Inaugural-Dissertation zur Erlangung der Doktorwürde
der Tierärztlichen Fakultät der Ludwig-Maximilians-Universität
München

Comparison between two vitrification methods for in vitro produced bovine embryos with an intact or a non-intact zona pellucida

von Manuel Nicolás Saucedo

aus Posadas, Argentinien

München 2016

Aus dem Veterinärwissenschaftlichen Department der Tierärztlichen
Fakultät der Ludwig-Maximilians-Universität München

Lehrstuhl für Molekulare Tierzucht und Biotechnologie

Arbeit angefertigt unter der Leitung von Univ.-Prof. Dr. E. Wolf

Gedruckt mit Genehmigung der Tierärztlichen Fakultät der
Ludwig-Maximilians-Universität München

Dekan: Univ.-Prof. Dr. Joachim Braun

Berichterstatter: Univ.-Prof. Dr. Eckhard Wolf

Korreferent: Univ.-Prof. Dr. Joachim Braun

Tag der Promotion: 06. Februar 2016

Meinen Eltern

TABLE OF CONTENTS

I. **INTRODUCTION** **1**

II. **REVIEW OF LITERATURE** **4**

1. **Vitrification of bovine embryos** **4**

1.1. Historical progress 4

1.2 Advantages of cryopreservation by vitrification for IVP embryos 7

1.3 Embryo quality and its parameters 8

1.3.1 Factors influencing embryo quality 10

1.3.1.1 In vitro maturation 10

1.3.1.2 In vitro culture 13

1.3.1.3 Lipid content 15

1.3.1.4 Superstimulatory treatments and their effect on follicular development 17

2. **Genetic evaluation bovine of embryos** **19**

2.1. Embryo biopsy and pre-implantation genetic diagnosis (PGD): background and advantages 19

3. **Vitrification of embryos** **22**

3.1. Principles of vitrification 25

3.2. Volume of the sample 27

3.3. Cooling/warming rates 28

3.4. Permeable and non-permeable cryoprotective agents 29

3.5. Damage caused by vitrification 31

4. **Conventional freezing of bovine embryos** **33**

4.1. Principles of conventional freezing 33

4.2. Freezing stages 33

4.3. Damage caused by slow freezing 34

III. **MATERIALS AND METHODS** **37**

1. **Materials** **37**

1.1. Apparatuses 37

1.2. Software 37

1.3. Consumables 37

1.4. Chemicals 38

1.5. Hormones 39

1.6. Media and solutions for IVP of bovine embryos 39
1.7. Vitrification media and solutions for bovine embryos..............................41

2. Methods 44
2.1. Experimental design 44
2.2. IVP of bovine embryos 45
2.2.1. COCs recovering from slaughterhouse derived ovaries............................45
2.2.2. In vitro fertilization 45
2.2.3. In vitro culture 46
2.3. Embryo evaluation 46
2.4. Vitrification of bovine IVP embryos...48
2.4.1. HFV method: vitrification and thawing of embryos 49
2.4.2. CVM method: Vitrification and thawing of embryos 53
2.4.3. Conventional freezing of bovine embryos 57
2.5. Biopsy of IVP embryos 59
2.6. Live-dead cell staining 60

3. Statistical analysis 62

IV. RESULTS..63

1. In vitro production of bovine embryos...63

2. Vitrification of IVP embryos 64
2.1. Overall results of vitrification 65

3. Conventional freezing of IVP embryos 78

3.1. Experiment 1..78

3.2. Experiment 2..78

3.3. Experiment 3..79

V. DISCUSSION 84

1. MVC concept and embryonic intrinsic factors determining vitrification outcomes...84

2. Splitting and biopsy of IVP embryos..89

VI. SUMMARY...93

VII. ZUSAMMENFASSUNG 95

VIII. APPENDIX 98

IX. REFERENCES 100

X. ACKNOWLEDGMENTS 129

LIST OF ABBREVIATIONS

AI	artificial insemination
BSA	bovine serum albumin
COC	cumulus oocyte complex
CVM	cryologic vitrification method
CPA	cryoprotective agent
DMSO	dimethyl sulfoxide
EG	ethylene glycol
GLY	glycerol
FSH	follicle-stimulating hormone
FCS	fetal calf serum
h	hours
hpi	hours post insemination
HFV	hollow fiber vitrification
ICM	inner cell mass
IVM	in vitro maturation
IVF	in vitro fertilization
IVC	in vitro culture
IVP	in vitro production
LH	luteinizing hormone
mg	milligram
µg	microgram
ml	milliliter
µl	microliter

M	molar
MVC	minimum volume cooling
OCS	estrous cow serum
PBS	phosphate-buffered saline
PVP	polyvinyl pyrrolidone
PVA	polyvinyl alcohol
SD	standard deviation
S.E.M.	standard error of the mean
SOF	synthetic oviductal fluid
TE	trophectoderm
ZP	*zona pellucida*

I. INTRODUCTION

Cryopreservation of embryos and the transfer of this technique to large animals might ease dissemination of animals of valuable genetic background (Whittingham 1971). Since the last century, a lot of progress has been made regarding animal breeding, but it was only in the last years that the use of genomic information in genetic evaluation was brought about revolutionary change in cattle selection programs. Cryopreservation of mammalian oocytes and embryos is a critical step in maximizing the efficiency in any breeding program so it plays an important role for the establishment of a breeding strategy involving reproductive biotechnologies.

To perform the genomic evaluation of bovine embryos, the zona pellucida (ZP) has to be opened in order to have access to the embryo proper. The ZP is a protein layer with pores on its surface, which acts as a barrier between the surrounding medium and the embryo as such. The disruption of its integrity comes with two different potential problems: on the one hand, once the ZP is opened, the embryo could come in contact with pathogens, and diseases might spread worldwide. Many researchers have been discussing ZP's role as a biological barrier against pathogens affecting the embryo proper and as a pathogen carrier as well. Van Soom, Wrathall et al. (2009) reviewed some of the factors to keep in mind a few years ago.

On the other hand, as stated above, with regard to cryopreservation this barrier-like layer acts as a filter, between the embryo proper and the medium in which it is handled, thus in a way regulating the passage of the cryoprotective agent (CPA) and therefore the time the embryo is exposed to it.

Since the availability of recipients at any given time is a limitation in the field, it was necessary to develop cryopreservation methods in order to preserve both in vivo-derived and in vitro-produced (IVP) embryos. Currently, there are two globally adopted embryo cryopreservation methods: conventional freezing methods (Whittingham 1971, Whittingham, Leibo et al. 1972) and vitrification (Rall and Fahy 1985). Basically, the conventional freezing methods are referred to as an equilibrium method, and vitrification is considered a non-equilibrium method. Briefly, when performing conventional freezing, controlled cooling takes

place and embryos are slowly dehydrated when exposed to solutions containing over 1 M solute concentration with a CPA. On the other hand, when performing vitrification embryos are exposed to high concentrations of permeable and non-permeable CPAs contained in a small volume of the sample. Afterwards, they are exposed to ultra-rapid cooling and warming rates, therefore avoiding chilling injuries (Vajta, Holm et al. 1998), in order to accomplish a glassy state (Mazur 1984, Leibo 1989, Mazur 1990, Leibo and Pool 2011). It might be stated that, for in vivo-derived embryos, conventional freezing with a programmable freezer and ethylene glycol (EG) as CPA is the most widely adopted cryopreservation method (Vajta 2000) when working with in vivo-derived embryos. This allows, after thawing the embryo being rehydrated within the straw, for a direct transfer into recipients (Voelkel and Hu 1992). On the other hand, for IVP embryos, vitrification has demonstrated to be a promising method (Massip, Van Der Zwalmen et al. 1987, Vajta, Holm et al. 1998). The fact that vitrification is less time consuming than the traditional conventional freezing method makes vitrification an interesting alternative for practitioners in the field. Besides, embryo biopsy gives animal breeders the possibility to perform preimplantation diagnosis regarding some characteristics of economic importance e.g. hereditary diseases and gender determination. Embryo cryopreservation by vitrification has been studied by many research groups worldwide and it is well-known that different factors can affect vitrification outcomes e.g. source of the oocyte, cattle breed, in vitro embryo production, embryo quality, embryo stage, and vitrification method among others.

Embryos from elite donors are highly desirable for breeders and play an important role regarding genetic improvement. However, inbreeding of certain high genetic merit cows may lead to an increase of autosomal recessive genes within a population. This fact might have long-term consequences i.e. introducing inherited diseases. That is one of the reasons why preimplantation genetic diagnosis of embryos has become a relatively recent indication in ET programs. Gender determination by detecting the male Y-chromosome at the preimplantation stage may be by far one of the most important traits, particularly in the dairy industry, where heifers are highly desirable as replacements of culling cows. Moreover, polled animals represent an alternative facilitating the breeder’s

daily work, particularly in dairy barns so that preimplantation genetic diagnosis can be used to select the polled merit as well.

By embryo splitting, demi-embryos are generated. One of them is biopsied in order to obtain enough material that can be used for genetic evaluation. Moreover, this is an attractive alternative for practitioners in the field since biopsied embryos can be cryopreserved and, later on transferred into recipients, while the embryo biopsy is used for genomic evaluation in the laboratory. However, there is not always a laboratory available that is able to process the sample and cryopreservation of the biopsied embryos becomes a crucial factor. There is evidence from several studies that cryopreservation of embryos and opening of the ZP affects embryo viability e.g. that the rate of dead cells is higher in biopsied embryos and that IVP embryos are more sensitive to chilling injuries than their in vivo counterparts. In any case, IVP embryos do represent an alternative (Bousquet, Twagiramungu et al. 1999) . Not just from the scientific point of view but also from the economic perspective. The gap between generations might be shortened and embryos from prepubertal heifers could be produced in vitro giving breeders the possibility to achieve pregnancies in the early life of their high genetic merit animals.

In order to determine whether the opening of the ZP has an influence on vitrification outcomes, bovine IVP embryos, either with intact or opened ZP, were subjected to cryopreservation in this study. Moreover, two different cryopreservation methods for in vitro produced embryos were evaluated. To evaluate the feasibility of these cryopreservation methods following embryo biopsy without affecting their developmental capacity as well as the outcomes regarding survival after thawing of biopsied embryos, the present study aimed to evaluate two different vitrification methods.

II. REVIEW OF LITERATURE

1. Vitrification of bovine embryos

1.1. Historical progress

It has been more than a century since vitrification was described by the German physicist Tammann (1898) for the first time. Back then, he discovered that when a liquid is cooled very rapidly, crystallization can be suppressed by achieving an amorphous “vitreous” state. In the late 1930s, with the focus on organic colloids, Luyet (1937) showed how a liquid can achieve a glassy state under extremely low temperatures, by modifying its physical properties in order to preserve cells and avoid damage caused by crystallization.

In the middle of the last century a few milestone events related to bovine embryo transfer were achieved: the first successful embryo transfer in cattle was reported in the early 1950’s by Jim Rowson in Cambridge, England; in 1951, Willett et al. in Wisconsin, USA reported the birth of a calf after oocyte fertilization and transfer [cited in (Hasler 2014)]; and two decades later Wilmut and Rowson reported the birth of Frosty II (1973). Despite all this progress, it was not until the late 1970s, with the non-surgical embryo transfer technique, that commercial bovine embryo transfer gained popularity by breeders in the USA and worldwide.

In the early 1960s, Mazur’s publication emphasized that intracellular ice crystal formation is reduced during cryopreservation if slow cooling is applied. This was a key factor determining cell survival (Mazur 1963). He pointed out that the temperature zone between -10°C and -60°C was critical for the cells and that they normally suffer cryoinjuries under these conditions (1984). A decade later, Whittingham et al. (1972) reported the survival of mice embryos after slow freezing and thawing. The temperature ranges between 25°C and 0°C, i.e. non-physiological temperatures but above 0º C, is known as the “dangerous zone”. Gametes might suffer chilling injuries in this range (Vajta, Holm et al. 1998). Since then, great efforts have been made to develop a cryopreservation method that avoids chilling injuries and intracellular ice crystal formation. Almost five decades after Luyet came up with the idea, Rall and Fahy described vitrification as a cryopreservation method when working with mouse embryos. Their

experiment was based on a combination of a high concentration of CPA and very high cooling rates. It was possible to achieve a glassy state of the solution without any ice crystal formation, i.e. vitrification was accomplished. The same year (1985), the successful cryopreservation of mouse embryos by vitrification was published by the same authors. Since then, a lot of work has been done by researchers, and cryopreservation of mammalian embryos and oocytes by vitrification was successfully achieved in many species. Massip et al. (1987) described vitrification as the solidification of a liquid achieved by an extreme elevation in viscosity during cooling so that the solution is said to turn into "glass". They reported the birth of two female calves upon transfer of vitrified-thawed embryos. This was the first time that vitrification of cattle embryos followed by an embryo transfer and normal offspring of calves were accomplished.

Over the last two decades, several vitrification methods were described by different authors (see Table 1), but it was not until the definition of the minimum volume cooling (MVC) concept (Hamawaki, Kuwayama et al. 1999) that vitrification became popular among research groups. This approach was based on the small volume of the sample, which led to ultra-rapid freezing, i.e. extremely high cooling and warming rates. The development of different vitrification devices made it possible to achieve cooling rates above 20000 °C/min (Vajta, Holm et al. 1998). These approaches have shown the benefits of vitrification regarding fundamental aspects of cryopreservation: reducing chilling injuries and decreasing the necessity of high CPA concentrations in order to minimize the toxicity of CPAs (Kuwayama 2007, Saragusty and Arav 2011).

The 2013 International Embryo Transfer Society (IETS) Statistics of Embryo Collection and Transfer in Domestic Farm Animals published in December 2014 reported 54622 dairy and 51283 beef cattle collections worldwide. The tally of in vivo-derived transferrable embryos collected was over 729246. The number of IVP bovine embryos globally produced, exceeded the half-million mark (546628 IVP bovine embryos) showing an increase of around 20% since the last report in 2012, with Brazil at the forefront. Brazil stands out, being responsible for 70.9% of IVP OPU bovine embryos worldwide. Another topic that needs to be highlighted is that the number of frozen embryos transferred, is still increasing, albeit slightly. Brazil's leadership is based, among others, on *Bos taurus indicus*

breeds, which yield a higher number of oocytes recovered/OPU session, as demonstrated by Pontes et al. (2010), who obtained on average 12.1 viable oocytes/OPU session from Gir (*Bos indicus*) compared with Holstein (*Bos taurus*) cows which yielded an average of 8.0 viable oocytes/OPU session. Moreover, Nelore cattle represents 80% of the Brazilian herd (Pontes, Melo Sterza et al. 2011), which might explain part the country's leading position.

Finally, regarding cryopreservation methods, a lot of information is available mainly on the traditional conventional freezing method. It can be considered the standard cryopreservation method for bovine embryos world-wide. Nonetheless, within the two decades since the technique was developed, cryopreservation by vitrification has gained territory world-wide, being applied first in the research field and afterwards in practice. The first important study trying to transfer and test the knowledge on vitrification and its advantages gained in the 1980s may be the one published by Van Wagtendonk-de Leeuw, Den Daas et al. (1997), Van Wagtendonk-de Leeuw, Aerts et al. (1998). Although, the embryos transferred were in vivo-derived, this study demonstrated the potential of the vitrification of embryos and its feasibility in the field. They achieved similar pregnancy rates upon transfer of vitrified embryos when compared as with frozen embryos. Unfortunately, not many studies have been published since then with such a number of recipients (n=393 recipients receiving vitrified embryos, 44.5% pregnancy rate; n=335 recipients receiving frozen embryos, 45.1% pregnancy rate).

In conclusion, regarding the importance that IVP embryos have gained in commercial programmes, it is necessary to mention the creation of a working group involving the OIE (World Organization for Animal Health), the private sector and the IETS (International Embryo Transfer Society) working together in order to establish sanitary standards for IVP embryos world-wide.

Table 1. Milestones in embryo transfer and cryopreservation

Year	Event	Authors	Country
1950	1st bovine non-surgical ET	Jim Rowson	England
1951	1st calf born from transferred bovine embryo	Willet, Black et al.	USA
1971	High survival rates after freezing and thawing of mouse embryos	Whittingham	England
1972	Live and normal offspring of mouse following transfer of cryopreserved embryos by slow-freezing	Whittingham, Leibo et al.	USA
1973	Birth of Frostie II, the first calf obtained from frozen-thawed embryo	Wilmut and Rowson	England
1985	Ice-free cryopreservation of mouse embryos	Rall and Fahy	USA
1987	Pregnancy and live-born calves following transfer of vitrified-thawed in vivo-derived bovine embryos	Massip, Van Der Zwalmen et al.	Belgium
1990	Pregnancy and twins offspring following transfer of frozen-thawed bovine in vitro-produced embryos	Fukuda, Ichikawa et al.	Japan

1.2 Advantages of cryopreservation by vitrification for IVP embryos

Vitrification has been described by many authors as the method of choice to cryopreserve IVP embryos. Currently, it was adopted worldwide in research field, and it was used to study and to fully – at least partly - understand, which factors play key roles when performing cooling and what the implications are for the further developmental capacity of the cells. On the other hand, it made it possible to analyse which kind of modifications the cells are going through at the structural and molecular level when they are subject to extremely low temperatures.

Although conventional freezing represents the most popular and most widely method adopted among practitioners in the field, this fact can be explained by two simple reasons. First of all, IVP embryos do not make up a large portion of the embryos being frozen; and secondly, conventionally frozen embryos are directly transferred into recipients avoiding the thawing steps that are necessary when subjecting them to vitrification in order to remove the CPA agents.

When working with bovine embryos, the vitrification outcomes achieved have made this “promising” method a reality. Post-thaw survival rates of 80% can be achieved when working with good quality embryos. Yet it can be stated that the technique has not been adopted by practitioners as expected. One of the most important characteristics of vitrification is that it represents a time-saving method and it has proven to be at least as good as the conventional freezing. Nevertheless, thawing is a critical aspect and achieving high warming rates is necessary to avoid intracellular ice crystal formation. However, the tally of IVP embryos transferred into recipients is still low, and so is the percentage subjected to cryopreservation. Recently, bovine IVP embryos have been vitrified using the Cryotop method and transferred into recipients after thawing, achieving a pregnancy rate of 43%, the highest achieved so far when working with IVP embryos (Sanches, Marinho et al. 2013).

Regarding vitrification, cellular toxicity due to the use of CPA is the major negative factor (Rall and Fahy 1985). However, advantages over the conventional freezing method were demonstrated by Stehlik et al. (2005) in human embryos subjected to vitrification, which have shown lower membrane damage. Also, a higher pregnancy rate upon transfer was achieved as compared to those embryos cryopreserved by the conventional freezing method. Metabolism judged by glucose, pyruvate, and the oxygen uptake of embryos subjected to conventional freezing was decreased when compared to those embryos that had undergone vitrification, which was due to stressful conditions (Kaidi, Bernard et al. 2001).

1.3 Embryo quality and its parameters

Although several evaluation protocols have been established, the parameters of evaluation of embryo quality and morphological assessment seem to differ among researchers. Currently, there are numerous characteristics related to the embryo itself in order to evaluate embryo quality, and it can be stated that morphology assessment (for in vivo and in vitro produced embryos) and the number of cells (for IVP embryos) have been by far the most widely adopted evaluation methods. However, embryo morphology still has many advantages over other evaluation parameters due to its feasibility.

In order to understand the importance of certain parameters when evaluating bovine embryos, a short description of physiological events taking place during

early embryonic stages might be useful. In the zygote, an S-phase of the cell cycle is completed after fertilization (Hyttel, Sinowatz et al. 2009). Once oocytes are fertilised they should still have the capacity to cleave and to further develop. Embryo kinetics has been demonstrated to be a reliable marker for predicting their developmental capacity (Lonergan, Khatir et al. 1999, Sugimura, Akai et al. 2012). It has been established that early-cleaving embryos, when compared to their slowly cleaving counterparts, are more likely to develop. Moreover, the slowly cleaving group of embryos have shown a higher incidence of chromosomal abnormalities than the rapidly cleaving ones. Embryo´s first cleavage, from the zygote up to the 2-cell stage, is characterized by the occurrence of three important events: the S, G_2, and M phase of the cell cycle (Parrish, Kim et al. 1992). The S phase of the cell cycle, a synthesis phase, corresponds to the phase in which DNA is replicated. This is followed by the G_2 phase, during which the cell grows and proteins necessary during mitosis are synthetized. Subsequently, the M phase (in which mitosis occurs) leads to the formation of two genetically identical cells. Moreover, the duration of the zygote's S-phase has been shown to be related to the fertility of the bull (Eid, Lorton et al. 1994). Embryos cleaving within the first 30 hpi achieve higher blastocyst rates (Lonergan, Khatir et al. 1999). In addition, these male effects were demonstrated in human embryos as well by Janny and Menezo (1994). The several following cleavages from the zygote up to the blastocyst stage are strongly related to the embryo's genomic activation and subsequent transcription which leads to specific protein synthesis. Up until the 8-cell stage, these cleavages are mainly determined by the maternal mRNA transcription, since proper embryo genomic activation only takes place at around 66-72 hpi (Wolf, Arnold et al. 2003). Embryos prone to develop up to the blastocyst stage have an earlier first, second, and third cleavage (Beck, Reichenbach et al. 2012, Orozco-Lucero, Dufort et al. 2014).

Furthermore, compaction and cavitation are key events during early embryonic life (Gordon 2003). Compaction occurs around the 32-cell stage both in ex vivo and IVP embryos and it represents the morphological result of protein synthesis and the formation of tight cellular junctions (Van Soom, Ysebaert et al. 1997). In human embryos, the delay of compaction of 24 h, is associated with a lower volume of the inner cell mass (ICM) (Ivec, Kovacic et al. 2011). This event is the first differentiation of the bovine embryo and denotes an essential event for its

subsequent development (Gordon 2003). However, regarding IVP embryos, compaction does not seem to occur every time and as distinctly as it happens in their in vivo counterparts (Soom, Boerjan et al. 1996).

It has been demonstrated that IVP embryos experience a delayed development as compared to their ex vivo counterparts, and the developmental rate has been associated with their further developmental capacity (Dinnyes, Lonergan et al. 1999, Lonergan, Khatir et al. 1999). Different authors have shown that embryo sex influences embryo kinetics, i.e. embryos that cleaved earlier tended to be male embryos (Avery, Madison et al. 1991, Xu, Yadav et al. 1992, Carvalho, Del Campo et al. 1996).

1.3.1 Factors influencing embryo quality

1.3.1.1 In vitro maturation

Currently, numerous factors are known to influence oocytes quality and developmental competence. These can be categorized into intrinsic (related to the oocyte itself) and extrinsic factors (Hendriksen, Vos et al. 2000). Among other factors, the sources of oocytes (in vivo or in vitro matured) emerge as one of the many issues determining their ability to develop to the blastocyst stage (Lonergan, Rizos et al. 2001). However, since many of these issues influence each other, they should not be analyzed separately. Nonetheless, in the past, much of the knowledge gained was accomplished by analyzing each one of them separately in order to understand their role and their importance within the process of maturation.

In mammals, oocytes remain arrested within the follicles in prophase of meiosis I (MI) until the LH surge before ovulation, which induces germinal vesicle breakdown (GVB) (Gordon 2003). Thereafter, one chromosome is extruded into the first polar body, whereas the other one aligns to the second metaphase (MII) plate. In order to obtain MII oocytes in vivo, different exogenous hormonal superovulation protocols have been developed (Humblot, Holm et al. 2005). In addition, it has been demonstrated that in vitro-matured oocytes, although varying in their developmental capacity, are able to progress up to the blastocyst stage following in vitro fertilization (Leibfried-Rutledge, Critser et al. 1987). The oocytes remain arrested in the MII stage once again until they are fertilized by a spermatozoon, which subsequently induces meiosis resumption (Gordon 2003).

Moreover, some of the oocytes with developmental capacity recovered in vitro from follicles undergo spontaneous meiosis resumption when they are in vitro matured, independently of their exposure to gonadotropins [for review see (Bilodeau-Goeseels 2012)], which leads to their incapacity to be fertilized. Follicle size has been proven to have an influence as well, since oocytes recovered from 2-6 mm follicles showed a lower developmental rate as compared to those obtained from follicles >6 mm in diameter (Lonergan, Monaghan et al. 1994). In addition, De Loos, van Beneden et al. (1992) found several deviations regarding cytoplasmic and cumulus maturation which were manifested either by a lack of synchronicity of the nuclear-cytoplasmic-cumulus maturation events or in some cases by structural disruptions.

Over the past decades, a lot of work has been done in order to improve and to mimic the physiological conditions under which oocytes develop, and it was postulated by Rose and Bavister (1992) that the oocyte's capacity to become an embryo after IVM/IVF might be the best indicator for assessing the maturation conditions in which they developed.

After in vitro maturation (IVM), bovine oocytes showed an increased ATP content (Stojkovic, Machado et al. 2001), which suggests further developmental competence. This occurs, in order to obtain the required energy for the following processes such as meiosis resumption and fertilization, mainly by mitochondrial metabolism (Somfai, Kaneda et al. 2011). As "fuel" for the oocytes and early embryo stages, lipids play an important role by providing the energy necessary for further development in mammalian species as shown by Sturmey, Reis et al. (2009).

Embryo lipid content differ among species, as shown by McEvoy, Coull et al. (2000). According to their experiments, pig oocytes contain significantly higher lipid content than those of cattle and sheep. Nevertheless, although oocytes with large enough energy reservoirs might be desirable, lipid content represents an unwanted characteristic to deal with when embryos are subjected to cryopreservation. Altering lipid metabolism pathways through the supplementation of the medium with lipid metabolic regulators either during IVM or in vitro culture (IVC) have shown to be a good strategy. During IVM, altering different oocyte metabolic pathways was shown to improve oocyte quality through the addition of certain amino acids to the maturation medium such as L-

carnitine (Takahashi, Inaba et al. 2013), cysteamine (Balasubramanian and Rho 2007), or linoleic acid (Lapa, Marques et al. 2011, Carro, Buschiazzo et al. 2013, Khalil, Marei et al. 2013, Absalón-Medina, Bedford-Guaus et al. 2014).

Structural differences e.g. abnormal distribution of the cortical granules and mitochondrial reorganisation (Hyttel, Greve et al. 1989) of in vitro matured oocytes were observed. Vacuolization (Zamboni, Thompson et al. 1972) and lipid accumulation (De Loos, van Beneden et al. 1992) have been associated with oocytes that have already undergone degeneration.

With regard to extrinsic factors, temperature and atmosphere have been shown to be key factors that must be kept close to the physiological values. In vitro, oocytes have shown higher maturation rates and were proven to still have the capacity of meiosis resumption after IVM at 39°C (Lenz, Ball et al. 1983, Katska and Smorag 1985). However, temperatures under 33°C (Katska and Smorag 1985) and above 41°C decreased maturation and fertilization rates in vitro (Lenz, Ball et al. 1983). Exposure of cumulus oocyte complexes (COCs) at the germinal vesicle (GV) stage to heat stress for 6 h did not affect the cleavage rate upon IVF, but decreased the number of embryos reaching the 8-cell stage. Moreover, exposure of oocytes to 41°C accelerated cytoplasmic maturation (Edwards, Saxton et al. 2005). Light (Rieger and Betteridge 1989), temperature (Lenz, Ball et al. 1983, Katska and Smorag 1985, Payton, Romar et al. 2004, Edwards, Saxton et al. 2005), gas atmosphere (Pinyopummintr and Bavister 1995, Thompson, McNaughton et al. 2000), and IVM (Leibfried-Rutledge, Critser et al. 1987, De Loos, van Beneden et al. 1992, Rose and Bavister 1992, Hendriksen, Vos et al. 2000, Watson, De Sousa et al. 2000, Rizos, Ward et al. 2002, Merton, De Roos et al. 2003, Mermillod, Dalbiès-Tran et al. 2008, McKeegan and Sturmey 2011), are just a few among many other influence factors well described in the literature. Studying temperature effects on oocyte maturation, Lenz et al. (1983) demonstrated that a greater percentage of oocytes matured in vitro between 35-39°C completed nuclear maturation as compared to those matured at 41°C. The latter ones were less likely to resume and complete meiosis, due to harmful temperature effects. The results from Lenz et al. (1983) were corroborated a few years later by Katska and Smorag (1985). Temperature effects during the hot and cold seasons on developmental capacity and oocyte quality were described for two subspecies in vivo (*Bos taurus*

taurus vs. *Bos taurus indicus*) as well. *Bos taurus taurus* breeds had lower oocyte quality during the hot season (Rocha, Randel et al. 1998).

In summary, embryo susceptibility to chilling injuries depends on the animal species, the embryo's developmental stage and the medium it developed in (Pollard and Leibo 1994).

1.3.1.2 In vitro culture

Just like IVM, IVC conditions have influence on the quality of bovine IVP embryos. Although many advances have been achieved regarding this kind of bovine embryos, there is still a difference between in vivo-derived embryos after a donor's artificial insemination (AI) and in vitro-produced after oocyte's in vitro fertilization (IVF). This was shown by different authors [see (Leibo and Loskutoff 1993, Massip, Mermillod et al. 1995, Abe, Yamashita et al. 1999)]. Both intrinsic and extrinsic factors play key roles in the advanced embryo stages, and the culture per se is one of the most important factors determining which kind of embryo can develop. It has been shown that nutritional requirements are most likely specific to the respective developmental stages, so the embryo responds to the addition of certain components to the culture medium.

Concerning extrinsic factors affecting embryo development, early studies focused on temperature, atmosphere, and culture medium. In this regard, zygotes cultured under reduced O_2 environment (5% O_2) have shown a developmental capacity similar to the that of zygotes developed in vivo, regarding embryo stage and day of development (Tervit, Whittingham et al. 1972). Higher O_2 concentrations inhibited the further development of IVP embryos.

In addition, regarding the culture medium, hardly any consistent results were achieved before the use of SOF (synthetic oviduct fluid), based on the biochemical composition of sheep oviduct fluid studied by Restall and Wales (1966). Brackett, Bousquet et al. (1982) reported in the early 1980s, although for in vivo-harvested oocytes, that the transfer of 4-cell stage embryo into a recipient following IVF ended in the birth of Virgil, the first calf born after oocyte IVF. Further IVC of zygotes were grown in Ham's F-10 medium supplemented with 10% oestrous cow serum (OCS), although embryos only reached the 8-cell stage.

The use of serum in IVC medium can be considered one of the most important factors affecting embryo quality. It has generated controversy among different

authors. Today it is known that its use leads to higher blastocysts rates. On the other hand, it has been shown to influence the embryo's lipid content, this, in turn, being a crucial factor determining embryo survival after cryopreservation. Serum contains and provides the IVC medium with vitamins, amino acids, growth factors, and chelators of heavy metals (Abe, Yamashita et al. 2002), In the past, the use of serum led to high rates of embryo loss upon transfer into recipients of IVP embryos (Reichenbach, Liebrich et al. 1992), abnormal placenta and foetal development (Kruip and Den Daas 1997, Farin, Piedrahita et al. 2006), placental atypical gene expression (Salilew-Wondim, Tesfaye et al. 2013), an increased incidence of perinatal mortality (Schmidt, Greve et al. 1996, Bonilla, Block et al. 2013), congenital malformation and augmented birth weight (Massip, Mermillod et al. 1996, Kruip and Den Daas 1997, Van Wagtendonk-de Leeuw, Aerts et al. 1998, Lazzari, Wrenzycki et al. 2002, Bonilla, Block et al. 2013), among other morphological characteristics reviewed by Young et al. (1998) and known as the "large offspring syndrome". The hatching rate was higher in those embryos cultured in medium supplemented with bovine serum albumin (BSA) and it was suggested the presence of serum in IVC medium negatively affect the hatching process (Mucci, Aller et al. 2006), but no difference was found as to which embryos reached the blastocyst stage when comparing medium with the presence of serum or without it (Rizos, Gutierrez-Adan et al. 2003).

The addition of glucose to the IVC medium has detrimental effects from the zygote stage up to the morula stage (Takahashi and First 1992), but was shown to have positive effects from the 8-16-cell stage (when embryonic genome activation takes place) up to the blastocyst stage (Rieger, Loskutoff et al. 1992), being the preferred nutrient from the 16-cell stage onwards up to the blastocyst stage (Gardner 1998). On the other hand, pyruvate and lactate were described as the main nutrient required by the embryo before reaching the 16-cell stage (Leese and Barton 1984, Khurana and Niemann 2000), although the addition of pyruvate to a ready-made medium containing lactate didn't show any advantages regarding the percentage of embryos reaching the morula and blastocyst stages (Rosenkrans, Zeng et al. 1993).

BSA was shown to have positive effects on embryos in culture environment, since it provided a higher water quality in the IVC medium. Thompson and Peterson (2000) postulated that BSA may contribute with amino acids to the medium after

suffering hydrolysis, which led to an increase in the intracellular concentration of amino acids in embryos cultured in BSA-supplemented medium (Orsi and Leese 2004).

In a way, studies have shown that embryos developing in protein-free media are compromised in their development. Essential and non-essential amino acids were shown to be very important components of the IVC medium in early embryo stages. Modifications of the traditional SOF medium described by Tervit, Whittingham et al. (1972), mSOF, were introduced by Takahashi and First (1992). The result was a successful culture from the 8-cell stage up to the blastocyst stage, but not from the one-cell stage up to the blastocyst stage but not from the one-cell stage up to the blastocyst stage. In their study, they demonstrated that the addition of amino acids to the IVC medium significantly improved the development of zygotes up to the morula stage. Furthermore, supplementation of the IVC medium with amino acids resulted in embryos with a greater cell number than those of the control group (71.5 cells versus 111.5 cells, control and supplemented group respectively).

