Comparison of two vitrification-systems, Cryotop® versus VitTrans, in biopsied bovine embryos

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ABBREVIATIONS

%	Percent
®	Registered Trademark
°C	Degree Celsius
BME	Basic amino acid solution
BSA	Bovine serum albumin
$CaCl_2H_2O$	Calcium chloride monohydrate
CI	Confidence interval
CIDR	Controlled internal drug release
CL	Corpus luteum
CO_2	Carbon dioxide
COCs	Cumulus-oocyte complexes
CPA	Cryoprotectant
CPS	Close-Pulled straw
CVM	Solid surface vitrification
CVSD	Cryotop® vitrification-straw dilution
CPS	Closed-Pulled straw
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EG	Ethylene glycol
ES	Equilibration solution
FCS	Foetal calf serum
FSH	Follicle-stimulating hormone
GMP	Glass micropipettes
h	Hour(s)
HEPES	N-2-Hydroxyethylpiperazineethanesulfonic acid
HM	Holding medium
ICM	Inner cell mass
i.e.	Latin: “Id est”, “that is”
IETS	International Embryo Technology Society
ISVD	In-straw vitrification dilution
IU	International unit
IVD	*In vivo* derived
IVF	*In vitro* fertilization

IVM	*In vitro* maturation
IVP	*In vitro* produced
KCl	Potassium chloride
KH2PO4	Potassium dihydrogen phosphate
LAMP	Loop-mediated isothermal amplification
LH	Luteinizing hormone
LMS	Least square means
LN_2	Liquid nitrogen
M	Molar
mg	Milligram(s)
min	Minute(s)
ml	Millilitre(s)
mm	Millimetre(s)
mM	Millimolar
MEM	Non-essential amino acids
MOET	Multiple ovulation embryo transfer
MPM	Modified Parker's Medium
MVC	Minimum volume cooling
NaCl	Sodium chloride
ng	Nanogram(s)
$NaH_2PO_2H_2O$	Sodium hypophosphite monohydrate
$NaH_2PO_4H_2O$	Sodium dihydrogen phosphate monohydrate
$NaHCO_3$	Sodium bicarbonate
O_2	Oxygen
OCS	Oestrus calf serum
OPS	Open-pulled straw
OPU	Ovum pick up
pH	Potential hydrogen
PBS	Phosphate buffered saline
p	Probability
pg	Picogram
PGF2α	Prostaglandin F2α
PVP	Polyvinylpyrrolidone
QTL	Quantitative trait loci in the genome

sec	Second
SEM	Standard error of the mean
SOF	Synthetic oviductal fluid
SSV	Solid-surface vitrification system
TALP	Tyrode's albumin-lactate-pyruvate
TCM	Tissue Culture Medium
TE	Trophectoderm
v/v	Volume per volume
VS	Vitrification solution
WS	Warming solution
xg	Times gravity
µl	Microlitre

I. INTRODUCTION

Embryo transfer technologies have been widely used to select high genetic merit donors and produce higher quantities of embryos to improve genetics. The transfer of only the best embryos by the selection of the best genotypes at the embryo stage accelerates the rate of genetic improvement and can reduce the costs associated with producing elite animals (PEDERSEN et al., 2012; GONZÁLEZ-RODRÍGUEZ et al., 2022). Performing biopsy in embryos provides a few cells that contain genomic DNA for embryo genotyping and epi-genotyping to determine the genomic values. Moreover, it allows to choose gender, viable embryos that will not lead to embryonic foetal losses (BERMEJO-ALVAREZ et al., 2011; OLIVEIRA et al., 2017) and eliminate embryos carrying known recessive genetic defects.

Cryopreservation of biopsied embryos has become essential because some embryos cannot be transferred due to a lack of recipients or the time it takes for genetic diagnosis. Tolerance of bovine embryos to cryopreservation depends on many factors, including method of production (*in vitro* vs. *in vivo* derived), embryo quality and developmental stage. According to numerous studies, *in vitro* produced (IVP) embryos show lower cryotolerance than their *in vivo* derived (IVD) counterparts (ENRIGHT et al., 2000; ABE et al., 2002; LONERGAN et al., 2003; ABDALLA et al., 2010). In fact, since there is no practical cryopreservation procedure that provides appropriate pregnancy rates, more fresh IVP bovine embryos are transferred to recipients than those cryopreserved (overall mean 60.5% - International Embryo Technology Society (IETS) Data Retrieval 2020, (VIANA, 2021)). Furthermore, the biopsy technique harms the embryo, which might lead to lower pregnancy rates.

Embryo cryopreservation is achieved by two different techniques, namely conventional slow freezing and vitrification (KASAI, 2002). Slow freezing involves slow cooling of embryos before storage in liquid nitrogen (LN_2). This method has been described in several studies (POLGE and WILLADSEN, 1978; YOUNGS, 2011) and has proven successful for the cryopreservation of transferable IVD embryos, although usually there is a 10% gap between the results after transfer of fresh vs. frozen IVD embryos (ABDULLAH et al., 2006). However, embryo viability is low on IVP embryos cryopreserved by the slow freezing method (NEDAMBALE et al., 2004; INABA et al., 2016) as there is cell membrane damage

by the formation of intracellular and extracellular ice crystals. Over the past years, vitrification has become a popular alternative method for cryopreservation of IVP embryos since there is a formation of a glass-like state in the rapid cooling process that prevents ice crystal formation. Additionally, it may also be useful for cryopreserving biopsied embryos with low cryotolerance (AKIYAMA et al., 2010), i.e. *in vitro* cultured and manipulated (ABDALLA et al., 2010), biopsied or cloned embryos (LONERGAN et al., 2007), resulting in a higher survival rate after cryopreservation than slow-freezing (TUCKER and LIEBERMANN, 2007; NAJAFZADEH et al., 2021).

Vitrification methods showed promising results for cryopreservation of IVP bovine embryos (NEDAMBALE et al., 2004; LOUTRADI et al., 2008; MORATO et al., 2010; KIM et al., 2012; RODRIGUEZ VILLAMIL et al., 2012; SANCHES et al., 2013) in particular the minimum volume cooling (MVC) application of the Cryotop® method (KUWAYAMA, 2007), that provides higher rates of cooling and warming (up to 40.000 °C/min), minimizes negative effects and improves survival rates, for both IVP and IVD embryos. Embryo vitrification can be performed using many available devices like plastic insemination straw (VAJTA et al., 1998a; VANDERZWALMEN et al., 2000; YAVIN et al., 2009), Cryoloop (LANE et al., 1999), solid surface vitrification (LINDEMANS et al., 2004), Cryotop® (KUWAYAMA, 2007), Hollow Fiber (MATSUNARI et al., 2012; UCHIKURA et al., 2016), Glass Pulled Micropipettes (KONG et al., 2000), and so on. Usually, the warming protocols for vitrification involve several steps and the need of handling embryos through the dilution media at the time of transfer, which makes performing vitrification under on-farm conditions a practical challenge.

A simple warming procedure is key to a successful commercial use of vitrification, allowing direct embryo transfer and ensuring high pregnancy rates. On the other hand, cryopreservation by conventional slow rate freezing is a widely adopted practice for the direct transfer of IVD bovine embryos since the manipulation of embryos before transfer is not necessary and embryos can be transferred in straws without dilution. Therefore, the simplification of the warming procedures to a 1-step dilution method is essential for the practical application of vitrification in the field, allowing the transfer of an embryo without the use of a microscope and high technical knowledge (HANSEN, 2020).

Some studies have been conducted in which the vitrification methodologies have

been adjusted for 1-step dilution from the warming protocols. For instance, VIEIRA et al. (2007) performed vitrification in hand-pulled glass micropipettes (GMP) and used 1-step cryoprotectant (CPA) dilution in 0.25 ml plastic straws after warming. INABA et al. (2011) and TAJIMI et al. (2018) researched vitrification using the Cryotop® method combined with a warming-straw dilution method. Some other modified warming protocols were studied, for example, a solid surface vitrification system and a simple method of in-straw dilution or a modified fibreplug that allowed in-straw CPA dilution (RODRIGUEZ-VILLAMIL et al., 2014; CAAMANO et al., 2015).

A new vitrification method, the VitTrans, was developed in 2014 for easy and rapid vitrification and in-straw warming of embryos for direct transfer in the field (MORATO and MOGAS, 2014). The main advantage of the design of the VitTrans is that it is comprised of a hard-plastic handle with an inner channel into which warming solution (WS) is rapidly introduced using a Luer connector. In this way, the embryos can be warmed using the VitTrans holder, a syringe carrying the WS and a plastic straw.

In many research studies, *in vitro* development parameters such as morphology, re-expansion and hatching of warmed vitrified embryos are used as a criterion for *in vitro* survival, but this will not always reflect the true survival of cryopreserved embryos. Moreover, an inconsistency of embryo quality among IVP embryos might also influence the diversity of results available from vitrified warmed IVP embryos with respect to *in vitro* embryonic re-expansion and pregnancy rates. The most rigorous criterion is the confirmation of pregnancy or the birth of calves resulting from the transfer of warmed vitrified embryos into recipient females (MASSIP et al., 1995).

In the present study, we attempted to test the VitTrans method for vitrification and warming of IVP and IVD bovine embryos that were vitrified after performing a biopsy. In a first experiment, a warming method suitable for 1-step IVP bovine embryo warming was evaluated. Then, the competence of the VitTrans protocol to be employed for vitrification and in-straw warming of biopsied IVP bovine embryos was tested. Finally, the ability of biopsied IVD embryos to establish pregnancies after vitrification/warming with the Cryotop® or VitTrans protocol was assessed. We compared the pregnancy rates of recipients receiving vitrified and biopsied IVD embryos using the VitTrans method, that enables direct transfer, with

the Cryotop®, an indirect transfer method. The pregnancy outcome obtained after transferring vitrified biopsied IVD embryos was also compared to those obtained after transferring fresh biopsied IVD embryos. Biopsies were carried out at the morula or blastocyst stage by dissociating cells from the embryo that were used for determining the sex. We determined whether female or male sex, quality of embryos prior to biopsy, and embryo development had any effect on the pregnancy outcome. The time between warming and transfer of each embryo with the two different vitrification methodologies was recorded, and the impact on pregnancy rates was analysed.

II. LITERATURE REVIEW

1. Cryopreservation of bovine embryos

Cryopreservation and long-term storage of embryos are important technologies for human and animal reproduction. When embryos are cryopreserved, their intracellular functions are stopped at a point where development cannot continue. Below the glass transition temperature of the cytoplasm and the suspending solution, which is approximately -130 °C metabolism stops. Traditionally, LN_2 (-196 °C) is used to maintain the temperature.

Frozen embryos must maintain their physical and chemical integrity, which will allow them to function upon warming (KRUSE, 2012). In order to achieve successful cryopreservation, dehydrating embryos using cryoprotective agents and controlling cooling rates are necessary to overcome thermal shock and ice formation. It is also important to note that excessive dehydration is associated with permanent damage to the cellular structures, pH changes that damage protein, and excessive ion concentration resulting in toxicity. In fact, freezing protocols depend on a variety of factors, including temperature, timing, volumes, containers, CPAs, diluents, and embryo stage. Since the availability of recipients at any given time is limited in the field, cryopreservation methods are needed to preserve IVD and IVP bovine embryos. Embryo cryopreservation is achieved by two different techniques, namely conventional slow freezing, and vitrification (KASAI, 2002) which are both primarily influenced by two factors, the addition of CPAs and cooling rate (VAJTA and KUWAYAMA, 2006).

1.1. Damage caused by cryopreservation

The freezing of oocytes and embryos of domestic animals and humans does not take place naturally under physiological conditions. They are large cell masses occupied in a high proportion by water and injuries can occur during the cryopreservation procedure by the formation of ice (VAJTA and NAGY, 2006; CUELLO et al., 2008). Therefore, the goal is to prevent these and avoid lethal or irreversible damage to the cells. Ice formation and chilling injuries are the most common causes of cryoinjuries. Cells can suffer thermal shock when they are cooled too quickly, and ice may form if they are not properly dehydrated. Even though during the cooling

process these two types of damage can occur, the chilling injury at +15 to +5 °C damages mainly cytoplasmic lipid droplets and microtubules, including the meiotic spindle in oocytes (ZENZES et al., 2001; VAJTA and NAGY, 2006; OZMEN and SAFAA, 2010; EROGLU et al., 2020); and between −5 and −80 °C, cellular disruption generally occurs due to the presence of both extra- and intracellular ice (VAJTA and NAGY, 2006).

The intracellular ice during the cryopreservation process is detrimental to its survival damaging the cell's intracellular membranes and plasma membrane during the thawing process (MAZUR, 2010). Due to the mechanical shearing and membrane shrinkage that occurs when extracellular ice is formed, the cytoplasm and plasma membrane may be damaged, however these distortions are reversible, returning to its normal shape after 3 to 4 minutes (min) after thawing (MAZUR et al., 2005). The phase of storage below −150 °C (typically in LN_2, at −196 °C) is probably the least dangerous unless accidental warming is present by the manipulation of the cryopreserved embryos (VAJTA and NAGY, 2006; TUCKER and LIEBERMANN, 2007).

During warming, as the solid ice melts, free water is released into the surrounding solution, reducing the osmolarity (JAIN and PAULSON, 2006). Slow warming can cause further cell damage by re-crystallization of free water. On the other hand, rapid warming can lead to osmotic shock when the extracellular osmotic pressure drops suddenly and therefore the water shifts quickly into cells, resulting in swelling and cell damage (MAZUR, 2010). Therefore, to prevent cryoinjuries, CPAs are used in all cryopreservation strategies (KASAI, 2002), but high concentrations are toxic to the cells (LIEBERMANN et al., 2003).

