermoë van gevitrifiseerde *in vitro* embrios om *in utero* te ontwikkel moet nog in gekontroleerde embrio-oorplasings programme bepaal word.

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