Finally, in order to avoid issues regarding the use of biological materials, chemically defined (Block, Bonilla et al. 2010) and semi-defined medium (Mucci, Aller et al. 2006) were described to avoid the problems of inconsistent results as described by McKiernan and Bavister (1992) in the past, thus demonstrating that developmental rates are not significantly affected.

1.3.1.3 Lipid content

It has been suggested that triacylglycerides, cholesterol, free fatty acids, and phospholipids (as membrane bilayer) are stored in embryos (Sudano, Paschoal et al. 2013). Lipid droplets are mainly composed of triacylglycerol in embryos (Diez, Heyman et al. 2001), this being the predominant lipid in the cytoplasm (McKeegan and Sturmey 2011). Lipids are necessary during early stages, serving as “fuel” for chemical reactions. It has been shown that lipid concentration decreases from the maturation of oocytes up to the 4-cell stage (Ferguson and Leese 1999). Triglycerides are believed to supply energy by undergoing mitochondrial β-oxidation and that way providing the necessary ATP for early embryonic stages. This fact was lately demonstrated by Dunning, Cashman et al. (2010). Their study, although performed with mice embryos, has shown that the

addition of an inhibitor of the enzyme carnitine palmitoyl transferase, etomoxir, significantly reduced the number of embryos achieving the 4-8 cell stage and the blastocyst stage. Contrarily, the addition of L-carnitine (with stimulatory effects on β-oxidation) significantly increased the number of embryos reaching the blastocyst stage. This was recently confirmed by Takahashi, Inaba et al. (2013) and Chankitisakul, Somfai et al. (2013). The last-mentioned authors, studying cryopreservation of oocytes and the effects of L-carnitine as an enhancer of β-oxidation, did not find any significant difference regarding lipid droplets density between the control group (non-treated) and the treated (L-carnitine) group. Surprisingly, a rearrangement of the lipid droplets was detected in the L-carnitine group, in which the lipid droplets were allocated in a diffuse manner in the oocyte; the study could not explain whether this fact or the redistribution of lipid droplets as an increase on metabolic activity, might be a reason for the high developmental competence of the oocytes after vitrification.

In addition, it has been over a decade since Visintin, Martins et al. (2002) made an interesting approach once more highlighting the differences between *Bos taurus* and *Bos indicus* subspecies with their work on ultrastructural differences between Holstein (*Bos taurus taurus*) and Nelore (*Bos taurus indicus*) in vivo-derived embryos. In this study, after controlled freezing, Holstein embryos morphologically evaluated had larger and more numerous cytoplasm lipid droplets than Nelore [which agrees with other observations reported by Sudano et al. (2012)]. Their cryosensitivity however was reduced when compared to Nelore embryos. Furthermore, the former showed fewer ultrastructural changes after vitrification (2002). Moreover, it was reported (Sudano, Paschoal et al. 2011) that Nelore in vivo embryos had a lower lipid content than their in vitro counterparts, and the lipid amount was correlated with survivability after cryopreservation in different studies (Pollard and Leibo 1994, Abe, Yamashita et al. 2002, Mucci, Aller et al. 2006). Interestingly, Sudano et al. (2012), studying phospholipids of the cellular membrane, reported that there is not only a difference between IVP embryos and in vivo-derived ones after superstimulatory treatment, but also concerning Simmental (*Bos taurus taurus*) and Nellore (*Bos taurus indicus*). They concluded that survival of cryopreservation, among others, is affected by the specific lipid composition of functional membranes.

However, and despite all efforts to alter the lipid metabolism in embryos, either through the addition of linoleic acid in order to stimulate the accumulation of neutral lipid in cytoplasm droplets; or through the addition of the previously mentioned L-carnitine to the medium as a booster of β-oxidation; the way how IVP embryos accumulate an abnormal amount of lipids in the cytoplasm still remains unknown.

1.3.1.4 Superstimulatory treatments and their effect on follicular development

In general, in vivo-derived embryos are obtained after the donor's superovulation scheme [for review see (Bó, Guerrero et al. 2009, Bó and Mapletoft 2014)], AI on the day of standing oestrous (day 0 = oestrous) and embryo recovery by flushing 7 days after the AI. Bó and Mapletoft (2014) described that the use of estradiol-17β or estradiol benzoate, within what they reported as the most common superstimulation scheme, leads to a new follicular wave growth, on average four days after treatment (Bó, Adams et al. 1995). The ova fertilization rate in heifers is influenced by not treating donors with estradiol right at the beginning of the superstimulation protocol. On the other hand, donor's superstimulation without using estradiol induces the growth of small follicles even in those that have already undergone atresia, but it does not lead to a new follicle wave, which, in turn, could result in low quality ova/embryos (Bó and Mapletoft 2014), i.e. many oocytes could be rescued by the superstimulatory treatment but their ovulatory capacity is not guaranteed. Recently, Fernandes, who is working with the Nelore breed [cited in (Barros, Ereno et al. 2009)] demonstrated that the mRNA expression pattern of LH receptor (LHR) isoforms of large superstimulated follicles (>10 mm) decreased as compared to those large follicles of non-superstimulated Nelore cows, thus compromising oocyte maturation and superovulatory response (Simões, Satrapa et al. 2012). Therefore, it can be inferred that it is not a matter of follicle size but that LHR expression patterns seem to be altered by superstimulatory treatments. Chu, Dufort et al. (2012) found alterations in gene expression profiles in oocytes upon superstimulation which compromised the oocyte's developmental capacity due to follicular environment alterations. Some imprinted genes that acquire their epigenetic marks during the oocytes' growth phase were altered in the placenta of mice that were superstimulated (Fortier, Lopes et al. 2008).

Although oocyte “maturation” was described as three events including: meiotic, cytoplasmic, and molecular maturation, it has not been clearly defined yet and little is known about this process (Sirard, Richard et al. 2006). Some ultra-structural changes and organelle rearrangements take place, events occurring during the last period before ovulation. It is known that at this stage previous to ovulation, LH peaks frequency is increased and that this issue is important for oocyte maturation. Hence, decreased expression of LH receptors might influence its further development.

Ova source and IVM are key factors which need to be improved for further development. Both procedures yield lower ova/embryo quality (Blondin, Coenen et al. 1996, Sirard and Blondin 1996, Hendriksen, Vos et al. 2000, Rizos, Ward et al. 2002, Lonergan, Rizos et al. 2003, Merton, De Roos et al. 2003, Sirard, Richard et al. 2006).

2. Genetic evaluation bovine of embryos

2.1. Embryo biopsy and pre-implantation genetic diagnosis (PGD): background and advantages

A lot of progress has been made over the last century regarding genetic improvement in dairy and beef cattle, among other mammalian species. However, there is a need for time-saving tests regarding phenotypic or genetic characteristics of certain animals without having to evaluate their next generation. Preimplantation genetic diagnosis (PGD) is performed at the early embryo stages and it has been described in many mammalian species including humans (Handyside, Kontogianni et al. 1990), cattle (Bondioli, Ellis et al. 1989), and horses (Peippo, Huhtinen et al. 1995, Herrera, Morikawa et al. 2014), among others.

In this regard, Jung, Reichenbach et al. (2014) recently performed a biopsy and genetic analysis of Simmental breed embryos for gender, polled status [by polymerase chain reaction (PCR) and gel electrophoresis] and eight inherited diseases (by 5′-exonuclease-based real-time PCR) particularly affecting Simmental, within 24 h after embryo recovery. This is clearly an advantage for those embryos that might not be eligible for cryopreservation. Moreover, the birth of correct genotyped calves was firstly reported by Peippo et al. (2007).

In embryos, every single cell contains one copy of the genome; therefore different biopsy techniques can be used in order to obtain material for the genetic analysis such as: zona drilling (Gordon and Talansky 1986, Gordon and Gang 1990), laser-assisted biopsy (Veiga, Sandalinas et al. 1997, Montag, van der Ven et al. 1998), splitting (Lopes, Forell et al. 2001), cell aspiration with a glass pipette (Roschlau, Roschlau et al. 1992), to name but a few methods which have been described; the biopsied material consisted or the polar body (Magli, Gianaroli et al. 2004), a single blastomere or a group of blastomeres (Park, Lee et al. 2001), a quarter-embryo (Polisseni, Sá et al. 2010) or demi-embryos (Lopes, Forell et al. 2001), which were subjected to the polymerase chain reaction (PCR) technique. PCR was described for the first time by Mullis, Erlich et al. (1987) and it is basically a enzymatic reaction which generates unlimited DNA copies beginning with a single molecule of DNA. In addition, bovine embryo sexing by PCR was achieved regardless of the size of the sample. In these experiments, the number of single

blastomeres dissociated from the embryo to perform the analysis, did not seem to affect PCR's effectiveness as was demonstrated by Park et al. (2001) who performed a PCR using groups of 1 to 8 blastomeres separated from the embryos showing an efficiency of 92% and 100% for the 1- and 8-blastomeres groups, respectively.

Furthermore, the ability of a single blastomere to develop up to blastocysts of bovine embryos is maintained up to the 8 cell stage (Tagawa, Matoba et al. 2008). Several research groups have shown that single blastomeres are able to progress into blastocysts with both ICM and trophectoderm (TE). Johnson et al. (1995) obtained 3 pregnancies after dissociating one 4-cell stage embryo that was able to further develop and 3 calves were born, thus providing the above-mentioned fact. Similar results were described by Loskutoff, Johnson et al. (1993) who obtained pregnancies upon transfer of IVP embryos after a 1-blastomere biopsy of 4-cell stage embryos. Working with IVP embryos and performing biopsies at early embryonic stages did not affect the embryo's development up to the blastocyst stage, this being relevant since this technique is performed before the formation of gap junctions (Park, Lee et al. 2001, Polisseni, Sá et al. 2010).

However, in order to perform molecular genetic tests, 2-200 ng are needed, with the amount on a single diploid cell being 6 pg (Jung, Reichenbach et al. 2014). In this regard, the importance of the embryo biopsy lies in the capacity to obtain enough material without causing any damage that could affect the embryo's further developmental capacity. Embryo-maternal recognition is a complex signalling pathway including both mother and conceptus [for review see (Wolf, Arnold et al. 2003)]. Interferon-tau (IFNT), a type-1 interferon, is secreted by the TE cells of embryos at the preimplantation stages (Roberts, Ealy et al. 1999, Roberts 2007) and it has an antiluteolytic effect by supressing prostaglandin F2 secretion by the endometrium, thus prolonging the lifespan of the corpus luteum (Meyer, Hansen et al. 1995). Hence, assuming that embryo splitting would generate two embryos with a reduced number of cells, it can be inferred that they would have a reduced secretion of IFNT, as demonstrated by Stojkovic et al. (1999) and this, in turn, could lead to a communication failure during preimplantation stages with the implantation failing subsequently. Demi-embryos have low viability (Chagas e Silva, Diniz et al. 2008) and this fact can explain - at least in part - why pregnancy rates after transfer into recipients of the whole

embryo are higher than if single demi-embryos are transferred. Nevertheless, it has been demonstrated recently that half-embryos transferred into recipients, as compared to the whole embryos, compensate by growing and by day 42 had almost, or even entirely, closed the gap to whole embryos (Chagas e Silva, Diniz et al. 2008, Lopes-da-Costa, Chagas e Silva et al. 2011). Polisseni, Sá et al. (2010) who performed biopsies at early embryonic stages didn't find any significant differences regarding the number of cells between the control group and the treatment group (biopsied ones) at different stages (blastocyst, expanded blastocysts and hatched blastocysts).

3. Vitrification of embryos

Since the availability of recipients at any given time is the main limiting-factor for the breeding, particularly ET, industry i.e. more embryos are produced from donors than recipients are available or eligible to receive an embryo on the day of the donor's flushing. Hence, embryo cryopreservation plays an important role providing an efficient system, i.e. matching the number of embryos produced by the donors to the number of embryos transferable/freezable per donor.

After successful vitrification had been achieved, researchers spent the last two decades of the past century focusing on morphological and ultrastructural changes induced by the vitrification itself. Numerous publications pointed out that the main advantage of this technique is the fact that it avoids ice crystal formation. Interesting observations were made by Leibo et al. (1978) using microscopy, who have shown physical damage to the cells due to the crystallisation of the sample. The advantages of vitrification as a cryopreservation method were clear enough; however, the method is not yet widely accepted in the field, even for frozen embryos (7-8% worldwide) according to Stroud (2012). Unfortunately, there is no official data available on the number of embryos being vitrified worldwide.

Back then, the first approaches were made with a French plastic straw. Van Wagtendonk-De Leeuw, Den Daas et al. (1995) vitrified embryos in a 0.25 ml plastic French straw obtaining a pregnancy rate of 44% upon transfer. However, poor quality embryos were included in this study, and they were loaded within the straws in a large volume of solution. According to Arav (1992), decreasing the volume of the sample would increase the probability of achieving ice-free cryopreservation. This was supported a few years later by (Vajta and Kuwayama 2006), who stated two other issues as determining factors in order to achieve the vitrified state: cooling/warming rate and viscosity of the sample. Because of the many studies showing the importance of the physical properties of the solution and the rate of temperature sinking, new devices have been described to serve as sample carriers. In order to characterize those methods developed during the past two decades, a simple classification can be done to categorize them in two groups differentiating by the volume of the sample: those with a large volume of the sample ($\geq$ 20 µl), and the ones with small volume of the sample [referred to as

minimum volume cooling (MVC) concept] (≤ 3 μl) (Vajta, Holm et al. 1998, Kuwayama 2007, Matsunari, Maehara et al. 2012).

Some others categorizations were proposed as follows: open and closed systems; with regard to the way the sample is subjected to extremely low temperatures, on pre-cooled surface (Dinnyes, Yang et al. 2001), in liquid nitrogen (LN_2) (-196 ºC) (Vajta, Holm et al. 1998), LN_2 vapour (~ -180 ºC) , or LN_2 slush (-210 ºC) (Yavin, Aroyo et al. 2009); regarding the use of a carrier for the sample, in those methods with or without a container (Lane, Bavister et al. 1999); with regard to the cooling rate achieved: slow-cooling, rapid-cooling and ultra-rapid cooling methods (Kuwayama 2007, Yavin, Aroyo et al. 2009).

Several vitrification methods, mainly clustered under the minimum volume cooling (MVC) concept have been described in the past two decades (see Table 2).

Table 2. Some vitrification methods

Method	Author	°C min^{-1}*	Carrier
Minimum Drop Size (MDS)	Arav and Zeron (1997)	~ 180000	Electron-Microscopic (EM) grids
Open pulled straw (OPS)	Vajta, Holm et al. (1998)	~ 20000	Pulled Plastic-Straw
Hemi-straw system	Vanderzwalmen, Bertin et al. (2000)		Plastic-straw
Cryoloop	Lane, Schoolcraft et al. (1999) (Isachenko, Isachenko et al. 2003)	~ 700000	Nylon loop
Cryologic Vitrification Method (CVM)	Lindemans, Sangalli et al. (2003)	~ 10000	Fiberplug
Cryotop	Kuwayama (2007)	~ 40000	Polypropylene strip
Solid Surface Vitrification (SSV)	Dinnyes, Yang et al. (2001)		
EM Grids	Martino, Songsasen et al. (1996)	~ 180000	EM grids
0,25 ml Straw	Rall (1987)	~ 2000	Plastic Straw
GPM	Vieira, Forell et al. (2007)		Glass Pulled Micropipettes
Hollow Fiber Vitrification	Matsunari, Maehara et al. (2012)	no data	Hollow Fiber
Super Pulled Open Straws (SOPS)	(Jelinkova, Selman et al.), Yavin, Aroyo et al. (2009)	~13000	Plastic straw
Sealed Pulled Straws (SPS)	Yavin, Aroyo et al. (2009)	~ 8000 - 32000	Plastic pulled straw
Nylon Mesh	Matsumoto, Jiang et al. (2001)	no data	Nylon mesh
Gel-loading tip	Tominaga and Hamada (2001)	no data	Plastic tip
Metal Mesh Vitrification (MMV)	Fujino, Kojima et al. (2008)	no data	Metal mesh
Plastic plate vitrification (PPV)	Fujino, Kojima et al. (2008)	no data	Plastic plate
Rapid-i® Vitrification	Hashimoto, Amo et al. (2013)	no data	Rapid-i® straw
Vit-Master®	Arav, Yavin et al. (2002)	~130000	-

*cooling rate (ºC min-1)

Cryotop (Kuwayama 2007), open-pulled straw (OPS) (Vajta, Holm et al. 1998), and Cryoloop (Lane, Bavister et al. 1999) can be stated as the most described methods in the literature.

The Solid-Surface Vitrification Method (Dinnyés, Dai et al. 2000) seems to be a good method, judged by the post-thawing survival rate of IVP embryos, and it has the advantage that the sample has no direct contact with the liquid nitrogen, avoiding a possible contamination of the sample, as reviewed by Bielanski (2012).

The Hollow Fiber Vitrification (HFV) Method (Matsunari, Maehara et al. 2012) was recently developed in Japan and results were shown for porcine, mouse (Maehara, Matsunari et al. 2012, Uchikura, Wakayama et al. 2014) and bovine embryos (Beck, Kurome et al. 2013).

Though vitrification is simple to perform, time-saving, and doesn't require a programmable freezer, it would not be fair not to mention that there are a few factors affecting the success rate and implementation of this cryopreservation technique in the field. Trained-personnel should be familiar with the micro-volume samples and carriers, which can be a crucial factor during embryo-handling. Moreover, although vitrification of embryos is a time-saving process, thawing can take longer, as described for several thawing-solutions and times of exposure. This fact can be an important issue when working with herds.

One possible explanation for it might be that in the beginning of the 21st century, research moved forward and focused on differences at the molecular level, more specifically gene expression profiles and metabolic activity. Interestingly, Leoni et al. (2003) have shown, that embryos need up to 18 h to fully-recover the metabolic activity, namely protein secretion, after thawing as compared to the non-vitrified ones.

3.1. Principles of vitrification

Vitrification has become the standard cryopreservation method in human assisted reproductive techniques (Vajta, Rienzi et al. 2015). The term vitrification comes from the Latin word *vitrum* (= glass) and its main goal is to achieve a glassy state without any ice crystal formation, making the cells pause in their developmental stage and metabolic activity. It is a physical event in which the solidification of fluids occurs without crystallization of the sample (Luyet and Hodapp 1938). The nucleation of small ice-crystals, called "seeding", within the cell leads to

apoptosis (Seki and Mazur 2009). According to Loewenstein (1981), integrity of the cell-membrane and gap junctions is essential, as they are responsible for the exchange of small metabolites and the maintenance of metabolic and electrical activity. Hence, preservation of the integrity of the cellular structure, by avoiding physical damage due to crystallization of the sample, is vital.

Accordingly, the sample (specifically containing the embryo) is exposed to different solutions containing greater concentrations of cryoprotectants, i.e. equilibration and vitrification solutions with an increasing osmolality for short periods of time. The embryos are in a way exposed to osmotic stress that leads to a dehydration process, i.e. the water escapes from the intracellular compartment through the cell-membrane in the extracellular direction, resulting in forced shrinkage of the blastocoel cavity - if already developed. Afterwards, the embryos are rapidly exposed to extremely low temperatures.

To achieve the glass-like or glass transition state liquid ("solution") must turn into solid, while the sample at a molecular level stops. The glass transition temperature of vitrification solutions is around -120 ºC ±10ºC (Wowk 2010) . Three important factors should be considered: cooling/warming rate, viscosity of the vitrification solution and volume of the sample (Saragusty and Arav 2011).

The likelihood of a sample to achieve the glassy state can be described by the following equation:

$$\text{Probability of vitrification} = \frac{\text{cooling/warming rate X viscosity of the solution}}{\text{volume of the sample}}$$

Several factors may influence the likelihood of vitrification, namely, achieving higher cooling rates or working with higher solute concentrations, i.e. high osmolality of the solution (Vajta and Kuwayama 2006), or by loading the sample in smaller volumes (Yavin and Arav 2007); each one of these factors by itself will increase the probability of attaining the glassy state.

The last but just as important factor is the purity of the sample. The presence of small particles leads to the formation of small ice-cores, hence crystallization occurs.

3.2. Volume of the sample

Many of vitrification methods have been described the past two decades, however since the mid-1990s mainly the sample's carrier has changed, going from the normal 0.25 ml French plastic-straw to the hook-like carrier of the CryoLogic®. The importance of reducing the volume of the sample relies on the possibility of reducing CPA's concentration, and in this manner minimizing the toxic effects of the CPA (Yavin and Arav 2007). Moreover, when working with small volume samples, thermal conductance through the same is improved since those with larger volume offer more resistance than smaller ones, thus maximizing cooling rates (Demirci and Montesano 2007).

Following the concept of minimum volume cooling (MVC), researchers focused on the development of different carriers (see Table 2. above) that have become more and more popular, mostly in the human assisted reproduction techniques (ART) and since the beginning of the 21st century widely accepted in the cryopreservation of other mammalian species. For those methods clustered under the MVC concept, the carrier's volume ranges from 0.05 µl to 3µl leading to greater cooling/warming temperature as compared to those methods containing larger volumes of vitrification solution, for instance French plastic straws.

However, an interesting approach was chosen by Stachecki, Willadsen et al. (2007) with the so-called S^3 vitrification. Using a 0.25 ml plastic straw as a container and working with human blastocysts, they obtained a 94% survival rate after vitrification. Recently, these results were corroborated by Reed, Said et al. (2014) who loaded human's biopsied -blastocysts (1 embryo/straw) into a 0.25 ml plastic-straw with 50 µl of commercial ready-made vitrification solution. The embryo was loaded as follows: three columns of 50 µl vitrification solution each separated by 50 µl air column, sealed and then plunged into liquid nitrogen. Survival rates obtained after warming obtained were 96% and embryos were transferred into the recipient. The same author reported a cooling rate of approx. 2000 ºC. Moreover, the good results were attributed to the DMSO-free freezing solution; however, media's components were not specified and the sample showed crystallization (Reed, Said et al. 2014).

In conclusion, the use of MVC concept methods requires trained personnel and although lab-workers are ready to apply it after a short-training period, this can be one important issue affecting the acceptance of these methods by practitioners.

3.3. Cooling/warming rates

There is controversy in the literature about whether extremely high cooling/warming rates are absolutely necessary or not. Furthermore, species-specific chilling sensitivity varies, and so do requirements for each embryo. However, as previously stated, the embryo's "dangerous zone", i.e. between 25ºC and 0ºC, should be overcome as quickly as possible. The initial cooling determines the success of the technique and according to Vajta et al. (1998) the ideal cooling rate/warming rate is about 20000 ºC/min.

However, a tiny droplet of pure water ($\leq$ 10 µl) vitrifies only at 106 Kelvin s-1 (Warkentin 2010), which makes vitrification impracticable in practice. Yet, the addition of certain components (CPAs) to the solution makes it easier to achieve a vitreous state of the sample working with rapid and ultra-rapid cooling rates.

Several vitrification methods have been described in the literature. They can be classified in two groups: the ones achieving ultra-rapid cooling rates and those attaining rapid cooling rates (see Table 2.). French plastic-straws, as described by Rall, belong to the first group as cooling rates described in the literature are approximately 1800 ºC–2800 ºC, depending on whether they are plunged into LN_2 (-196 ºC) or into LN_2-slush (-210 ºC; VitMaster, IMT, Israel). By contrast, methods clustered within the ultra-rapid cooling group, such as Cryotop and Minimum Drop Size (MDS) achieve extremely high cooling rates (40000 ºC and 180000 ºC, Cryotop and MDS respectively). Yet, with regard to thermal conductance, it can be assumed that the kind of carrier plays an important role.

Interestingly, different studies have shown ambiguous results. Recently, when focusing on warming rate and its effect on survival rate of vitrified oocytes, it was assumed that slow-warming rates led to a re-crystallization of the sample and this served as an explanation why almost 80% of oocytes survived (group rapid-warming), as compared to the 0% survival rate in the slow-warming group. In the same study, an interesting approach did not show any significant difference; oocytes were cooled at 522 ºC/ min but warmed at 2950 ºC/min. Later on, the same authors, this time working with mouse embryos corroborated the importance of achieving rapid-warming rates over cooling rates (Seki, Jin et al. 2014).

In conclusion, the importance of achieving ultra-rapid cooling and warming rates lies in the possibility of reducing the use of cytotoxic CPAs (Arav 1992, Arav,

Shehu et al. 1993). This issue can be very well established since the vitrification of embryos was achieved by Rall and Fahy (1985) for the very first time. Before, experiments had been based on very high concentrations of CPAs and slow-cooling/warming rates. Nowadays, in turn, there is a tendency to decrease or even withdraw the use of CPAs by increasing cooling/warming rates.

3.4. Permeable and non-permeable cryoprotective agents

Embryos and cells are normally in equilibrium with their surrounding environment. Solutes and ions present in the medium and needed by the cells for their metabolism are actively exchanged in order to maintain the intra-cellular machinery working. As previously stated, vitrification is a non-equilibrium method, and its fundamentals are: ultra-rapid cooling/warming rates, minimum volume of the sample, and increased viscosity of the solution (namely high osmolality of the solution) due to the use of high concentration of permeable and non-permeable cryoprotectants, so-called CPAs.

The efficacy of vitrification as a cryopreservation method has already been shown; however, the most important fact that needs to be improved is related to the use of CPAs. As stated above, vitrification implies the exposure of the embryo to equilibration and vitrification solution, normally containing increasing concentrations of permeable and non-permeable CPAs. During these processes embryos undergo dehydration and are under osmotic stress.

Moreover, permeable CPAs enter the cells while non-permeable CPAs facilitate dehydration as water is withdrawn from the intracellular compartment. Furthermore, as stated above, pure water must be cooled at extremely high cooling rates; therefore, another function of CPAs is to reduce the cooling rate at which the sample must be chilled to achieve a vitreous state. However, several permeable CPAs used are toxic. Among them, EG is considered a CPA with minimal toxicity, and was shown to be the most permeable CPA (Széll, Shelton et al. 1989). Moreover, glycerol (GLY) appeared to be more permeable than DMSO, propylene glycol or acetamide (Voelkel and Hu 1992, Kasai 1996, Massip 2001). This combination of minimal toxicity and high permeability represents an excellent alternative. Dimethyl sulfoxide, in turn, is considered very toxic. Additionally, it was shown by other authors that CPA’s permeability differs between species and embryonic stages. At room temperature, blastocysts need up

to 15 minutes to equilibrate in 1M GLY solution (Mazur 1976); while unfertilised oocytes needed more than 3 h to equilibrate (Jackowski, Leibo et al. 1980).

Early studies made by Massip, Van Der Zwalmen et al. (1987) showed the importance of sucrose and trehalose as key factors in maintaining the functionality and structure of the cellular membrane in a water-reduced environment. According to Campos-Chillon, Walker et al. (2006), galactose acts as not only as an osmotic agent but also protects the cell- membrane of the cells. The loss of integrity of the cell-membrane was reported by several authors as the main damage to be avoided when performing cryopreservation. Moreover, the addition of sugars to the thawing solution acts as a buffer, avoiding the possibility of embryo's osmotic shock due to the extremely rapid rehydration (Liebermann, Dietl et al. 2003).

Worldwide, different CPAs are used, and they can be classified into permeable and non-permeable ones (see Table 3.). They are used as single components or associated with others in order to avoid cytotoxicity. Moreover, macromolecules showed extracellular cryoprotective activity (Checura and Seidel 2007).

Table 3. Permeable, non-permeable and macromolecules used in vitrification

Non-permeable Cryoprotectant	Permeable Cryoprotectant	Macromolecule
Sucrose	EG	PVP
Trehalose	DMSO	BSA
Galactose	1, 2- Propanediol	PVA
Glucose	GLY	Ficoll 70
Fructose	Propylene glycol	Ficoll 400
Manitol	Acetamide	Dextran

Adapted from Moore and Bonilla (2006)

As previously stated, the most important fact related to vitrification is the physical transformation of the liquid sample ("solution") into an amorphous solid state once the glass-transition temperature is achieved. However, each liquid or solution has its own glass-transition temperature, which was estimated at - 120ºC for common vitrification solutions (Wowk 2010). Studying vitrification solutions and the amount of CPA needed to avoid ice-damage, an interesting approach was made by Fahy et al. (1987). In their study, they quantified the amount of GLY needed to avoid crystallisation and damage at any sub-zero temperature.

Theoretically, it is possible to calculate which percentage of liquid of an organ can be transformed (liquid to solid) without any ice-damage. Taken from their calculation, a combination of 68% GLY and 32% pure water will vitrify entirely. With this regard, several combinations of CPAs were described (see Table 4.)

Table 4. Some CPA combinations described in the literature

Method	Vitrification Solution	Reference
Cryotop	20% EG + 20% DMSO + 0.5M Sucrose	Sanches, Marinho et al. (2013)
Cryotop	15% EG + 15% DMSO + 0.5 M Sucrose	Asgari, Hosseini et al. (2012)
CVM	30% EG + 1 M Trehalose	Villamil, Lozano et al. (2012)
CVM	16.5% EG + 16.5% DMSO + 0.5 M Sucrose	Trigal, Gómez et al. (2012)
OPS	16.5% EG +16.5% DMSO + 0.5 M Sucrose	Vajta, Holm et al. (1998)
Closed Pulled-Straw	20% EG + 20% DMSO + 18% Ficoll 70 + 0.5 M Sucrose	Yu, Deng et al. (2010)

3.5. Damage caused by vitrification

Although vitrification has become the method of choice for IVP embryos, it is known to cause injuries at different levels. Morphological, functional, and metabolic cell alterations were described by several authors.

With regard to structural injuries, within the first hours after thawing morphological alterations were present (Vajta, Hyttel et al. 1997). Furthermore, other authors found cytoskeleton disruption (Dalcin, Silva et al. 2013), extensive cytoplasmic disarrangement (Overstrom, Duby et al. 1993), cell membrane alteration of oocytes (Hotamisligil, Toner et al. 1996) after exposure for 5 min to 4M EG solution. Moreover, it was recently demonstrated to cause damage at the molecular level (Stinshoff, Wilkening et al. 2011). Furthermore, decreased mitochondrial activity and damage at the ultra-structural level were reported by Dalcin, Silva et al. (2013).

According to Gomez, Muñoz et al. (2009), embryos presented ICM and the same authors attributed this to vitrification Vajta, Hyttel et al. (1997) have shown that the intercellular junction and cytoskeleton were disrupted and attributed those ultra-structural changes to the vitrification process. Although injuries were more evident at 4 h after thawing, they were barely noticeable 24 h post thawing.

Furthermore, the ZP was altered by the vitrification procedure as described by several authors. Decrease of the pore diameter (Moreira da Silva and Metelo 2005), and ZP hardening (Vanderzwalmen, Bertin et al. 2003) were observed. The effect of thermal stress and its relationship with damages of the ZP were described by several authors (Rall and Meyer 1989, Kasai, Zhu et al. 1996, Van den Abbeel and Van Steirteghem 2000). ZP's structure alteration (Moreira da Silva and Metelo 2005) and cracking of the same (Kasai, Zhu et al. 1996, Dinnyés, Dai et al. 2000) were reported in other studies.

The use of high concentrations of CPAs was pointed out as one of the most controversial factors. Their toxicity, even if applied for a short period of time is well known and it has been described by Fahy (1986).

4. Conventional freezing of bovine embryos

Since the first mammalian cell was cryopreserved (Polge, Smith et al. 1949), a lot of work has been done in order to improve cryopreservation methods. The very well-known and traditional conventional freezing can be considered the cryopreservation technique most widely accepted by practitioners in field. Conventional freezing, or better called controlled cooling, was first described in mouse embryos in the early 1970s by Whittingham (1971), Whittingham, Leibo et al. (1972).

4.1. Principles of conventional freezing

Briefly, this technique is based on controlled cooling of the sample. It normally contains solutes with a more than 1M concentration and EG as a permeable-cryoprotectant. Hence, at some point during controlled cooling the crystallization of small ice nuclei containing pure water will take place. The space remaining between the small ice crystals is the so-called “unfrozen” part of the sample containing cells and solutes. As a result, the concentration of solutes and cryoprotectants (namely, EG) increases as the “unfrozen” fraction of the sample decreases. This issue leads to an increase in solute concentration causing an efflux of water from the intracellular compartment, i.e. dehydration of the cell. Water efflux and permeable-cryoprotectant influx will stop at some point when the intracellular and extracellular compartments reach an equilibrium (Schneider 1986). Moreover, as previously stated, this technique is considered an equilibrium method, as it allows the water to leave the cell in a controlled way, thus minimizing the possibility of ice crystal formation. At some point, the viscosity of the “unfrozen” fraction becomes too high so that the formation of small ice crystals is not possible any more, and this fraction then turns into an amorphous ice crystal-free fraction.

4.2. Freezing stages

Freezing begins at room temperature and ends with embryos plunged into LN_2 at -196 ºC. It can be divided into four stages: (1) controlled cooling until “seeding” takes place; (2) “seeding”; (3) controlled cooling down to -32ºC (intermediate temperature); and finally (4) sample is plunged into LN2.

Accordingly, the embryo is loaded in 0.25 ml French plastic-straws with a solution containing 1.5 M EG as a permeable cryoprotectant and is allowed to equilibrate for 10 minutes at room temperature. Following, the straw is moved to a controlled freezing machine and cooling begins until a temperature of -6ºC. After 4 minutes at -6ºC "seeding" is performed with a cotton swab, and the straw remains at -6ºC for another 6 minutes. In the meantime, the plastic straw is cooled at 0.3 ºC/min down to -32 ºC, and finally the plastic straw is plunged into liquid nitrogen.

For thawing frozen IVP bovine embryos, the plastic straw is recovered from the LN_2, it is held in the air for 5 seconds and finally plunged into a water-bath at 25º for 15 seconds.