1.2. Slow Freezing

Slow freezing is the standardized technique used in the routine of cryopreservation of cattle embryos. Ready-to use media for freezing and programmable freezing machines are available and after a brief training period, it can be performed (VAJTA and NAGY, 2006). Slow freezing is the process whereby cells are chilled gradually (typical cooling rate is around 0.3-0.5 °C/min). As embryos contain large amount of water, they require adequate dehydration in CPA solutions and slow cooling, to allow the transfer of intracellular fluids to extracellular spaces without serious osmotic effects and deformation of the cells (MAZUR et al., 2005; VAJTA

and NAGY, 2006; OZMEN and SAFAA, 2010).

Before freezing, embryos are typically equilibrated in 1-2 mol/l solutions of permeable and non-permeable CPAs, as ethylene glycol and sucrose, for 8-10 min. After embryos reach osmotic equilibrium, they are loaded into 0.25 ml straws, sealed, and cooled relatively rapidly to -6 or -7 °C by placing the straws into the controlled-rate freezer. The toxic and osmotic damage caused by the relatively low concentration of CPA solutions is limited. However, the low concentration of CPAs is insufficient to avoid ice crystal formation and an additional manipulation is required to minimize the damage (VAJTA and NAGY, 2006). Therefore, after 5 min holding at -6 °C, seeding is performed in order to induce formation of ice crystals in the solution preferably far from where the embryo is located (YOUNGS, 2011) and embryos are hold at seeding temperature for 10 min before further cooling. Controlled growth of ice in extracellular solution is a result of slow cooling and seeding. The subsequent steps for freezing are performed by the machine. The controlled rate cooling, around 0.3-0.5 °C/ min, continues to around -35 °C. At these temperatures, straws are immersed into LN_2 for final cooling and storage.

For thawing, the straw containing the embryo to be thawed is quickly and carefully removed from the goblet. The straw is hold in air for 3-5 seconds to reduce the incidence of a cracked zona pellucida (RALL and MEYER, 1989), and then it is submerged into a 25-30 °C water bath for an additional 15 seconds. After removing the straw from the water bath, it is important to wipe the straw and either remove the plugging rod or use a straw cutting device to snip off the heat-sealed non-cotton plug end of the straw. For direct transfer, the straw can be loaded into an embryo transfer gun and transferred as quickly as possible to a synchronous recipient cow.

1.3. Vitrification

Vitrification as a method of cryopreserving embryos appeared on the scene in the middle 1980s as an alternative to the traditional slow freezing of cattle embryos (ZAYADAN et al., 2020). Vitrification is an ultra-rapid cooling technique that produces the solidification of a solution induced by extreme elevation in viscosity during cooling (FAHY et al., 2004; TUCKER and LIEBERMANN, 2007; KRUSE, 2012). This cryopreservation technique is based on the close contact between the vitrification solution (VS) containing CPA agents and LN_2. In the vitrification process the cooling rate is high enough to induce the glassy stage (MAZUR and

SEKI, 2011) and ice-crystal formation is completely prevented by using high concentrations of CPAs (VAJTA et al., 1998a). The foremost advantage of vitrification over conventional freezing is the ice-free cryopreservation (FAHY et al., 2004). However, the elevated level of CPAs approaches the limits of osmotic stress and potential toxicity to cells. Therefore, to minimise the adverse effects from CPA toxicity, the exposure of embryos to the vitrifying solution with high concentration of CPAs needs to be brief and embryos must be cooled quickly (TUCKER and LIEBERMANN, 2007).

VSs include permeable and non-permeable CPAs and single or multiple type of macromolecular solutes. Using a combination of different permeable CPAs permits the concentration of each CPA to be reduced while still enabling the induction of vitrification (PUGH et al., 2000). CPAs are used to penetrate cells and replace cytoplasmic water, ethylene glycol (EG) and dimethyl sulfoxide (DMSO) are common CPAs utilised, both together as a mixture of CPAs in vitrification protocols (KELLY et al., 2003; VAJTA and NAGY, 2006; CUELLO et al., 2008; INABA et al., 2011; MOGAS, 2019). High warming rates are also required to avoid ice crystal formation when the temperature returns to normal during the warming procedures (TUCKER and LIEBERMANN, 2007; MOGAS, 2019). Upon warming, CPAs are typically removed in the presence of sucrose or galactose, and it is usually performed stepwise, with highly controlled increase of the temperature (KRUSE, 2012). CPA composition, concentration, and removal, as well as vitrification and warming methodologies are described further below (VAJTA and NAGY, 2006). Many variables have been reported that can profoundly influence the efficiency of vitrification like the type and concentrations of CPAs, the medium used as base medium, the temperature of the VS at exposure, the time of exposure to CPAs, the volume of CPA solution, the device used for vitrification, the technical proficiency of the embryologist, the quality and developmental stage of the embryo and the contamination of the sample by direct contact with LN_2 (LIEBERMANN et al., 2003; TUCKER and LIEBERMANN, 2007; KRUSE, 2012; DO et al., 2014).

In commercial bovine embryo transfer, there has been little application of the vitrification technique, even though hundreds of vitrification studies have been published (VAJTA and NAGY, 2006), but in human assisted reproduction, vitrification protocols are successfully applied obtaining several birth reports (EL-DANASOURI and SELMAN, 2001; MUKAIDA et al., 2001;

VANDERZWALMEN et al., 2002; KRUSE, 2012).

1.4. Cooling rates in vitrification procedures

Cooling rates may vary depending on the cryopreservation method. From moderate or stepwise cooling between the physiological temperature to -6 °C and highly controlled cooling rates (0.3-0.5 °C/min) when cryopreserving by slow freezing, to either rapid (around 200 °C/min) or ultra-rapid cooling (up to 20.000-100.000 °C/min) (KASAI, 2002; VAJTA and NAGY, 2006). The cooling rate required to induce vitrification is around 20.000 °C/min when cells are stored in open-pulled straw (VAJTA et al., 1998a) and around 117.000 °C/min on Cryotop® (SEKI and MAZUR, 2009).

Many protocols for vitrification allow cells to be placed into the CPAs and then plunged directly into LN_2 (MOGAS, 2019). The concentration of permeable CPA changes is depending on the cooling rate used, allowing for use of lower levels of CPAs if the cooling rate is faster and conversely higher concentrations of CPAs when cooling rate is slower (VAJTA and NAGY, 2006; TUCKER and LIEBERMANN, 2007). The cooling rate is also affected by the volume of the VS surrounding the embryo, the thickness of the straw or container and the layer of LN_2 gas that is formed when submerging into LN_2 (KRUSE, 2012). The smaller the volume of VS and the closer the contact to LN_2, the greater the cooling rate and warming rate, so that embryos are cooled and warmed rapidly, and embryo viability is maintained after vitrification (MATSUNARI et al., 2012). High cooling rates (from 15.000 to 30.000 °C/min) can be achieved by using a small volume (< 1 µl) of highly concentrated CPAs (MARTINO et al., 1996). Moreover, the use of high cooling rates combined with small sample volumes during vitrification also reduces the CPA concentrations, exposure time and subsequent CPA toxicity (YAVIN et al., 2009).

1.5. Effect of warming rates when using vitrification procedures

In addition to cooling, a successful cryopreservation also depends on the warming rate. Embryos cooled appropriately must also be warmed at an optimum rate to avoid injury caused by osmotic shock or recrystallization of ice during the warming process, regardless of what the cooling rate is (HOCHI et al., 2001; SEKI and MAZUR, 2009).

High warming rates are necessary to avoid recrystallisation during the warming process, which can occur at -80 °C (SEKI and MAZUR, 2009) and detrimentally affects the viability of cells (MAZUR and SEKI, 2011). Moreover, if there is insufficient CPA in the VS, ice crystals can be formed while warming, thus compromising cell viability (JIN et al., 2008). Increasing the rate of warming is also advantageous as this facilitates the reduction in the concentration of permeable solutes. A warming rate of 117.500 °C/min enables the concentration of CPAs to be halved while maintaining morphological and functional viability of 8-cell mouse embryos following vitrification (SEKI et al., 2014).

Lowering the CPA level is a key consideration in reducing cell toxicity. High warming rates could be achieved by directly plunging the vitrified cells into a WS (from -196 °C to 37 °C) (LIEBERMANN et al., 2003). DO et al. (2019) stated that although research has highlighted the importance of the warming rates for vitrification procedures of mouse and human embryos, this has not yet been assessed with IVP bovine oocytes and embryos which are more sensitive to cryopreservation (TEIXEIRA, 2018; MOGAS, 2019).

2. Factors affecting the results of vitrification and pregnancy rates

There are several variables that can determine the outcomes of vitrification and pregnancy rates such as the loading device used and its methodology, media protocol, pre-vitrification embryo quality selection, pre-vitrification embryo stage and embryo micromanipulation.

2.1. Vitrification loading devices and methodologies

During vitrification, the embryo is placed in a loading device surrounded by vitrification media. The device is then placed into LN_2, where it is stored. Nowadays, there are a variety of loading devices for bovine embryos in which very small volumes of the sample are used to be vitrified. The first vitrification experiments were performed in the traditional vessels of cryopreservation, i.e. cryovials or 0.25 ml plastic insemination straws (MASSIP, 1987; VAJTA and NAGY, 2006). These tools were not specially designed for vitrification as they had a thick wall and required a relatively large amount of solution for loading (5-7 µl for the 0.25 ml straw, and more than 10 µl for cryovials) (TUCKER and LIEBERMANN, 2007). Consequently, the achievable cooling and warming rates

were limited (approximately 2.000ºC/min for straws, and much less for cryovials) (VAJTA and NAGY, 2006).

An alternative approach was the minimum drop size method (ARAV, 1992; ARAV and ZERON, 1997), which involves immersing a solid surface in LN_2 and placing a small droplet of VS containing the embryo or oocyte on it. As another method for direct contact with small samples, copper electron microscopic grids were used as carriers (MARTINO et al., 1996). The MVC concept has been the foundation of most recent developments in ultrarapid vitrification methods (HAMAWAKI et al., 1999).

Various devices and strategies have been developed to put MVC protocols into practical use such as the open-pulled straw (OPS) method, designed by VAJTA et al. (1998a). It is possible to shorten and manually decrease the wall thickness and inner diameter of insemination straws (0.25 mL) after heating them. After cutting the straws, embryos were loaded with approximately 1 µL of VS and the estimated cooling rate was around 20.000 °C/min. LANE et al. (1999) then developed the Cryoloop, another MVC method, which consisted of a small nylon loop mounted on a stainless-steel pipe inserted into the lid of a cryovial. The embryos were loaded over a thin film of VS into the loop and it was then directly immersed into LN_2. VANDERZWALMEN et al. (2000) devised the hemi-straw carrier system, essentially an open ended 0.25 mL straw, which allowed successful vitrification of oocytes and embryos. Other devices were developed, i.e. the solid surface vitrification (CVM) (DINNYÉS et al., 2000), gel-loading tips (TOMINAGA and HAMADA, 2001), closed-pulled straws (CP) (CHEN et al., 2017), Flexipet denuding pipette (LIEBERMANN et al., 2002), and so on. Maybe one of the most important contributions to vitrification carriers was made by KUWAYAMA et al. (2005), who developed the Cryotop® method, in which cells are contained in < 0.1 µL of VS on the surface of a thin plastic film strip attached to a plastic handle.

The use of MVC and direct contact with LN_2 result in rapid cooling and warming rates. However, they all introduce contamination problems by direct contact with LN_2. To solve this problem, filtered or UV-sterilized LN_2 can be used followed by placing the sample and the carrier tool into a container before storage (VAJTA et al., 1998b). Another concern is that many vitrification methods do not allow direct transfer which present challenges as it requires a microscope for placing embryos into a straw for transferring the embryo into a recipient cow.

Some studies, however, developed a modified version of the vitrification device that enabled direct transfer or semi-direct transfer procedures of vitrified embryos. For instance, the OPS method was modified to a CPS enabling the direct transfer of embryos (YU et al., 2010). Straws could be used to warm embryos when utilising various vitrification carriers such as the Cryotop® (INABA et al., 2011), Cryotec (TAJIMI et al., 2018), GMP (VIEIRA et al., 2007) and the fibreplug (RODRIGUEZ-VILLAMIL et al., 2014; CAAMANO et al., 2015) to allow a semi-direct or direct transfer. Although these modifications can be used for cryopreservation by vitrification and semi-direct or direct transfer, the viability of the embryos after the warming seems to be reduced. YU et al. (2010) reported that when using CPS, the hatching rate of IVP embryos was 35.2%. CAAMANO et al. (2015) reported a 59.9% hatching rate of IVP embryos when using an in-straw warming dilution method for direct transfer. VIEIRA et al. (2007) reported a 20.0% pregnancy rate of recipients after transfer. INABA et al. (2011) reported a 40.0% and 29.4% pregnancy rate of recipients after in-direct and direct transfer method respectively. See Table 1, which summarizes publications regarding survival, hatching and pregnancy rates of recipients after transfer of vitrified bovine embryos. In most of the cited studies, the embryos used were selected for morphological normality.

Table 1: The effect of loading device on survival, hatching and pregnancy rates

	Loading device	Sample size	Survival rate (%)	Hatching rate (%)	Pregnancy rate (%)	Transfer method
(YU et al., 2010)	OPS	153	58.0	35.2	-	In-direct
	CPS	158	56.3	34.9	-	In-direct
(INABA et al., 2011)	CVSD	44	100.0	93.2	40.0	In-direct
	ISVD	37	100.0	97.3	29.4	Direct
(TAJIMI et al., 2018)	Cryotec	30	100.0	-	-	Direct
(VIEIRA et al., 2007)	GMP	58	84.5	55.2	20.0	Direct
(RODRIGUEZ-VILLAMIL et al., 2014)	GMP	250	67.8	40.8	-	Direct
	CVM	249	69.0	46.0	-	In-direct
(CAAMANO et al., 2015)	Modified fibreplug	112	82.2	59.9	-	Direct
(PUGH et al., 2000)	Straw	20	32.5	-	55.0	In-direct

Abbr: OPS (Open-pulled straw), CPS (Closed-pulled straw), Cryotop® vitrification-straw dilution (CVSD) method, In-straw vitrification dilution (ISVD), GMP (Glass micropipettes), CVM (Solid surface vitrification)

2.2. Media protocols: Cryoprotectants

CPAs are substances used to protect cells from freezing damage that can also be found in nature as antifreeze compounds and proteins created by Arctic and Antarctic insects, fish, and amphibians. CPAs increase the solute concentration in the cells while dehydrating them. Unfortunately, most CPAs have some negative effects, including toxicity and osmotic injuries (VAJTA and NAGY, 2006; MARTINEZ-RODERO et al., 2021). Toxicity is usually proportional to the concentration of the CPAs and the time of exposure (at physiological temperatures). Mixtures of CPAs have less toxicity and are more effective than a single CPA (BEST, 2015).