4.3. Damage caused by slow freezing

As previously mentioned, by "seeding" the operator induces the crystallization of the sample. Two phases during slow-freezing can be distinguished: ice-nucleation and ice-growth. Therefore, crystallization of the sample induces physical damage to the cells, as shown by Leibo and Pool [Figure 1. in (2011) and Mazur (1977).

Damage caused to the ZP by the slow-freezing procedure was demonstrated in human embryos as well. In a study, Van den Abbeel and Van Steirteghem (2000) found a relationship between ZP damage and embryo viability by analysing blastomere survival after thawing. Moreover, the double-layer membrane of the cell was damaged during the chilling process (Arav, Zeron et al. 1996), leading to cell death.

In another study, a greater intracellular concentration of CPAs was shown after vitrification as compared to those oocytes submitted to slow-freezing (Vanderzwalmen, Connan et al. 2013). Yet, the last study was performed in mouse oocytes and, as previously mentioned, differences in CPA permeability between species have been reported. However, this study denotes the importance of exposure time to CPAs.

Furthermore, it is well-known that IVP embryos are more sensitive to chilling injuries than their ex vivo counterparts, as it was reviewed in (Leibo and Loskutoff 1993, Pollard and Leibo 1994). Recently, Villamil, Lozano et al. (2012) reported a 40% re-expansion rate of IVP embryos after slow-freezing as compared

to 69% for those embryos submitted to vitrification. However, no difference was found in re-expansion rates when ex vivo-derived embryos were submitted to slow-freezing. Cryosensitivity of IVP embryos, as previously stated was related to their abnormal lipid accumulation.

In Figure 1 below, taken from Mazur (Mazur 1977), shows a simple scheme of physical events taking place during cooling.

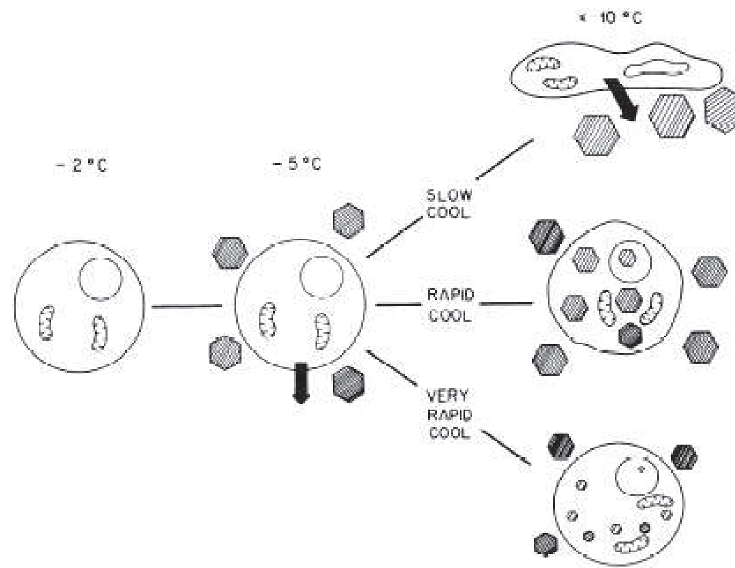


Figure 1. Physical events during slow-, rapid-, and ultra-rapid cooling
Adapted from Mazur (1977)

Table 5. Summary pregnancy rates after transfer of vitrified ZP intact IVP embryos

Breed	Embryos	Method	Stage	Preg. rate %	Calv. rate (%)	Reference
Bos taurus	15		4	40%	--	
	33	Cryotop	6	36.4%	--	Loparatova et al., (2012)
	20		7	40%	--	
Bos indicus	1908	Cryotop	6 & 7	43,2%	--	Sanches, Marinho et al. (2013)
Bos taurus	141	OPS	7	27,7%	--	Block, Bonilla et al. (2010)
Bos taurus	279	OPS	7	25,7%	20,1%	Stewart, Block et al. (2011)
Bos taurus	23	APV	6	17,4%	--	赤上正貴, 脇本亘 et al. (2013)
	30		6	46,7%		
Bos Taurus	58	OPS	6	24%	17%	Rasmussen, Block et al. (2013)
Bos taurus			7	32,5%	--	Pugh, Tervit et al. (2000)
Bos taurus	15	0,25ml Straw	6	60%	--	Agca, Monson et al. (1998)
Bos taurus	16	0,25ml Straw	6	43,7%	--	Martinez, De Matos et al. (1998)
Bos taurs and	60		-	47%	--	Fry, Earl et al. (2004)
Bos indicus	44	CVM		48%		
	18			61%		
Bos taurus	54	0,25ml Straw	6	6,5%	--	Al-Katanani, Drost et al. (2002)
Bos taurus	70	0,25ml Straw	4 & 6	28,6%	--	Block, Bonilla et al. (2009)
	78		7	29,5%		
					--	

III. MATERIALS AND METHODS

1. Materials

1.1. Apparatuses

Axiovert 135 Inverted Microscope	Zeiss, Germany
5171 Micromanipulator	Eppendorf, Germany
MS 5 Stereomicroscope	Leica, Germany
AXIOLAB drb KT Microscope	Zeiss, Germany
Fluorescence microscope Axiovert 200 M	Zeiss, Germany
CO_2- Incubator	Binder GmbH, Germany
CO_2-O_2 Incubator	Medcenter Einrichtungen GmbH, Germany
CVM™ Vitrification System	CryoLogic , Australia
Megafuge 1.0 R Centrifuge	Heraus Spatech GmbH, Germany
Vortex Genie 2™	Zurich, Switzerland
Follicle aspiration unit Aspirator 4014	Labotect, Germany
Heatplate HT 200	Minitüb GmbH, Germany

1.2. Software

SPSS Version 21	IBM Inc.
GraphPad Prism® Version 5.02	GraphPad Software, Inc.
Axiovision 4.8.2 SP3	

1.3. Consumables

4-Well dish	Nunc, Germany
Petridish (40 mm diameter)	Nunc, Germany
Petridish (40 mm diameter)	Nunc, Germany

Coverslips	Hecht Assistant
Centrifuge tubes (10 ml)	Nunc, Germany
Pipette tips (50-1000 µl)	Eppendorf, Germany
Pipette tips (0.1-10 µl) Biohit SafetySpace Filter Tip	Consartic GmbH, Germany
Cellulose triacetate hollow fiber (Dialyzer Sureflux-150FH)	NIPRO Corporation, Japan
Hypodermic needle	Medical Planning Corporation, Japan
Sperm, Sire “RADAU” 9972288	B.V.N Lindenhof, Germany
BD Syringe Luer-Lok™ Tip 10 ml	BD, USA
Millex® GP Filter Unit 0,22 µm	Merck Millipore Ltd, Ireland
Ophtalmic blade	

1.4. Chemicals

Albumin, from bovine serum	Sigma, Germany
BME amino acids solution	Sigma, Germany
MEM Non-essential amino acids solution	Sigma, Germany
Heparin	Sigma, Germany
Pyruvic acid	Sigma, Germany
$MgCl_2$	
NaCl 0,9%	Sigma, Germany
Polyvinylpyrrolidone	Sgima, Germany
Trition X-100	Sigma, Germany
Propidium Iodide	Sigma, Germany
Hoechst 33258	Sigma, Germany
Sucrose	Sigma, Germany

1.5. Hormones

Follicle-stimulating Hormone (FSH) 50 Units	Sioux
Luteinizing Hormone (LH) 25 Units	Sioux

1.6. Media and solutions for IVP of bovine embryos

All chemicals were purchased from Sigma unless stated otherwise.

Maturation medium

Modified Parker's Medium (MPM)

Solution 1 (100 ml)	600.0 mg	Lactic acid
	100.0 ml	Aqua Bidest
Solution 2 (1000 ml)	1000 ml	TCM 199 (Life Technologies, Germany)
	100.0 mg	L-glutamine
	800.0 mg	$NaHCO_3$
	1400.0 mg	Hepes
	250.0 mg	Pyruvic acid
	1100.0 µl	Gentamycin stock solution
Supplementation (10 ml)	5%	OCS
	50.0 µl	(0.025 U/ml maturation medium) FSH
	50.0 µl	(0.0125 U/ml maturation medium) LH

Swim-up medium for sperm capacitation

Sperm TALP (500 ml)	2900.0 mg	NaCl
	1045.0 mg	$NaHCO_3$
	20.0 mg	$NaH_2PO_2H_2O$
	1190.0 mg	Hepes
	5.0 mg	Phenol red
	1825.0 µl	Na lactate sirup (60%)
	155.0 mg	$Mg_2Cl_2H_2O$
	192.0 mg	$CaCl_2H_2O$
Supplementation (10 ml)	60.0 mg	Bovine serum albumin (BSA)
	500.0 ml	Pyruvate stock

In vitro fertilization medium

Fert TALP (500 ml)	3330.0 mg	NaCl
	117.5 mg	KCl
	1051.5 mg	$NaHCO_3$
	23.5 mg	$NaH_2PO_4H_2O$
	32.5 mg	Penicilline
	5.0 mg	Phenol red
	930.0 mg	Na lactate sirup (60%)
	50.0 mg	$MgCl_2H_2O$
	198.5 mg	$CaCl_2H_2O$
Supplementation (10 ml)	60.0 mg	BSA
	100.0 µl	Pyruvate stock
	250 µl	Heparin stock

In vitro culture medium

Synthetic oviduct fluid (SOF, 500 ml)	31460.0 mg	NaCl
	267.0 mg	KCl
	81.0 mg	KH_2PO_4
	123.9 mg	$CaCl_2H_2O$
	48.3 mg	$MgCl_2H_2O$
	1053.0 mg	$NaHCO_3$
	0.7 mg	Phenol red
	181.5 mg	Pyruvate
	2500.0 mg	L-glutamine stock
	235.3 µl	Na lactate syrup (60%)
Supplementation (10 ml)	400 µl	BME amino acid solution
	100 µl	MEM aminoacid solution
	5.0 %	OCS

1.7. Vitrification media and solutions for bovine embryos

Equilibration solutions

Basic solution (BS)
(4 ml)

	Amount	Component	Supplier
	3200 µl	20 mM Hepes 199 Earle's	Biochrom AG
	800 µl	Fetal calf serum (FCS)	

Equilibration solution 1
(ES1) (4 ml)

	Amount	Component	Supplier
	2600 µl	20 mM Hepes 199 Earle's	Biochrom AG
	800 µl	FCS	
	300 µl	EG	
	300 µl	Dimethyl sulfoxide (DMSO)	

Equilibration solution 2
(ES2) (4 ml)

	Amount	Component	Supplier
	2600 µl	20 mM Hepes 199 Earle's	Biochrom AG
	800 µl	FCS	
	300 µl	EG	
	300 µl	DMSO	
	342.3 mg	Sucrose	

Equilibration solution 3
(ES3) (4 ml)

	Amount	Component	Supplier
	2600 µl	20 mM Hepes 199 Earle's	Biochrom AG
	800 µl	FCS	
	300 µl	EG	
	300 µl	DMSO	
	684.6 mg	Sucrose	

Vitrification solution (VS)
(4 ml)

2000 µl	20 mM Hepes 199 Earle's	Biochrom AG
800 µl	FCS	
600 µl	EG	
600 µl	DMSO	
684.6 mg	Sucrose	

Thawing solution (TS)
(4 ml)

3840 µl	20 mM Hepes 199 Earle's	Biochrom AG
160 µl	FCS	
1095.36 mg	Sucrose	

Dilution Solution 1 (DS1)

(4 ml)

3600 µl	20 mM Hepes 199 Earle's	Biochrom AG
400 µl	FCS	
684 mg	Sucrose	

Dilution Solution 2

(DS2)

(4 ml)

3400 µl	20 mM Hepes 199 Earle's	Biochrom AG
600 µl	FCS	
342 mg	Sucrose	

Washing Solution 1 (WS1)

(4 ml)

3200 µl	20 mM Hepes 199 Earle's	Biochrom AG
800 µl	FCS	

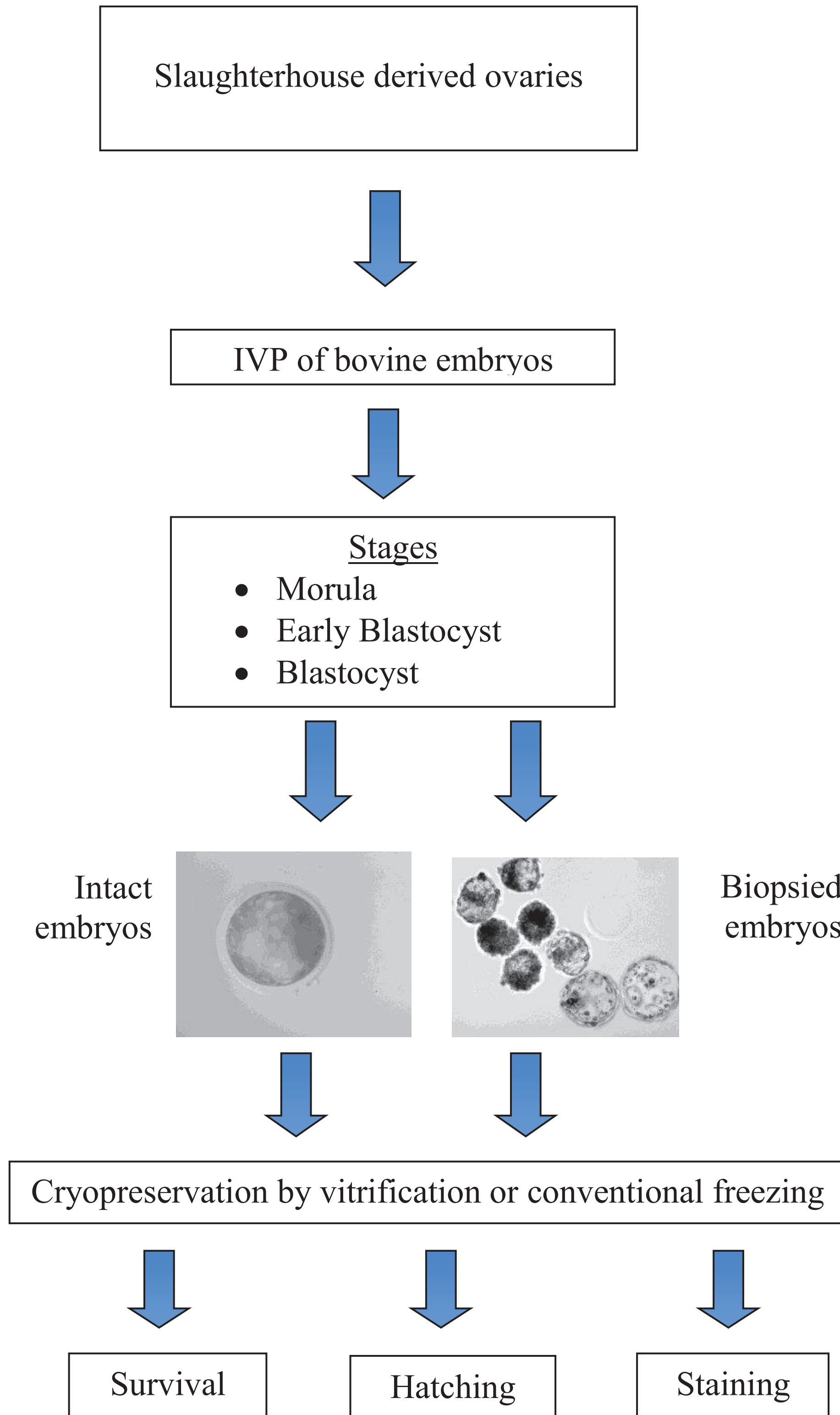
Washing Solution 2 (WS2)

(4 ml)

3200 µl	20 mM Hepes 199 Earle's	Biochrom AG
800 µl	FCS	

2. Methods

2.1. Experimental design



2.2. IVP of bovine embryos

2.2.1. COCs recovering from slaughterhouse derived ovaries

Ovaries were collected from the slaughterhouse in Munich and were transported to the laboratory in phosphate buffered saline (PBS) at 25°C. Once in the laboratory, ovaries were rinsed with fresh PBS at 25°C. Ovaries presenting cysts on the surface were discarded. Follicles with a diameter greater than 2 mm (millimetres) were aspirated with a vacuum pump (80-100 mm Hg) and follicular fluid was collected in a Falcon tube. By transferring the sediment at the bottom of the Falcon tube into a Petri dish, COCs were recovered. Only class I and II COCs, according to Berg and Brem (1989) were used for the experiments. Class I oocytes presented more than 5 layers of cumulus cells, with homogeneous colour and non-granular cytoplasm. COC class II presented 3-5 complete layers of cumulus cells, with homogenous colour of their cytoplasm.

Once selected, COCs were washed three times in MPM (modified Parker's medium) and cultured in four-well dishes with 400 µl of MPM in groups of 40 COCs each. IVM time was 22 h at 39°C with 5% CO_2 in air at maximum humidity.

2.2.2. In vitro fertilization

In order to perform IVF, oocytes were washed three times in Fert-TALP and consequently transferred to four-well plates in groups of 40 COCs. Each well of the four-well plate was previously loaded with 400 µl of Fert-TALP solution and was allowed to equilibrate for an hour at 39ºC with 5% CO_2 in air at maximum humidity.

For sperm capacitation, semen from a bull of known high fertility stored in LN_2 at -196ºC in plastic straw was thawed in a water bath at 39ªC for 10 seconds. Following this, semen and diluent were recovered, and 100 µl immediately layered on the bottom of each of the four centrifugation tubes containing 1 ml Fert-TALP medium each. The four centrifugation tubes previously described were allowed to equilibrate at 39ºC with 5% CO_2 for 1.5 h. After incubation time, the supernatant fraction was taken from all four centrifugation tubes and submitted to centrifugation at 1800 rpm for 10 minutes at 28ºC. After centrifugation, the supernatant fraction was carefully removed, and the remaining approximately 50 µl of pellet were diluted with Fert-TALP medium. Estimation of sperm

concentration was determined by counting three replicates of two sperm dilutions in a Neubauer improved counting chamber. This procedure was standardized and for every experiment. Each well containing 40 COCs received a concentration of 1 x 10^6 sperms/ml of Fert-TALP medium. Finally, matured oocytes and sperm were co-incubated for 18 h at 39ºC in an atmosphere of 5% CO_2 in air at maximum humidity.

2.2.3. In vitro culture

For IVC, synthetic oviduct fluid supplemented with essential and non-essential amino acids (SOFaa) medium was used. Following this, presumptive zygotes were recovered after 18 h of IVF, transferred into 1 ml SOFaa, and mechanically denuded by vortexing for 3 minutes. Afterwards, the presumptive zygotes were recovered and washed three times in SOFaa medium and transferred into four-well dishes, each well containing 400 µl of SOFaa medium covered with 400 µl mineral oil. Each well contained groups of 40 presumptive zygotes. Presumptive zygotes presenting grey-coloured non-homogeneous or granulated cytoplasm were discarded. Presumptive zygotes were cultured in the incubator at 39ºC, in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. IVC was performed for twelve days (time of IVF = 0 h).

2.3. Embryo evaluation

In order to be able to compare the results of the experiments performed in this study with those published by other authors, the same evaluation system regarding quality and stage of development as described in the IETS Manual (Chapter 9 and illustrated in Appendix D, 4th Edition IETS Manual, 2009) was used (see Table 4. and Figure 2.). The time point at which the sperms were added to the 4-well dishes containing the in vitro-matured oocytes was defined as 0 h. The embryos' cleavage rate was assessed 48-72 h after IVF and recorded. No additional information about early cleavage events from embryos was generated, since the main purpose of the experiments was the production of embryos for cryopreservation. An additional observation of embryos was performed on days 5, 6, 7, 8, and 9 after IVF, and stage and day of development were recorded. Good (grade I) and fair (grade II) quality embryos at the morula, early blastocyst, or blastocyst stage were selected for cryopreservation.

Regarding IVP sessions, some experiments were only performed in order to obtain results for a control group (neither cryopreservation nor biopsy treatment was performed). Since some experiments provided a limited number of embryos, all of those that had developed up to the morula, early blastocyst, or blastocyst stage were subjected to cryopreservation experiments. Finally, in some experiments, good and fair quality embryos were selected for cryopreservation, while the rest of the embryos (control embryos, non-treated) were cultured until day 12.

Table 6. Morphological criteria for embryo quality grading according IETS recommendations (IETS Manual, 4th Edition, 2009)

Grade I	Excellent or good	Spherical, with a minimum of 85% of their cells intact, homogenously coloured and viable embryonic mass. ZP with smooth surface
Grade II	Fair	Irregularities in colour, shape or size of the cell mass. At least 50% of the cellular mass viable, embryonic mass viable.
Grade III	Poor	Denotable irregularities in size, embryonic mass, colour and density. Still present 25% of viable embryonic mass.
Grade IV	Dead or degenerating	Non-fertilized oocytes, blocked one-cell embryos or degenerated ones.

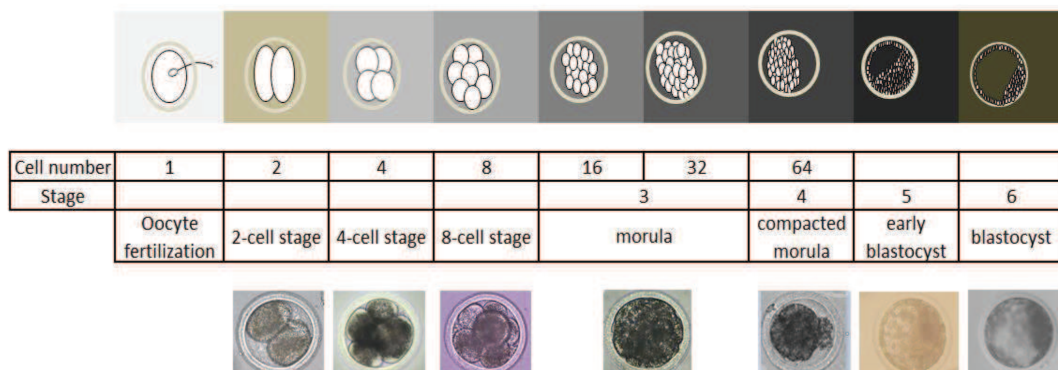


Figure 2. Developmental stages of bovine embryos

Adapted from the 4th Edition of the IETS Manual (2009)

Embryo evaluation was performed at different days of development. The compacted morula stage was defined as the stage in which blastomeres achieve a firm and compact structure. Following this, the early blastocyst stage was characterized by the appearance of the blastocoel cavity on one side of the cells mass, which is relatively easy to differentiate at the beginning, since the rest of the embryo, especially of IVP ones, is black in color. The blastocyst stage was defined as the stage in which the blastocoel cavity expanded, occupying the whole cavity within the ZP. Once the embryo remained expanding, the ZP got thinner as the blastocoel cavity increased in volume. This event symbolized the commencing of the expanded blastocyst stage. The final stage that embryos were allowed to reach was the hatching blastocyst stage, which was represented by the embryo coming out of the ZP. The morula, early blastocyst or blastocyst stages were achieved from day 5 onwards until day 8 of development. Those embryos suitable regarding their quality were submitted to vitrification (either vitrified with intact or opened ZP). Upon selection experiments were performed.

2.4. Vitrification of bovine IVP embryos

The solutions used for either the Hollow Fiber Vitrification Method (HFV) or the CVM were prepared as described by (Beck, Kurome et al. 2013). Hepes (20mM)-buffered tissue culture medium 199 (Biochrom AG, Germany) supplemented with 20% FCS was used as a basal solution. The vitrification solution contained 15% (v/v) DMSO (Sigma, Germany) and 15% (v/v) EG (Sigma, Germany) as permeable cryoprotectants and 0.5 M sucrose (Sigma, Germany) as a non-permeable cryoprotectant.

The morulae, early blastocysts, and blastocysts obtained in 49 experiments at day 4-6 after IVF (IVF = day 0) were assigned to either the vitrification or non-vitrification group. Embryos assigned to the non-vitrification group were in vitro-cultured until day 12, while the remaining embryos (vitrification group) were cryopreserved either by the HFV method or the CVM. Following this, embryos were vitrified in a first set of experiments by the HFV method as described by Matsunari et al. (2012).

2.4.1. HFV method: vitrification and thawing of embryos

In vitro-cultured embryos were first placed in 4 ml holding medium (base solution) in a 35 mm plastic dish and washed in order to remove the remaining oil from the IVC medium. Subsequently, the embryos were aspirated in a cellulose triacetate hollow fiber (approximately 185 µm inner diameter, 215 µm outer diameter, FB-150FH; Nipro Corporation, Osaka, Japan) attached to a hypodermic needle (Fig. 4A) (length 5 mm; outer diameter 0.15 mm; inner diameter 0.1mm; Medical Planning Corporation, Miyagi, Japan) using a 1ml syringe and a mouth-sucker tube (Fig. 4B; Fig. 5A).

The embryos (up to 15 embryos) were loaded into the hollow fiber in a 3-4 mm column of holding solution with air bubbles on each side (Fig.4C; Fig. 5B). Using a stainless steel dissection forceps, the hollow fiber was detached from the hypodermic needle (Fig.4D) and transferred to the equilibration solution (ES) for 3 min (Fig. 5C). Following this, embryos were transferred into the vitrification solution (VS) at room temperature (approx. 25ºC) using a forceps (Fig.4E). The hollow fiber was kept in VS for 40 seconds and it was gently moved around in the 35 mm dish with the forceps to facilitate contact with permeable CPAs. After 40 seconds in the VS, the hollow fiber containing the embryos was plunged into liquid nitrogen (LN_2) while being held vertically with the forceps (Fig. 4F; Fig. 5D). The hollow fiber was kept in LN_2 for a short period of time (2-3 minutes) or placed in a 0.5 ml plastic straw (Fig. 5E) for long-term storage (up to 6 weeks) in an LN_2 tank.

The volume in which the embryos were loaded was calculated as described by Yavin and Arav (2007) in the following manner:

$$V = L \pi r^2$$

V being the volume contained in the hollow fiber, *L* the length of the hollow fiber, and *r* the radius (approximately 92.5 µm in the case of the hollow fiber). The calculation of the volume containing the embryos resulted in values ranging from 0.05-0.1µl.

In case the hollow fiber was not stored in LN_2 tanks, it was rewarmed by rapidly immersing it in 4 ml thawing solution (TS) containing 1 M sucrose at 38.5ºC for 1 minute in a 35 mm plastic dish in which the rehydration process took place. Afterwards, embryos were transferred into the dilution solution (DS) containing 0.5 M sucrose where the embryos were kept for 3 minutes before being transferred to the washing solution (WS) where they remained for 5 minutes each. This was performed as described above (Fig. 4E), with a stainless steel dissection forceps and the embryos being handled as a unit. In the second washing solution, embryos were recovered from the hollow fiber by carefully squeezing the hollow fiber from one end to the other with a second dissection forceps until the embryos were expelled from the hollow fiber (Fig. 4G). Both the dilution solutions and the washing solution were kept at room temperature. Once the embryos were recovered they were transferred into an IVC medium in 4-well plates.

The long-term stored embryos were recovered as follows. Holding the plastic-straw above the liquid nitrogen, a self-made shortened plunger of an AI gun was inserted on the opposite side of the plastic straw containing the hollow fiber on the inside. Following this, the plastic straw was rapidly transferred into the thawing solution and the side containing the hollow fiber was immersed in it. Then, the plunger pushed the plastic straw's cotton-plug (Fig. 5G) from the opposite direction, so that the hollow fiber was recovered (Fig. 5F) and immersed into the thawing solution. The hollow fiber containing the embryos was then transferred into the DS and finally into the WS with a stainless steel dissection forceps as previously described.

Vitrification medium (Cow) 4 ml					
		VITRIFICATION SOLUTION			
	BS	ES1	ES2	ES3	VIT SOL
Medium	20 mM Hepes TCM-199 solution with 20% calf serum	calf serum 800 µL DMSO+EG 600 µL TCM-199 2600 µL	calf serum 800 µL DMSO+EG 600 µL TCM-199 1600 µL **1M** SUC TCM-199 1000 µL	calf serum 800 µL DMSO+EG 600 µL TCM-199 600 µL **1M** SUC TCM-199 2000 µL	calf serum 800 µL DMSO+EG 1200µL **1M** SUC TCM-199 2000µL
T°, exposure time		RT, 60 sec	RT, 60 sec	RT, 60 sec	RT, 40 sec
		20 mM TCM-199 20% CS (BS) + 7,5% EG +7,5% DMSO	20 mM TCM-199 20% CS (BS) +7,5% EG +7,5% DMSO + 0,25M sucrose	20 mM TCM-199 20% CS (BS) +7,5% EG +7,5% DMSO + 0,5M sucrose	20% calf serum +15% EG +15% DMSO + 0,5M sucrose
		THAWING SOLUTION			
	TS	DS1	DS2	WS1	WS2
Medium	calf serum 160 µL TCM-199 640 µL 1,25M SUC TCM-199 3200 µL	calf serum 400 µL TCM-199 1600µL 1M SUC TCM-199 2000µL	calf serum 600 µL TCM-199 2400µL 1M SUC TCM-199 1000µL	calf serum 800 µL TCM-199 3200µL	calf serum 800 µL TCM-199 3200µL
T°, exposure time	38.5°C, 60 sec	RT, 3 min	RT, 3 min	RT, 5 min	RT, 5 min
	BS + 1M SUC	BS + 0,5M SUC	BS + 0,25M SUC	BS	BS

Figure 3. Solutions used for vitrification and thawing

DMSO = dimethyl sulfoxide; EG = ethylene glycol; SUC = sucrose; RT = room temperature.

Vitrification. BS = basic solution; ES1 = equilibration solution 1; ES2 = equilibration solution 2; ES3 = equilibration solution 3; VS = vitrification solution

Thawing. Thawing solution (TS); DS1 = dilution solution 1; DS2 = dilution solution 2; WS1 = washing solution 1; WS2 = washing solution 2.

Adapted from *Kuwayama, Vajta et al. (2005)*, *Maehara, Matsunari et al. (2012), Beck, Kurome et al. (2013)*

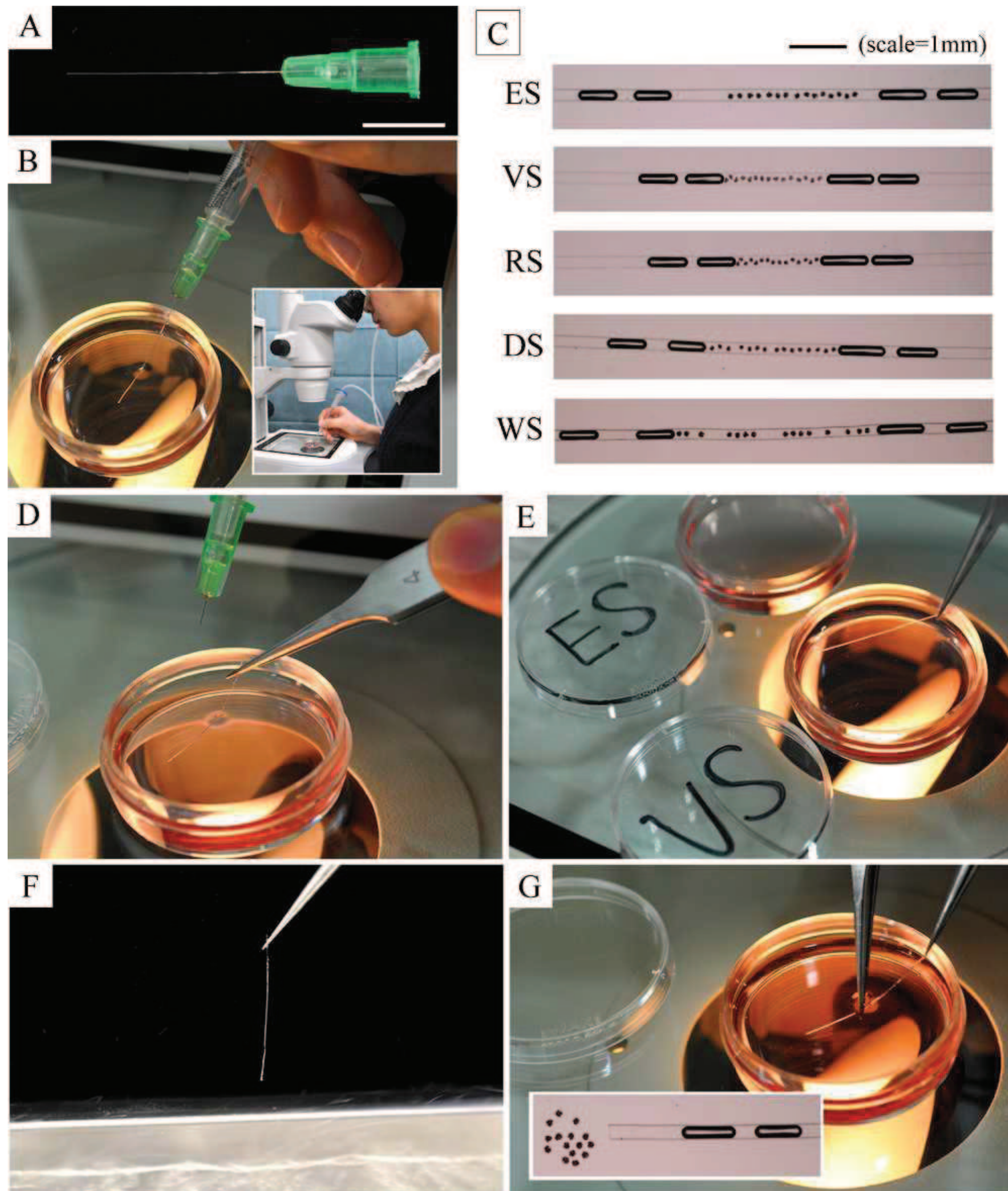


Figure 4. Vitrification and re-warming steps of porcine IVM/IVF-derived morulae with the HFV Method

Maehara, Matsunari et al. (2012) with kind permission of the Society for the Study of Reproduction, Inc.

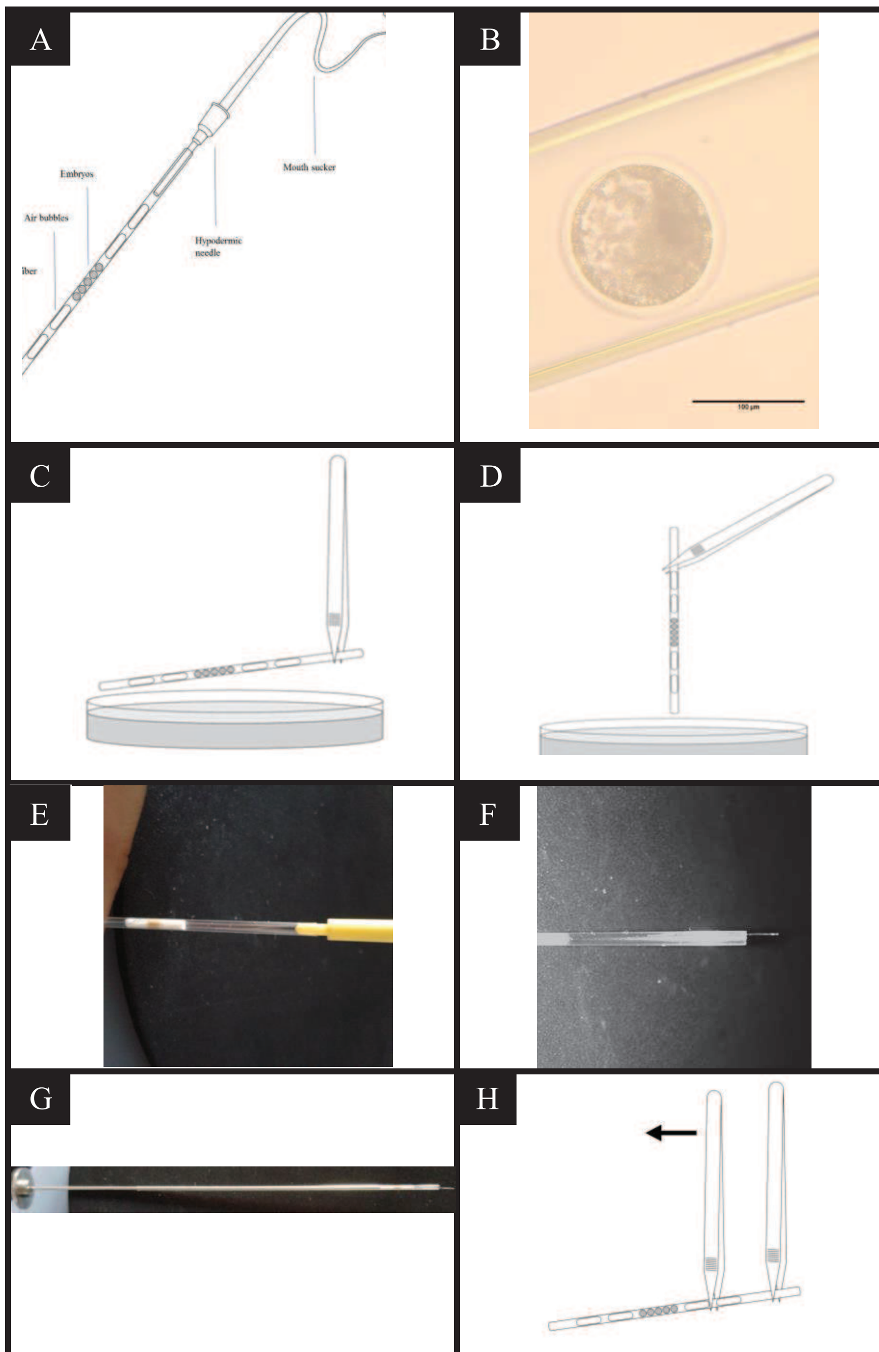


Figure 5. Vitrification and in-straw hollow fiber's storage

Adapted from Maehara, Matsunari et al. (2012) and Beck, Kurome et al. (2013)

2.4.2. CVM method: Vitrification and thawing of embryos

Embryos vitrified with CVM were handled in the same way as those vitrified with the HFV method during the washing and equilibration steps. Instead of a 35 mm Petri dish, vitrification solution was prepared and embryos were handled in a 4-well plate, each one containing 400 µl of the corresponding solution for every step. The CVM® Vitrification Kit was used to perform vitrification (see Figure 6).

Once embryos had been exposed to the vitrification solution for 40 seconds, they were loaded (up to 5 embryos/demi-embryos) with 0.7-1 µl in the fibreplug by gently touching the ring's fibreplug (see Figure 8. A-B). Subsequently, once the small droplet had gotten in contact with the ring the droplet-embryo unit remains attached to it due to surface tension. Finally, the fibreplug containing the sample was rapidly moved into the CryoBath containing the pre-cooled metal-block (see Figure 8. C) in order to carefully let it touch the cooled metal-block surface. As a result the droplet vitrified (see Figure 8. D-E). As soon as the droplet had achieved the glassy state (remained approx. 10 seconds in contact with the metal block surface), the fibreplug was inserted into the plastic straw (Kryohalm, Consartic® GmbH, Germany) with the ring facing towards the bottom of the CryoBath. Consequently, each fibreplug was pushed further to the bottom and the protecting plastic straw acting as a container to avoid any direct contact with LN_2 (see Figure 8. F-G) was heat-sealed with a Straw Sealer [(HAS-1 Straw Sealer, Biotherm™, CVM) see Figure 8. H]. Finally, the unit plastic straw-fibreplug was stored in LN_2 tanks for up to 1 week.

For the thawing (re-warming) procedure, each straw-fibreplug unit was quickly transferred from LN_2 tanks into the CVCup containing LN_2 (see Figure 8. I) and the plastic straw was cut with a cutting-plier (see Figure 8. J) in order to recover the fibreplug. Then, the fibreplug was pulled out and rapidly transferred to a 4-well plate containing 400 µl of thawing solution at 38.5 ºC; thus, the fibreplug was immersed into the thawing solution (TS) and embryos were recovered and allowed to remain in TS for1 minute. Consequently, embryos were transferred into dilution (DS1 and DS2) and washing solution (WS1 and WS2) similar to the HFV method.

Finally, after thawing of the embryos vitrified by HFV and CVM, they were either in vitro-cultured in SOF medium under normal IVC conditions until day 12 or stained. Observation of thawed embryos took place during the 24-48 h post-thawing and results were recorded. Moreover, several embryos once re-expanded were selected and stained for live-dead cell counting.

Figure 6. CryoLogic Vitrification Method

CVM^{TM} Vitrification Kit. CryoLogic®

Picture from CryoLogic's website

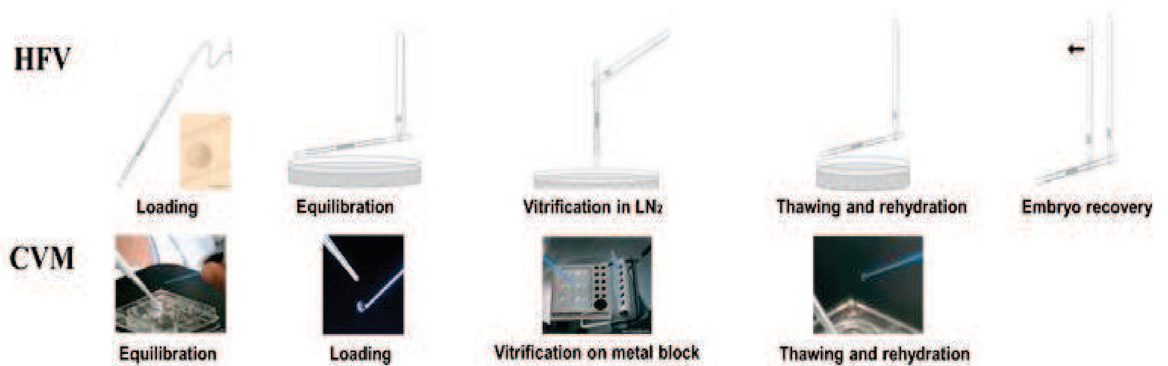


Figure 7. Embryo handling and steps towards vitrification with the CVM and HFV Method. HFV. Hollow Fiber Vitrification Method.

Adapted from Beck, Kurome et al. (2013)

CVM. CryoLogic Vitrification Method. CVM^{TM} Vitrification Kit. CryoLogic®

Pictures from CryoLogic's website

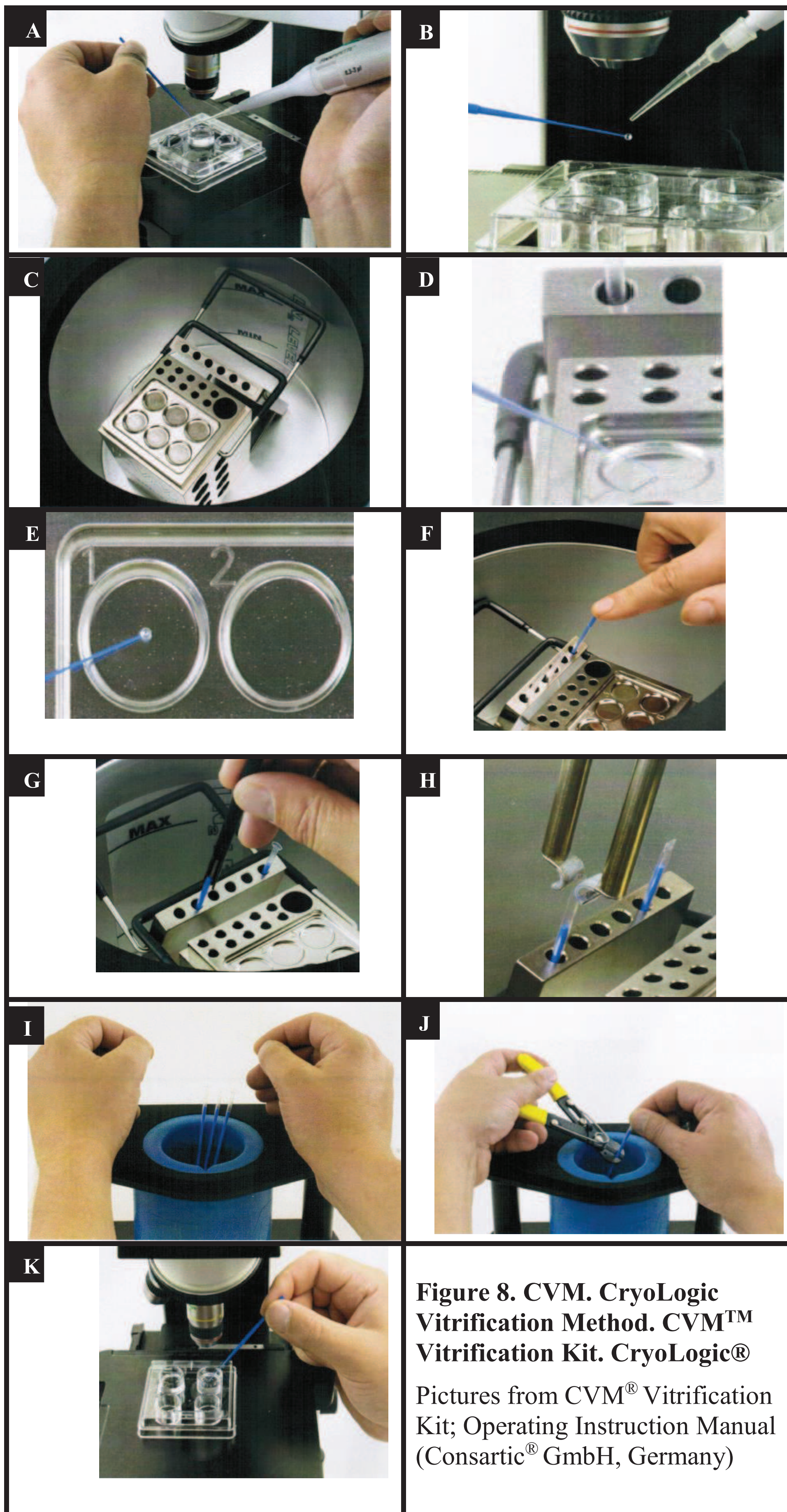


Figure 8. CVM. CryoLogic Vitrification Method. CVM™ Vitrification Kit. CryoLogic®

Pictures from CVM® Vitrification Kit; Operating Instruction Manual (Consartic® GmbH, Germany)

2.4.3. Conventional freezing of bovine embryos

For comparison, bovine intact and biopsied in vitro produced embryos were cryopreserved with a standard method for slow freezing. Embryos, demi embryos and biopsied embryos were calibrated in 1.5 mMol ethylene glycol (EG) and aspirated in embryo transfer straws for direct transfer. After 12-15 minutes equilibration, freezing started at -6°C. Seeding was done after 4 minutes and freezing curve started 6 minutes later with a rate of 0.5 °C/minute. At -32°C, straws were placed in liquid nitrogen for storage. Three experiments were done.

In experiment 1, embryos of excellent quality were splitted (Figure 9.) and one-half was vitrified and the other half slowly frozen in EG.

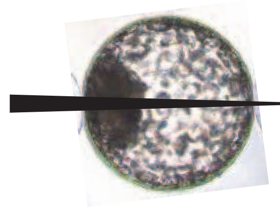


Figure 9. Splitting of in vitro produced embryos

In a second experiment in vitro produced embryos were biopsied (small piece of the trophoblast; Figure 10.) and frozen direct, 3 h and 24 h after treatment.

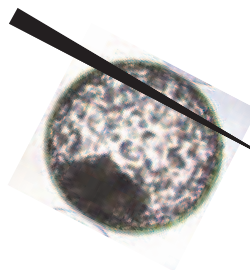


Figure 10. Biopsy of in vitro produced embryos

In a third experiment, on day seven grade 1 in vitro produced blastocysts were frozen in LN2 using a conventional protocol in 1.5 M EG. Thawed embryos (5 sec in air, 15 sec at 26°C water bath) were splitted in PBS+BSA with a steel micro blade into two equal sized-parts (M1: method 1=½+½; n=21, replicates=2) or into two unequal sized-parts M2: Method 2=½+⅓ and a biopsy part (⅙); n=25, replicates=2). Controls consisted of intact frozen-thawed blastocysts (n=37,

replicates=4), non-frozen intact (n=64, replicates=4) and non-frozen splitted embryos (M1fresh) (n=21, replicates=2). Embryo developmental rates were evaluated after 48 h of culture in SOF+5% OCS to assess embryo viability.

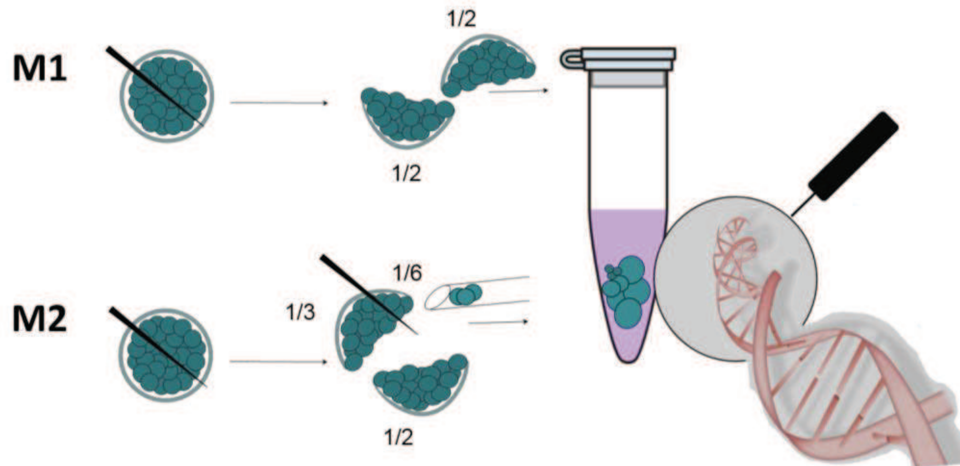


Figure 11. Biopsy methods for frozen–thawed embryos

2.5. Biopsy of IVP embryos

Good (grade I) and fair (grade II) quality embryos were selected either at the morula (stage code: 4), early blastocyst (stage code: 5), or blastocyst (stage code: 6) stage and a biopsy was performed following embryo-splitting.

In short, on days 5-8 IVP embryos at different developmental stages were picked from the IVC and used for the experiments. First, embryos were washed in SOFaa medium (modified SOF supplemented with amino acids) without BSA in a 35 mm plastic dish and embryos were handled at room temperature (approx. 25ºC). The same SOF medium was used for washing and handling all embryos but each stage group of embryo was handled in different 35 mm plastic dishes. Until the embryos were split, they were maintained at 25ºC with a warming plate (Control Unit HT 200, Minitüb, Germany) out of the incubator.

At that moment, one single embryo was loaded into SOFaa medium with a Transferpettor (Brand, Germany) and transferred into another 35 mm plastic dish used for biopsies. This plastic dish contained a 30 µl drop of SOFaa on the left side approximately half-way between the center and the edge of the dish, as the ophthalmic blade of the micromanipulator was presented from the right side. As the SOF medium was BSA-free, the embryo (Figure 12 A) stuck to the plastic surface. Following this, the ophthalmic blade was manipulated with a joystick in order to slowly take it down and be located right above the center of the embryo. By then and once the ophthalmic blade and the embryo got in contact, the embryo started to increase in size and expand due to the pressure on the ZP. By pressing the blade down, the embryo collapsed and moving the blade resulted in the splitting of the embryo (Figure 12. B).The embryo was divided in a way that both demi-embryos remained with ICM and TE. Subsequently, a small number of cells from the demi-embryo's TE were removed (Figure 12. C). As a result, 2 demi-embryos (Figure 12. D) and a sample of cells, referred to as a biopsy were produced (Figure 12. E).

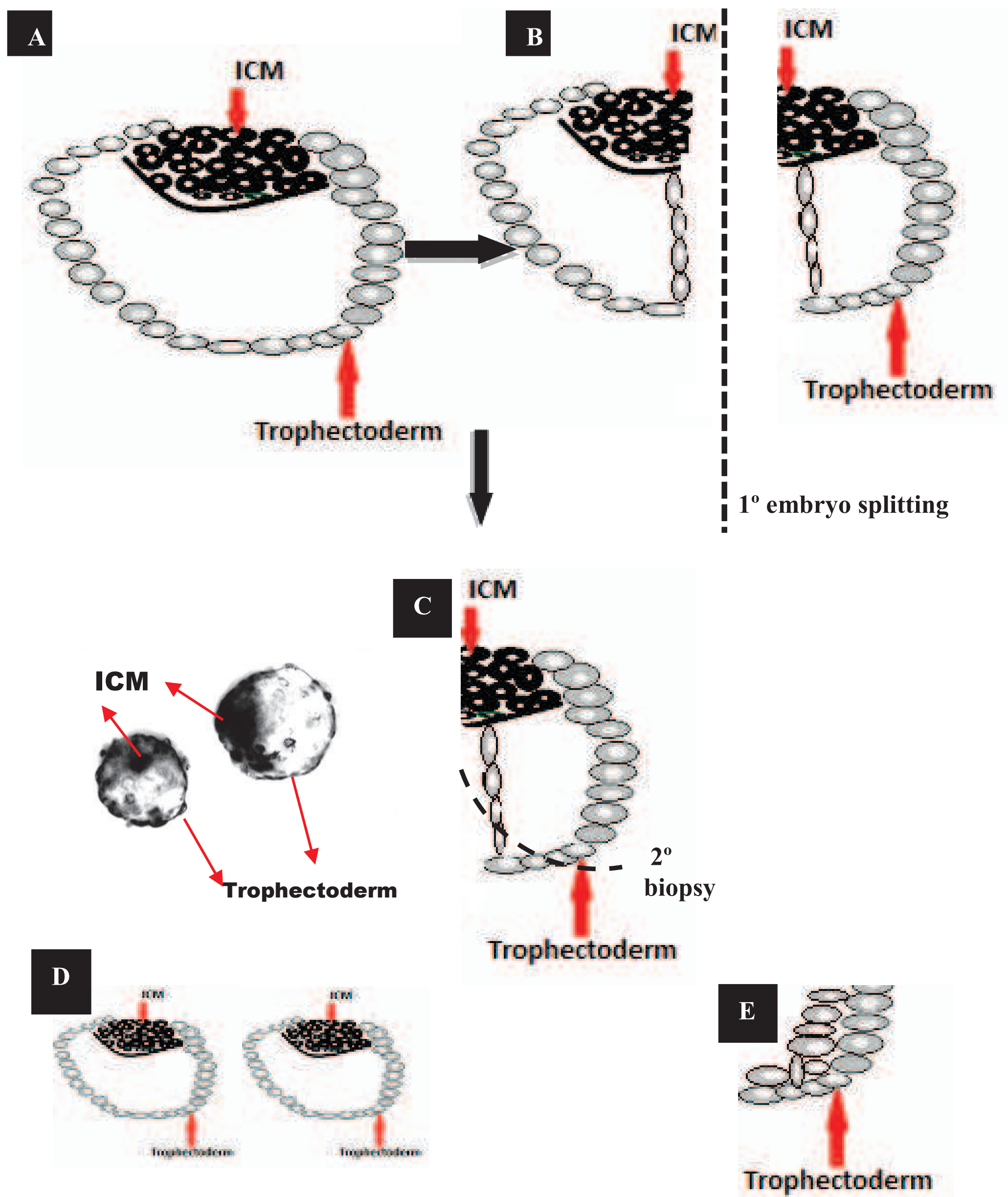


Figure 12. Embryo splitting and biopsy procedure

2.6. Live-dead cell staining

To evaluate the cryopreservation method's effect on embryonic cells, all embryos selected for live-dead cell staining, either coming from the control group (no cryopreservation) or the vitrification group (HFV or CVM) were taken from an IVC 4-well dish and washed three times with SOFaa medium. Live-dead cell staining followed the laboratory's own standards and was performed as described in the study by Beck, Kurome et al. (2013).

In short, hatched embryos derived from the control group (days 8-10 from IVC) were washed in 4 ml PBS-PVP ("washing solution") [(10 mM PBS in 9% NaCl

with 1 mg/ml polyvinyl pyrrolidone (PVP; Sigma, Germany)], adding Triton X-100 at a final concentration of 0.2% (v/v). In a second 35 mm plastic dish containing 1ml PBS-PVP 0.2% Triton X-100 (v/v) solution, Hoechst 33342 dye Stock (5 mg /ml); 4 µl of Hoechst 33342 Stock was added to the solution in order to obtain a final concentration of 25 µg/ml ("Hoechst 33342 solution"). Subsequently, propidium iodide (PI) (Sigma, Germany) was used at a final concentration of 0.2 µg/ml ("PI solution") in a third 35 mm plastic dish containing 1 ml PBS-PVP 0.2% Triton X-100 (v/v) solution. Finally, another 35 mm plastic dish containing 1956 µl of PBS/PVP 0.2% (v/v) Triton X-100, 4 µl of Hoechst 33342 Stock and 40 µl of PI Stock was added ("Hoechst 33342/PI solution"). Hoechst 33342 is a blue-fluorescence dye that binds to the DNA of the cells; while PI is a red-fluorescence dye that only penetrates the membrane of dead cells.

After the first step (embryo washing), the embryo was transferred to the Hoechst 33342 solution for 5 minutes; then, it was moved to the PI solution and remained there for 1 minute. Finally it was transferred into a Hoechst 33342/PI solution and remained there for 15 minutes. All steps took place at room temperature (approx. 25ºC). Thereafter, the embryo was removed from the "Hoechst 33342/PI solution", placed in a 200 µl drop of PBS-PVP (washing solution) in a 35 mm plastic dish covered with mineral-oil and observed under a fluorescence inverted microscope using the same excitation wavelength and barrier filter to visualize both dyes. Thus, PI penetrated the cells that with damages to the cell membrane (appearing red with UV excitation), while Hoechst 33342 entered all cells. However, in cells presenting membrane damage, PI suppressed Hoechst's blue-dye and cells appeared red. Cell-counting of dead (red) and live (blue) cells was made three times for each embryo, moving up and down the fine adjustment knob of the microscope.

3. Statistical analysis

Statistical analyses were performed with the software SPSS Version 20 and with GraphPad Prism Version 5.02; while graphics were made with GraphPad Prism Version 5.02. Moreover, data is presented as Mean Value ± or Standard Error of the Mean (S.E.M.). Differences in survival rate by cryopreservation methods, developmental stage, embryo quality, and effect of biopsy on vitrification outcomes were compared using the Mann-Whitney test and Kruskal-Wallis test. The Pearson survival rate-day of development was used to analyze bivariate correlation. P-values lower than 0.05 were considered significant. If embryos were lost during handling or the vitrification procedure, it was recorded and they were excluded from the statistical analyses. Due to the main goal of the study, i.e. to evaluate cryopreservation methods, low-quality embryos (grade III and grade IV) were excluded from the cryopreservation experiments and were not included in the statistical analysis. Moreover, in the second part of the study where biopsied embryos were vitrified, those severely damaged during the biopsy or not qualifying as grade I (good) or grade II (fair) quality were not vitrified and excluded from the statistical analysis as well.

IV. RESULTS

1. In vitro production of bovine embryos

In total, 6051 presumptive zygotes were cultured during 49 IVP sessions. Developmental capacity represented by the cleavage, blastocyst, and hatching rates are shown in Table 7. Moreover, the best IVP session resulted in 82.5% of zygotes showing cleavage; while the IVP session with the lowest cleavage yielded only 43.5%. With regard to the blastocyst rate, the best IVP led to 39.9%, while the lowest IVP showed just 6.67% of embryos reaching the blastocyst stage.

Table 7. Developmental rates of IVP bovine embryos

IVP (n)	Presumptive zygotes IVC (n)	Cleavage rate (%)	Blastocyst rate (%)	Hatching rate (%)
49	6051	65.70 ± 0.48	23.43 ± 0.38	66.61± 3.21

Mean (± S.E.M.) of embryos' cleavage and blastocyst rate

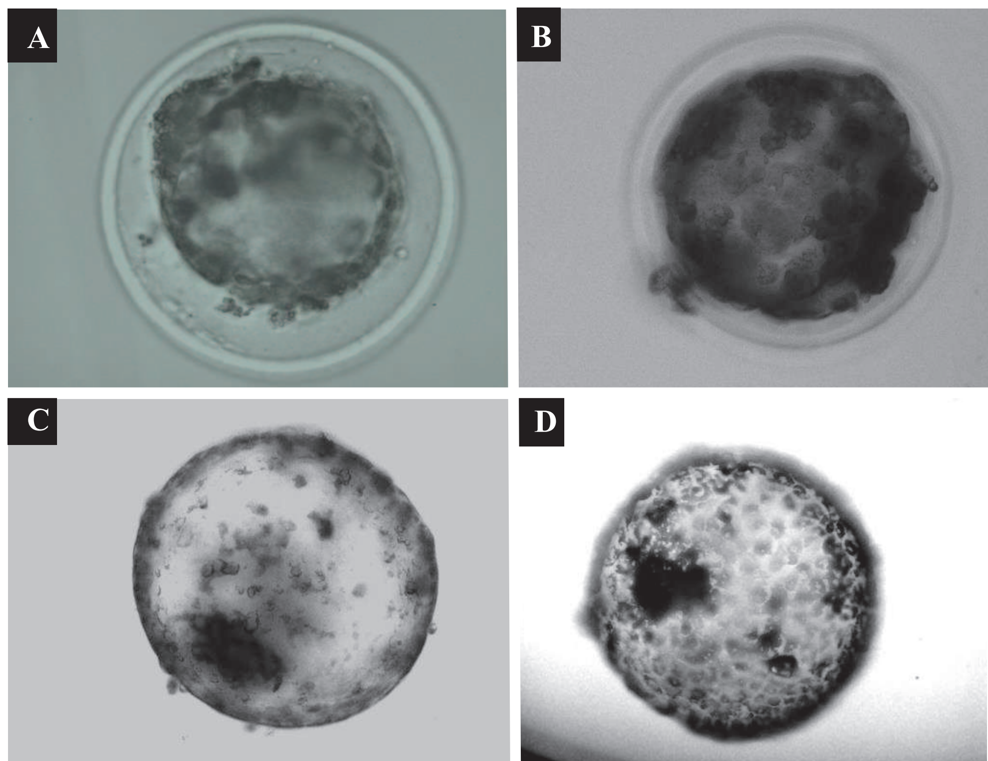


Figure 13. Non-hatched (A-B) and hatched embryos (C-D)

A-B: day 8 control embryos not hatched, showing shrinkage of the blastocoel cavity (A)

B-C: hatched-control embryos

2. Vitrification of IVP embryos

As previously stated, only grade I (good) and grade II (fair) quality embryos were vitrified. In the first part of the study, only embryos with an intact ZP (no biopsy group) were vitrified. In the second part, embryos with an opened ZP were cryopreserved. A simple classification regarding quality and stage of embryos vitrified is presented in Table 8 and Table 9.

Table 8. Vitrification of bovine IVP embryos/demi embryos

Group	Biopsy	Embryos (n)	Vitrified embryos (n)	Non-recovered / lost (%)
HFV -	-	275	273	0.7 %
HFV +	+	55	50	9.0 %
CVM -	-	282	256	9.2 %
CVM +	+	373	312	16.4 %

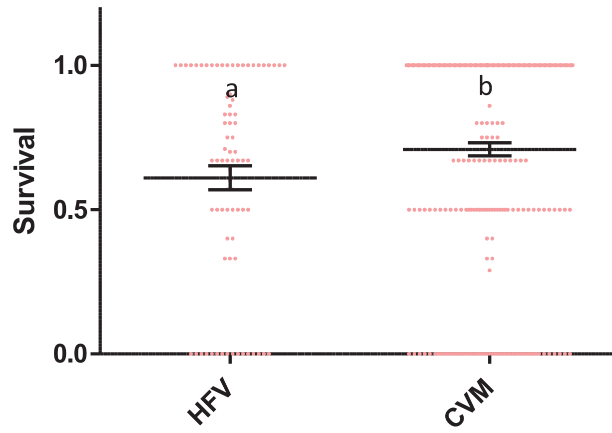
Table 9. Classification of embryos regarding quality

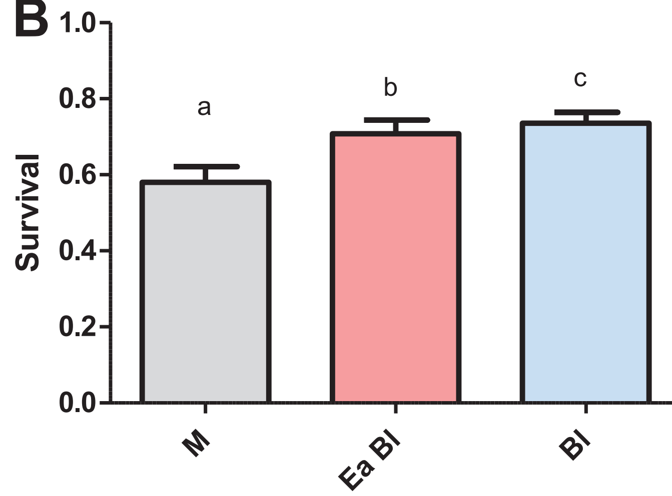
Stage	Biopsy	Grade I (n)	Grade II (n)
Morula	-	104	46
Morula	+	85	8
Early Blastocyst	-	107	38
Early Blastocyst	+	111	11
Blastocyst	-	177	55
Blastocyst	+	140	7
Total		724	165

2.1. Overall results of vitrification

In the following, data on the overall outcomes after vitrification are presented. Overall survival rate outcomes for both vitrification methods, CVM versus HFV, are presented in Figure 14A.

A large variety of outcomes can be seen, ranging from 0% (none of the vitrified embryos survived) to 100% (all of them survived). However, this occurred for both methods. Furthermore, one quarter of the samples showed survival rates of less than or equal to 38.25% and 50%, for HFV and CVM respectively.


A
Survival
1.0
0.5
0.0
a
b
HFV
CVM


B
Survival
1.0
0.8
0.6
0.4
0.2
0.0
a
b
c
M
Ea Bl
Bl

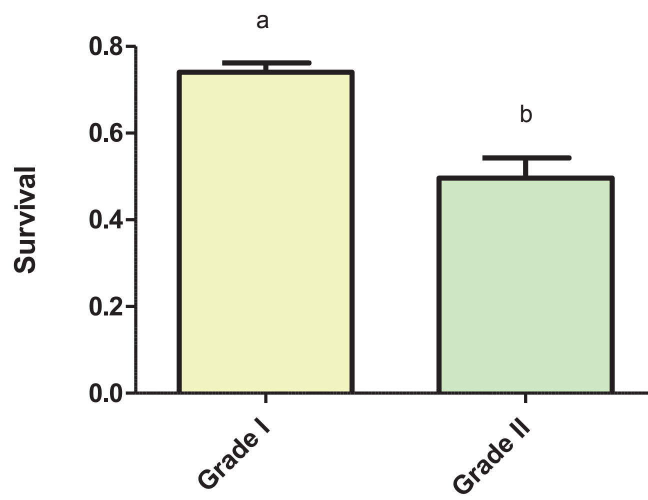

C
Survival
0.8
0.6
0.4
0.2
0.0
a
b
Grade I
Grade II

Figure 14. Vitrification: overall outcomes, influence of method, stage and quality

A: Survival outcomes comparing HFV and CVM methods

B: Survival outcomes regarding embryo stage

C: Survival outcomes regarding embryo quality

Columns without a common superscript letter differed (P < 0.05)

As shown in Figure 14. A, CVM produced significantly higher survival rates than HFV, 70.83% vs. 60.97%, respectively (P=0.005, Mann-Whitney test). Regarding the developmental stage in which embryos were vitrified (Figure 14. B), significantly higher survival rates were achieved when working with blastocysts (B) than with early blastocysts (EaBl) and morula (M) (B=73.55%; EaB=70.77%; M=58.02%) (P=0.004, Kruskall-Wallis test). Moreover, the influence of embryo quality on vitrification outcomes was analyzed as shown in Figure 14. C. Significantly higher survival rates were accomplished with grade I embryos (good) than with grade II (fair); 74.04% vs. 49.64% survived after thawing, for grade I and grade II, respectively (P < 0.0001, Mann-Whitney test).