CPAs are divided into two categories, permeating and non-permeating. Permeable CPAs penetrate the cell membrane, form hydrogen bonds with intracellular water molecules, lower the freezing temperature of the mixture, and prevent ice crystallization (PEREIRA and MARQUES, 2008). Also, permeable CPAS dilutes residual electrolytes by remaining in solution and protect the cells from solution effects (JAIN and PAULSON, 2006). EG, propylene glycol, DMSO, glycerol, formamide, methanol, and butanediol are permeable CPAs. Widespread use of DMSO, glycerol and propylene glycol as CPAs was common until about 1990 (MASSIP, 2001) when use of EG became dominant (KRUSE, 2012). EG´s characteristic features of low molecular weight, high permeation ability, and low toxicity allows no stepwise dilution post warming and direct transfer of embryos.

Non-permeable CPAs remain extracellular and cause intracellular dehydration by removing free water from the cell. Although there are very few examples in which non-permeable CPAs alone protect cells against freezing damage, they are used in combination with permeable CPAs to increase the net concentration of permeable CPAs inside the cell and also prevent ice crystal formation (JAIN and PAULSON, 2006; VAJTA and NAGY, 2006). There are non-permeable CPAs with low molecular weight such as galactose, glucose, sucrose and trehalose, and with high molecular weight like polyvinylpyrrolidone (PVP), polyvinyl alcohol and Ficoll. Most used non-permeable CPAs are trehalose and sucrose. The addition of a sugar like sucrose or trehalose, that do not penetrate the cell membrane, to an EG-based VS can significantly reduce the toxicity of EG by decreasing the concentration required to achieve a successful cryopreservation of human oocytes and embryos (AMBROSINI et al., 2006; OZMEN and SAFAA, 2010; DO et al., 2014; MIN et

al., 2016; GONZÁLEZ-RODRÍGUEZ et al., 2022). By including non-permeating compounds into the vitrifying solution and incubating the cells in this solution prior to cryopreservation, non-permeable compounds help dehydrate the cells and decrease their exposure to toxic CPAs. The sucrose also acts as an osmotic buffer to reduce the osmotic shock that might otherwise result from the dilution of the CPA after warming (KULESHOVA et al., 1999; TUCKER and LIEBERMANN, 2007).

Both permeable and non-permeable CPAs may also have additional protective mechanisms, for example to stabilize intracellular structures and the cell membrane (BEST, 2015). KUWAYAMA et al. (1992) proposed that stepwise addition of CPA, although inconvenient, minimized osmotic stress and damage to cells. CPA solutions are usually prepared in buffered media with a stable pH between 7.2-7.4. Although, Dulbecco's phosphate buffered saline (PBS) is most used, other HEPES (N-2-Hydroxyethylpiperazineethanesulfonic acid) buffered culture media such as TCM-199 have also been used successfully.

2.3. Embryo quality

Embryo quality has been demonstrated to be a significant factor in embryo survival after freezing since the development of the first successful embryo transfer techniques (HAN et al., 1994) and pregnancy rates post embryo transfer (BO and MAPLETOFT, 2013). Therefore, the primary goal is to identify the embryo with the highest quality and potential to implant, establish and maintain a pregnancy.

The current method for embryo assessment is based on the embryo classification system that is recommended by the IETS, which evaluates morphological quality in three grades: excellent or good I, fair II or poor III (BO and MAPLETOFT, 2013). These morphological criteria to determine embryo quality are based on metrics including fragmentation, symmetry of blastomeres, zona pellucida thickness, dead and extruded cells, irregularity of shape, heterogeneity of colour and the presence of vacuoles (MORI et al., 2002). These determinations are based on the visual analysis performed by stereomicroscopy and the grading of the embryo depends on the accuracy and experience of the embryologist. Although the selection of the embryo that has the highest potential embryo quality for transfer is clear, there is a largely subjective and a high grading variance between embryologists (RICHARDSON et al., 2015). Regardless of subjectivity, visual

analysis of embryo morphology is commonly used to determine embryo quality.

Stage and quality of embryos are extremely important in their ability to survive cryopreservation. Embryos of quality grades I and II yield higher post-thaw pregnancy rates than quality grade III embryos. Embryos which quality grade is III are normally transferred fresh into recipient females and not cryopreserved due to the low post-thaw pregnancy rates. Also, pregnancy rates are higher for grades I and II fresh embryos than for grade III (HASLER, 2001). There is not a significant difference in pregnancy rates between excellent and good embryos, which are both in the quality grade I (CENARIU et al., 2012; ROCHA et al., 2016).

Although embryo quality based on morphology is of predominant importance for selecting bovine embryos, it may still hide undetectable genetic or epigenetic defects that may compromise an embryo's developmental capacity (NEL-THEMAAT and NAGY, 2011). Moreover, embryo quality can be affected and yield lower pregnancy rates when embryos are mishandled during the embryo freezing or embryo warming procedures, i.e. maintaining embryos in diluted VS for longer periods (CAMPOS-CHILLON et al., 2006) or when embryos are produced *in vitro* or manipulated i.e., biopsied, cloned or bisected (SKRZYSZOWSKA et al., 1997).

2.4. In vivo derived versus in vitro produced embryos

Despite all the improvements, it is well documented that IVP still differ from their IVD counterparts; IVP embryos yield lower pregnancy rates after transfer (HASLER, 2001) and have lower cryotolerance (ENRIGHT et al., 2000; ABE et al., 2002; LONERGAN et al., 2003; ABDALLA et al., 2010). Production of embryos *in vitro* is a three-step process involving *in vitro* oocyte maturation, *in vitro* fertilization, and subsequent culture of the presumptive zygote to the blastocyst stage (MORI et al., 2002; HANSEN, 2020; CHARPIGNY et al., 2021). Embryos produced *in vitro* need to develop under the specific steps in different microenvironments that try to mimic the *in vivo* situation that occurs in the oviduct (CHARPIGNY et al., 2021). Although, *in vitro* production techniques have improved in the last decades and proved that it is possible to bypass the oviduct during early development, they do not adequate well enough the *in vivo* unique and dynamic microenvironments (BESENFELDER et al., 2012).

IVP embryos have darker cytoplasm and a lower buoyant density (POLLARD,

1993), a higher lipid content (FAIR et al., 2001), a more fragile zona pellucida, differences in metabolism (KHURANA and NIEMANN, 2000) and a higher incidence of chromosomal abnormalities (SLIMANE et al., 2000), compared to IVD embryos. In addition, many differences at the level of gene expression were reported (RIZOS et al., 2002a; WRENZYCKI et al., 2005). Furthermore, at the ultrastructural level, IVD blastocysts exhibit a denser and continuous cover of microvilli, with the trophectoderm (TE) cells closely connected by tight junctions, the inner cell mass (ICM) cells surrounded by smaller intercellular spaces and much less lipid droplets (RIZOS et al., 2002b; ZAYADAN et al., 2020; GONZÁLEZ-RODRÍGUEZ et al., 2022) than IVP embryos. All these morphological differences at the ultrastructural level may in part explain the higher sensitivity to cryoinjury exhibited by IVP embryos (FAIR et al., 2001).

2.5. Embryo biopsy

The embryo biopsy consists of removing a small number of cells for the sampling of DNA for further analysis (LEONI et al., 2000). Embryo biopsy together with the assessment of genomic estimated breeding values and sex determination of embryos enables the very early diagnosis of various genetic disorders and allows the selection of desired embryos for further transfer (CENARIU et al., 2012).

Biopsy can be performed on embryos in different developmental stages; cleavage by collecting a single blastomere, at the morula stage by collecting 2 to 3 blastomeres or at the blastocyst stage by excising 5 to 15 TE cells (CENARIU et al., 2012; NAJAFZADEH et al., 2021). In bovine embryos, an embryonic biopsy is generally performed at the blastocyst or morula stage. The decision for the optimal biopsy condition will influence the results of the subsequent implantation of the embryo and the accuracy and reliability of the genetic analysis from the DNA obtained (LACAZE et al., 1996; HASLER et al., 2002; KAGEYAMA and HIRAYAMA, 2012). There are several advantages to collecting TE cells over blastomeres, including increased number of cells for analysis, greater reliability, and less invasive procedure as the biopsy is taken from the extra-embryonic part of the blastocyst, leaving the embryo's ICM intact (ADLER et al., 2014; NAJAFZADEH et al., 2021).

Usually, three biopsy methods are used in cattle which differs on their invasiveness and practicability for ordinary use: the aspiration technique, the needle technique,

and the microblade biopsy technique (CENARIU et al., 2012). In the aspiration and the needle biopsy technique the embryo is held in place by a holding pipette. With an aspiration pipette of 20 µm in diameter or a fine needle, the zona pellucida is perforated and cells are gently aspirated through the aspiration pipette (CENARIU et al., 2012). For the microblade biopsy method, a microblade is placed on the edge of the embryo and moved down in order to extract 5 to 15 embryo cells from the embryo. At the morula stage, cells can be cut from any edge of the embryo. However, when an embryo is biopsied at the blastocyst stage, TE cells are excised (HANSEN, 2020). With this technique, the embryo can be stabilised either using a holding pipette, a dish with scratches, as suggested by BREDBACKA and BREDBACKA (1996), or using a special medium without protein (PEREZ et al., 2012). To prevent genomic DNA cross-contamination between embryos, the microblade and holding pipette must be rinsed between biopsies (THIBIER and NIBART, 1995).

When performing a TE biopsy, between 5 to 15 cells per embryo should be removed. A single diploid cell contains only 6 pg of DNA and amounts of 2-200 ng are needed for single-nucleotide polymorphism genotyping based on microarray platforms (JUNG et al., 2014). Therefore, whole-genome amplification technology needs to be performed to amplify and generate microgram quantities of DNA. Number of cells or size of the biopsy has a direct relationship with the efficiency of sex determination in bovine embryos. LACAZE et al. (1996) observed a lower efficiency of sexing when fewer than five cells were used per biopsy (GUIGNOT et al., 2009). Thus, the removal of enough numbers of cells in the biopsy procedure is required without decreasing embryo viability as the viability of biopsied embryos is related to the size of the biopsy (GUIGNOT et al., 2009; LAURI et al., 2013; PONSART et al., 2013).

Embryo biopsy is an invasive procedure that could cause damage leading to reduced embryo viability and pregnancy rates after transfer (CENARIU et al., 2012). Large variations with regards to pregnancy rates after transferring fresh biopsied embryos (33.0% - 63.0%) and biopsied cryopreserved embryos (30.0% - 62.0%) have been reported using different biopsy methods (EL-SAYED et al., 2006; GHANEM et al., 2011; CENARIU et al., 2012; FISHER et al., 2012; PONSART et al., 2013). However, the microblade technique remains the most practical and has been associated with good pregnancy results under field conditions (CENARIU et al.,

2012).

The biopsied embryo must be cryopreserved until its corresponding biopsy is analysed (NAJAFZADEH et al., 2021; GONZÁLEZ-RODRÍGUEZ et al., 2022). However, in addition to the extraction of cellular material from the embryo, the zona pellucida of the embryo must be opened partially. The zona pellucida is a protein layer with pores on its surface, which act as a filter between the embryo itself and the medium in which it is handled, in a way regulating the passage of the CPAs and therefore the time the embryo is exposed to it. The main concern with vitrification, is exposing the biopsied embryos with high concentrations of CPAs, which may cause osmotic shock and thus affect embryo survival (LOUTRADI et al., 2008; NAJAFZADEH et al., 2021).

III. MATERIAL AND METHODS

1. Animal use

IVD embryos were collected from donors during routine embryo transfer activities of Bayern-Genetik GmbH. Embryo flushing and embryo transfer were performed by trained veterinarians and technicians. Animals did not show any clinical abnormalities or fertility problems and showed regular oestrus cyclicity without any signs of clinical uterine infections. Cows were used after natural oestrus had occurred or after intravaginal progestogens and PGF2α had been used to synchronize the oestrus. All animals were kept indoors during embryo collection.

2. Chemicals and Suppliers

Unless stated differently, chemicals and reagents that were used for *in vitro* production of embryos, were purchased from Sigma-Aldrich (Taufkirchen, Germany). Chemicals, reagents, and composition of the media can be found in the Appendix (chapter IX, p. 83)

3. In vitro production of bovine embryos

The following protocol is based on that reported previously (Berg and Brem 1989).

3.1. COCs recovering from slaughterhouse derived ovaries

Ovaries of Simmental prepubertal heifers and cows that were used in this experiment for oocyte collection were collected from a local slaughterhouse and transported to the laboratory in sodium chloride (NaCl, 0.9%) at 38.5 °C within 1 hour (h). The ovaries were washed off blood with 0.9% NaCl solution (three times, 37 °C).

Cumulus cell-oocytes (COCs) were aspirated from 3-10 mm follicles using an aspirator connected to a 19 G needle. The aspiration rate and vacuum negative pressure were 80-100 mm Hg. Follicular fluids with COCs were collected in 50 ml conical tubes which were maintained at 30-35 °C at the time of oocyte search. The follicular aspirates were placed into a Petri dish and COCs were searched and washed in maturation medium. COCs were counted and graded. Quality was graded

according to classic morphological criteria (see Table 2). Briefly, class A COCs had compact, multi-layered cumulus cells with homogeneously organized ooplasm; class B COCs had compact cumulus cells with homogeneously organized ooplasm; class C COCs had less compact cumulus cells with irregular ooplasm containing dark clusters in the ooplasm; and class D COCs were without cumulus cells or had over-expanded cumulus cells and a jelly-like matrix. Class A and B COCs, those with cytoplasmic uniformity and at least three layers of cumulus cells surrounding the oocyte were selected for maturation in four-well dishes containing 500 µl of maturation medium (50 COCs/well).