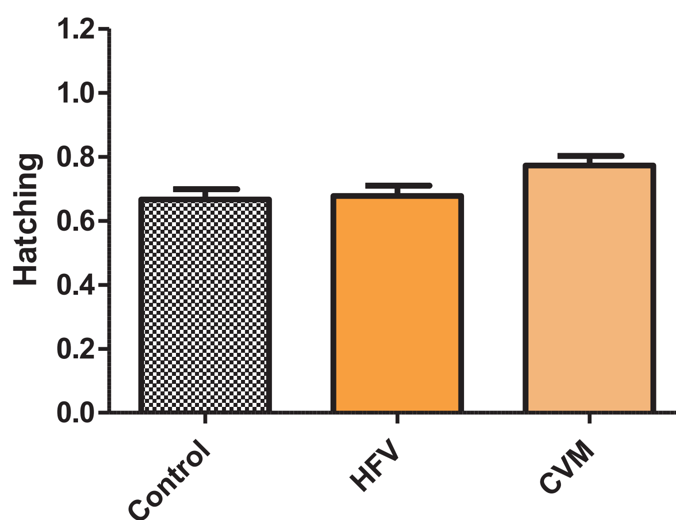


Figure 15. Hatching rate after vitrification of intact embryos

Control= untreated embryos; HFV= embryos vitrified with HFV; CVM= embryos vitrified with CVM

Regarding hatching rates of control embryos, intact embryos vitrified with HFV and intact embryos vitrified with CVM, no significant difference was found (P= 0.0614, Kruskal Wallis test) between the groups.

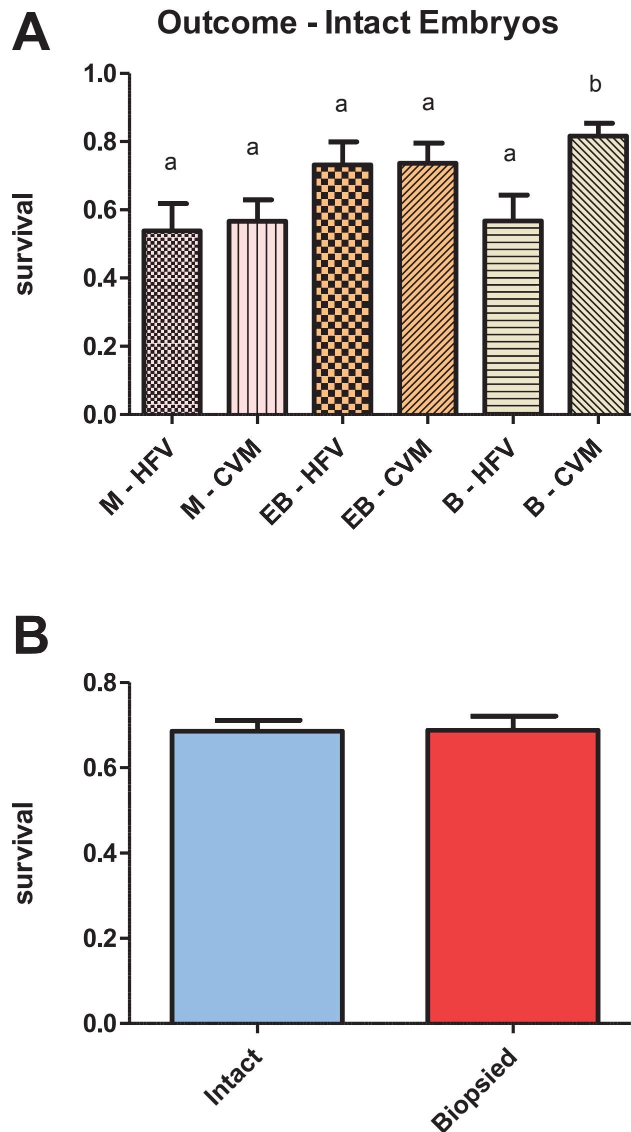


Figure 16. Vitrification outcomes

A: Outcome comparing embryo stage for each vitrification method. M=morula; EB=early blastocyst; B=blastocyst; HFV=Hollow Fiber Vitrification; CVM= Cryologic Vitrification Method

B: Outcome regarding intact or opened (biopsied) ZP. Intact=embryos with intact ZP; Biopsied=embryos biopsied (opened ZP)

Columns without a common superscript letter differed ($P < 0.05$)

In the figures shown above, vitrification outcomes of intact embryos regarding stage for both vitrification methods are compared (Figure 16. A). Significantly higher survival rates were attained with CVM at the blastocyst stage (P=0.0005; Kruskal-Wallis test) as compared to all other groups.

As shown in Figure 16B, opening of the ZP was analyzed. No significant difference was found when working with intact (68.6%) or biopsied (68.8%) embryos (P=0.835; Mann-Whitney test).

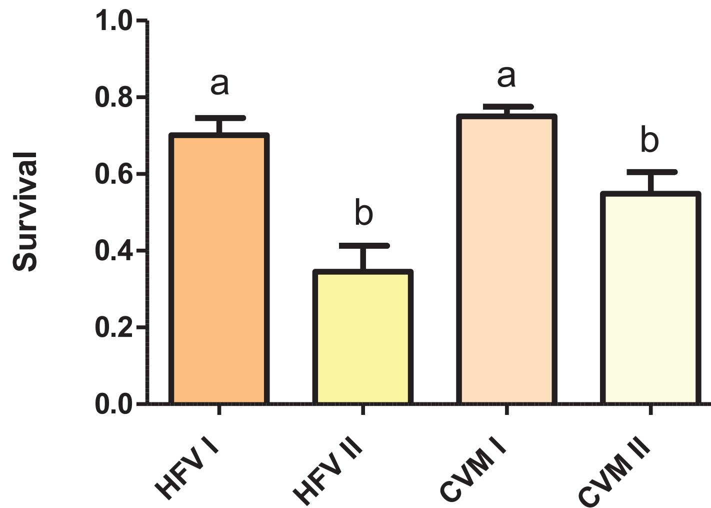


Figure 17. Vitrification outcomes regarding embryo quality

HFV I: Survival outcomes of embryos grade I vitrified with HFV

CVM I: Survival outcomes of embryos grade II vitrified with CVM

HFV II: Survival outcomes of embryos grade II vitrified with HFV

CVM II: Survival outcomes of embryos grade II vitrified with CVM

Columns without a common superscript letter differed (P < 0.05)

In the following, vitrification outcomes of intact and biopsied embryos regarding quality are presented. As previously stated, both HFV and CVM are MVC techniques and one of the main goals of the study was to compare their outcomes. In this regard, embryo quality was pre-assessed and embryos were classified into good (grade I) and fair (grade II) quality. Grade I embryos showed significantly higher survival rates than grade II embryos, regardless of the vitrification method used (P < 0.0001, Kruskal-Wallis test). Moreover, with regard to the vitrification method and survival outcomes, no significant difference was found between HFV and CVM, neither for grade I (HFV I vs. CVM I, 70.1% and 75.1%, respectively; P=0.063, Mann-Whitney test) nor for grade II embryos (HFV II vs. CVM II, 34.6% and 54.8%, respectively; P=0.051, Mann-Whitney test).

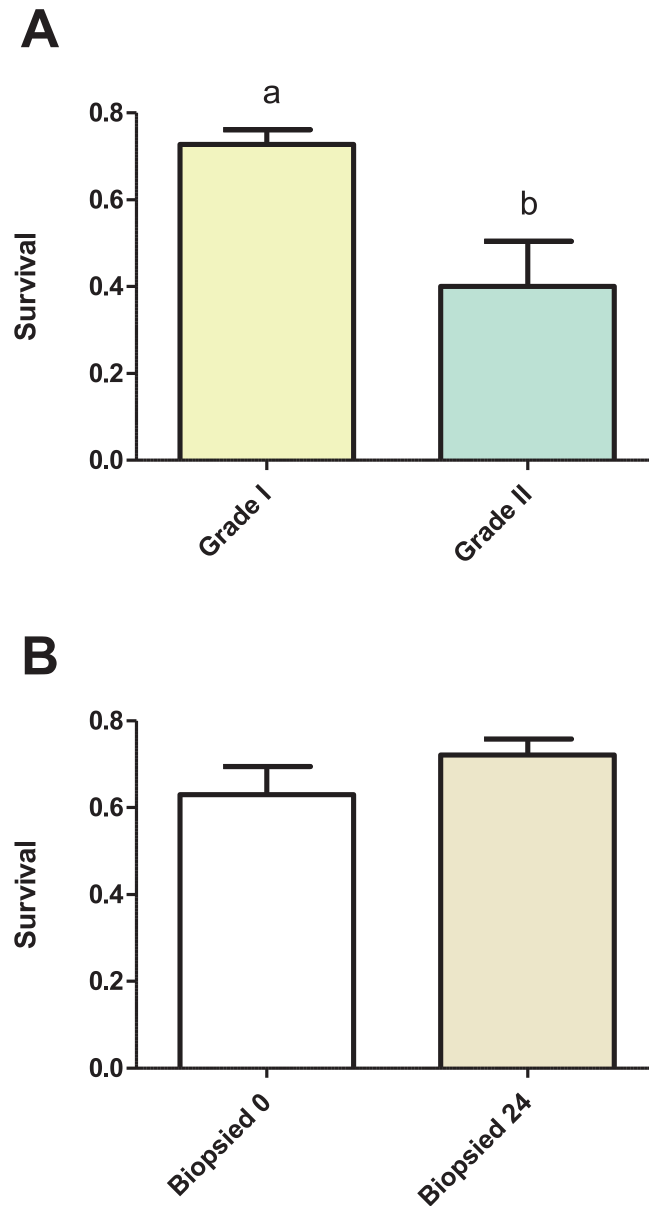


Figure 18. Effect of quality and biopsy's time-point on vitrification outcomes

A: Survival outcomes regarding embryo quality before biopsy

B: Survival outcomes regarding time-point of vitrification (Biopsy 0=approx. 3 hours; or Biopsy 24=approx. 20 h) after biopsy

Columns without a common superscript letter differed ($P < 0.05$)

In the following, the effect of embryo quality before biopsy and the vitrification time-point after biopsy were analyzed. As shown in Figure 18A, significantly higher survival rates were attained with grade I than with grade II embryos (72.8% vs. 40.0%, respectively; P=0.0053, Mann-Whitney test). Moreover, as

shown in Figure 18B, no significant difference was found regarding the vitrification time-point after biopsy (63.0% vs. 72.1%, for 3 h and 20 h respectively; $P=0.8075$, Mann-Whitney test).

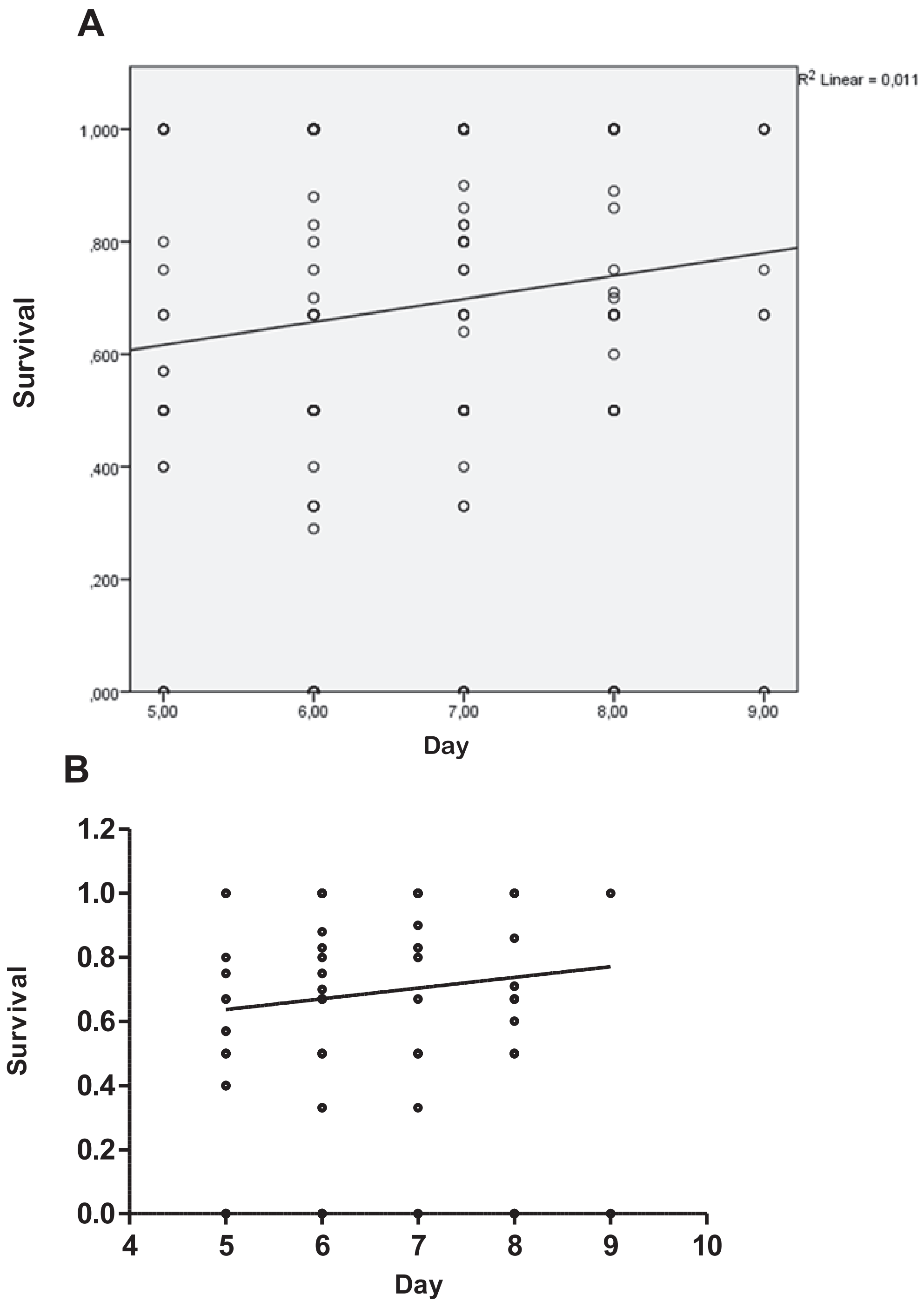


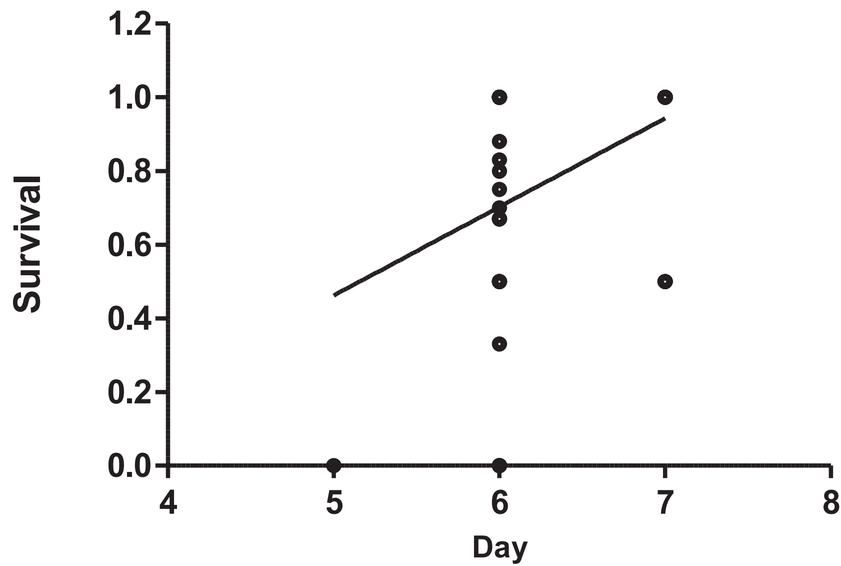
Figure 19. Correlation between day of development and survival rate of intact embryos

A: Survival outcomes of intact and biopsied embryos regarding developmental day

B: Survival outcomes of intact embryos regarding developmental day

As shown in Figure 19A, the correlation between developmental day (as a quality parameter) and survival rate was evaluated. In this analysis, intact and biopsied embryos were included. Although no significant difference was found (P=0.051), a very weak positive correlation exists (Pearson correlation's value = 0.103; R^2 = 0.011).

Moreover, in order to avoid a biopsy effect on the first analysis, only intact embryos were included in the second evaluation (Figure 19B). No significant difference was found (P = 0.193). As seen in the first group, a very weak positive correlation exists (Pearson's correlation value = 0.08; R^2 = 0.007).



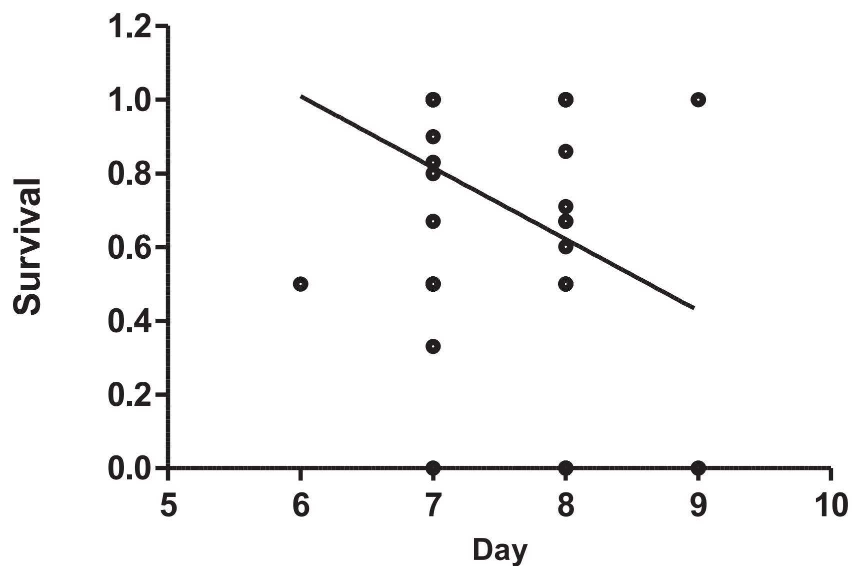


Figure 20. Correlation between day of development and survival rate of early blastocyst and blastocysts

A: Survival outcomes of early blastocysts at different days of development

B: Survival outcomes of blastocysts at different days of development

In these two graphics, the correlation between the embryo's day of development, i.e. early blastocysts (stage 5) and blastocysts (stage 6) reaching these stages at some specific day, and survival rate is presented. Only intact embryos were analyzed. It cannot be concluded that early blastocysts developed earlier showed greater survival rates (Figure 20A), as only a significant positive correlation was found (Pearson's r = 0.31; $R^2 = 0.094$). Contrarily, significantly decreased survival rates were found in embryos reaching the blastocyst stage later as compared to the ones who reached that stage earlier; i.e. slow-developed blastocysts showed poor results after vitrification (Figure 20B). In this group, a significant negative correlation was found (Pearson's r = - 0.31; P = 0.002; $R^2 = 0.095$).

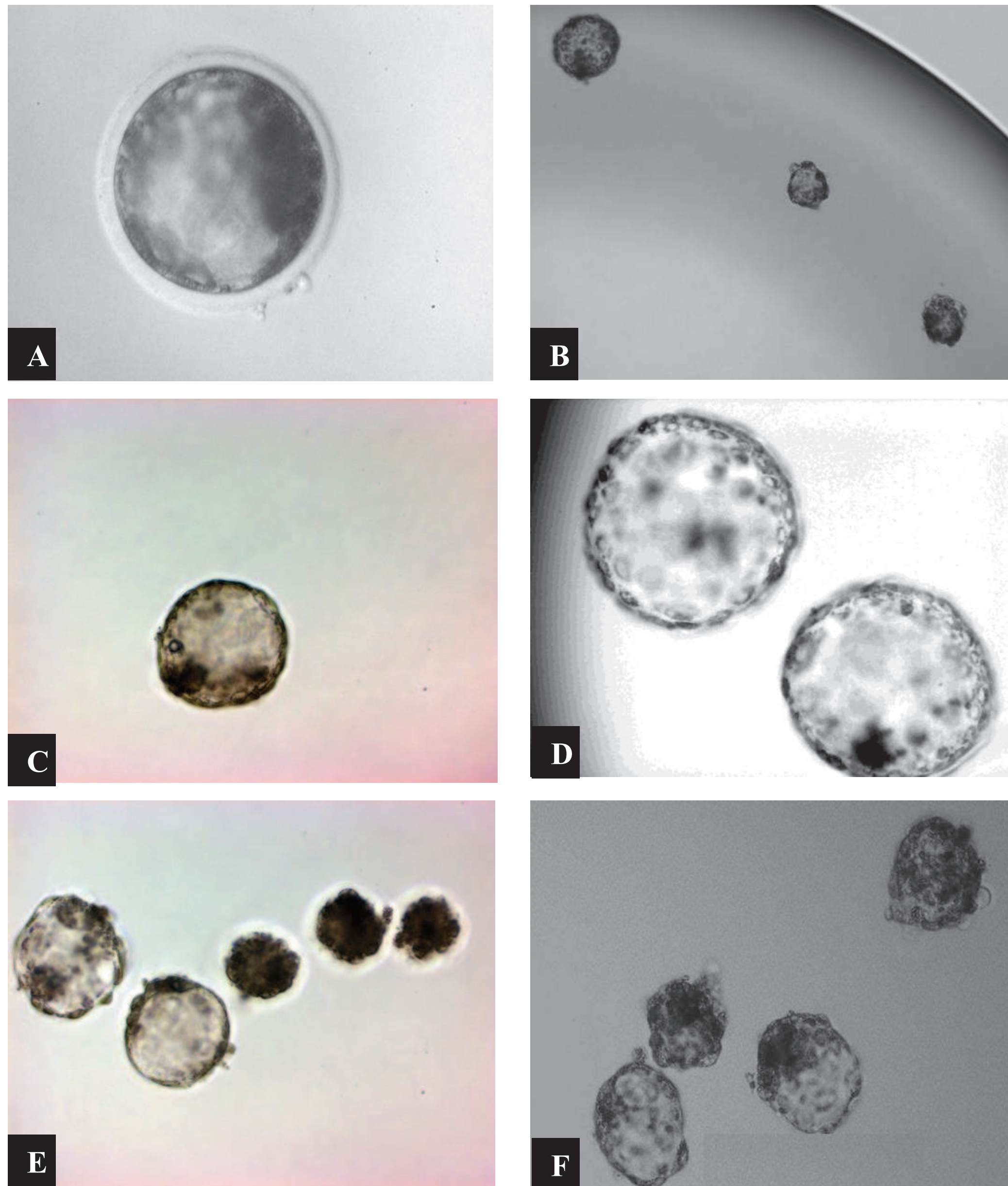


Figure 21. Embryo biopsy and post-thawing assessment

A: Good-quality blastocyst selected for splitting, biopsy, and vitrification

B: Demi-embryos and biopsy produced by splitting technique

C- D: Demi-embryo before vitrification

E: Demi-embryos post-thawing

F: Re-expanded demi-embryos

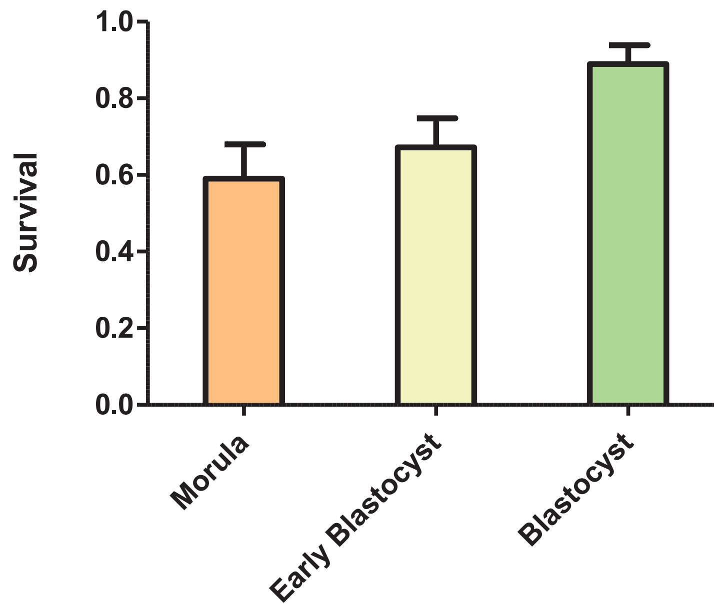


Figure 22. Effect of embryo stage on biopsy outcomes

As shown in Figure 22, demi-embryo survival and the effect of the embryo's stage on biopsy outcomes were evaluated. No significant difference was found between the stages [59.0%, 67.2%, and 90.0%, for morula, early blastocyst and blastocyst, respectively; P=0.0568 Kruskal-Wallis test] with regards to survival after splitting and biopsy. However, it can be seen that blastocysts gave better results as there is a tendency of advanced embryo stages to overcome the splitting and biopsy treatment.

3. Conventional freezing of IVP embryos

3.1. Experiment 1

The comparative conventional freezing and vitrification of demi embryos (N=22) showed no differences in survival after 4 hours of culture (Figure 23.) Both groups resulted in 77.3% re-expanded embryos. After 24 hours in culture, the conventional freezing group showed a slightly less survival rate than the vitrified group (63.6% vs. 72.7%).

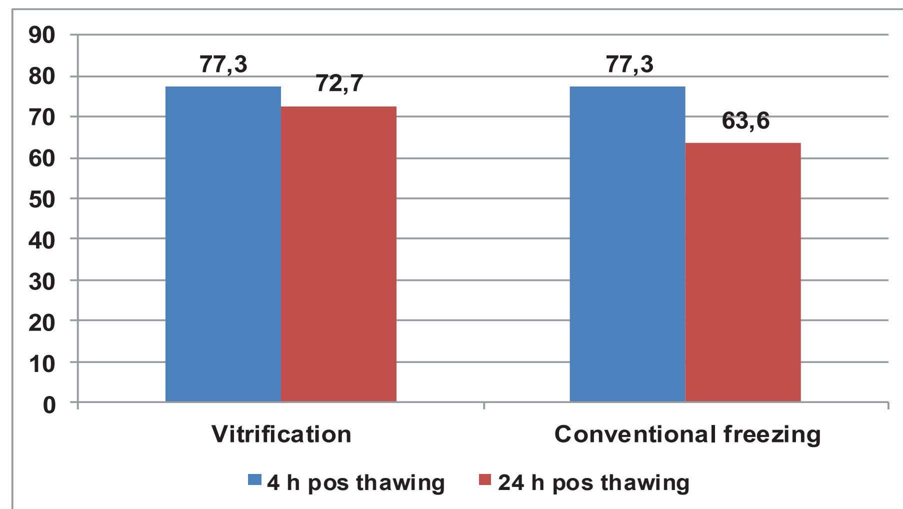


Figure 23. Survival rates of vitrified vs. conventional frozen demi embryos

Method	Survival after 4 h culture (%)	Survival after 24 h culture (%)
Vitrification CVM	77.3	77.3
Conventional freezing	72.7	63.6

3.2. Experiment 2

Results for conventional freezing of in vitro produced untreated embryos (N=27) showed survival rates of 73.3% which was similar to the biopsied groups frozen after 0 (N=29) and 3 (N=29) hours after treatment. Only the embryos frozen after 24 hours (N=29) showed a reduced survival (52.9%). After 24 h of post frozen culture all groups, except the ones frozen after three hours showed a reduced survival rate (76.5% vs. 60.0% vs. 58.8% vs. 52.9%) (Figure 24).

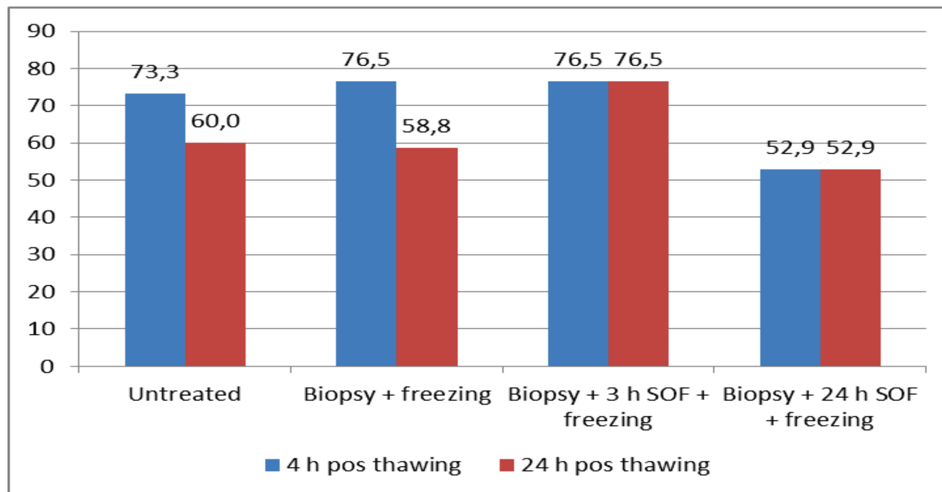


Figure 24. Survival rate of conventional frozen biopsied embryos

3.3. Experiment 3

After conventional freezing/thawing, developmental rates in vitro achieved with only bisected (G1: 66.7% at least one embryo part; 33.3% both parts) or bisected and biopsied embryos (G2) embryos (72% at least one embryo part; 32% both parts) were similar (development of at least one embryo part) or lower (both parts) to the controls (fresh bisected embryos) (81% at least one embryo part; 71.4% both parts). Developmental rates with intact frozen/thawed embryos (64.9%) were lower than non-frozen intact embryos (100%).

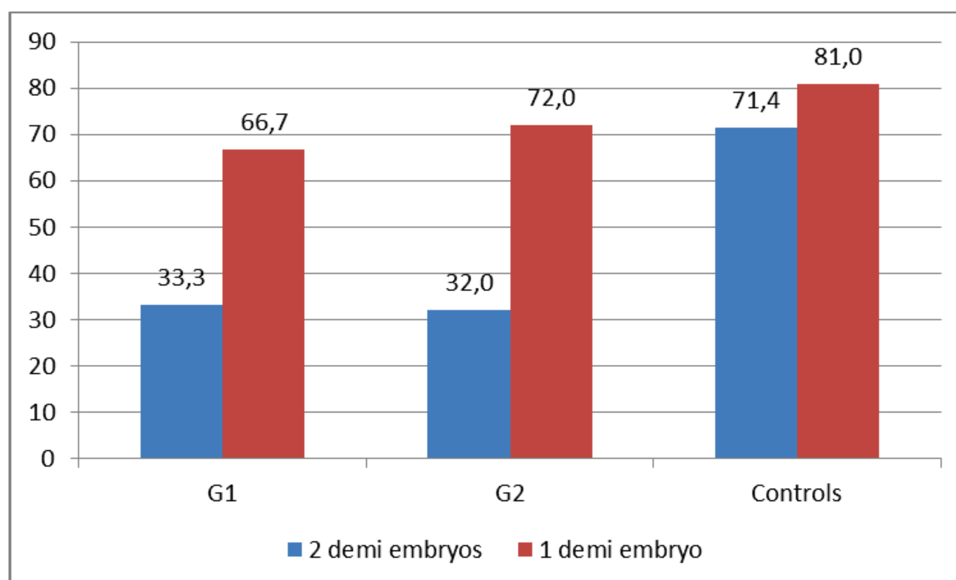


Figure 25. Developmental rates of frozen and fresh splitted embryos

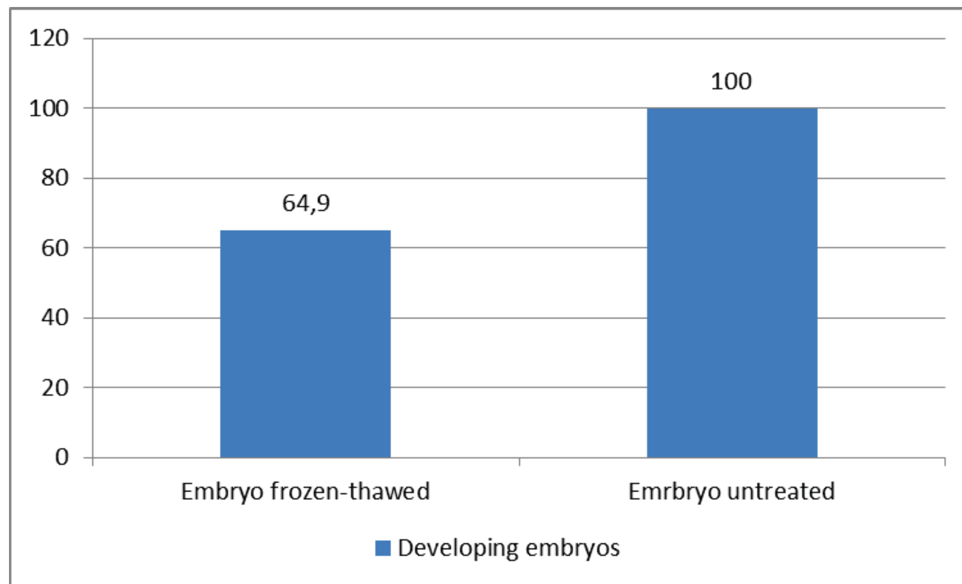


Figure 26. Developmental rates of frozen-thawed and untreated embryos

4. Embryo staining and cell-counting

Embryo staining was performed in randomly picked ones of the control group (non- vitrified), intact (no splitting) and demi-embryos. Vitrified embryos were assessed post-thawing, and several hatched embryos were stained. The vitrified demi-embryos, once re-expanded post-thawing, were also stained.

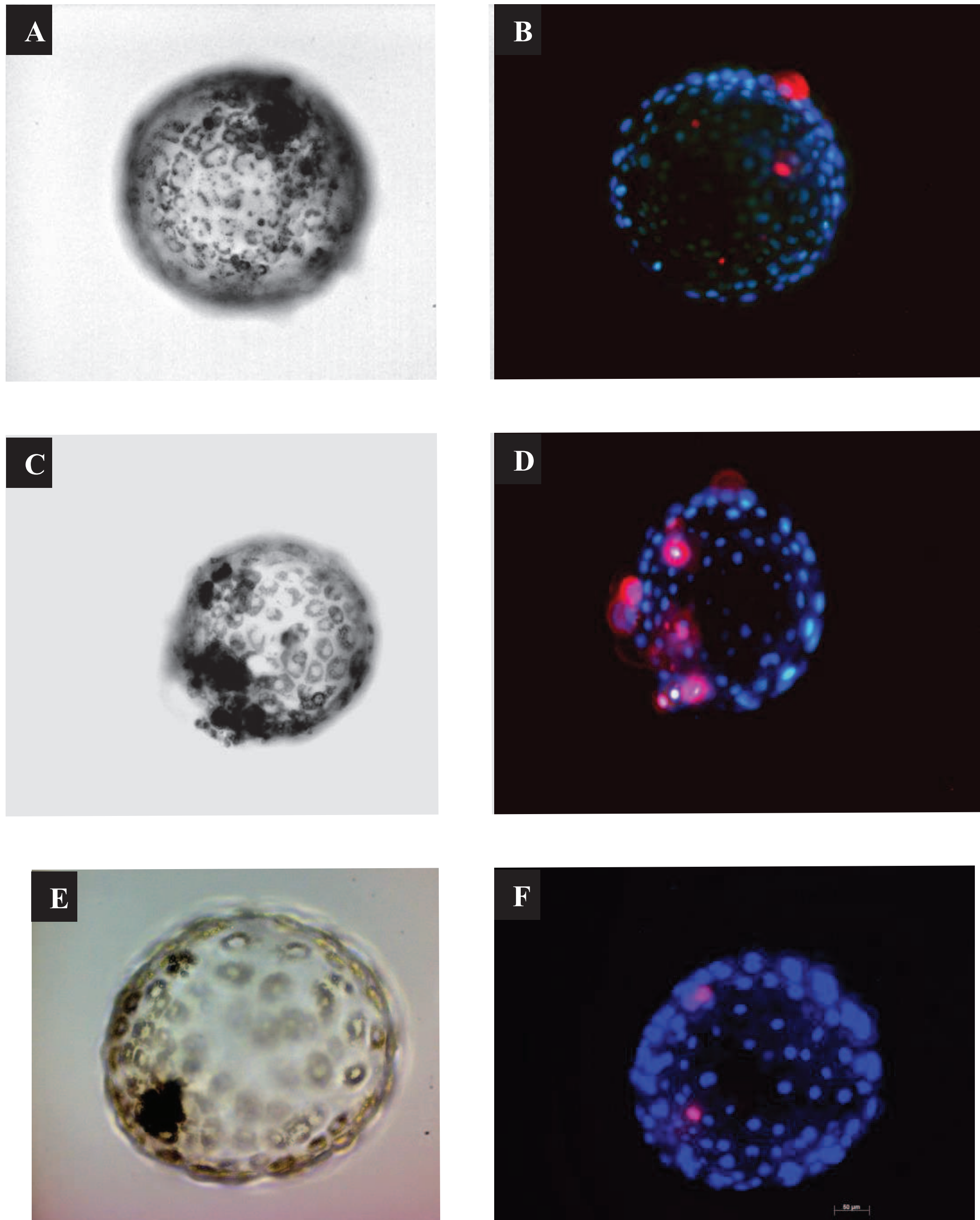


Figure 27. Live-dead cell staining

A-B: non-treated hatched embryo

C-D: thawed demi-embryos

E-F: vitrified embryo after hatching

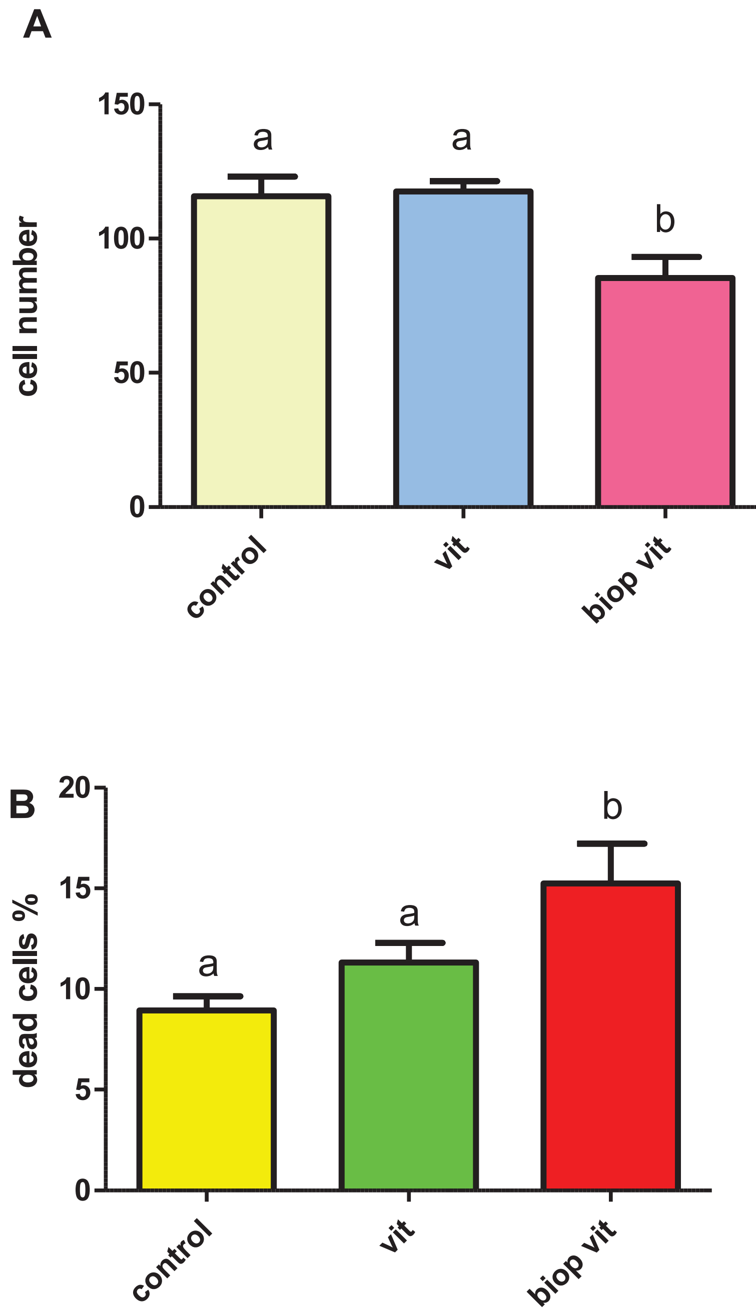


Figure 28. Cell counting and percentage of dead cells after vitrification

A: Cell counting of embryos made under fluorescence microscope

B: Percentage of dead cells

control = untreated embryos; **vit** = intact- vitrified embryos; **biop vit** = biopsied-vitrified embryos

Columns without a common superscript letter differed ($P < 0.05$)

As shown in Figure 28A, there was no significant difference regarding the total number of cells between control hatched embryos which were randomly picked from IVP sessions and intact-vitrified embryos after hatching (115.8 ± 7.3 vs 117.5 ± 3.9, control and intact-vitrified, respectively). However, as expected due to splitting, biopsied demi-embryos showed a significantly decreased cell number (85.3 ± 7.9) as compared to control- and intact-vitrified groups (P=0.0024, Kruskal-Wallis test). Regarding the percentage of dead cells observed in embryos (Figure 28. B), there was no significant difference between control- and intact-vitrified embryos (8.9% vs. 11.3%, control- and intact vitrified embryos respectively). Nevertheless, biopsied demi-embryos showed a significantly greater percentage of dead cells (15.2%) post-thawing (P=0.029, Kruskal-Wallis test) than the other groups.

V. DISCUSSION

1. MVC concept and embryonic intrinsic factors determining vitrification outcomes

To test the suitability of two different vitrification methods, bovine embryos were produced in vitro, and good (grade I) and fair (grade II) quality embryos were submitted to vitrification. The recently developed Hollow Fiber Vitrification Method (HFV) was tested and compared to the already-proved CVM. Accordingly, bovine IVP embryos vitrified with CVM showed significantly higher survival rates as compared to the embryos vitrified with HFV (70.83% vs. 60.97%, P=0.005). However, classification of embryos regarding quality did not bring any significant difference between both vitrification methods, neither for grade I embryos (70.1 % vs. 75.1%, HFV and CVM respectively, P=0.063) nor for grade II (34.55% vs. 54.84%, HFV and CVM respectively, P=0.051). The benefits of the “self-made” HFV Method were already shown by Matsunari et al (2012) who achieved healthy offspring of piglets after in vitro-developed blastocysts’ transfer into a sow from previously vitrified-thawed in vivo-derived morulae. The advantage of HFV lies in the possibility to load a certain amount of embryos in a very small volume, thus maintaining the advantages of MVC concept methods (Maehara, Matsunari et al. 2012). Moreover, embryos loaded into the hollow fiber are managed as a unit which is very important with regard to embryo handling in extremely small volumes of the sample. This can be seen in the results section as both vitrification methods were compared. In this study, a greater percentage of embryos and demi-embryos, lost or non-recovered, were attained with CVM as compared to HFV. Nonetheless, this might be related to the operator as training is a prerequisite for both methods. On the one hand, the long-term storage of the hollow fiber in 0.5 ml plastic straws seems to be possible; nonetheless, the main problem of the hollow fiber is the handling 2-3 cm above the LN_2 surface and the possibility of breaking it up. In order to avoid this problem, hollow fiber long-term storage in cryotubes has already been described by Matsunari et al. (2012). On the other hand, working with <1 µl samples in CVM did not ease the loading step. So far, no studies have reported embryo loss/non-recovery with CVM, this being a critical step as the Fibreplug is loaded. Difficulties controlling the size of the droplet and loading the sample are

permanent. While loading, embryos tend to either remain attached to the walls of the plastic loading tips or in a small volume that remains within the plastic tip. Nonetheless, CVM stands out with its user-friendly device. Furthermore, another advantage of the CVM is that there is no direct contact between the sample and the LN_2 , this being an important issue regarding contamination of the sample as reviewed by Bielanski (2012). Yet, HFV represents an alternative as well, since it is possible to vitrify the sample in LN_2 vapour.

Compared to other authors, this study achieved similar or slightly better results with CVM with regard to survival rates of vitrified good quality (grade I) bovine IVP blastocysts [85.27% survived in this study vs. 90% (Fry, Earl et al. 2004), 92% (Lindemans, Sangalli et al. 2003), 73.8% (Trigal, Gómez et al. 2012), 79.3% (Trigal, Muñoz et al. 2012), 69% (Villamil, Lozano et al. 2012)]. These variations might be explained due to differences in vitrification and thawing solutions. With regard to HFV, there is not so much data as it is a relatively new vitrification method. Still, this study's results are in line with those previously reported by Matsunari et al.(2012) with IVM-derived porcine parthenogenetic morulae (73.8% vitrified morulae developed to blastocysts), Maehara et al. (2012) with IVP porcine morulae (77.6% vitrified morulae developed to blastocysts), and Uchikura et al. (2014) with bovine IVP morulae (80.1% vitrified developed further to blastocysts). Nonetheless, lower survival rates (53.9% of bovine IVP morulae survived vitrification with HFV) were achieved as compared to those reported by the previously named authors. Moreover, working with good quality early blastocysts and blastocysts, a 75.9% re-expansion rate was attained; which is slightly lower than the results reported by Beck et al. (2013).

In order to evaluate developmental capacity, the hatching rate was recorded as well. As hatching is an active process (Massip and Mulnard 1980) which would give the developmental capacity of the embryo, no significant difference was found between groups (59.96%, 61.28%, and 71.40%, for control untreated group, HFV and CVM respectively; P=0.061), although there was a slight increase with CVM. With regard to CVM, these results confirm and are similar to those reported by other authors (Lindemans, Sangalli et al. 2003, Leroy, Van Hoeck et al. 2010, Trigal, Gómez et al. 2012, Trigal, Muñoz et al. 2012, Villamil, Lozano et al. 2012). With regard to HFV, hatching rates achieved in this study are higher than those reported by Matsunari et al. (2012) (32% vitrified morulae developed

to hatched blastocysts) and Maehara et al. (2012) (10.6% developed to hatched blastocyst stage) with porcine IVP embryos. Nevertheless, it was shown that hatching is influenced, among others by IVC conditions (Krisher, Lane et al. 1999, Rizos, Ward et al. 2002) and structural differences between different species have been described. Both in vivo and in vitro, not every expanded blastocyst ends up with the proper embryo hatching of the ZP; however, this is a crucial and necessary event for an embryo's implantation, and greater hatching rates of in vitro-cultured bovine embryos were reported by Rizos et al (2002). Moreover, it was suggested that vitrification alters the ZP's structure (Moreira da Silva and Metelo 2005). It remained unclear in this study whether this fact was related, although no significant difference, to slightly higher hatching rates of vitrified embryos with CVM. In addition, assisted hatching was shown to increase implantation rates of fair and poor quality embryos (Cohen, Alikani et al. 1992, Taniyama, Watanabe et al. 2011). Furthermore, blastocysts and expanded blastocysts have a thinner ZP which might have led to slightly higher hatching rates. In some embryos, cracking of the ZP was observed in this study and support by observations made by Rall and Meyer (1989). In addition, the ZP of IVP embryos is thinner and more porous as compared to their in vivo counterparts (Abe, Otoi et al. 1999). Solidification of the sample by achieving an amorphous "glassy" state implies mechanical stress. While in opened containers liquid solutions can contract without any stress to decrease in volume, a solid (vitrified) solution will suffer mechanical stress as it is in contact with container walls (Wowk 2010). According to Wowk's statement, thermally-induced fracture of the sample due to dramatic temperature changes (thermally-induced mechanical stress) can occur. Nevertheless, according to IETS' sanitary standards and recommendations for handling in vivo-derived embryos, integrity of the ZP should be guaranteed in order to avoid the spreading of pathogens as a result of the worldwide trade of embryos.

In addition, whether hatching is related to the further developmental capacity or not is doubtful, as most experiments and observations take place in vitro until day 12 of the embryo's development. In this study, morphology, re-expansion, and hatching evaluation was performed; however, embryo "quality" is a more complex characteristic and is often evaluated subjectively rather than objectively. In sheep cryopreserved embryos, alterations at the structural level (Dalcin, Silva

et al. 2013) were shown. Moreover, it was shown recently that vitrification has a negative effect at the molecular level (Stinshoff, Wilkening et al. 2011). Among others, the expression of Aquaporin 3, a gene which is considered important for embryo development and implantation (Huang, He et al. 2006), was shown to be altered (Camargo, Boite et al. 2011). Another interesting approach by Sudano et al. (2013) showed some similar effects at the molecular level. Furthermore, the importance of changes at the molecular level relies on the assumption that embryo-maternal communication might be affected, this resulting in no pregnancy. It can be concluded that the attainment of the blastocyst stage says much about the in vitro developmental environment but little about the embryo's fitness (McEvoy, Sinclair et al. 2000).

Embryo differential staining is useful in order to evaluate cell-membrane damage; thus, in this study the embryos were stained after hatching. With regard to the embryo's cell number, slightly lower results were attained as compared to those reported by other authors (Lonergan, Khatir et al. 1999, Leroy, Van Hoeck et al. 2010, Trigal, Muñoz et al. 2012), who used 10% (v/v) FCS. However, it is difficult to compare the cell number of embryos as it is strongly influenced by IVC conditions (Rizos, Gutierrez-Adan et al. 2003) and every laboratory has its own standards. In this study, a concentration of 5% (v/v) OCS was used for the IVC medium, as embryo quality (morphological assessment) seemed to be better than when higher concentrations of OCS is used. Embryo development followed day-developmental stage patterns as described in other studies. Still, as expected, a significantly higher dead-cell's percentage (dead cells/total cells) was found in biopsied-vitrified embryos as compared to the intact-vitrified and control embryos. Nevertheless, with regard to the control group, the percentage of dead cells is much higher than the one reported by other authors, ranging from 0.7-2.1 % (Kaidi, Bernard et al. 2001, Mucci, Aller et al. 2006, Rios, Mucci et al. 2010). However, Abdalla et al. (2010) reported 6 - 8% of dead cells in fresh blastocysts using a similar IVC medium, similar percentage of dead cells of the control group in this study. Nonetheless, it can be assumed that hatched embryos without any treatment will have a certain number of dead cells and that this might be related to IVP conditions and is also part of normal developmental in early embryonic stages.

Intact-vitrified embryos' percentage of dead cells in this study was 11.3%, slightly higher than the one reported by Beck et al. (2013) with HFV intact-vitrified embryos. Yet, a lower percentage of cells showed an altered cell membrane as compared to those reported by Mucci et al. (2006) using OPS, who reported 14.4% of cells showing alterations of the cell membrane. Moreover, in this regard the percentage attained in this study is higher than the one reported by Abdalla, Shimoda et al. (2010) with Cryotop, who reported 2–4 % of cells with an altered cell membrane. Furthermore, Caamaño et al. (2015), working with CVM, reported 4-6% of dead cells after in-straw dilution of bovine IVP embryos. The higher percentage of dead cells of intact-vitrified embryos attained in this study, as compared to the one previously mentioned, might be due to handling difficulties. It was expected that in this study, using similar concentration of CPAs in vitrification solutions, to attain a lower percentage of dead cells. However, some problems were identified in two steps. On the one hand, while recovering the hollow fiber stored into the 0.5 ml plastic straw, it was exposed to room temperature for about 5 seconds until plunging it into the thawing solution. This might have led to ice-nucleation and growth of the sample, or at least facilitated this event. On the other hand, in the CVM method, difficulties during loading the sample could have led to cellular toxicity due to long exposure of the embryo to high concentrations of CPAs in the vitrification solution. Although in this study CPAs were mixed in order to reduce their toxicity, DMSO is known for its high toxicity as it was shown in mouse embryos (Mukaida, Wada et al. 1998). In addition, it can be speculated that a certain number of live and active cells is necessary to assure enough Na^{+}-K^{+} -ATPase activity, thus producing sufficient intracellular fluid for the embryo's expansion and hatching (Van Soom, Ysebaert et al. 1997)

In this study, embryo quality was assessed and vitrified embryo's age was recorded before vitrification. It was suggested that the age of an embryo might affect its survival after cryopreservation (Saha, Rajamahendran et al. 1996). However, the blastocyst reaching this stage on day 6 was related to components of the medium. Yet, there was no improvement in terms of cryotolerance as compared to the in vivo counterparts. Therefore, it is difficult to conclude if an embryo's age will dramatically improve cryotolerance or not. In the present study, a negative correlation was found in slow-developed blastocysts reaching this stage

on day 8-9 of IVC, i.e. the later they reached the blastocyst stage, the lower the survival rates post-thawing were found. It was shown that day 8 blastocysts presented a decreased total and ICM cell number, and were classified as low quality embryos (Van Soom, Ysebaert et al. 1997). In turn, in early blastocysts of 6-7 days of age a positive correlation was found.

In conclusion, both vitrification methods tested in this study seem to be suitable for bovine IVP embryos. Moreover, the selection of only good quality embryos for vitrification seems to determine its outcomes. It should be kept in mind that morphological assessment of bovine IVP embryos is a restrictive technique regarding embryo fitness. It provides the operator with little information and long-term effects of vitrification on the proper embryo.

2. Splitting and biopsy of IVP embryos

Preimplantation genetic diagnosis (PGD) is a very valuable technique. It was shown to be suitable to certain mammalian species such as rabbit (Gardner and Edwards 1968), human (Munné, Sandalinas et al. 2003), equine (Herrera, Morikawa et al. 2014), ovine (Naitana, Loi et al. 1996), and bovine (Jung, Reichenbach et al. 2014). In livestock species, it contributed in the past with gender's determination analysis, and so does it until nowadays. Peippo et al. (2007) reported the birth of correct genotyped calves after biopsy with a microblade. Furthermore, it was recently shown in bovine species that it is possible to perform biopsy, sexing, polled-status determination, and analysis of certain inherited diseases within 24 h with a high correlation between calves born and the results obtained from biopsied embryos (Jung, Reichenbach et al. 2014). Thus, the second part of the present study was aimed to analyze if vitrification has an influence on survival rate outcomes of bovine IVP embryos with an opened ZP.

Overall, there was no significant difference in survival rates between intact-vitrified embryos and splitted-vitrified embryos (with an opened ZP).

Regarding the impact of developmental stages, the results achieved in this study confirm findings reported by other authors working with ex vivo-derived (Williams, Elsden et al. 1984, Naitana, Loi et al. 1996) and IVP bovine embryos

(Rho, Johnson et al. 1998). It seems that IVP embryos at the blastocyst stage overcome biopsies more successfully than morula stage embryos. Efficiency of embryo splitting in this study is similar to the one reported by Rho et al. (1998) using an inverted microscope and a microsurgical steel blade. They reported 90% of IVP blastocysts showing re-expansion and 60% of morula, vs. 59.01% and 89.91% achieved in this study for morula and blastocysts, respectively. Similar results were reported by Peippo et al. (2007), who reported 94% survival of day 7 IVP bovine embryos 24 h post-biopsy. Moreover, in other studies it was shown that embryo biopsy at an early embryonic stage is possible without affecting its developmental capacity (Polisseni, Sá et al. 2010). However, in this study the aim was to evaluate the viability after vitrification of biopsied embryos at a certain stage that normally would be recovered after superstimulatory treatments, AI of donors, and embryo recovery on day 7 after AI, i.e. morula stage onwards. Yet, it can be stated that splitting and biopsy of IVP embryos, particularly morula, is not the best way to handle embryos, since many of them did not even re-expand after splitting. This can be related to damages caused to the tight cellular junctions that might have existed before, altering in this manner embryo's further developmental capacity. As reported by Rizos et al. (2002), IVP bovine embryos show a lack of desmosomal junctions as compared to their in vivo counterparts. Therefore, a fine biopsy technique, particularly with IVP embryos, i.e. the use of a micromanipulator with a holding pipette and a biopsy needle (aspiration technique) is certainly useful, although this technique can have two major disadvantages: first, it can only be performed in laboratories and it will not be possible to apply it under field conditions; and second, it was shown in other studies that a great percentage of the biopsy (sample) was lost due to handling difficulties (Chrenek, Boulanger et al. 2001).

The results of timing of cryopreservation after biopsy attained in this study are in concordance with those reported by Ito, Sekimoto et al. (1999) who suggested that a short IVC before cryopreservation would increase embryo viability post-thawing. Although only a moderate effect could be observed, splitted-biopsied embryos vitrified 18-20 h after manipulation showed better results than those vitrified approximately 3 h following splitting and biopsy. The collapse of the blastocoel cavity by splitting and biopsy cannot be avoided and it can take some time for embryos to re-expand (up to 24 h). However, it was shown that a short

IVC can have positive effects on embryos as they were allowed to remain IVC with BSA or FCS after biopsy as demonstrated by Yotsushima, Sakaguchi et al. (2004). Whether this effect has any physiological background remains unclear, but it can be assumed that once the splitting has been performed, the embryo will need some time to re-organize its own structure, and nutrients are going to be demanded by the cells in order to restore the cellular activity.

Concerning survival rates of embryos with an opened ZP, overall in this study 73% of embryos vitrified by CVM and HFV showed re-expansion. These results are similar to those reported in other studies. Still, none of the vitrification methods described in this study was used to vitrify embryos with an opened ZP. Pereira et al. (2008) vitrified biopsied-embryos at the blastocyst stage in a 0.25 ml plastic-straw in 20-30 µl of vitrification solution, after removing approximately 8-10 blastomeres using a micromanipulator with a micro blade. Vajta et al. (1997) reported 75% re-expanded blastocysts post-thawing following vitrification and in-straw dilution in a 0.25 ml plastic-straw, loading embryos in 8 µl of vitrification solution, performing biopsy of day 7-8 blastocysts with a razor blade. Nevertheless, results in this study are slightly lower than those reported by Yotsushima et al. (2004), who biopsied blastocysts with a micro razor blade attached to a micromanipulator, one-tenth of the TE was removed, and then embryos were vitrified in a 0.25 ml plastic-straw. In other study, embryos were biopsied with a fine glass needle at days 3-4 of development (Tominaga and Hamada 2001, Tominaga and Hamada 2004), and then vitrified in gel-loading tips obtaining over 90% of survival rate of in biopsied groups.

Unfortunately, as previously stated no studies reporting vitrification of biopsied embryos either with CVM or HFV was found, so that a comparison is difficult to make. However, it was shown once more that embryo quality influences survival rate outcomes. Embryos of better quality showed better re-expansion rates after splitting and biopsy, and this in turn will influence vitrification outcomes.

In addition, the careful and correct selection of embryos after biopsy would determine either success or failure. Embryos at the blastocyst stage showed re-expansion and their size was significantly greater than the one of those at the morula stage, which might be better for cryopreservation concerning biopsy and the resulting small-size embryos with the splitting-biopsy technique. The latter might explain why a greater percentage of embryos were lost with CVM.

Difficulties occurred and more embryos were lost due to the minimum volume of the sample which made it extremely difficult to manipulate them. In this regard, HFV gave the operator more security. The handling of small embryos and demi-embryos and their loading in medium containing a high concentration of CPA are difficult steps, turning this into an even more difficult process when embryos are very small. Moreover, split and biopsy of morula, if they survived, always led to small embryos as compared to blastocysts. On the one hand, there is some doubt as to whether demi-morula were able to increase their cell number, as they resulted in embryos with less than 50 cells when already re-expanded. In these cases, it can be assumed that they have no further developmental capacity. As previously stated, the embryo must have a certain total number of cells and ICM and expansion or hatching (Van Soom, Ysebaert et al. 1997) does not mean that embryos are ready and suitable for implantation into the uterus. Bisected embryos with a low cell number were associated with low pregnancy rates after transfer (McEvoy and Sreenan 1990). Moreover, interferon-tau, secreted by TE cells as reviewed in (Roberts 2007), is very important to avoid luteolysis. Thus, it can be assumed that embryo-maternal communication might be altered.

In conclusion, in the present study two different vitrification methods, HFV and CVM were tested for bovine IVP embryo vitrification, either with an intact or with an opened ZP (splitted-biopsied). Both vitrification methods were shown to be suitable, CVM standing out for its user-friendly Fibreplug® and HFV for being an easy-handling self-made vitrification device. Once more, it was shown that the importance of a very careful selection of only good quality embryos, determining a very big part of the success or failure of the technique. Furthermore, working with IVP embryos at the blastocyst stage resulted in better outcomes. With regard to the biopsy of IVP bovine embryos, the use of a micromanipulator with a holding pipette and aspiration needle might allow for a more careful handling of the embryo in order to avoid any kind of structural damage. Regarding the time-point of the cryopreservation, allowing the embryo to recover for a short period of time after splitting and biopsy may have positive effects on its survival after vitrification.

VI. SUMMARY

Comparison between two vitrification methods for in vitro produced bovine embryos with an intact or a non-intact zona pellucida

The possibility to genotyping bovine embryos at the time of collection, or at the blastocyst stage for embryos produced in vitro offers many advantages for animal breeding. Embryo genotyping and sexing may be a strategy to increase the number of elite heifers in dairy herds by transferring only high-merit female embryos to recipients. Embryo genotyping has enabled the determination of breeding values and allowed the genetic diagnosis of some hereditary defects already on pre-implantation stages, i.e., before embryo transfer. Moreover, embryo genotyping makes it possible to accurately detect genes of interest such as, for example for the trait of being polled. However, since calculating the breeding value of pre-transfer embryos might take days or weeks after embryo biopsy it is usually necessary to cryopreserve the biopsied embryo until the genotyping results and genomic breeding estimates are obtained. Therefore, freezing of biopsied embryos since to be crucial in ensuring success of embryo-based genomic selection programs and improvement are still necessary particularly for biopsied in vitro produced embryos. In the context of genomic selection, other benefits for breeding can arise equally from a combined application of embryo splitting before biopsy in order to obtain identical high-valuable twins. However, the bottle neck in the routinely use of embryo genotyping is how to manipulate the embryo, above all to remove only a small but representative number of blastomeres by biopsy and to cryopreserve the biopsied embryo without decreasing its viability.

To determine whether the opening of the zona pellucida has an influence on embryo freezing results, bovine IVP embryos were subjected to cryopreservation in the present study. Moreover, two vitrification methods for in vitro produced embryos either with an intact or a non-intact zona pellucida were evaluated. For this purpose whole, bisected and biopsied in vitro produced embryos at different stages of development and quality grades were used. To evaluate the results of both vitrification methods, 323 embryos were vitrified with HFV (Matsunari,

Maehara et al. 2012) and 568 embryos were vitrified with CVM (CVM™, Cryologic®, Australia).

Overall, significantly higher survival rates were achieved with the CVM compared to the HFV method (70.83% vs 60.97%, respectively; P=0.005). Moreover, with regard to the embryo stage at vitrification, significantly higher survival rates were achieved when working with blastocysts than with early blastocysts and morulae (P=0.004). Furthermore, significantly higher survival rates were accomplished with grade I (good) than with grade II (fair) embryos (P<0.0001). Overall, working with bisected and biopsied embryos, no significant difference was found in survival rates as compared to intact-vitrified embryos (P=0.835). Moreover, significantly higher survival rates after biopsy were attained with grade I than with grade II embryos (P=0.0053). Furthermore, no significant difference was found regarding the vitrification time-point after biopsy when embryos were vitrified approximately 3 or 20 h after biopsy (P=0.8075), yet a positive effect was found in those embryos that remained IVC longer before vitrification as compared to those vitrified after a short IVC post-biopsy. With regard to the dead-cell percentage (dead cells/ total cells), no significant difference between control- and intact-vitrified embryos was found. Nevertheless, biopsied demi-embryos showed a significantly higher percentage of dead cells, post-thawing as compared to the other groups (P=0.029).

Both vitrification methods are reliable. CVM stands out with its user-friendly device and HFV allows the handling of up to 15 embryos within a hollow fibre, giving more security to non-trained technicians.

In a further study the CVM- and the conventional freezing cryopreservation method by 1.5 M ethylene glycol were compared using in vitro produced demi-embryos. There were no differences in results of in vitro embryo development rates for 4 h and 24 h after thawing between vitrified (77.3% and 72.7%) and conventional frozen (77.3% and 63.6%) demi-embryos. A higher in vitro demi-embryo development rate after biopsy was observed when conventional freezing was performed 3 h after biopsy (75.9% vs 55.2% by freezing the embryos immediately after biopsy vs 58.6% by freezing 24 h after biopsy and in vitro culture).

VII. ZUSAMMENFASSUNG

Vergleich zweier Verfahren zur Vitrifikation in vitro produzierter Rinderembryonen mit intakter oder eröffneter Zona Pellucida

Mit der Möglichkeit, Rinderembryonen zum Zeitpunkt ihrer Entnahme oder im Blastozystenstadium bei in vitro produzierten Embryonen zu Genotypisieren sind zahlreiche züchterische Vorteile verbunden. Durch die Geschlechtsbestimmung schon am Embryo können mehr wertvolle Färsen zur Remontierung der weiblichen Nachzucht erzeugt werden. Hinzu können die genomischen Zuchtwerte am Embryo ermittelt und auch unerwünschte Erbfehlerträger noch vor der Implantation bzw. vor der Übertragung auf Empfängertiere identifiziert werden. Darüber hinaus erlaubt die Genotypiserung von Embryonen die Identifikation von einzelnen züchterisch interessanten Genen, wie beispielsweise die für die natürliche Hornlosigkeit. Nachdem aber die genomischen Zuchtwerte erst nach mehreren Tagen oder Wochen nach der Embryo-Biopsie bekannt werden, ist es meist notwendig, die biopsierten Embryonen einzufrieren. Damit dabei keine Verluste nach dem Auftauen entstehen, müssen die Tiefgefrierverfahren für biopsierte Embryonen weiter optimiert werden. Zusätzliche Vorteile ergeben sich durch die mikrochirurgische Teilung der zu untersuchenden Embryonen für die Erzeugung von züchterisch wertvollen Zwillingen mit bekannten genomischen Zuchtwerten. Ein wichtiger Schritt für die Nutzung der genomischen Evaluation von Rinderembryonen in Zuchtprogrammen ist demzufolge die Tiefgefrierkonservierung biopsierter bzw. geteilter Embryonen.

In der vorliegenden Arbeit wurde der Einfluss der Zona pellucida auf die Tiefgefrierkonservierung von in vitro produzierten Rinderembryonen untersucht. Dabei wurden zwei Verfahren zur Vitrifikation von Embryonen mit intakter oder eröffneter Zona pellucida verglichen. Es wurden hierfür ganze, geteilte und biopsierte Embryonen in verschiedenen Entwicklungsstadien und Qualitätsklassen verwendet.