In vitro maturation medium consisted in TCM-199 medium supplemented with 5% oestrus cow serum (OCS), FSH (50 IU/ml) and LH (25 IU/ml). No more than 20 min passed from completion of aspiration to placement of oocytes in maturation media because prolonged time outside of the ovary and not in its synthetic environment causes stress to the oocyte and could compromise its further development. COCs were matured for 20-22 h in the maturation medium at 38.5 °C in a 5% CO_2 humidified air atmosphere.

Table 2: Morphological classification of COCs modified from (MARC et al., 2014)

Category	Cumulus investment	Ooplasm
Class A	> 5 compact cell layers	Homogenous colour, not granulated
Class B	3-5 compact cell layers	Homogenous colour
Class C	Few cell layers, breaks	Granulated and not homogenous
Class D	Denuded or expanded	Small, granulated and not homogenous

3.2. In vitro fertilization

After *in vitro* maturation, matured COCs were rinsed three times in Fert-TALP medium (Tyrode's albumin-lactate-pyruvate (TALP) medium supplemented with 0.2 mM pyruvate, 3 mg/ml fatty acid-free BSA, and 2 mM heparin-sodium salt) and transferred in groups of 50 into 4-well dishes containing 400 µl of Fert-TALP medium. For semen preparation, motile spermatozoa were obtained by swim-up incubation of frozen-thawed spermatozoa from Simmental bulls (Bayern-Genetik GmbH, Kumhausen, Germany) in Sperm-TALP medium (TALP medium supplemented with 0.2 mM pyruvate and 3 mg/ml fatty acid-free BSA) for 1-2 h at

38.5 °C. After that, supernatant was centrifugated for 10 min at 328 xg and pellet was re-suspended in Fert-TALP. Oocytes were co-incubated with spermatozoa at a concentration of $2x10^6$ sperm/ml for 20h at 38.5 °C in a 5% CO_2 humidified air atmosphere.

3.3. In vitro culture

For *in vitro* culture, synthetic oviductal fluid (SOF) medium supplemented with 5% OCS, 40µl/ml basal medium Eagle and 10 µl/ml non-essential amino acid solution was filtered through a 0.22 µm nitrocellulose membrane, placed in a quantity of 500 µl in four-well dishes with oil overlay and CO_2 equilibrated for a minimum of 2 h at 38.5 °C in a humidified atmosphere of 5% CO_2, 5% O_2.

After fertilization, the presumptive zygotes were denuded from cumulus cells by vortexing for 3 min in a tube containing 1ml of SOF medium. The content was transferred with a Pasteur pipette in an empty petri dish and groups of 50 putative zygotes were then cultured in 500 µl of supplemented SOF medium in four-well dishes (Nunc, Roskilde, Denmark) for 7 days at 38.5 °C in a 5% CO_2, 5% O_2 humidified air atmosphere.

Only Grade I of expanded blastocysts were used for the experimental treatments.

4. Bovine embryo in vivo recovery

IVD embryos were derived from Simmental cow donors subjected to superstimulatory protocols and artificial insemination. A total of 10 cows, between the ages of 3 and 11, were used as donors to collect embryos by uterine flushing. The animals were kept in individual stalls in Kammerlehen, the embryo transfer facility of Bayern-Genetik GmbH. Hay ad libitum and mineral feed were provided along with concentrates, ß-carotene, and deep straw bedding. Animals did not show any clinical abnormalities or fertility problems and they showed regular oestrus cyclicity without any signs of clinical uterine infections.

The oestrus detection program BayernWatch (Fa. ENGS, Rosh Pina, Israel) was used. Donors' ovaries were examined by ultrasound on day 8 -14 after oestrus detection and before superstimulatory protocols were started to ensure that a functional corpus luteum (CL) (> 15 mm in diameter) was present on one of the ovaries. In some cases, a controlled internal drug release (CIDR, Zoetis, Berlin,

Germany) device was used in order to deliver progesterone in a controlled manner into the animal's bloodstream and prolong the luteal phase. All animals were kept indoors when embryo collections were performed. Embryo recovery by uterine flushing was performed by trained veterinarians and technicians.

In cow donors the superstimulatory treatment was started on day 8 -14 after oestrus detection, using a total of 700 IU of FSH/LH (Folltropin, Vetoquinol, USA) administered in decreasing doses via intramuscular injection, twice daily for 4 days with an interval of 12 h. At the time of the seventh injection, cows also received a luteolytic dose of 25 mg PGF2α (Dinolytic, Zoetis, Berlin, Germany) intramuscularly. All females were artificially inseminated with frozen/thawed semen 0, 12, 24 and 36 h after oestrus detection, using one straw of frozen sperm from bulls of proven fertility.

Embryos were recovered non-surgically by uterine flushing 7 days after artificial insemination using low epidural anaesthesia with 5 ml of procaine hydrochloride 5% (Procamidor, Richter Pharma Ag, Wels, Austria). The uterine horns were irrigated individually with the flushing medium (EmXcell, IMV, L'Aigle, France). The flushing medium together with the recovered embryos was filtered through a commercial embryo filter (EmSafe, Minitube, Tiefenbach, Germany) before being scanned under a stereomicroscope (Nikon SMZ 745T, Nikon Europe, Amsterdam, The Netherlands) at 15x magnification.

The quality and stage of development of the recovered embryos were classified according to the IETS Manual criteria (BO and MAPLETOFT, 2013). Only grades I and II of compacted morula, early blastocysts, blastocysts, and expanded blastocysts were used for the experimental treatments. Repeated embryo collections were performed in 45-to-60-day intervals.

5. Embryo evaluation

Only excellent or good quality grade I day 7 blastocysts of IVP embryos according to the IETS Manual criteria were assigned either as controls or for biopsies. On the other hand, less strict selection parameters were used when choosing IVD embryos, and grades I and II in stages of compacted morulae, early blastocysts, blastocysts, and expanded blastocysts were biopsied and assigned either to fresh or vitrification groups. These morphological evaluations were always done by the same

embryologist based on visual analysis performed by stereomicroscopy.

6. Biopsy procedure

All procedures were done with the use of surgical gown, disposable surgical gloves, facemask, and hair cover to prevent risk of contamination. All working areas, as well as the equipment involved in the process, were cleaned thoroughly with laboratory disinfectant before starting. The access of the area was limited only to the operators involved in the procedure.

Culture medium was prepared and placed in four-well dishes, as many wells as number of embryos available (one embryo per well). All dishes and wells were labelled with numbers and donor information. The dishes were incubated at 38.5 °C for at least 1 h before performing the biopsy procedure. For the preparation of the biopsy procedure, the Microfeather K-715 15° microblade (FEATHER Safety Razor Co., Ltd., Japan) was connected to the Eppendorf automated console (Micromanipulator 5171 and Transjector 5246, Eppendorf, Hamburg, Germany) and aligned under the stereoscope. Two dishes with biopsy medium, containing NaCl 0.9% supplemented with 0.15% PVP, were prepared for the rinsing of the embryos before the biopsy procedure. For the preparation of the tubes, disposable surgical gloves were used and all surfaces under the laminar flow were pre-cleaned. Tubes of 0.5 ml were labelled and prepared with 3.5 µl Tris-EDTA-Buffer and 3.5 µl Denaturation Buffer for immediate sexing.

Biopsies were carried out as described previously (PEREZ et al., 2012; TASKIN et al., 2016) with some modifications. Embryos were rinsed twice in the biopsy medium. After rinsing, the embryos became more adhesive to the surface of the petri dish decreasing their mobility, which limited the movement of the embryos during the micromanipulation. Embryos were then placed individually in a 0.5 ml drop of biopsy medium on a plastic culture dish without holding pipette. Blastocysts were rotated until ICM was clearly visible. The microblade pressed gently against a portion of TE cells that had been placed at the opposite pole of the ICM and slowly moved left and right until a small portion of the embryo (5-15 TE cells) was cut off (see Figure 1). At the morula stage, a small portion of cells (2-3 blastomeres) was detached from any edge of the embryo.

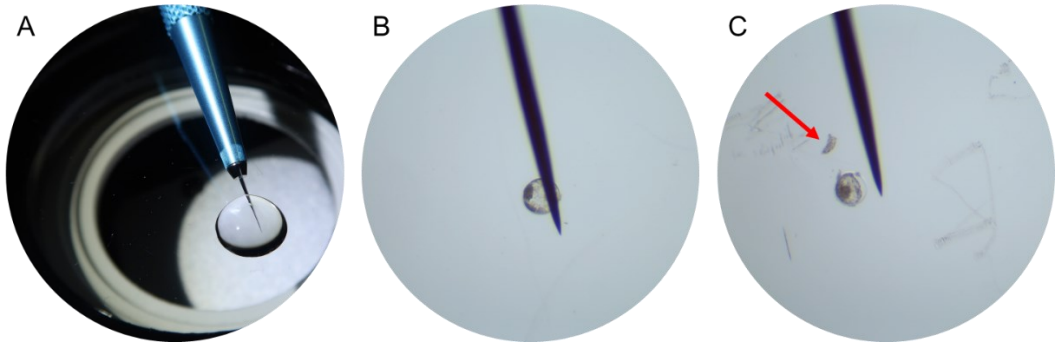

Figure 1: **Trophectoderm biopsy procedure performed using the microblade technique.** A) Human eye view of the microscalpel blade. B) Microscope view of TE biopsy using the microblade technique in a blastocyst. Note the ICM positioned away from the biopsy side. C) Microscope view of the blastocyst together with the excised TE cells (arrow) after the biopsy procedure.

The biopsied cells were gently loaded using a low retention tip (LoRetention, Eppendorf, Hamburg, Germany) with an Eppendorf pipette (Eppendorf Research plus, Eppendorf, Hamburg, Germany) set at 0.5 µL and placed at the bottom of the tubes containing 3.5 µl Tris-EDTA-Buffer and 3.5 µl Denaturation Buffer for immediate sex determination. The tubes were hold on a rack on ice.

The biopsied embryo was gently taken with a pipette and transferred into the pre-equilibrated embryo culture media in the four-well dish, previously labelled. The four-well dish was kept on a warming plate until 4 embryos were individually placed in each well, and then put back in the incubator in a controlled atmosphere at 38.5 °C with 5% CO_2, 5% O_2 humidified air.

This procedure was repeated for each embryo and biopsied cells, using a new tip and petri dish every time. To prevent DNA cross-contamination, the microblade was washed and disinfected following the biopsy procedure.

6.1. Loopamp sex determination

Biopsied cells were placed in tubes containing 3.5 µl Tris-EDTA-Buffer (10 mM Tris-HCl-1mM EDTA; pH 8.0, Sigma Aldrich, Taufkirchen, Germany) and 3.5 µl Denaturation Buffer (REPLI-g single cell kit, Qiagen, Hilden, Germany) for cell lysis and immediate sex determination. Sex identification of the samples was conducted by loop-mediated isothermal amplification (LAMP) method using the bovine sexing kit for commercial use (Loopamp Bovine Embryo Sexing Kit, Eiken Chemical Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions.

In LAMP, DNA amplification is done in isothermal condition using a DNA polymerase and four sets of specific DNA primers for DNA synthesis along with a set of primer for accelerating the LAMP reaction. The amplification of target DNA is estimated by measuring the turbidity due to a white precipitate of magnesium pyrophosphate with a turbidimeter (Loopamp End Point, Eiken Chemical Co., Ltd., Tokyo, Japan) and can be completed in 40 min.

7. Vitrification protocol

Vitrification was performed following the Cryotop® method described by MORATO et al. (2010) and the VitTrans procedure described by MORATO and MOGAS (2014) with some modifications. The Cryotop® method (Kitazato Supply, Fujinomiya, Japan) comprises a fine, transparent carrier where the embryo is loaded in a very small volume (0.1µL) attached to a plastic handle (see Figure 2). The VitTrans (MORATO and MOGAS, 2014) allows vitrified embryos to be directly heated in the straw by introducing a warming solution into an inner channel. The solution dilutes the cryoprotectant and transports the embryo to the straw (see Figure 3).

Figure 2: **Cryotop® cryodevice** comprises an (A) embryo carrier where an embryo is placed, attached to a (B) plastic handle.

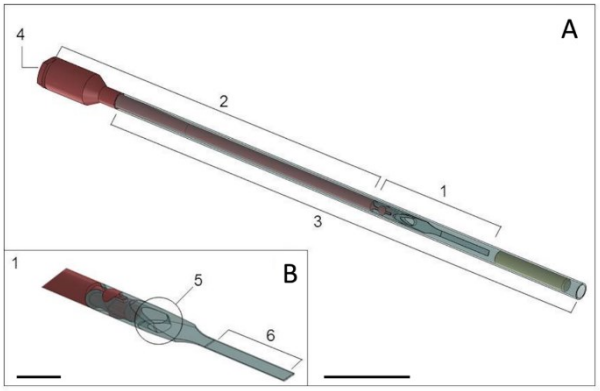

Figure 3: **The VitTrans** (A) comprises a carrier in which an embryo is placed (1), handle made of hard plastic with an inner channel (2) into which the WS is injected to dilute the CPAs and transport the embryo to the transfer straw (3), and a luer syringe connector (4) for connecting the device to the syringe containing the WS. The straw (3) serves as a cover for the device, protecting it from mechanical damage while it is being stored. A new 0.5 ml straw for sample dilution and direct embryo transfer after warming is placed for the transfer. Scale bar: 2 cm (B) Closer view of the end of the device (1) showing the outflow of the inner channel (5) and embryo attachment piece (6). Scale bar: 1 cm.

The vitrification of bovine embryos was performed in two steps. Media were kept at room temperature. Every step was visualised using a stereomicroscope. Prior to vitrification, embryos were initially equilibrated for at least 1 h in holding medium (HM: HEPES-buffered TCM199 medium supplemented with 20% foetal calf serum (FCS)) at room temperature in order to remove any previous medium. Then, embryos were incubated in equilibration solution (ES: 7.5% (v/v) ethylene glycol and 7.5% (v/v) dimethyl sulfoxide in HM) for 6 min (biopsied embryos) or 8 min (intact embryos).

In experiment 1 and 2, the equilibration in ES was performed for 4-5 embryos at the same time using one media set-up in a Petri dish, positioning the embryos at the edge of the dish. In experiment 3, embryos were equilibrated individually in a 4 well dish, one embryo per well. Embryos were then transferred to one 15µL drop of vitrification solution (VS) consisting of 15% (v/v) EG, 15% (v/v) DMSO, and 0.5 M sucrose dissolved in the HM and moved again into a second drop of VS. After incubation for 30 s in VS, each embryo was individually loaded onto the embryo attachment part of the Cryotop® or the VitTrans device. Then, most of the solution was removed, leaving only a thin layer on the blastocyst (0.1µL), and the

sample was quickly plunged into LN_2. Subsequently, the Cryotop® or VitTrans cryodevice was covered with a protective straw. The entire process from immersion in the VS to plunging into LN_2 took less than 1 min (see Figure 4). The devices were stored in LN_2 until further use.

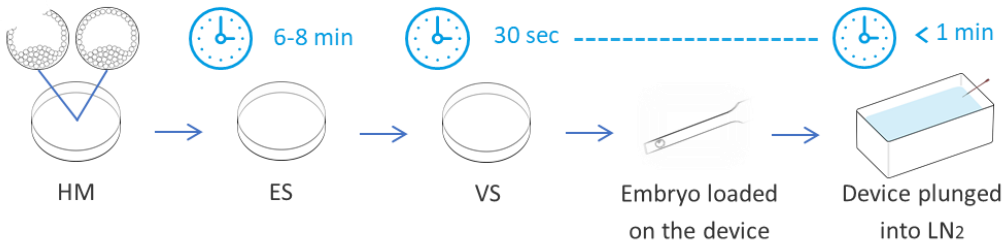

Figure 4: **Schematic presentation of vitrification procedure.** During vitrification, an embryo is exposed to ES containing DMSO/EG from 6 to 8 min. Then the embryo is placed in VS containing higher concentrations of DMSO/EG and 0.5 M sucrose for 30 seconds to dehydrate, loaded onto a Cryotop® or VitTrans cryodevice with a very small volume of vitrification medium, and plunged into LN2 within less than 1 min.

Abbr: HM: Holding medium, ES: Equilibration solution, VS: Vitrification solution, LN2: Liquid nitrogen.

Representative sequences of the osmotic response of biopsied and intact IVP blastocysts exposed to the ES and VS are pictured in Figure 5.

Figure 5: **Representative sequences of the osmotic responses** of an (A) IVP biopsied embryo and an (B) intact embryo exposed to HM, ES and VS. (C) Image of an embryo loaded onto the embryo attachment part of the Cryotop® or the VitTrans cryodevice.

Abbr: HM: Holding medium, ES: Equilibration solution, VS: Vitrification solution.

7.1. Cryotop® warming protocol

All warming steps were performed at 38.5 °C using a stereomicroscope to visualise each step. During warming, the protective straw was removed from the Cryotop® while it was still submerged in LN_2. Embryos were then subjected to different warming protocols: (1) three-step procedure: the loaded Cryotop® was plunged directly into the WS containing 1M sucrose dissolved in HM. After 1 min, embryos were transferred to 0.5 M sucrose dissolved in HM for 3 min and to HM for 5 min (see Figure 6); (2) 1-step procedure: the loaded Cryotop® was plunged directly into the WS containing 0.5 M sucrose dissolved in HM for 5 min (see Figure 7). In experiment 1, IVP blastocysts warmed by one- or three-step warming procedure were transferred to SOF culture medium and incubated at 38.5 °C in 5% CO_2 and 5% O_2 in air for 24 h. In experiment 3, IVD embryos were loaded in HM into a 0.25 ml French straw for transfer into recipients.

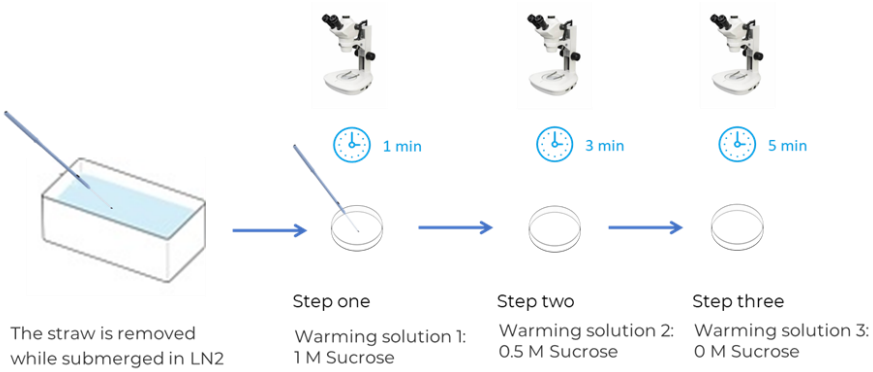

Figure 6: Schematic presentation of the three-step warming procedure using Cryotop®.

Abbr: Liquid nitrogen (LN2), molar (M).

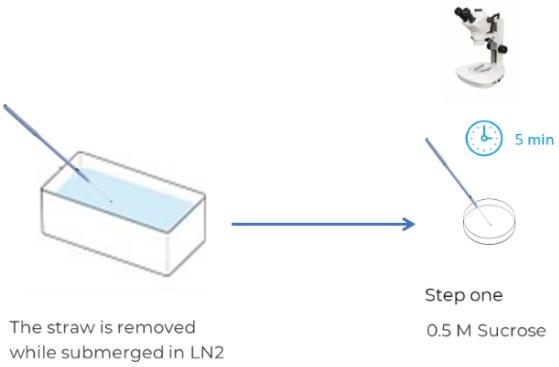

Figure 7: Schematic presentation of the 1-step warming procedure using Cryotop®.

Abbr: Liquid nitrogen (LN2), molar (M).

7.2. VitTrans warming protocol

Embryos in the VitTrans group were warmed in-straw as described previously (MORATO and MOGAS, 2014) with some modifications.

For warming, the VitTrans was first twisted off within LN_2 to release pressure and a new 0.5 ml equine embryo transfer plastic straw (IMV technologies, L'Aigle, France) was placed onto the VitTrans cryodevice. Then the entire VitTrans cryodevice (including the plastic straw) was removed of the LN_2 and immediately attached using the luer connector to a syringe carrying the CPA WS (300 µl of 0.5 M sucrose dissolved in HM) at 40 °C. By entering the WS into the VitTrans device, the embryo is flushed into the plastic straw. The straw containing the warmed IVD embryo (experiment 3) was readily used for transfer after the VitTrans was removed (see Figure 8). For IVP embryos (experiment 2), the cotton plug end of the straw was cut, and the contents of the straw released into a Petri dish. Blastocysts were then moved to SOF media and *in vitro* cultured for 24 h at 38.5 °C in 5% CO_2 and 5% O_2 in air.

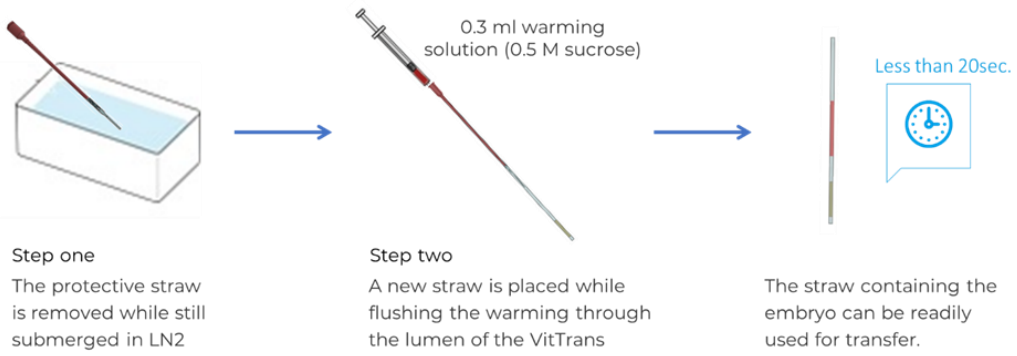

Figure 8: Schematic presentation of the warming procedure using VitTrans.

Abbr: Liquid nitrogen (LN2).

8. Embryo transfer

A total of 61 Simmental cows and heifers were used as recipients. Recipient animals did not show any clinical abnormalities or fertility problems and they showed regular oestrus cyclicity without any signs of clinical uterine infections. The recipient cows and heifers were from a wide range of parities and different farms, but they had a good corporal condition. Animals were used after natural oestrus had occurred or after intravaginal progestogens and PGF2a had been used to

synchronize oestrus. Embryo transfers were performed on day 7 or 8 after the onset of oestrus by trained veterinarians and technicians. Recipients' ovaries were examined by transrectal palpation or ultrasound on the day of embryo transfer to ensure that a functional CL (>15 mm in diameter) was present on one of the ovaries.

Embryos vitrified using the Cryotop® method were warmed in the laboratory and loaded with holding medium into a 0.25 ml embryo transfer plastic straw (IMV technologies, L'Aigle, France). Straws were transported to the clients' farm with the embryo transporter TREO (WTA, Cravinhos, SP, Brazil) and non-surgically transferred into the uterine horn ipsilateral to the existing CL (> 15 mm in diameter) under low epidural anaesthesia (5 ml procaine hydrochloride 5%) using a bovine embryo transfer gun (IMV Technologies, L'Aigle, France).

Embryos vitrified using the VitTrans protocol were warmed directly at the client´s farm under field conditions. The embryo was transferred using a 0.5 ml straw, the same straw that was used to warm it. The presence of the embryo inside the straw was always confirmed using a stereomicroscope when embryos were warmed via the VitTrans procedure. Each embryo was non-surgically transferred into the uterine horn ipsilateral to the existing CL (> 15 mm in diameter) under low epidural anaesthesia (5 ml procaine hydrochloride 5%) using an equine embryo transfer gun (IMV Technologies, L'Aigle, France).

For each embryo transfer, the time from the introduction of the straws inside the transferring gun until the embryo transfer was recorded. Pregnancy was diagnosed by transrectal palpation or using a portable ultrasound imaging device equipped with a transrectal probe around 60 days after embryo transfer.

9. Experimental design

The overall objective of the present study was to assess the applicability of a vitrification method suitable for direct warming of biopsied bovine embryos. Three experiments were done to determine *in vitro* embryo viability of IVP biopsied bovine embryos and pregnancy rates of IVD biopsied bovine embryos, using two different vitrification, and warming protocols. The VitTrans, that enables direct embryo transfer to the uterus of the recipient animal, was compared to the Cryotop® method.

In the first experiment, a 1-step warming method for IVP bovine embryos was evaluated. Grade I day 7 IVP expanded blastocysts were biopsied and immediately vitrified onto Cryotops®. Warming was carried out by the 1-step or 3-step warming procedure. Embryo survival was expressed as proportion of blastocysts showing signs of re-expansion at 24 h post-warming.

In the second experiment, the competence of the VitTrans protocol to be employed for vitrification and in-straw warming of biopsied IVP bovine embryos was tested. Grade I day 7 IVP expanded blastocysts were randomly assigned to three different groups: intact, non-biopsied blastocysts, biopsied 0 h, blastocysts vitrified immediately after the biopsy procedure, and biopsied 3 h, blastocysts vitrified 3 h after the biopsy procedure. Blastocysts from each group were vitrified and warmed by the VitTrans methodology. Embryo survival was expressed as proportion of blastocysts showing signs of re-expansion at 24 h post-warming.

In the third experiment, the ability of IVD biopsied embryos to establish pregnancies after vitrification/warming with the Cryotop® or VitTrans protocol was assessed. Grade I and II day 4, 5, 6 and 7 IVD embryos were biopsied and randomly distributed into three groups: control, fresh biopsied embryos; VitTrans, biopsied embryos were cultured for 3 h after the biopsy procedure and vitrified by the VitTrans protocol; Cryotop®, biopsied embryos were cultured for 3 h after the biopsy procedure and vitrified by Cryotop®, 3 step warming protocol. Fresh, not vitrified, embryos were transferred 3 h after the biopsy procedure to synchronous recipients. Vitrified embryos were warmed before being transferred to recipients.

The time between insertion of the straws into the transferring gun and transfer of each embryo was recorded. The LAMP method was used to determine the sex of the embryos using biopsied cells.

Pregnancy was diagnosed by transrectal palpation or ultrasound examination around 60 days after embryo transfer (see Figure 9) The impact of vitrification method, embryo quality, embryo developmental stage and time from warming to transfer of biopsied IVD embryos on pregnancy rate was assessed.

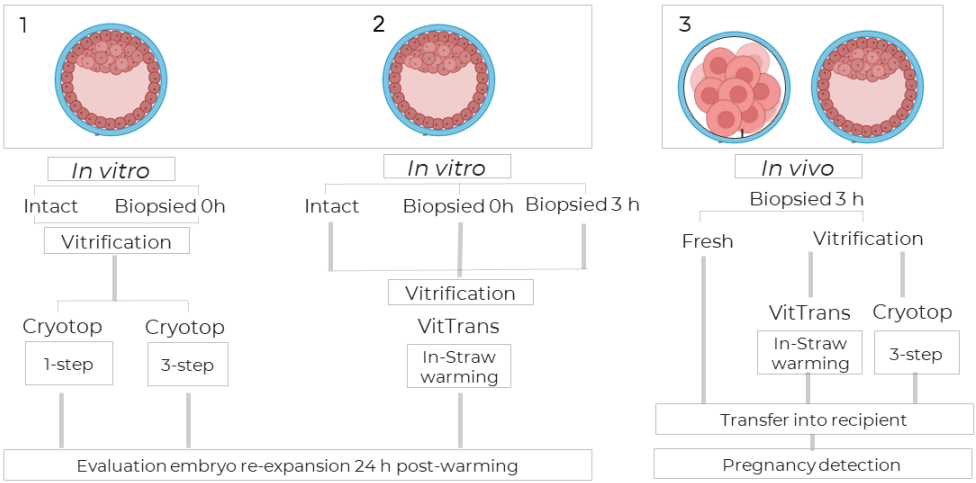

Figure 9: Schematic representation of the experimental design.