Für den Vergleich wurden 323 Embryonen mit einem neuartigen Vitrifikationsverfahren in durchlässigen porösen Schläuchen (HFV-Verfahren

nach Matsunari, Maehara et al. 2012) und weitere 568 Embryonen mit dem CVM-Verfahren (CVMTM, CryoLogic®, Australia) tiefgefroren.

Insgesamt waren mit dem CVM-Verfahren die in vitro Weiterentwicklungsraten der Embryonen nach dem Auftauen höher als mit dem HFV-Verfahren (70,83% vs. 60,97%, p=0,005). Bezüglich des Entwicklungsstadiums wurden mit frühen Blastozysten und Blastozysten bessere Ergebnisse als mit Morulae erreicht (p=0,004). Darüber hinaus wurden höhere Überlebensraten mit Embryonen Klasse I (gut) als mit Klasse II (mittelmäßig) beobachtet (p<0,0001). Die Weiterentwicklungsraten nach Vitrifikation mit geteilten bzw. biopsierten Embryonen unterscheiden sich nicht von denen nicht biopsierter Embryonen (mit intakter Zona pellucida) (p=0,835). Höhere Weiterentwicklungsraten nach Biopsie wurden mit Embryonen der Klasse I im Vergleich zu Klasse II erreicht (p=0,0053). Bezüglich des Zeitpunktes der Tiefgefrierkonservierung (3 Std. oder 20 Std. nach Biopsie) wurden keine Unterschiede beobachtet (p=0,8075), wenn auch eine kurzzeitige in vitro Kultivierung nach Biopsie vor dem Einfrieren sich positiv auf die Ergebnisse auswirkte. Bei den Untersuchungen mittels Lebend-tot-Färbung wurden keine Unterschiede zwischen den Gesamtzellzahlen von frischen (nicht tiefgefrorenen) und vitrifizierten Blastozysten beobachtet (115,8 ± 7,3 bzw. 117,5 ± 3,9). Auch der Anteil abgestorbener Zellen unterschied sich zwischen den beiden Gruppen nicht (8,9% bzw. 11,3%). Bei biopsierten Embryohälften war der Anteil der abgestorbenen Zellen nach dem Auftauen mit 15,2% höher als bei den anderen Versuchsgruppen (p=0,029).

Beide Vitrifikationsverfahren erwiesen sich in der vorliegenden Untersuchung gleichermaßen als gut für die Tiefgefrierkonservierung von biopsierten Embryonen geeignet. Das CVM-Verfahren war handlicher, hingegen konnte man mit dem HFV-Verfahren gleichzeitig bis zu 15 Embryonen innerhalb eines einzigen Hollow Fibers tiefgefrieren.

In einer weiteren Versuchsreihe wurden das HFV-Verfahren und das konventionelle Einfrierverfahren in 1,5 M Ethylenglykol mit Embryo-Hälften aus in vitro Embryoproduktion verglichen. Es wurden keine signifikanten Unterschiede zwischen den in vitro Weiterentwicklungsraten 4 Std. und 24 Std. nach dem Auftauen mit vitrifizierten (77,3 % bzw. 72,7%) und konventionell tiefgefrorenen (77,3% bzw. 63,6%) Embryo-Hälften beobachtet. Die höhere Weiterentwicklungsrate in vitro beim konventionellen Einfrieren von biopsierten

Blastozysten (Trophektodermbiopsie) wurden beim Einfrieren ca. 3 Std. nach Biopsie (75,9% vs. 55,2% nach sofortiger Tiefgefrierkonservierung nach Biopsie vs. 58,6% bei 24 Std. nach Biopsie und anschließender in vitro Kultur) erzielt.

VIII. APPENDIX

INDEX OF TABLES

Table 1. Milestones in embryo transfer and cryopreservation 7
Table 2. Some vitrification methods 24
Table 3. Permeable, non-permeable and macromolecules used in vitrification... 30
Table 4. Some CPA combinations described in the literature............................ 31
Table 5. Summary pregnancy rates after transfer of vitrified ZP intact IVP embryos.. 36
Table 6. Morphological criteria for embryo quality grading according IETS recommendations (IETS Manual, 4th Edition, 2009) 47
Table 7. Developmental rates of IVP bovine embryos.. 63
Table 8. Vitrification of bovine IVP embryos/demi embryos............................. 64
Table 9. Classification of embryos regarding quality 64

INDEX OF FIGURES

Figure 1. Physical events during slow-, rapid-, and ultra-rapid cooling............. 35
Figure 2. Developmental stages of bovine embryos 47
Figure 3. Solutions used for vitrification and thawing....................................... 51
Figure 4. Vitrification and re-warming steps of porcine IVM/IVF-derived morulae with the HFV Method 52
Figure 5. Vitrification and in-straw hollow fiber's storage................................. 53
Figure 6. CryoLogic Vitrification Method... 55
Figure 7. Embryo handling and steps towards vitrification with the CVM and HFV Method. HFV. Hollow Fiber Vitrification Method.................................... 55
Figure 8. CVM. CryoLogic Vitrification Method. CVMTM Vitrification Kit. CryoLogic® 56
Figure 9. Splitting of in vitro produced embryos.. 57
Figure 10. Biopsy of in vitro produced embryos 57
Figure 11. Biopsy methods for frozen–thawed embryos 58
Figure 12. Embryo splitting and biopsy procedure... 60
Figure 13. Non-hatched (A-B) and hatched embryos (C-D)................................ 63

Figure 14. Vitrification: overall outcomes, influence of method, stage and quality 67
Figure 15. Hatching rate after vitrification of intact embryos 67
Figure 16. Vitrification outcomes 68
Figure 17. Vitrification outcomes regarding embryo quality........ 69
Figure 18. Effect of quality and biopsy's time-point on vitrification outcomes .. 70
Figure 19. Correlation between day of development and survival rate of intact embryos 72
Figure 20. Correlation between day of development and survival rate of early blastocyst and blastocysts 74
Figure 21. Embryo biopsy and post-thawing assessment 76
Figure 22. Effect of embryo stage on biopsy outcomes 77
Figure 23. Survival rates of vitrified vs. conventional frozen demi embryos 78
Figure 24. Survival rate of conventional frozen biopsied embryos 79
Figure 25. Developmental rates of frozen and fresh splitted embryos........ 79
Figure 26. Developmental rates of frozen-thawed and untreated embryos.......... 80
Figure 27. Live-dead cell staining........ 81
Figure 28. Cell counting and percentage of dead cells after vitrification 82

IX. REFERENCES

Abdalla, H., M. Shimoda, H. Hara, H. Morita, M. Kuwayama, M. Hirabayashi and S. Hochi (2010). "Vitrification of ICSI-and IVF-derived bovine blastocysts by minimum volume cooling procedure: effect of developmental stage and age." Theriogenology **74**(6): 1028-1035.

Abe, H., T. Otoi, S. Tachikawa, S. Yamashita, T. Satoh and H. Hoshi (1999). "Fine structure of bovine morulae and blastocysts in vivo and in vitro." Anatomy and embryology **199**(6): 519-527.

Abe, H., S. Yamashita, T. Itoh, T. Satoh and H. Hoshi (1999). "Ultrastructure of bovine embryos developed from in vitro–matured and–fertilized oocytes: Comparative morphological evaluation of embryos cultured either in serum-free medium or in serum-supplemented medium." Molecular reproduction and development **53**(3): 325-335.

Abe, H., S. Yamashita, T. Satoh and H. Hoshi (2002). "Accumulation of cytoplasmic lipid droplets in bovine embryos and cryotolerance of embryos developed in different culture systems using serum-free or serum-containing media." Molecular reproduction and development **61**(1): 57-66.

Absalón-Medina, V., S. Bedford-Guaus, R. Gilbert, L. Siqueira, G. Esposito, A. Schneider, S. Cheong and W. Butler (2014). "The effects of conjugated linoleic acid isomers cis-9, trans-11 and trans-10, cis-12 on in vitro bovine embryo production and cryopreservation." Journal of dairy science.

Agca, Y., R. Monson, D. Northey, O. Abas Mazni, D. Schaefer and J. Rutledge (1998). "Transfer of fresh and cryopreserved IVP bovine embryos: normal calving, birth weight and gestation lengths." Theriogenology **50**(1): 147-162.

Al-Katanani, Y., M. Drost, R. Monson, J. Rutledge, C. Krininger III, J. Block, W. Thatcher and P. Hansen (2002). "Pregnancy rates following timed embryo transfer with fresh or vitrified in vitro produced embryos in lactating dairy cows under heat stress conditions." Theriogenology **58**(1): 171-182.

Arav, A. (1992). "Vitrification of oocytes and embryos." Embryonic development and manipulation in animal production trends in research and applications/editors, A. Lauria, F. Gondolfi.

Arav, A., D. Shehu and M. Mattioli (1993). "Osmotic and cytotoxic study of vitrification of immature bovine oocytes." Journal of reproduction and fertility **99**(2): 353-358.

Arav, A., S. Yavin, Y. Zeron, D. Natan, I. Dekel and H. Gacitua (2002). "New trends in gamete's cryopreservation." Molecular and cellular endocrinology **187**(1): 77-81.

Arav, A. and Y. Zeron (1997). "Vitrification of bovine oocytes using modified minimum drop size technique (MDS) is effected by the composition and the concentration of the vitrification solution and by the cooling conditions." Theriogenology **47**(1): 341-341.

Arav, A., Y. Zeron, S. Leslie, E. Behboodi, G. Anderson and J. Crowe (1996). "Phase transition temperature and chilling sensitivity of bovine oocytes." Cryobiology **33**(6): 589-599.

Asgari, V., S. Hosseini, M. Forouzanfar, M. Hajian and M. Nasr-Esfahani (2012). "Vitrification of in vitro produced bovine embryos; effect of embryonic block and developmental kinetics." Cryobiology.

Avery, B., V. Madison and T. Greve (1991). "Sex and development in bovine in-vitro fertilized embryos." Theriogenology **35**(5): 953-963.

Balasubramanian, S. and G.-J. Rho (2007). "Effect of cysteamine supplementation of in vitro matured bovine oocytes on chilling sensitivity and development of embryos." Animal reproduction science **98**(3): 282-292.

Barros, C. M., R. L. Ereno, R. A. Simões, P. Fernandes, J. Buratini and M. F. Nogueira (2009). "Use of knowledge regarding LH receptors to improve superstimulatory treatments in cattle." Reproduction, Fertility and Development **22**(1): 132-137.

Beck, A., M. Kurome, H. Nagashima, M. Reichenbach, H. Reichenbach and E. Wolf (2013). "Hollow fiber vitrification of biopsied< i> in vitro</i> produced bovine blastocysts." Reproductive Biology **13**: 57.

Beck, A., M. Reichenbach, H. Reichenbach, F. Habermann, G. Arnold and E. Wolf (2012). "126 Time of first cleavage and development to blastocyst of bovine IVF embryos monitored by time-lapse imaging after in vitro or in vivo maturation." Reproduction, Fertility and Development **25**(1): 210-210.

Berg, U. and G. Brem (1989). "In vitro production of bovine blastocysts by in vitro maturation and fertilization of oocytes and subsequent in vitro culture." Reproduction in Domestic Animals **24**(3): 134-139.

Bielanski, A. (2012). "A review of the risk of contamination of semen and embryos during cryopreservation and measures to limit cross-contamination during banking to prevent disease transmission in ET practices." Theriogenology **77**(3): 467-482.

Bilodeau-Goeseels, S. (2012). "Bovine Oocyte Meiotic Inhibition Before In Vitro Maturation and Its Value to In Vitro Embryo Production: Does it Improve Developmental Competence?" Reproduction in Domestic Animals **47**(4): 687-693.

Block, J., L. Bonilla and P. Hansen (2009). "Effect of addition of hyaluronan to embryo culture medium on survival of bovine embryos in vitro following vitrification and establishment of pregnancy after transfer to recipients." Theriogenology **71**(7): 1063-1071.

Block, J., L. Bonilla and P. Hansen (2010). "Efficacy of in vitro embryo transfer in lactating dairy cows using fresh or vitrified embryos produced in a novel embryo culture medium." Journal of dairy science **93**(11): 5234-5242.

Blondin, P., K. Coenen, L. Guilbault and M.-A. Sirard (1996). "Superovulation can reduce the developmental competence of bovine embryos." Theriogenology **46**(7): 1191-1203.

Bo, G., G. Adams, R. Pierson and R. Mapletoft (1995). "Exogenous control of follicular wave emergence in cattle." Theriogenology **43**(1): 31-40.

Bó, G. A., D. C. Guerrero, A. Tríbulo, H. Tríbulo, R. Tríbulo, D. Rogan and R. J. Mapletoft (2009). "New approaches to superovulation in the cow." Reproduction, Fertility and Development **22**(1): 106-112.

Bó, G. A. and R. J. Mapletoft (2014). "Historical perspectives and recent research on superovulation in cattle." Theriogenology **81**(1): 38-48.

Bondioli, K., S. Ellis, J. Pryor, M. Williams and M. Harpold (1989). "The use of male-specific chromosomal DNA fragments to determine the sex of bovine preimplantation embryos." Theriogenology **31**(1): 95-104.

Bonilla, L., J. Block, A. Denicol and P. Hansen (2013). "Consequences of transfer of an in vitro-produced embryo for the dam and resultant calf." Journal of dairy science.

Bousquet, D., H. Twagiramungu, N. Morin, C. Brisson, G. Carboneau and J. Durocher (1999). "In vitro embryo production in the cow: an effective alternative to the conventional embryo production approach." Theriogenology **51**(1): 59-70.

Brackett, B., D. Bousquet, M. Boice, W. Donawick, J. Evans and M. Dressel (1982). "Normal development following in vitro fertilization in the cow." Biology of reproduction **27**(1): 147-158.

Caamaño, J. N., E. Gómez, B. Trigal, M. Muñoz, S. Carrocera, D. Martín and C. Díez (2015). "Survival of vitrified in vitro–produced bovine embryos after a one-step warming in-straw cryoprotectant dilution procedure." Theriogenology **83**(5): 881-890.

Camargo, L. S. A., M. C. Boite, S. Wohlres-Viana, G. B. Mota, R. V. Serapiao, W. F. Sa, J. H. M. Viana and L. A. G. Nogueira (2011). "Osmotic challenge and expression of aquaporin 3 and Na/K ATPase genes in bovine embryos produced in vitro." Cryobiology **63**(3): 256-262.

Campos-Chillon, L., D. Walker, J. De La Torre-Sanchez and G. Seidel Jr (2006). "In vitro assessment of a direct transfer vitrification procedure for bovine embryos." Theriogenology **65**(6): 1200-1214.

Carro, M., J. Buschiazzo, G. Ríos, G. Oresti and R. Alberio (2013). "Linoleic acid stimulates neutral lipid accumulation in lipid droplets of maturing bovine oocytes." Theriogenology **79**(4): 687-694.

Carvalho, R., M. Del Campo, A. Palasz, Y. Plante and R. Mapletoft (1996). "Survival rates and sex ratio of bovine IVE embryos frozen at different developmental stages on day 7." Theriogenology **45**(2): 489-498.

Cohen, J., M. Alikani, J. Trowbridge and Z. Rosenwaks (1992). "Implantation enhancement by selective assisted hatching using zona drilling of human embryos with poor prognosis." Human Reproduction **7**(5): 685-691.

Chagas e Silva, J., P. Diniz and L. Lopes da Costa (2008). "Luteotrophic effect, growth and survival of whole versus half embryos and, their relationship with plasma progesterone concentrations of recipient dairy heifers." Animal Reproduction Science **104**(1): 18-27.

Chankitisakul, V., T. Somfai, Y. Inaba, M. Techakumphu and T. Nagai (2013). "Supplementation of maturation medium with L-carnitine improves cryo-tolerance of bovine in vitro matured oocytes." Theriogenology **79**(4): 590-598.

Checura, C. and G. Seidel (2007). "Effect of macromolecules in solutions for vitrification of mature bovine oocytes." Theriogenology **67**(5): 919-930.

Chrenek, P., L. Boulanger, Y. Heyman, P. Uhrin, J. Laurincik, J. Bulla and J.-P. Renard (2001). "Sexing and multiple genotype analysis from a single cell of bovine embryo." Theriogenology **55**(5): 1071-1081.

Chu, T., I. Dufort and M.-A. Sirard (2012). "Effect of ovarian stimulation on oocyte gene expression in cattle." Theriogenology **77**(9): 1928-1938.

Dalcin, L., R. C. Silva, F. Paulini, B. D. Silva, J. P. Neves and C. M. Lucci (2013). "Cytoskeleton Structure, Pattern of Mitochondrial Activity and Ultrastructure of Frozen or Vitrified Sheep Embryos." Cryobiology.

De Loos, F., T. van Beneden, T. Kruip and P. van Maurik (1992). "Structural aspects of bovine oocyte maturation in vitro." Molecular reproduction and development **31**(3): 208-214.

Demirci, U. and G. Montesano (2007). "Cell encapsulating droplet vitrification." Lab on a chip **7**(11): 1428-1433.

Diez, C., Y. Heyman, D. Le Bourhis, C. Guyader-Joly, J. Degrouard and J. Renard (2001). "Delipidating in vitro-produced bovine zygotes: effect on further development and consequences for freezability." Theriogenology **55**(4): 923-936.

Dinnyés, A., Y. Dai, S. Jiang and X. Yang (2000). "High developmental rates of vitrified bovine oocytes following parthenogenetic activation, in vitro fertilization, and somatic cell nuclear transfer." Biology of Reproduction **63**(2): 513-518.

Dinnyes, A., P. Lonergan, T. Fair, M. P. Boland and X. Yang (1999). "Timing of the first cleavage post-insemination affects cryosurvival of in vitro–produced bovine blastocysts." Molecular reproduction and development **53**(3): 318-324.

Dinnyes, A., X. Yang, X. Li, H. Bagis, G. Presicce and B. Gasparrini (2001). "Solid Surface Vitrification (SSV): An efficient method for oocyte and embryo cryopreservation in cattle, pig and mouse." Cryobiology **43**: 332.

Dunning, K. R., K. Cashman, D. L. Russell, J. G. Thompson, R. J. Norman and R. L. Robker (2010). "Beta-oxidation is essential for mouse oocyte developmental competence and early embryo development." Biology of reproduction **83**(6): 909-918.

Edwards, J., A. Saxton, J. Lawrence, R. Payton and J. Dunlap (2005). "Exposure to a physiologically relevant elevated temperature hastens in vitro maturation in bovine oocytes." Journal of dairy science **88**(12): 4326-4333.

Eid, L., S. Lorton and J. Parrish (1994). "Paternal influence on S-phase in the first cell cycle of the bovine embryo." Biology of reproduction **51**(6): 1232-1237.

Fahy, G. M. (1986). "The relevance of cryoprotectant "toxicity" to cryobiology." Cryobiology **23**(1): 1-13.

Fahy, G. M., D. I. Levy and S. E. Ali (1987). "Some emerging principles underlying the physical properties, biological actions, and utility of vitrification solutions." Cryobiology **24**(3): 196-213.

Farin, P. W., J. A. Piedrahita and C. E. Farin (2006). "Errors in development of fetuses and placentas from in vitro-produced bovine embryos." Theriogenology **65**(1): 178-191.

Ferguson, E. and H. Leese (1999). "Triglyceride content of bovine oocytes and early embryos." Journal of reproduction and fertility **116**(2): 373-378.

Fortier, A. L., F. L. Lopes, N. Darricarrère, J. Martel and J. M. Trasler (2008). "Superovulation alters the expression of imprinted genes in the midgestation mouse placenta." Human molecular genetics **17**(11): 1653-1665.

Fry, R., C. Earl, K. Fry and W. Lindemans (2004). "243 Pregnancy rates in the field after the transfer of bovine IVP embryos vitrified by the cryologic vitrification method." Reproduction, Fertility and Development **17**(2): 272-272.

Fujino, Y., T. Kojima, Y. Nakamura, H. Kobayashi, K. Kikuchi and H. Funahashi (2008). "Metal mesh vitrification (MMV) method for cryopreservation of porcine embryos." Theriogenology **70**(5): 809-817.

Fukuda, Y., M. Ichikawa, K. Naito and Y. Toyoda (1990). "Birth of normal calves resulting from bovine oocytes matured, fertilized, and cultured with cumulus cells in vitro up to the blastocyst stage." Biology of reproduction **42**(1): 114-119.

Gardner, D. K. (1998). "Changes in requirements and utilization of nutrients during mammalian preimplantation embryo development and their significance in embryo culture." Theriogenology **49**(1): 83-102.

Gardner, R. and R. Edwards (1968). "Rabbit by transferring Sexed Blastocysts." Nature **218**.

Gomez, E., M. Muñoz, A. Rodríguez, J. Caamaño, N. Facal and C. Díez (2009). "Vitrification of bovine blastocysts produced in vitro inflicts selective damage to the inner cell mass." Reproduction in Domestic Animals **44**(2): 194-199.

Gordon, I. R. (2003). Laboratory production of cattle embryos [electronic resource]: I. Gordon, CABI.

Gordon, J. and I. Gang (1990). "Use of zona drilling for safe and effective biopsy of murine oocytes and embryos." Biology of reproduction **42**(5-6): 869-876.

Gordon, J. W. and B. E. Talansky (1986). "Assisted fertilization by zona drilling: a mouse model for correction of oligospermia." Journal of Experimental Zoology **239**(3): 347-354.

Hamawaki, A., M. Kuwayama and S. Hamano (1999). "Minimum volume cooling method for bovine blastocyst vitrification." Theriogenology **51**(1): 165.

Handyside, A. H., E. H. Kontogianni, K. Hardy and R. Winston (1990). "Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification." Nature **344**(6268): 768-770.

Hashimoto, S., A. Amo, S. Hama, K. Ohsumi, Y. Nakaoka and Y. Morimoto (2013). "A closed system supports the developmental competence of human embryos after vitrification : Closed vitrification of human embryos." J Assist Reprod Genet **30**(3): 371-376.

Hasler, J. F. (2014). "Forty years of embryo transfer in cattle: A review focusing on the growth of the industry in North America, and personal reminisces." Theriogenology **81**(1): 152-169.

Hendriksen, P., P. Vos, W. Steenweg, M. Bevers and S. Dieleman (2000). "Bovine follicular development and its effect on the in vitro competence of oocytes." Theriogenology **53**(1): 11-20.

Herrera, C., M. I. Morikawa, M. B. Bello, M. von Meyeren, J. Eusebio Centeno, P. Dufourq, M. M. Martinez and J. Llorente (2014). "Setting up equine embryo gender determination by preimplantation genetic diagnosis in a commercial embryo transfer program." Theriogenology **81**(5): 758-763.

Hotamisligil, S., M. Toner and R. D. Powers (1996). "Changes in membrane integrity, cytoskeletal structure, and developmental potential of murine oocytes after vitrification in ethylene glycol." Biology of Reproduction **55**(1): 161-168.

Huang, H.-F., R.-H. He, C.-C. Sun, Y. Zhang, Q.-X. Meng and Y.-Y. Ma (2006). "Function of aquaporins in female and male reproductive systems." Human reproduction update **12**(6): 785-795.

Humblot, P., P. Holm, P. Lonergan, C. Wrenzycki, A. S. Lequarré, C. G. Joly, D. Herrmann, A. Lopes, D. Rizos, H. Niemann and H. Callesen (2005). "Effect of stage of follicular growth during superovulation on developmental competence of bovine oocytes." Theriogenology **63**(4): 1149-1166.

Hyttel, P., T. Greve and H. Callesen (1989). "Ultrastructural aspects of oocyte maturation and fertilization in cattle." Journal of reproduction and fertility. Supplement **38**: 35.

Hyttel, P., F. Sinowatz, M. Vejlsted and K. Betteridge (2009). Essentials of domestic animal embryology, Elsevier Health Sciences.

Isachenko, E., V. Isachenko, I. I. Katkov, S. Dessole and F. Nawroth (2003). "Vitrification of mammalian spermatozoa in the absence of cryoprotectants: from past practical difficulties to present success." Reproductive biomedicine online **6**(2): 191-200.

Ito, K., A. Sekimoto, M. Hirabayashi, S. Hochi, K. Kimura, M. Ueda and Y. Nagao (1999). "Effect of Time Interval between Biopsy and Vitrification on Survival of In Vitro-Produced Bovine Blastocysts." Journal of Reproduction and Development **45**(5): 351-355.

Ivec, M., B. Kovacic and V. Vlaisavljevic (2011). "Prediction of human blastocyst development from morulas with delayed and/or incomplete compaction." Fertility and Sterility **96**(6): 1473-1478.e1472.

Jackowski, S., S. Leibo and P. Mazur (1980). "Glycerol permeabilities of fertilized and unfertilized mouse ova." Journal of Experimental Zoology **212**(3): 329-341.

Janny, L. and Y. J. Menezo (1994). "Evidence for a strong paternal effect on human preimplantation embryo development and blastocyst formation." Molecular reproduction and development **38**(1): 36-42.

Jelinkova, L., H. A. Selman, A. Arav, E. Strehler, N. Reeka and K. Sterzik (2002). "Twin pregnancy after vitrification of 2-pronuclei human embryos." Fertility and sterility **77**(2): 412-414.

Johnson, W., N. Loskutoff, Y. Plante and K. Betteridge (1995). "Production of four identical calves by the separation of blastomeres from an in vitro derived four-cell embryo." The Veterinary record **137**(1): 15-16.

Jung, S., M. Reichenbach, R. Fries, E. Wolf, C. Gschoederer, J. Scherzer, T. Grupp and H.-D. Reichenbach (2014). "316 GENOMIC EVALUATION OF BOVINE EMBRYOS WITHIN 24 HOURS." Reproduction, Fertility and Development **27**(1): 247-247.

Kaidi, S., S. Bernard, P. Lambert, A. Massip, F. Dessy and I. Donnay (2001). "Effect of conventional controlled-rate freezing and vitrification on morphology and metabolism of bovine blastocysts produced in vitro." Biology of Reproduction **65**(4): 1127-1134.

Kasai, M. (1996). "Simple and efficient methods for vitrification of mammalian embryos." Animal Reproduction Science **42**(1): 67-75.

Kasai, M., S. Zhu, P. Pedro, K. Nakamura, T. Sakurai and K. Edashige (1996). "Fracture damage of embryos and its prevention during vitrification and warming." Cryobiology **33**(4): 459-464.

Katska, L. and Z. Smorag (1985). "The influence of culture temperature on in vitro maturation of bovine oocytes." Animal Reproduction Science **9**(3): 205-212.

Khalil, W. A., W. F. Marei and M. Khalid (2013). "Protective effects of antioxidants on linoleic acid–treated bovine oocytes during maturation and subsequent embryo development." Theriogenology **80**(2): 161-168.

Khurana, N. K. and H. Niemann (2000). "Energy metabolism in preimplantation bovine embryos derived in vitro or in vivo." Biology of reproduction **62**(4): 847-856.

Krisher, R. L., M. Lane and B. D. Bavister (1999). "Developmental competence and metabolism of bovine embryos cultured in semi-defined and defined culture media." Biology of reproduction **60**(6): 1345-1352.

Kruip, T. A. and J. Den Daas (1997). "In vitro produced and cloned embryos: effects on pregnancy, parturition and offspring." Theriogenology **47**(1): 43-52.

Kuwayama, M. (2007). "Highly efficient vitrification for cryopreservation of human oocytes and embryos: the Cryotop method." Theriogenology **67**(1): 73-80.

Kuwayama, M., G. Vajta, O. Kato and S. P. Leibo (2005). "Highly efficient vitrification method for cryopreservation of human oocytes." Reproductive biomedicine online **11**(3): 300-308.

Lane, M., B. D. Bavister, E. A. Lyons and K. T. Forest (1999). "Containerless vitrification of mammalian oocytes and embryos." Nature biotechnology **17**(12): 1234-1236.

Lane, M., W. B. Schoolcraft, D. K. Gardner and D. Phil (1999). "Vitrification of mouse and human blastocysts using a novel cryoloop container-less technique." Fertility and Sterility **72**(6): 1073-1078.

Lapa, M., C. Marques, S. Alves, M. Vasques, M. Baptista, I. Carvalhais, M. Silva Pereira, A. Horta, R. Bessa and R. Pereira (2011). "Effect of trans-10 cis-12 conjugated linoleic acid on Bovine Oocyte Competence and Fatty Acid Composition." Reproduction in Domestic Animals **46**(5): 904-910.

Lazzari, G., C. Wrenzycki, D. Herrmann, R. Duchi, T. Kruip, H. Niemann and C. Galli (2002). "Cellular and molecular deviations in bovine in vitro-produced embryos are related to the large offspring syndrome." Biology of Reproduction **67**(3): 767-775.

Leese, H. and A. M. Barton (1984). "Pyruvate and glucose uptake by mouse ova and preimplantation embryos." Journal of reproduction and fertility **72**(1): 9-13.

Leibfried-Rutledge, M., E. Critser, W. Eyestone, D. Northey and N. First (1987). "Development potential of bovine oocytes matured in vitro or in vivo." Biology of reproduction **36**(2): 376-383.

Leibo, S. (1989). "Equilibrium and nonequilibrium cryopreservation of embryos." Theriogenology **31**(1): 85-93.

Leibo, S. and N. Loskutoff (1993). "Cryobiology of in vitro-derived bovine embryos." Theriogenology **39**(1): 81-94.

Leibo, S., J. McGrath and E. Cravalho (1978). "Microscopic observation of intracellular ice formation in unfertilized mouse ova as a function of cooling rate." Cryobiology **15**(3): 257-271.

Leibo, S. and T. B. Pool (2011). "The principal variables of cryopreservation: solutions, temperatures, and rate changes." Fertility and sterility **96**(2): 269-276.

Lenz, R., G. Ball, M. Leibfried, R. Ax and N. First (1983). "In vitro maturation and fertilization of bovine oocytes are temperature-dependent processes." Biology of reproduction **29**(1): 173-179.

Leoni, G., F. Berlinguer, I. Rosati, L. Bogliolo, S. Ledda and S. Naitana (2003). "Resumption of metabolic activity of vitrified/warmed ovine embryos." Molecular reproduction and development **64**(2): 207-213.

Leroy, J., V. Van Hoeck, M. Clemente, D. Rizos, A. Gutierrez-Adan, A. Van Soom, M. Uytterhoeven and P. Bols (2010). "The effect of nutritionally induced hyperlipidaemia on in vitro bovine embryo quality." Human reproduction **25**(3): 768-778.

Liebermann, J., J. Dietl, P. Vanderzwalmen and M. J. Tucker (2003). "Recent developments in human oocyte, embryo and blastocyst vitrification: where are we now?" Reproductive biomedicine online **7**(6): 623-633.

Lindemans, W., L. Sangalli, A. Kick, C. Earl and R. Fry (2003). "105 Vitrification of bovine embryos using the CLV method." Reproduction, Fertility and Development **16**(2): 174-174.

Loewenstein, W. (1981). "Junctional intercellular communication." Physiological Reviews **69**(29).

Lonergan, P., H. Khatir, F. Piumi, D. Rieger, P. Humblot and M. Boland (1999). "Effect of time interval from insemination to first cleavage on the developmental characteristics, sex ratio and pregnancy rate after transfer of bovine embryos." Journal of reproduction and fertility **117**(1): 159-167.

Lonergan, P., P. Monaghan, D. Rizos, M. P. Boland and I. Gordon (1994). "Effect of follicle size on bovine oocyte quality and developmental competence following

maturation, fertilization, and culture in vitro." Molecular Reproduction and Development **37**(1): 48-53.

Lonergan, P., D. Rizos, A. Gutierrez-Adan, T. Fair and M. Boland (2003). "Oocyte and embryo quality: effect of origin, culture conditions and gene expression patterns." Reproduction in Domestic Animals **38**(4): 259-267.

Lonergan, P., D. Rizos, F. Ward and M. P. Boland (2001). "Factors influencing oocyte and embryo quality in cattle." Reprod Nutr Dev **41**: 427-437.

Lopes-da-Costa, L., J. Chagas e Silva, M. C. Deloche, N. Jeanguyot, P. Humblot and A. E. M. Horta (2011). "Effects of embryo size at transfer (whole versus demi) and early pregnancy progesterone supplementation on embryo growth and pregnancy-specific protein bovine concentrations in recipient dairy heifers." Theriogenology **76**(3): 522-531.

Lopes, R. F. F., F. Forell, A. T. D. Oliveira and J. L. Rodrigues (2001). "Splitting and biopsy for bovine embryo sexing under field conditions." Theriogenology **56**(9): 1383-1392.

Loskutoff, N., W. Johnson and K. Betteridge (1993). "The developmental competence of bovine embryos with reduced cell numbers." Theriogenology **39**(1): 95-107.

Luyet, B. J. (1937). "The vitrification of organic colloids and of protoplasm." Biodynamica **1**(29): 1-14.

Luyet, B. J. and E. L. Hodapp (1938). "Revival of frog's spermatozoa vitrified in liquid air." Experimental Biology and Medicine **39**(3): 433-434.

Maehara, M., H. Matsunari, K. Honda, K. Nakano, Y. Takeuchi, T. Kanai, T. Matsuda, Y. Matsumura, Y. Hagiwara and N. Sasayama (2012). "Hollow Fiber Vitrification Provides a Novel Method for Cryopreserving In Vitro Maturation/Fertilization-Derived Porcine Embryos 1." Biology of reproduction **87**(6).

Magli, M. C., L. Gianaroli, A. P. Ferraretti, M. Toschi, F. Esposito and M. C. Fasolino (2004). "The combination of polar body and embryo biopsy does not affect embryo viability." Human Reproduction **19**(5): 1163-1169.