(1) Experiment 1: Bovine IVP blastocysts were assigned to intact or biopsied 0h group prior to vitrification onto Cryotop®, and they were warmed by a 1- or 3-step procedure. (2) Experiment 2: Bovine IVP blastocysts were assigned to different groups prior to vitrification using the VitTrans vitrification/in-straw warming method: Intact, biopsied 0h, biopsied 3 h period of culture group. Embryos in the experiment 1 and 2 were evaluated 24 h post-warming. (3) Experiment 3: Bovine IVD embryos were biopsied and distributed between three groups prior to transfer into recipients: fresh, VitTrans, Cryotop®. Pregnancy was diagnosed 60 days after embryo transfer.

9.1. Experiment 1 - Vitrification of intact or biopsied IVP blastocysts using the Cryotop® method and warmed by 1-step or 3-step procedure

A total number of 154 grade I day 7 IVP blastocysts were randomly assigned either to an intact (n=96) or a biopsied 0h group (n=58). The blastocysts allocated to the biopsied 0h group were biopsied using the microscalpel blade biopsy technique. Embryos from both groups, intact and biopsied 0h, were vitrified on Cryotops®.

All blastocysts were exposed to an ES containing 7.5% (v/v) EG + 7.5% (v/v) DMSO in HM for 6 min (biopsied embryos) or 8 min (intact embryos), transferred to the VS consisting of 15% (v/v) EG + 15% (v/v) DMSO + 0.5 M sucrose dissolved in the HM for 30 s, placed in a microdroplet on the Cryotop® and then plunged into LN_2. The devices were labelled and stored in LN_2 for at least 24 h. Intact and biopsied vitrified blastocysts were warmed by: (1) 1-step warming procedure (intact, n=21; biopsied 0h, n=28) or (2) 3-step procedure (intact, n=75; biopsied 0h,

n=30). After warming, the blastocysts were placed in four well dishes in culture medium for 24 h. All embryos were evaluated for re-expansion and morphology 24h post-warming.

9.2. Experiment 2 – Vitrification and in-straw warming of intact and biopsied 0 h/3 h IVP blastocysts using the VitTrans method

A total number of 113 grade I day 7 IVP blastocysts were randomly assigned to three different groups prior to vitrification: intact (n=34), biopsied 0h (n=34), and biopsied 3 h of culture (n=45). The blastocysts allocated to the biopsy group were biopsied using the microscalpel blade biopsy technique. The blastocysts assigned to the biopsy 0h were vitrified immediately after performing the biopsy treatment. The blastocysts assigned to biopsied 3 h of culture, were left in culture medium and placed in the incubator set at 38.5 °C with 5% CO_2, 5% O_2 humidified air for 3 h before performing the vitrification procedure.

Embryos from all groups, intact, biopsied 0h and biopsied 3 h of culture, were vitrified on the VitTrans cryodevice using the same vitrification protocol. All blastocysts were exposed to an ES containing 7.5% (v/v) EG + 7.5% (v/v) DMSO in HM for 6 min (biopsied embryos) or 8 min (intact embryos), transferred to the VS consisting of 15% (v/v) EG + 15% (v/v) DMSO + 0.5 M sucrose dissolved in the HM for 30 s and then placed in a microdroplet onto the embryo attachment piece of the VitTrans cryodevice. The VitTrans was plunged into LN_2 and covered with a 0.5 ml straw.

For warming, the protective cover was removed from the VitTrans while still submerged in LN_2. Subsequently, a new 0.5 ml embryo transfer straw was placed on the VitTrans while flushing the CPA solution (300 µl of 0.5 M sucrose in HM), warmed at 40 °C, with a syringe through the lumen of the VitTrans device. By entering the WS into the VitTrans device, the embryo is flushed into the plastic straw. The cotton plug end of the straw was cut, and the contents of the straw released into a Petri dish. Blastocysts were then moved to SOF media and *in vitro* cultured for 24 h at 38.5 °C in 5% CO_2 and 5% O_2 in air. Re-expansion and morphology were evaluated 24h post-warming.

9.3. Experiment 3 - Effects of vitrification on pregnancy outcome of biopsied IVD embryos

In this third experiment, pregnancy outcome was evaluated. A total number of 61 IVD embryos with 11 replicates, in which each replicate corresponded to a day of embryo recovery, were used. Recovered grade I and II stage 4, 5, 6 and 7 IVD embryos were biopsied using the microblade biopsy technique and randomly distributed between three groups: control, fresh biopsied embryos (n=26), VitTrans (n=15), and Cryotop® (n=20).

Embryos assigned to the vitrification groups and were left in culture medium at 38.5 °C with 5% CO_2, 5% O_2 humidified air for 3 h before performing the vitrification procedure. After the 3 h period of culture, embryos were exposed to an ES containing 7.5% (v/v) EG and 7.5% (v/v) DMSO in HM for 6 min, transferred to the VS consisting of 15% (v/v) EG + 15% (v/v) DMSO + 0.5 M sucrose in HM for 30 s and then placed in a microdroplet onto the embryo attachment piece of the VitTrans or Cryotop® cryodevice. The loaded cryodevice was then plunged into LN_2 and covered with a protective straw.

Embryos in the VitTrans group, were non-surgically transferred to synchronized recipients after in-straw dilution with 0,5 M sucrose in HM at 40 °C in a 0.5 ml equine embryo transfer straw (IMV technologies, France) under field conditions. Embryos in the Cryotop® group, were warmed by a 3-step procedure in the laboratory, loaded into a 0.25 ml straw (IMV technologies, France), transported to the client´s farm and transferred to synchronized recipients. The embryos assigned to the group of fresh embryos were transferred fresh 3 h after the biopsy procedure to synchronous recipients. The LAMP method was used to determine the sex of the embryos using biopsied cells.

Pregnancy was diagnosed by palpation via the rectum or ultrasonography after day 60 of embryo transfer. The impact of the embryo sex on pregnancy rates was evaluated. Also, we determined whether embryo quality and embryo development prior to biopsy had any effect on the pregnancy outcome. The time between warming and transfer of each embryo with the two different vitrification methodologies was recorded, and the impact on pregnancy rates was analysed.

10. Statistical analysis

The software IBM SPSS Version 25.0 (IBM Corp., USA) was used to perform all statistical analysis.

In experiment 1 and 2, embryo survival after 24 h of *in vitro* culture was assessed and the percentages were calculated per group considering replicates. Normal distribution was checked with Shapiro-Wilk test. Homogenity of variances was checked with Levene test. Survival and re-expansion rates were also compared by ANOVA and Bonferroni test for pair-waise comparisons.

In experiment 3, the conception rates at 60 days of pregnancy were analysed both by binomial logistic regression and Chi-square test. The Chi-square test was chosen to examine whether the cryopreservation method influenced pregnancy rates. Also, the comparison of quality and embryo development on pregnancy rates were analysed using logistic regression models. The period of time between the warming and the transfer and its influence on pregnancy outcome was examined for the two different cryopreservation methodologies by Chi-square test.

The level of statistical significance was set at $p < 0.05$. P-values between 0.01 and 0.05 were considered significant results, and between 0.05 and 0.1 as tendency.

Descriptive analysis of the data was shown on tables or using graphical representations as floating bars charts and bar charts with error bars. GraphPad software (GraphPad Software, USA) was used to prepare the graphs. In experiment 1 and 2, differences between groups were expressed as least square means (LMS) ± standard error of the mean (SEM). In experiment 3, the data is reported based on a 95% confidence interval (CI).

IV. RESULTS

Many warming protocols for vitrified embryos involve warming embryos in a multi-step dilution procedure through certain concentrations of sucrose to gradually remove CPAs. However, under field conditions, this can be challenging as it requires a microscope for the dilution steps and placing embryos into a straw before embryo transfer. The simplification of the warming procedures to a 1-step dilution method is essential for the practical application of vitrification in the field, thus allowing direct embryo transfer.

1. Vitrification of intact or biopsied IVP blastocysts using the Cryotop® method and warmed by 1-step or 3-step procedure

The aim of the first experiments was to assess the *in vitro* survival rates of intact and biopsied bovine blastocysts that had been warmed by 1-step or 3-step warming procedure. The Cryotop® method, was chosen because it is the industry standard for vitrification of human embryos. IVP blastocysts were biopsied and immediately vitrified onto Cryotops®. Warming was carried out by the 1-step or 3-step warming procedure. The embryo survival was recorded 24 h post-warming as indicated by re-expansion.

Figure 10 shows the results of embryo survival 24 h after warming of intact or biopsied IVP blastocysts using the 1-step or 3-step warming procedure. Results indicate that neither the warming method nor the biopsy procedure had any effect on embryo survival of day 7 IVP expanded blastocyst at 24 h post-warming. After 1-step warming, there were no differences in embryo survival in intact (69.8% ± 8.7) or biopsied embryos (65.0% ± 5.5, $p = 0.897$). Similarly, after 3-step warming, intact embryos showed similar survival rates (75.8% ± 6.9) than biopsied embryos (61.7% ± 16.2, $p = 0.367$).

Representative pictures of intact and biopsied blastocysts before vitrification, after 1- or 3-step warming and after re-expansion are pictured in Figure 11.

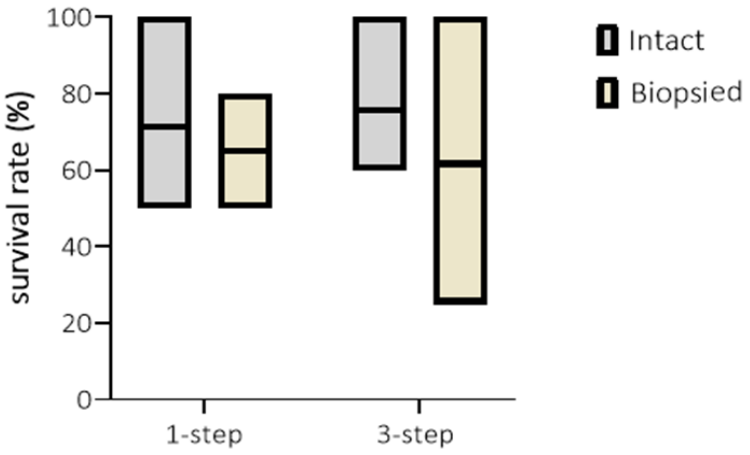

Figure 10: **Post warming survival (%) of intact or biopsied embryos vitrified using the Cryotop® method and warmed by 1-step (intact, n=21; biopsied, n=28) or 3-step-(intact, n=75; biopsied, n=30) warming procedure.** The solid line indicates the mean, and floating bars represent minimum to maximum values. Experiments were replicated 6 times.

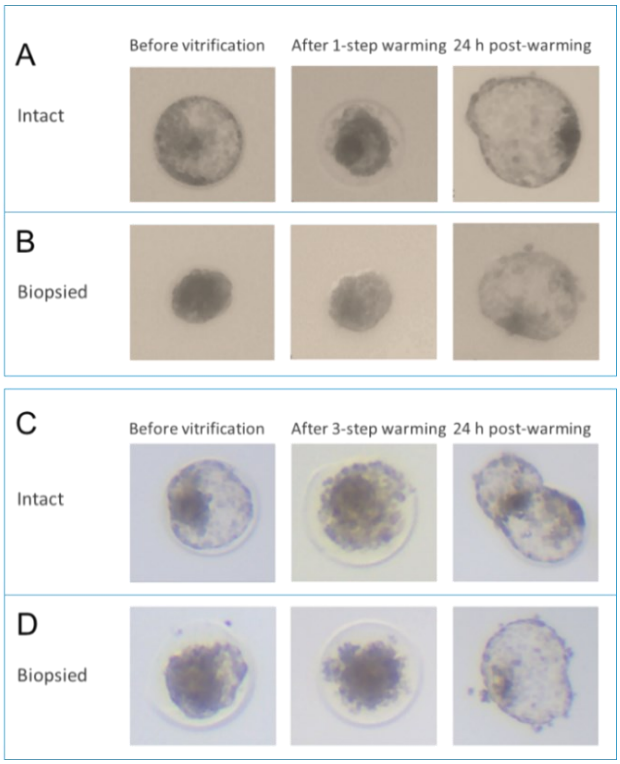

Figure 11: **IVP blastocysts after 1- and 3-step warming using the Cryotop®.**

(A) Intact embryo before, right after 1-step warming and 24 h post-warming. (B) Embryo subjected to the biopsy procedure before, right after 1-step warming and 24 h post-warming. (C) Intact embryo before, right after 3-step warming and 24 h post-warming. (D) Embryo subjected to the biopsy procedure before, right after 3-step warming and 24 h post-warming.

2. Vitrification and in-straw warming of intact and biopsied 0 h/3 h IVP blastocysts using the VitTrans method

The aim of this second experiment was to determine the *in vitro* survival rates of intact and biopsied IVP blastocysts using the VitTrans cryodevice for vitrification and in-straw warming. IVP blastocysts that were intact, biopsied without culture, and biopsied with 3 h of culture were vitrified and warmed in-straw by the VitTrans method.

Post-warming survival of intact and biopsied IVP blastocysts cultured for 0 or 3 h before vitrification using the in-straw VitTrans method are shown in Figure 12. The post-warming survival rates of in-straw warmed embryos were not significantly different between groups, though there was a positive tendency for higher survival rates in intact embryos (84.6% ± 2.4) when compared with those of biopsied embryos (57.1%, 62.8%, p = 0.058). However, there was no significant difference in survival rates of biopsied blastocysts vitrified after 0 h (57.1% ± 11.9) or 3 h in culture (62.8% ± 7.4; p = 0.632).