Martinez, A., D. De Matos, C. Furnus and G. Brogliatti (1998). "In vitro evaluation and pregnancy rates after vitrification of in vitro produced bovine embryos." Theriogenology **50**(5): 757-767.

Martino, A., N. Songsasen and S. Leibo (1996). "Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling." Biology of Reproduction **54**(5): 1059-1069.

Massip, A. (2001). "Cryopreservation of embryos of farm animals." Reproduction in Domestic Animals **36**(2): 49-55.

Massip, A., P. Mermillod and A. Dinnyes (1995). "Morphology and biochemistry of in-vitro produced bovine embryos: implications for their cryopreservation." Human Reproduction **10**(11): 3004-3011.

Massip, A., P. Mermillod, A. Van Langendonckt, H. Reichenbach, P. Lonergan, U. Berg, C. Carolan, R. De Roover and G. Brem (1996). "Calving outcome following transfer of embryos produced in vitro in different conditions." Animal Reproduction Science **44**(1): 1-10.

Massip, A. and J. Mulnard (1980). "Time-lapse cinematographic analysis of hatching of normal and frozen—thawed cow blastocysts." Journal of reproduction and fertility **58**(2): 475-478.

Massip, A., P. Van Der Zwalmen and F. Ectors (1987). "Recent progress in cryopreservation of cattle embryos." Theriogenology **27**(1): 69-79.

Matsumoto, H., J. Jiang, T. Tanaka, H. Sasada and E. Sato (2001). "Vitrification of large quantities of immature bovine oocytes using nylon mesh." Cryobiology **42**(2): 139-144.

Matsunari, H., M. Maehara, K. Nakano, Y. Ikezawa, Y. Hagiwara, N. Sasayama, A. Shirasu, H. Ohta, M. Takahashi and H. Nagashima (2012). "Hollow Fiber

Vitrification: A Novel Method for Vitrifying Multiple Embryos in a Single Device." The Journal of reproduction and development.

Mazur, P. (1977). "The role of intracellular freezing in the death of cells cooled at supraoptimal rates." Cryobiology **14**(3): 251-272.

Mazur, P. (1984). "Freezing of living cells: mechanisms and implications." American Journal of Physiology-Cell Physiology **247**(3): C125-C142.

Mazur, P. (1990). "Equilibrium, quasi-equilibrium, and nonequilibrium freezing of mammalian embryos." Cell biophysics **17**(1): 53-92.

Mazur, R., Jackowski SC, Leibo SP (1976). "Preliminary estimates of the permeability of mouse ova and early embryos to glycerol." Biophysics J **16**(232 a).

McEvoy, T., G. Coull, P. Broadbent, J. Hutchinson and B. Speake (2000). "Fatty acid composition of lipids in immature cattle, pig and sheep oocytes with intact zona pellucida." Journal of reproduction and fertility **118**(1): 163-170.

McEvoy, T. and J. Sreenan (1990). "Effect of embryo quality and stage of development on the survival of zona pellucida-free cattle demi-embryos." Theriogenology **33**(6): 1245-1253.

McEvoy, T. G., K. D. Sinclair, L. E. Young, I. Wilmut and J. J. Robinson (2000). "Large offspring syndrome and other consequences of ruminant embryo culture in vitro: relevance to blastocyst culture in human ART." Human Fertility **3**(4): 238-246.

McKeegan, P. J. and R. G. Sturmey (2011). "The role of fatty acids in oocyte and early embryo development." Reproduction, Fertility and Development **24**(1): 59-67.

McKiernan, S. H. and B. D. Bavister (1992). "Different lots of bovine serum albumin inhibit or stimulate in vitro development of hamster embryos." In Vitro Cellular & Developmental Biology-Animal **28**(3): 154-156.

Mermillod, P., R. Dalbiès-Tran, S. Uzbekova, A. Thélie, J. M. Traverso, C. Perreau, P. Papillier and P. Monget (2008). "Factors affecting oocyte quality: who is driving the follicle?" Reproduction in Domestic Animals **43**(s2): 393-400.

Merton, J., A. De Roos, E. Mullaart, L. De Ruigh, L. Kaal, P. Vos and S. Dieleman (2003). "Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry." Theriogenology **59**(2): 651-674.

Meyer, M., P. Hansen, W. Thatcher, M. Drost, L. Badinga, R. Roberts, J. Li, T. Ott and F. Bazer (1995). "Extension of Corpus Luteum Lifespan and Reduction of Uterine Secretion of Prostaglandin F2α of Cows in Response to Recombinant Interferon-τ." Journal of dairy science **78**(9): 1921-1931.

Montag, M., K. van der Ven, G. Delacrétaz, K. Rink and H. van der Ven (1998). "Laser-assisted microdissection of the zona pellucida facilitates polar body biopsy." Fertility and sterility **69**(3): 539-542.

Moore, K. and A. Q. Bonilla (2006). "Cryopreservation of mammalian embryos: the state of the art." Annual Review of Biomedical Sciences.

Moreira da Silva, F. and R. Metelo (2005). "Relation between Physical Properties of the Zona Pellucida and Viability of Bovine Embryos after Slow-freezing and Vitrification." Reproduction in Domestic Animals **40**(3): 205-209.

Mucci, N., J. Aller, G. G. Kaiser, F. Hozbor, J. Cabodevila and R. Alberio (2006). "Effect of estrous cow serum during bovine embryo culture on blastocyst development and cryotolerance after slow freezing or vitrification." Theriogenology **65**(8): 1551-1562.

Mukaida, T., S. Wada, K. Takahashi, P. Pedro, T. An and M. Kasai (1998). "Vitrification of human embryos based on the assessment of suitable conditions for 8-cell mouse embryos." Human Reproduction **13**(10): 2874-2879.

Mullis, K. B., H. A. Erlich, N. Arnheim, G. T. Horn, R. K. Saiki and S. J. Scharf (1987). One of the first Polymerase Chain Reaction (PCR) patents, Google Patents.

Munné, S., M. Sandalinas, T. Escudero, E. Velilla, R. Walmsley, S. Sadowy, J. Cohen and D. Sable (2003). "Improved implantation after preimplantation genetic diagnosis of aneuploidy." Reproductive BioMedicine Online **7**(1): 91-97.

Naitana, S., P. Loi, S. Ledda, P. Cappai, M. Dattena, L. Bogliolo and G. Leoni (1996). "Effect of biopsy and vitrification on in vitro survival of ovine embryos at different stages of development." Theriogenology **46**(5): 813-824.

Orozco-Lucero, E., I. Dufort, C. Robert and M. A. Sirard (2014). "Rapidly cleaving bovine two-cell embryos have better developmental potential and a distinctive mRNA pattern." Molecular reproduction and development **81**(1): 31-41.

Orsi, N. M. and H. J. Leese (2004). "Amino acid metabolism of preimplantation bovine embryos cultured with bovine serum albumin or polyvinyl alcohol." Theriogenology **61**(2): 561-572.

Overstrom, E., R. Duby, J. Dobrinsky, J. Robl, A. Baguisi, P. Lonergan, P. Duffy, J. Walsh, J. Roche and M. Boland (1993). "Cytoskeletal damage in vitrified or frozen bovine embryos." Theriogenology **39**(1): 276.

Park, J. H., J. H. Lee, K. M. Choi, S. Y. Joung, J. Y. Kim, G. M. Chung, D. I. Jin and K. S. Im (2001). "Rapid sexing of preimplantation bovine embryo using consecutive and multiplex polymerase chain reaction (PCR) with biopsied single blastomere." Theriogenology **55**(9): 1843-1853.

Parrish, J., C. Kim and I. Bae (1992). "Current concepts of cell-cycle regulation and its relationship to oocyte maturation, fertilization and embryo development." Theriogenology **38**(2): 277-296.

Payton, R. R., R. Romar, P. Coy, A. M. Saxton, J. L. Lawrence and J. L. Edwards (2004). "Susceptibility of bovine germinal vesicle-stage oocytes from antral follicles to direct effects of heat stress in vitro." Biology of reproduction **71**(4): 1303-1308.

Peippo, J., M. Huhtinen and T. Kotilainen (1995). "Sex diagnosis of equine preimplantation embryos using the polymerase chain reaction." Theriogenology **44**(5): 619-627.

Peippo, J., S. Viitala, J. Virta, M. Räty, N. Tammiranta, T. Lamminen, J. Aro, H. Myllymäki and J. Vilkki (2007). "Birth of correctly genotyped calves after multiplex marker detection from bovine embryo microblade biopsies." Molecular reproduction and development **74**(11): 1373-1378.

Pereira, R., I. Carvalhais, J. Pimenta, M. Baptista, M. Vasques, A. Horta, I. Santos, M. Marques, A. Reis and M. S. Pereira (2008). "Biopsied and vitrified bovine embryos viability is improved by trans10, cis12 conjugated linoleic acid supplementation during in vitro embryo culture." Animal reproduction science **106**(3): 322-332.

Pinyopummintr, T. and B. Bavister (1995). "Optimum gas atmosphere for in vitro maturation and in vitro fertilization of bovine oocytes." Theriogenology **44**(4): 471-477.

Polge, C., A. Smith and A. Parkes (1949). "Revival of spermatozoa after vitrification and dehydration at low temperatures." Nature **164**(4172): 666.

Polisseni, J., W. F. d. Sá, M. d. O. Guerra, M. A. Machado, R. V. Serapião, B. C. d. Carvalho, L. S. d. A. Camargo and V. M. Peters (2010). "Post-biopsy bovine embryo viability and whole genome amplification in preimplantation genetic diagnosis." Fertility and sterility **93**(3): 783-788.

Pollard, J. and S. Leibo (1994). "Chilling sensitivity of mammalian embryos." Theriogenology **41**(1): 101-106.

Pontes, J., F. Melo Sterza, A. Basso, C. Ferreira, B. Sanches, K. Rubin and M. Seneda (2011). "Ovum pick up, in vitro embryo production, and pregnancy rates from a large-scale commercial program using Nelore cattle (Bos indicus) donors." Theriogenology **75**(9): 1640-1646.

Pontes, J., K. Silva, A. Basso, A. Rigo, C. Ferreira, G. Santos, B. Sanches, J. Porcionato, P. Vieira and F. Faifer (2010). "Large-scale in vitro embryo production and pregnancy rates from Bos taurus, Bos indicus, and indicus-taurus dairy cows using sexed sperm." Theriogenology **74**(8): 1349-1355.

Pugh, P., H. Tervit and H. Niemann (2000). "Effects of vitrification medium composition on the survival of bovine in vitro produced embryos, following in

straw-dilution, in vitro and in vivo following transfer." Animal Reproduction Science **58**(1): 9-22.

Rall, W. (1987). "Factors affecting the survival of mouse embryos cryopreserved by vitrification." Cryobiology **24**(5): 387-402.

Rall, W. and T. Meyer (1989). "Zona fracture damage and its avoidance during the cryopreservation of mammalian embryos." Theriogenology **31**(3): 683-692.

Rall, W. F. and G. M. Fahy (1985). "Ice-free cryopreservation of mouse embryos at− 196 C by vitrification."

Rasmussen, S., J. Block, G. Seidel Jr, Z. Brink, K. McSweeney, P. Farin, L. Bonilla and P. Hansen (2013). "Pregnancy rates of lactating cows after transfer of in vitro produced embryos using X-sorted sperm." Theriogenology **79**(3): 453-461.

Reed, M. L., A.-H. Said, D. J. Thompson and C. L. Caperton (2014). "Large-volume vitrification of human biopsied and non-biopsied blastocysts: a simple, robust technique for cryopreservation." Journal of assisted reproduction and genetics: 1-8.

Reichenbach, H., J. Liebrich, U. Berg and G. Brem (1992). "Pregnancy rates and births after unilateral or bilateral transfer of bovine embryos produced in vitro." Journal of reproduction and fertility **95**(2): 363-370.

Restall, B. J. and R. G. Wales (1966). "The fallopian tube of the sheep. 3. The chemical composition of the fluid from the fallopian tube." Aust J Biol Sci **19**(4): 687-698.

Rho, G.-J., W. H. Johnson and K. J. Betteridge (1998). "Cellular composition and viability of demi-and quarter-embryos made from bisected bovine morulae and blastocysts produced in vitro." Theriogenology **50**(6): 885-895.

Rieger, D. and K. Betteridge (1989). Principles and practice of embryo culture. Proceedings of the 8th Annual Meeting of the American Embryo Transfer Association.

Rieger, D., N. Loskutoff and K. Betteridge (1992). "Developmentally related changes in the metabolism of glucose and glutamine by cattle embryos produced and co-cultured in vitro." Journal of reproduction and fertility **95**(2): 585-595.

Rios, G., N. Mucci, G. Kaiser and R. Alberio (2010). "Effect of container, vitrification volume and warming solution on cryosurvival of in vitro-produced bovine embryos." Animal reproduction science **118**(1): 19-24.

Rizos, D., T. Fair, S. Papadopoulos, M. P. Boland and P. Lonergan (2002). "Developmental, qualitative, and ultrastructural differences between ovine and bovine embryos produced in vivo or in vitro." Molecular reproduction and development **62**(3): 320-327.

Rizos, D., A. Gutierrez-Adan, S. Perez-Garnelo, J. De La Fuente, M. Boland and P. Lonergan (2003). "Bovine embryo culture in the presence or absence of serum: implications for blastocyst development, cryotolerance, and messenger RNA expression." Biology of reproduction **68**(1): 236-243.

Rizos, D., F. Ward, P. Duffy, M. P. Boland and P. Lonergan (2002). "Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality." Molecular reproduction and development **61**(2): 234-248.

Roberts, R., A. Ealy, A. Alexenko, C.-S. Han and T. Ezashi (1999). "Trophoblast interferons." Placenta **20**(4): 259-264.

Roberts, R. M. (2007). "Interferon-tau, a Type 1 interferon involved in maternal recognition of pregnancy." Cytokine & growth factor reviews **18**(5): 403-408.

Rocha, A., R. Randel, J. Broussard, J. Lim, R. Blair, J. Roussel, R. A. Godke and W. Hansel (1998). "High environmental temperature and humidity decrease oocyte quality in Bostaurus but not in Bostaurus cows." Theriogenology **49**(3): 657-665.

Roschlau, D., K. Roschlau, R. Roselius, U. Dexne, U. Michaelis, R. Strehl and P. Unicki (1992). "Experiences in sexing of bovine embryos commercial programs." 8th Réunion Association Europeenne de Transfert Embryonnaire.

Rose, T. A. and B. D. Bavister (1992). "Effect of oocyte maturation medium on in vitro development of in vitro fertilized bovine embryos." Molecular reproduction and development **31**(1): 72-77.

Rosenkrans, C., G. Zeng, G. McNamara, P. Schoff and N. First (1993). "Development of bovine embryos in vitro as affected by energy substrates." Biology of Reproduction **49**(3): 459-462.

Saha, S., R. Rajamahendran, A. Boediono, C. Sumantri and T. Suzuki (1996). "Viability of bovine blastocysts obtained after 7, 8 or 9 days of culture in vitro following vitrification and one-step rehydration." Theriogenology **46**(2): 331-343.

Salilew-Wondim, D., D. Tesfaye, M. Hossain, E. Held, F. Rings, E. Tholen, C. Looft, U. Cinar, K. Schellander and M. Hoelker (2013). "Aberrant placenta gene expression pattern in bovine pregnancies established after transfer of cloned or in vitro produced embryos." Physiological genomics **45**(1): 28-46.

Sanches, B., L. Marinho, J. Pontes, A. Basso, M. Meirinhos, K. Silva-Santos, C. Ferreira and M. Seneda (2013). "Cryosurvival and pregnancy rates after exposure of IVF-derived Bos indicus embryos to forskolin before vitrification." Theriogenology **80**(4): 372-377.

Saragusty, J. and A. Arav (2011). "Current progress in oocyte and embryo cryopreservation by slow freezing and vitrification." Reproduction **141**(1): 1-19.

Schmidt, M., T. Greve, B. Avery, J.-F. Beckers, J. Sulon and H. Hansen (1996). "Pregnancies, calves and calf viability after transfer of in vitro produced bovine embryos." Theriogenology **46**(3): 527-539.

Schneider, U. (1986). "Cryobiological principles of embryo freezing." Journal of in vitro Fertilization and Embryo Transfer **3**(1): 3-9.

Seki, S., B. Jin and P. Mazur (2014). "Extreme rapid warming yields high functional survivals of vitrified 8-cell mouse embryos even when suspended in a half-strength vitrification solution and cooled at moderate rates to −196°C." Cryobiology **68**(1): 71-78.

Seki, S. and P. Mazur (2009). "The dominance of warming rate over cooling rate in the survival of mouse oocytes subjected to a vitrification procedure." Cryobiology **59**(1): 75-82.

Simões, R. A., R. A. Satrapa, F. S. Rosa, M. Piagentini, A. Castilho, R. L. Ereno, L. A. Trinca, M. F. Nogueira, J. Buratini Jr and C. M. Barros (2012). "Ovulation rate and its relationship with follicle diameter and gene expression of the LH receptor (LHR) in Nelore cows." Theriogenology **77**(1): 139-147.

Sirard, M.-A., F. Richard, P. Blondin and C. Robert (2006). "Contribution of the oocyte to embryo quality." Theriogenology **65**(1): 126-136.

Sirard, M. and P. Blondin (1996). "Oocyte maturation and IVF in cattle." Animal Reproduction Science **42**(1): 417-426.

Somfai, T., M. Kaneda, S. Akagi, S. Watanabe, S. Haraguchi, E. Mizutani, T. Q. Dang-Nguyen, M. Geshi, K. Kikuchi and T. Nagai (2011). "Enhancement of lipid metabolism with L-carnitine during in vitro maturation improves nuclear maturation and cleavage ability of follicular porcine oocytes." Reproduction, Fertility and Development **23**(7): 912-920.

Soom, A. V., M. Boerjan, M. T. Ysebaert and A. de Kruif (1996). "Cell allocation to the inner cell mass and the trophectoderm in bovine embryos cultured in two different media." Molecular reproduction and development **45**(2): 171-182.

Stachecki, J., S. Willadsen, K. Wiemer, J. Garrisi and J. Cohen (2007). "S 3 vitrification: a safe, simple, and successful method for blastocyst vitrification." Fertility and Sterility **88**: S347-S348.

Stehlik, E., J. Stehlik, K. Paul Katayama, M. Kuwayama, V. Jambor, R. Brohammer and O. Kato (2005). "Vitrification demonstrates significant improvement versus slow freezing of human blastocysts." Reproductive biomedicine online **11**(1): 53-57.

Stewart, B., J. Block, P. Morelli, A. Navarette, M. Amstalden, L. Bonilla, P. Hansen and T. Bilby (2011). "Efficacy of embryo transfer in lactating dairy cows during summer using fresh or vitrified embryos produced in vitro with sex-sorted semen." Journal of dairy science **94**(7): 3437-3445.

Stinshoff, H., S. Wilkening, A. Hanstedt, K. Brüning and C. Wrenzycki (2011). "Cryopreservation affects the quality of in vitro produced bovine embryos at the molecular level." Theriogenology **76**(8): 1433-1441.

Stojkovic, M., M. Büttner, V. Zakhartchenko, J. Riedl, H. D. Reichenbach, H. Wenigerkind, G. Brem and E. Wolf (1999). "Secretion of interferon-tau by bovine embryos in long-term culture: comparison of in vivo derived, in vitro produced, nuclear transfer and demi-embryos." Animal Reproduction Science **55**(3–4): 151-162.

Stojkovic, M., S. A. Machado, P. Stojkovic, V. Zakhartchenko, P. Hutzler, P. B. Gonçalves and E. Wolf (2001). "Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after in vitro maturation: correlation with morphological criteria and developmental capacity after in vitro fertilization and culture." Biology of Reproduction **64**(3): 904-909.

Stroud, B. (2012) "IETS Statistics and Data Retrieval Committee Report. The year 2011 worldwide statistics of embryo transfer in domestic farm animals." **30 (4)**, 16-26.

Sturmey, R. G., A. Reis, H. J. Leese and T. G. McEvoy (2009). "Role of Fatty Acids in Energy Provision During Oocyte Maturation and Early Embryo Development." Reproduction in Domestic Animals **44**: 50-58.

Sudano, M., D. Paschoal, R. Maziero, T. Rascado, M. Guastali, L. Crocomo, L. Magalhães, B. Monteiro, A. M. Jr and R. Machado (2013). "Improving postcryopreservation survival capacity: an embryo-focused approach." Anim. Reprod **10**(3): 160-167.

Sudano, M. J., E. S. Caixeta, D. M. Paschoal, A. Martins, R. Machado, J. Buratini and F. D. Landim-Alvarenga (2013). "Cryotolerance and global gene-expression patterns of Bos taurus indicus and Bos taurus taurus in vitro-and in vivo-produced blastocysts." Reproduction, Fertility and Development.

Sudano, M. J., D. M. Paschoal, T. da Silva Rascado, L. C. O. Magalhães, L. F. Crocomo, J. F. de Lima-Neto and F. da Cruz Landim-Alvarenga (2011). "Lipid

content and apoptosis of in vitro-produced bovine embryos as determinants of susceptibility to vitrification." Theriogenology **75**(7): 1211-1220.

Sudano, M. J., V. G. Santos, A. Tata, C. R. Ferreira, D. M. Paschoal, R. Machado, J. Buratini, M. N. Eberlin and F. D. Landim-Alvarenga (2012). "Phosphatidylcholine and Sphingomyelin Profiles Vary in Bos taurus indicus and Bos taurus taurus In Vitro-and In Vivo-Produced Blastocysts 1." Biology of reproduction **87**(6).

Sugimura, S., T. Akai, Y. Hashiyada, T. Somfai, Y. Inaba, M. Hirayama, T. Yamanouchi, H. Matsuda, S. Kobayashi and Y. Aikawa (2012). "Promising system for selecting healthy in vitro–fertilized embryos in cattle." PloS one **7**(5): e36627.

Széll, A., J. Shelton and K. Széll (1989). "Osmotic characteristics of sheep and cattle embryos." Cryobiology **26**(3): 297-301.

Tagawa, M., S. Matoba, M. Narita, N. Saito, T. Nagai and K. Imai (2008). "Production of monozygotic twin calves using the blastomere separation technique and Well of the Well culture system." Theriogenology **69**(5): 574-582.

Takahashi, T., Y. Inaba, T. Somfai, M. Kaneda, M. Geshi, T. Nagai and N. Manabe (2013). "Supplementation of culture medium with l-carnitine improves development and cryotolerance of bovine embryos produced in vitro." Reproduction, Fertility and Development **25**(4): 589-599.

Takahashi, Y. and N. First (1992). "In vitro development of bovine one-cell embryos: influence of glucose, lactate, pyruvate, amino acids and vitamins." Theriogenology **37**(5): 963-978.

Tammann (1898). "Über die Abhängigkeit der Zahl der Kristalle, welche sich in verschiedenen unterkühlten Flüssigkeiten bilden, von der Temperatur." Z Phys Chem(25): 441-479.

Taniyama, A., Y. Watanabe, Y. Nishino, T. Inoue, Y. Taura, M. Takagi, C. Kubota and T. Otoi (2011). "Assisted hatching of poor-quality bovine embryos increases pregnancy success rate after embryo transfer." Journal of Reproduction and Development **57**(4): 543-546.

Tervit, H., D. Whittingham and L. Rowson (1972). "Successful culture in vitro of sheep and cattle ova." Journal of Reproduction and Fertility **30**(3): 493-497.

Thompson, J., C. McNaughton, B. Gasparrini, L. McGowan and H. Tervit (2000). "Effect of inhibitors and uncouplers of oxidative phosphorylation during compaction and blastulation of bovine embryos cultured in vitro." Journal of reproduction and fertility **118**(1): 47-55.

Tominaga, K. and Y. Hamada (2001). "Gel-loading tip as container for vitrification of in vitro-produced bovine embryos." Journal of Reproduction and Development **47**(5): 267-273.

Tominaga, K. and Y. Hamada (2004). "Efficient production of sex-identified and cryosurvived bovine in-vitro produced blastocysts." Theriogenology **61**(6): 1181-1191.

Trigal, B., E. Gómez, J. N. Caamaño, M. Muñoz, J. Moreno, S. Carrocera, D. Martín and C. Diez (2012). "In vitro and in vivo quality of bovine embryos in vitro produced with sex-sorted sperm." Theriogenology **78**(7): 1465-1475.

Trigal, B., M. Muñoz, E. Gómez, J. Caamaño, D. Martin, S. Carrocera, R. Casais and C. Diez (2012). "Cell Counts and Survival to Vitrification of Bovine In Vitro Produced Blastocysts Subjected to Sublethal High Hydrostatic Pressure." Reproduction in Domestic Animals.

Uchikura, A., T. Wakayama, S. Wakayama, H. Matsunari, M. Maehara, Y. Matsumura, K. Nakano, E. Sasaki, J. Okahara and H. Tsuchiya (2014). "49 Practical application of the hollow fiber vitrification method for cryopreservation of mammalian embryos." Reproduction, Fertility and Development **26**(1): 138-139.

Vajta, G. (2000). "Vitrification of the oocytes and embryos of domestic animals." Animal Reproduction Science **60**: 357-364.

Vajta, G., P. Holm, T. Greve and H. Callesen (1997). "Comparison of two manipulation methods to produce in vitro fertilized, biopsied and vitrified bovine embryos." Theriogenology **47**(2): 501-509.

Vajta, G., P. Holm, M. Kuwayama, P. J. Booth, H. Jacobsen, T. Greve and H. Callesen (1998). "Open pulled straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos." Molecular reproduction and development **51**(1): 53-58.

Vajta, G., P. Hyttel and H. Callesen (1997). "Morphological changes of in-vitro-produced bovine blastocysts after vitrification, in-straw direct rehydration, and culture." Molecular reproduction and development **48**(1): 9-17.

Vajta, G. and M. Kuwayama (2006). "Improving cryopreservation systems." Theriogenology **65**(1): 236-244.

Vajta, G., L. Rienzi and F. M. Ubaldi (2015). "Open versus closed systems for vitrification of human oocytes and embryos." Reproductive BioMedicine Online **30**(4): 325-333.

Van den Abbeel, E. and A. Van Steirteghem (2000). "Zona pellucida damage to human embryos after cryopreservation and the consequences for their blastomere survival and in-vitro viability." Human Reproduction **15**(2): 373-378.

Van Soom, A., A. Wrathall, A. Herrler and H. Nauwynck (2009). "Is the zona pellucida an efficient barrier to viral infection?" Reproduction, Fertility and Development **22**(1): 21-31.

Van Soom, A., M. T. Ysebaert and A. de Kruif (1997). "Relationship between timing of development, morula morphology, and cell allocation to inner cell mass and trophectoderm in in vitro-produced bovine embryos." Molecular reproduction and development **47**(1): 47-56.

Van Wagtendonk-de Leeuw, A., B. Aerts and J. Den Daas (1998). "Abnormal offspring following in vitro production of bovine preimplantation embryos: a field study." Theriogenology **49**(5): 883-894.

Van Wagtendonk-De Leeuw, A., J. Den Daas, T. A. Kruip and W. F. Rall (1995). "Comparison of the efficacy of conventional slow freezing and rapid cryopreservation methods for bovine embryos." Cryobiology **32**(2): 157-167.

Van Wagtendonk-de Leeuw, A., J. Den Daas and W. Rall (1997). "Field trial to compare pregnancy rates of bovine embryo cryopreservation methods: vitrification and one-step dilution versus slow freezing and three-step dilution." Theriogenology **48**(7): 1071-1084.

Vanderzwalmen, P., G. Bertin, C. Debauche, V. Standaart and E. Schoysman (2000). "“In Vitro” Survival of Metaphase II Oocytes (MII) and Blastocysts After Vitrification in a Hemi-Straw (HS) System." Fertility and Sterility **74**(3): S215-S216.

Vanderzwalmen, P., G. Bertin, C. Debauche, V. Standaert, N. Bollen, E. Van Roosendaal, M. Vandervorst, R. Schoysman and N. Zech (2003). "Vitrification of human blastocysts with the Hemi-Straw carrier: application of assisted hatching after thawing." Human Reproduction **18**(7): 1504-1511.

Vanderzwalmen, P., D. Connan, L. Grobet, B. Wirleitner, B. Remy, S. Vanderzwalmen, N. Zech and F. Ectors (2013). "Lower intracellular concentration of cryoprotectants after vitrification than after slow freezing despite exposure to higher concentration of cryoprotectant solutions." Human Reproduction **28**(8): 2101-2110.

Veiga, A., M. Sandalinas, M. Benkhalifa, M. Boada, M. Carrera, J. Santaló, P. N. Barri and Y. Ménézo (1997). "Laser blastocyst biopsy for preimplantation diagnosis in the human." Zygote **5**(04): 351-354.

Vieira, A., F. Forell, C. Feltrin and J. Rodrigues (2007). "In-straw cryoprotectant dilution of IVP bovine blastocysts vitrified in hand-pulled glass micropipettes." Animal reproduction science **99**(3): 377-383.

Villamil, P. R., D. Lozano, J. Oviedo, F. Ongaratto and G. Bó (2012). "Developmental rates of in vivo and in vitro produced bovine embryos cryopreserved in ethylene glycol based solutions by slow freezing or solid surface vitrification." Anim. Reprod **9**(2): 86-92.

Visintin, J., J. Martins, E. Bevilacqua, M. Mello, A. Nicácio and M. Assumpção (2002). "Cryopreservation of bos taurus vs bos indicus embryos: are they really different?" Theriogenology **57**(1): 345-359.

Voelkel, S. and Y. Hu (1992). "Direct transfer of frozen-thawed bovine embryos." Theriogenology **37**(1): 23-37.

Warkentin, M. A. (2010). Behavior Of Biological And Aqueous Systems At Low Temperature, Cornell University.

Watson, A. J., P. De Sousa, A. Caveney, L. C. Barcroft, D. Natale, J. Urquhart and M. E. Westhusin (2000). "Impact of bovine oocyte maturation media on oocyte transcript levels, blastocyst development, cell number, and apoptosis." Biology of reproduction **62**(2): 355-364.

Whittingham, D. (1971). "Survival of mouse embryos after freezing and thawing."

Whittingham, D., S. Leibo and P. Mazur (1972). "Survival of mouse embryos frozen to-196 and-269 C." Science **178**(4059): 411-414.

Wilmut, I. and L. Rowson (1973). "Experiments on the low-temperature preservation of cow embryos." Veterinary Record **92**(26): 686-690.

Williams, T., R. Elsden and G. Seidel (1984). "Pregnancy rates with bisected bovine embryos." Theriogenology **22**(5): 521-531.

Wolf, E., G. Arnold, S. Bauersachs, H. Beier, H. Blum, R. Einspanier, T. Fröhlich, A. Herrler, S. Hiendleder and S. Kölle (2003). "Embryo-Maternal Communication in Bovine–Strategies for Deciphering a Complex Cross-Talk." Reproduction in Domestic Animals **38**(4): 276-289.

Wowk, B. (2010). "Thermodynamic aspects of vitrification." Cryobiology **60**(1): 11-22.

Xu, K., B. Yadav, W. King and K. Betteridge (1992). "Sex-related differences in developmental rates of bovine embryos produced and cultured in vitro." Molecular reproduction and development **31**(4): 249-252.

Yavin, S. and A. Arav (2007). "Measurement of essential physical properties of vitrification solutions." Theriogenology **67**(1): 81-89.

Yavin, S., A. Aroyo, Z. Roth and A. Arav (2009). "Embryo cryopreservation in the presence of low concentration of vitrification solution with sealed pulled straws in liquid nitrogen slush." Human Reproduction **24**(4): 797-804.

YOTSUSHIMA, K., M. SAKAGUCHI, M. SHIMIZU, T. OKIMURA and Y. IZAIKE (2004). "Effects of fatty acid-free bovine serum albumin and fetal calf serum supplementing repair cultures on pre-and post-warm viability of biopsied bovine embryos produced in vitro." Journal of Reproduction and Development **50**(4): 471-476.

Young, L. E., K. D. Sinclair and I. Wilmut (1998). "Large offspring syndrome in cattle and sheep." Reviews of Reproduction **3**(3): 155-163.

Yu, X., W. Deng, F. Liu, Y. Li, X. Li, Y. Zhang and L. Zan (2010). "Closed pulled straw vitrification of in vitro–produced and in vivo–produced bovine embryos." Theriogenology **73**(4): 474-479.

Zamboni, L., R. Thompson and D. M. Smith (1972). "Fine morphology of human oocyte maturation in vitro." Biology of reproduction **7**(3): 425-457.

赤上正貴, 脇本亘, 山口大輔, 渡辺晃行 and 足立憲隆 (2013). "ウシ体外操作胚盤胞のアルミプレートガラス化法." Nihon Chikusan Gakkaiho **84**(1): 35-41.

X. ACKNOWLEDGMENTS

First of all I would like to thank Univ.-Prof. Dr. Eckhard Wolf for providing me with the opportunity to carry out this work at the chair for Molecular Animal Breeding, Ludwig-Maximilian-University, Munich, and his support.

Special thanks to my mentors Myriam and Horst Reichenbach, for their patient and active support in the laboratory, which was crucial for the success of this study. It was a pleasure to work with them. I am also very thankful to Mayuko Kurome, who always supported me, and to my other colleagues at the Moor- and Versuchsgut.

I would like to acknowledge the Bayerische Forschungsstiftung to support my work financially.

I also would like to thank Tuna Gungör for giving me helpful advice and support in the laboratory.

Furthermore I want to give my thanks to Peter Rieblinger for the excellent care of the animals.

Finally I would like to thank my parents for their support and understanding during the last three years. Many thanks to Amelie and her family for their support, and for believing in me.