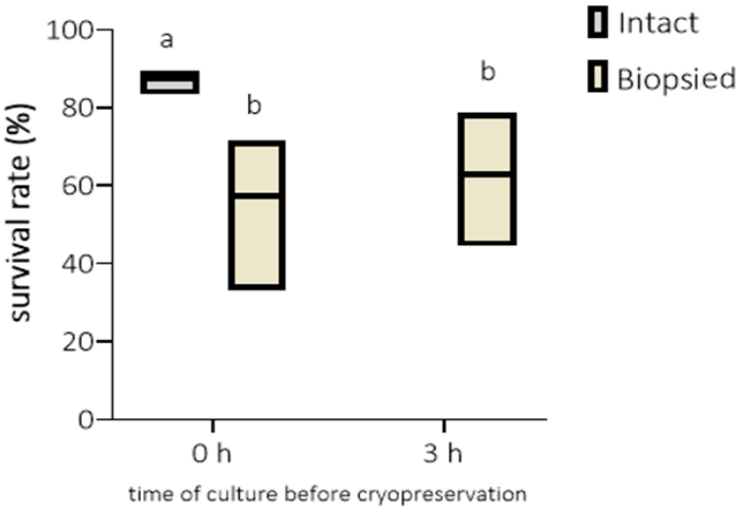

Figure 12: **Post warming survival (%) of recovered/warmed intact (n = 34) or biopsied embryos cultured for 0 h (n = 34) or 3 h (n = 45) before vitrification/in-straw warming by the VitTrans method were compared.** The solid line indicates the mean, and floating bars represent minimum to maximum values. [a,b] Values within columns with different superscripts show a tendency (p = 0.058). Experiments were replicated 8 times.

Some embryos were lost during warming using the VitTrans method in all groups. They were found attached to the inside of the wall of the straw after flushing the

media into the lumen of the VitTrans or on the embryo attachment of the cryodevice. A total number of 14 embryos, resulting in 3 intact group (10.3% ± 7.5), 6 biopsied 0 h (16.8% ± 3.6) and 5 biopsied 3 h (10.5% ± 4.2) period of culture, were lost. The rate of lost embryos during the warming were not significantly different between groups (p = 0.643).

3. Effects of vitrification on pregnancy outcome of biopsied IVD embryos

The aim of the third experiment, was to assess the ability of IVD biopsied embryos to establish pregnancies after vitrification/warming using the Cryotop® or VitTrans method. Not only was the effect of the vitrification method analysed, but also the influence of the quality of the embryo, its developmental stage, the sex and period of time between warming and transfer.

A total of 61 transferrable IVD embryos of quality I and II according to the IETS criteria were collected from 10 cows. Out of these embryos, 32 were quality grade I and 29 grade II. In terms of developmental stages, 29 embryos were compact morulae, 7 were early blastocysts, 18 were blastocysts, and 7 were expanded blastocysts. There was no difference in conception rates after transferring IVD embryos fresh (53.8%, 95% CI: 33.3 - 74.4), vitrified/warmed using the Cryotop® (35.0%, 95% CI: 12.1 - 57.9) or VitTrans (33.3%, 95% CI: 16.3 - 60.6) methods (p = 0.280, see Figure 13).

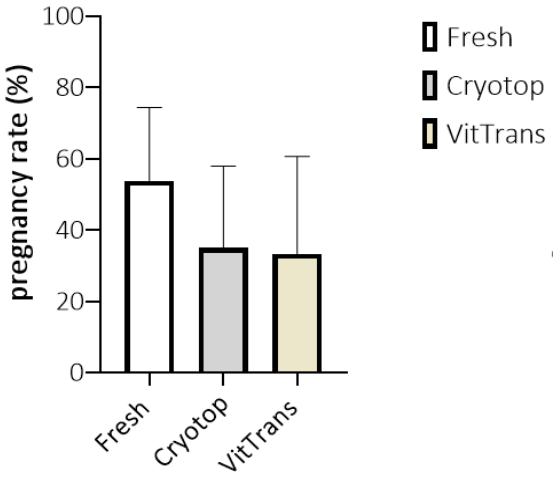

Figure 13: Comparison of pregnancy outcome among biopsied IVD embryos transferred fresh (n = 26), cryopreserved on Cryotop® (n = 20) or VitTrans (n = 15). The bars indicate 95% CI.

To assess the effect of vitrification, biopsied IVD embryos from the VitTrans and Cryotop® groups were pooled together as cryopreserved embryos group, and compared with non-cryopreserved, fresh embryos. However, there was a positive tendency for higher pregnancy rate after transfer of fresh embryos compared to cryopreserved embryos (34.3%, 95% CI: 17.7 - 50.4, p = 0.103, Figure 14)

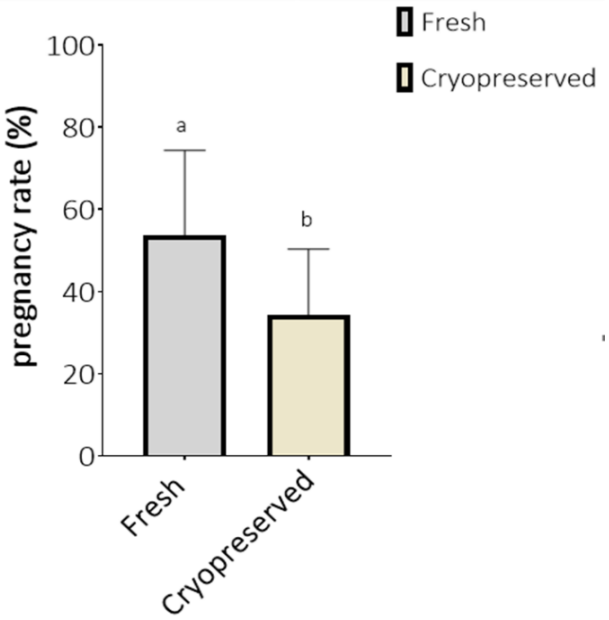

Figure 14: **Comparison of pregnancy outcome among biopsied IVD embryos transferred fresh (n = 26) or after cryopreservation (VitTrans and Cryotop®, n = 35).** The bars indicate 95% CI. [a,b] Values within columns with different superscripts show a tendency (p = 0.103).

The pregnancy outcome after the transfer of embryos with different quality or stages of development was evaluated. There was no significant difference in pregnancy rates of IVD embryos classified as grade I (35.3%, 95% CI: 10.0 - 74.4) and grade II (33.3%, 95% CI: 9.2 - 57.5) in the cryopreserved group (p = 0.592). The pregnancy rate of IVD embryos that were transferred fresh showed a positive tendency towards embryos classified as grade I (60.0 %, 95% CI: 31.9 - 88.1) compared to embryos graded as grade II (45.5%, 95% CI: 10.4 - 80.5) (p = 0.108) (see Figure 15).

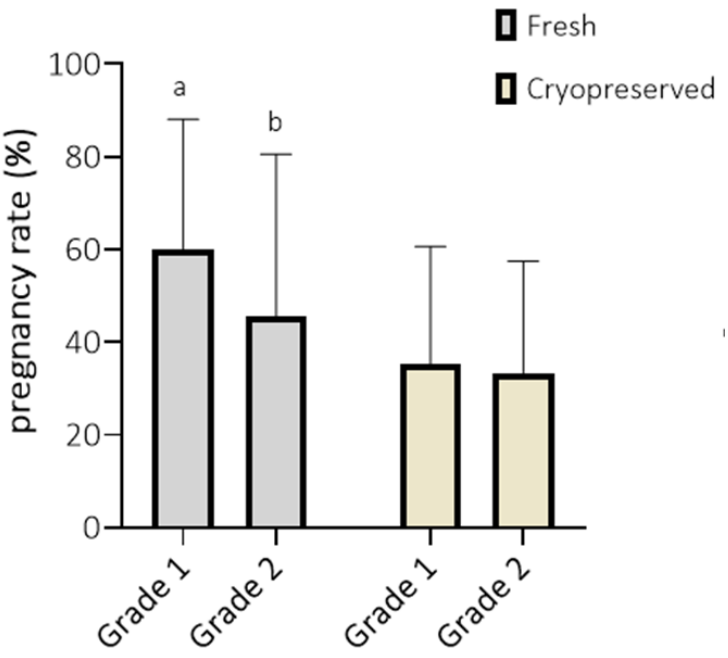

Figure 15: **Pregnancy rate 60 days after transfer of fresh or cryopreserved IVD biopsied embryos classified as grade I (fresh, n = 15; cryopreserved, n = 17) and grade II (fresh, n = 11; cryopreserved, n = 18).** The bars indicate 95% CI. [a,b] Values within columns with different superscripts show a tendency (p = 0.108).

Likewise, no significant differences in pregnancy rates were observed when embryos were biopsied at different stages of development and transferred fresh or after cryopreservation. Pregnancy rates ranged from 40.0% to 100.0% after transfer of fresh IVD embryos (p = 0.720), and from 25.0% to 50.0% after transfer of those cryopreserved (p > 0.719) (see Table 3).

Representative pictures of biopsied IVD embryos producing a successful pregnancy after transfer, at different developmental stages and quality before biopsy, after 3 h of culture post-biopsy and prior to vitrification, are shown in Figure 16.

Table 3: Pregnancy rate 60 days after transfer of IVD biopsied embryos at different development stages

Treatment	Developmental stage	n	Pregnancy rate (60 d) %	95% CI
Fresh	Morulae	14	57.1	27.5 - 86.8
	Early blastocyst	1	100.0	-
	Blastocyst	6	50.0	37.5 - 74.8
	Expanded blastocyst	5	40.0	28.0 - 60.0
Cryopreserved	Morulae	15	33.3	6.3 - 60.3
	Early blastocyst	6	50.0	22.3 - 81.8
	Blastocyst	12	25.0	3.7 - 53.7
	Expanded blastocyst	2	50.0	5.8 - 68.5

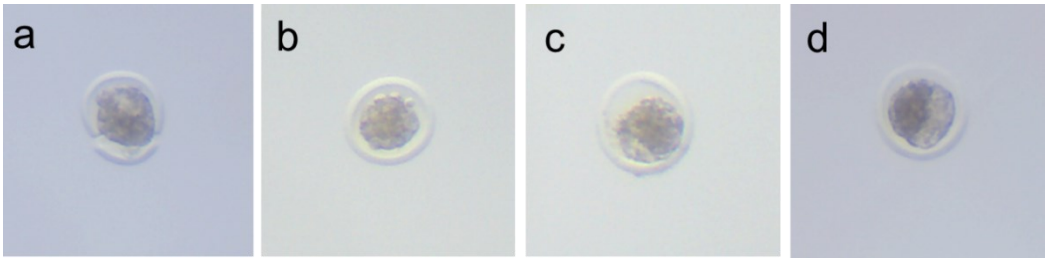

Figure 16: Biopsied IVD embryos with different developmental stage and quality before biopsy that resulted in a positive pregnancy diagnosis after transfer (a) morulae grade II and (b) early blastocyst grade I (c) blastocyst grade I and (d) expanded blastocyst grade II; after 3 h of culture post-biopsy and prior to vitrification.

When embryos were sorted for sex, transfer of female IVD biopsied embryos produced significantly higher pregnancy rates (70.6%, 95% CI: 46.4 - 94.7) than male (33.3%, 95% CI, 0.0 - 33.3) when embryos were transferred fresh ($p = 0.027$). There was no relation between the sex of embryos and their potential to establish a pregnancy in IVD biopsied embryos that were vitrified and transferred into recipients (XX = 41.70%, 95% CI: 9.0 - 74.4; XY = 38.90%, 95% CI: 13.9 - 63.8; $p = 0.588$, see Figure 17). The success rate of sex identification by LAMP method in all groups was 81%, which confirmed that enough DNA sample was taken and efficiently amplified.

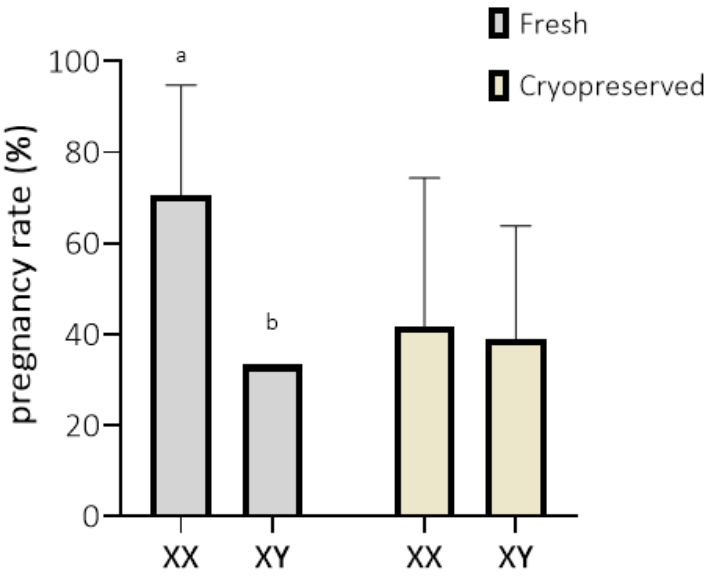

Figure 17: **Biopsied IVD embryos of both sexes and the pregnancy outcome. (XX: fresh, n = 17, cryopreserved, n = 12; XY: fresh, n = 3, cryopreserved, n = 18).** The bars indicate 95% CI. [a,b] Values within columns with different superscripts show a significant difference (p = 0.027).

Pregnancy rates of biopsied embryos transferred after different intervals between warming and transfer are shown in Table 4 and Figure 18. The interval between warming and placing the straws into the transferring gun and embryo transfer ranged from 5 to 45 min, with an average time of 8 min warming in the VitTrans and 20 min warming using the Cryotop® and has an influence on pregnancy rates (p = 0.003), as well as the method used for warming (Cryotop or VitTrans, (p = 0.002)). Embryos vitrified on VitTrans and warmed in-straw resulted in significantly higher pregnancy rates when they were transferred immediately after warming (up to 10 min). However, pregnancy rates were significantly higher when embryos vitrified using the Cryotop® method were transferred at 20 and 35 min after warming rather than immediately after warming.

Table 4: Pregnancy outcome of biopsied IVD embryos transferred after different times after warming, while remaining in the transferring gun

Device	Time between warming and transfer (min)	n	Pregnancy rate % (60 d)
VitTrans	5	11	18.1 [a,*]
	10	3	100.0 [b,*]
	30	1	0.0 [c,*]
Cryotop®	5	4	0.0 [a;#]
	10	3	0.0 [b,#]
	15	6	33.3 [c,#]
	20	1	100.0 [d,#]
	35	1	100.0 [d,#]
	45	5	60.0 [e,#]

[a,b,c,d,e] Values within columns with different superscripts indicate a significant difference in times between warming and transfer ($p = 0.003$). [*,#] Values within columns with different superscripts indicate a significant difference between cryopreservation devices (VitTrans, Cryotop®) ($p = 0.002$).

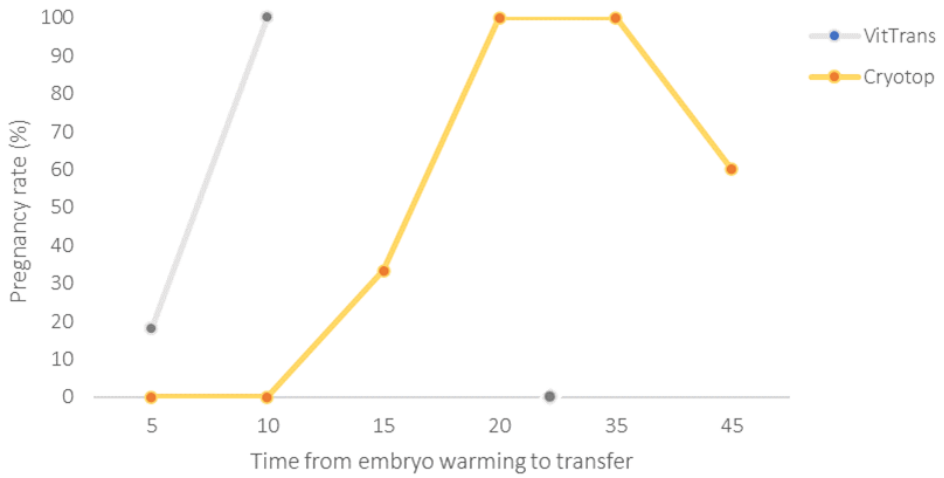

Figure 18: Pregnancy outcome of biopsied IVD embryos transferred at different times after warming, while remaining in the transferring gun.

The first calf born after direct transfer of a sexed biopsied IVD embryo that was vitrified on VitTrans and warmed by the in-straw methodology, is pictured in Figure 19.

Figure 19: **Calf born after transfer of a biopsied vitrified embryo using the VitTrans method.**

V. DISCUSSION

The main objective of the present study was to determine if a vitrification/in-straw warming method was suitable for cryopreservation and direct transfer of biopsied bovine embryos. Embryo biopsy provides the material for early genetic selection and early sex determination, but the biopsy technique harms the embryo, making it more sensitive to cryopreservation (MOGAS, 2019; GONZÁLEZ-RODRÍGUEZ et al., 2022). Vitrification and slow freezing are the two commonly used embryo cryopreservation methods. In a recent report, NAJAFZADEH et al. (2021) showed that vitrification provided a higher cryo-survival rate than slow freezing in biopsied bovine IVP blastocysts, but warming protocols often involve multi-step dilutions that make transferring vitrified embryos difficult in field conditions.

Therefore, in a first experiment in this study, we aimed to evaluate *in vitro* viability of vitrified IVP biopsied bovine embryos warmed using a 1-step warming protocol, as this will be essential to simplify the warming procedure and enable direct embryo transfer in the field. This was done by comparing the 1-step warming with a 3-step warming procedure using the Cryotop® method. In a second experiment, we determined *in vitro* viability of intact and biopsied bovine embryos that were vitrified and subsequently warmed in-straw using the VitTrans cryodevice, which allows direct transfer. We also evaluated the optimal time interval between biopsy and vitrification in terms of embryo *in vitro* survival. All this allowed us to select the best conditions to perform direct transfer of cryopreserved biopsied embryos under field conditions for the third study. In this final part of the study, pregnancy outcome of IVD biopsied embryos vitrified/warmed using the VitTrans method, which enables direct transfer of embryos to the uterus of a recipient animal, was compared to the Cryotop® method, that consists of a 3-step warming procedure and in-direct transfer method.

1. Vitrification of intact or biopsied IVP blastocysts using the Cryotop® method and warmed by 1-step or 3-step procedure

In the first experiment, we first compared the survival of biopsied IVP embryos vitrified/warmed using the Cryotop® method but using two different warming protocols (multi-step vs. 1-step). The survival rates obtained suggest that warming

and dilution by a 1- or 3-step procedure did not lead to any differences in embryo viability. After 1-step warming, there were no significant differences in embryo survival in intact or biopsied embryos and the same results were found in vitrified intact and biopsied embryos after 3-step warming. So far, previous research has found no negative effects on the developing potential of vitrified intact embryos when warmed directly using a 1-step warming protocol (INABA et al., 2011; MORATO and MOGAS, 2014; ZHANG et al., 2015)

Similarly, the biopsy treatment did not influence survival rates after vitrification/warming using the different dilution steps. These results suggest that the presence or absence of the zona pellucida, which normally acts as a barrier to the movement of water and CPAs (DINNYES et al., 2007; SARAGUSTY and ARAV, 2011) does not appear to have a significant impact on the osmotic response to the vitrification/warming process. Similarly, warming by a 1-step dilution in 0.5 M sucrose is sufficiently effective to protect intact or biopsied embryos from osmotic swelling shock during dilution (rehydration) or higher embryo death in comparison to the 3-step during warming.

2. Vitrification and in-straw warming of intact and biopsied 0 /3 h IVP blastocysts using the VitTrans method

The aim of this second experiment was to determine the *in vitro* survival of intact and biopsied IVP blastocysts when using the VitTrans cryodevice for vitrification and in-straw warming.

IVP blastocysts that were intact, biopsied without culture, and biopsied with 3 h of culture showed good *in vitro* viability after in-straw warming. No significant differences were observed between groups though there was a tendency for higher survival in intact embryos (84.6%) when compared with those of biopsied embryos ($p = 0.058$). In contrast, survival rates in biopsied blastocysts vitrified/warmed after 0 h (57.1%) or 3 h in culture (62.8%) showed no significant differences ($p = 0.632$).

The TE epithelium regulates exchange and accumulation of small molecules and fluid during blastocoel formation (COCKBURN and ROSSANT, 2010; CHEN et al., 2017) as well as the exchange of water and CPA during the vitrification/warming process. TE biopsy typically alters the integrity of tight junctions and causes morphological changes (blastocyst collapse and cells loss)

because of decreased pressure that could impair the tolerance of biopsied embryos to cryopreservation (ITO et al., 1999). CHEN et al. (2017) suggested that culturing embryos for ≥ 3 h might alleviate tight junction impairment in the biopsied human blastocysts and further enhance the equilibration efficiency during vitrification. Similarly, biopsied bovine embryos cultured for a period of 2.5 to 5 h regained morphology and recovered at the cellular and structural level improving embryo survival after vitrification (ROSCHLAU et al., 1997; ITO et al., 1999).

ALBIHN et al. (1990) reported that bovine demi-embryos that were cultured for a period of 30 min were found to have restored their morphology such as cell polarization, cell-to-cell contact between the trophoblastic cells and the presence of mitosis. Experiments carried out in ovine demonstrated that the stage of development influences the subsequent in vitro viability of biopsied and vitrified ovine embryos. While vitrified ovine embryos biopsied at precompacted morula stages can be improved by a short period of in vitro culture between cell sampling and vitrification, no differences were found at compacted morula and blastocyst stages (NAITANA et al., 1996).

In our study, however, vitrification of biopsied expanded blastocysts after a period of 3 h of culture did not yield a higher *in vitro* survival compared to immediate vitrification after biopsy, possibly due to the fact that they were vitrified at the expanded blastocyst stage. Also, the time of culture (0h or 3h) between the biopsy procedure and the vitrification had no significant influence on embryos losses.

However, the amount of embryo losses during warming using the in-straw VitTrans method was inadequate. Embryos were lost during the warming procedure; they were found attached to the inside of the wall of the straw after expelling the content into a Petri dish, or they were attached to the embryo holder of the device. Embryos that have been biopsied may adhere easily due to the loss or damage of the zona pellucida following the biopsy procedure (LEONI et al., 2000; GONZALEZ et al., 2019).

Because of the embryo loss, we decided to culture biopsied embryos for 3 h before vitrification in the third experiment.

The microblade biopsy technique used in this investigation resulted in embryos losing the zona pellucida or extensive holes in this structure, which may increase the risk of adhesion of the embryonic cells to the straw or device. Bovine demi-

embryos that were cultured for a period of 30 min restored their pre-bisection morphology such as cell polarization, cell-to-cell contact between the trophoblastic cells and the presence of mitosis (CHESNÉ et al., 1987; ITO et al., 1999). Therefore, the risk of adhesion may be reduced after 3 h of culture because the membrane and structure of the cell of biopsied embryos damaged by biopsy treatment may recover during these culture periods.

However, in this investigation, in comparison to the intact embryos, the biopsy procedure had no significant effect on the number of embryos lost during warming ($p = 0.643$).

3. Effects of vitrification on pregnancy outcome of biopsied IVD embryos

In many research studies, *in vitro* development parameters such as morphology, re-expansion and hatching of warmed vitrified embryos are used as a criterion for *in vitro* survival (MARTINEZ-RODERO et al., 2021; ORDÓÑEZ-LEÓN et al., 2022). The prediction of embryo cryo-survival by the subjective characterization based mainly on embryo re-expansion is an insufficient predictor for embryo survival. However, it is still the most used method, and it was the method selected in the two previous experiments to score survival rate.

The ability to maintain and accomplish the pregnancy and the birth of a healthy live offspring is the most reliable criterion for scoring survival rate of a biopsied vitrified/warmed embryo (MASSIP et al., 1995).

For the successful application of vitrification in field conditions, a simple warming process that enables direct embryo transfer and ensures high pregnancy rates is needed, since in conventional slow rate freezing the embryos can be transferred without requiring a microscope or high levels of expertise (MORATO and MOGAS, 2014; MARTINEZ-RODERO et al., 2021; GONZÁLEZ-RODRÍGUEZ et al., 2022). Therefore, the warming procedure should allow in-straw dilution and transfer of embryos without manipulating them beforehand.

The aim of this on-farm experiment was to compare pregnancy outcomes after vitrification/warming with the VitTrans, in-straw warming, direct transfer method and the Cryotop® method, multi-step dilution, in-direct transfer method.

3.1. Pregnancy rates after transfer of fresh or cryopreserved IVD biopsied embryos

The pregnancy outcome of biopsied IVD embryos did not differ between the two vitrification methods, embryos in the Cryotop® (35.0%) group were warmed using a 3-step warming procedure, and in the VitTrans group they were warmed in-straw (33.3%, p = 0.280). Nevertheless, the pregnancy outcome of biopsied IVD embryos tended to be reduced following vitrification in comparison with fresh embryos (53.8%, p = 0.103).

Previous research found that deep freezing (10 % glycerol) reduced the pregnancy rate of biopsied IVD embryos when compared to frozen/thawed control embryos (44.4% vs 49.5%), but this difference was not statistically significant (ROSCHLAU et al., 1997). When IVD embryos were slow-frozen, the pregnancy rate of biopsied embryos (46%) was comparable to that of intact (non-biopsied) embryos (54%) (CAAMANO et al., 2015). AGCA et al. (1998) observed lower pregnancy rates after transfer of slowly frozen biopsied IVP embryos (23%) than after 391 transfers of vitrified (44%) or fresh (50%) embryos, though these differences were not significant. Moreover, CAAMANO et al. (2015) reported good pregnancy rates (41.6%) after transfer of vitrified intact IVP bovine embryos that had been vitrified and warmed using a 1-step warming procedure. In this study, we did not transfer a control group of IVD intact vitrified embryos for comparison, so we cannot determine whether the biopsy procedure combined with vitrification negatively affected the pregnancy outcome.

The biopsy technique can have a major impact on later embryonic development and survival after cryopreservation. Each biopsy technique has advantages and disadvantages (MULLAART, 2002). The damage of the zona pellucida seemed to be reduced when embryos are biopsied using the needle or the aspiration technique (THIBIER and NIBART, 1995; CENARIU et al., 2012). CENARIU et al. (2012) showed in their study that the biopsy method that yielded higher pregnancy rates was the needle biopsy (57.0%) followed by 43.0% in the aspiration technique group and 31.0% using the microblade technique. Similarly, THIBIER and NIBART (1995) described that fresh transferred IVD embryos biopsied by aspiration yielded a higher pregnancy rate (55.0%) that those biopsied using a microblade (28.0%).

The microblade biopsy technique used in this study, is invasive to the embryo.

However, it is also the most practical method, as it limits the equipment needed to a microscope and a microscalpel blade connected to a micromanipulator. Nevertheless, according to our results, the pregnancy outcome achieved with IVD embryos that had been biopsied by the microblade method and transferred fresh (53.8%) was comparable with other reported studies. HIRAYAMA et al. (2004) reported a 57.4% pregnancy rate after transfer of IVD embryos biopsied by microblade technique. Also, a 62.0% pregnancy rate after transfer of fresh IVD embryos biopsied with a microblade was described by HASLER et al. (2002). LACAZE et al. (2009) reported 59.0% of pregnancy rates after transfer of fresh IVD embryos after microblade biopsy technique. Similarly, GHANEM et al. (2014) reported a 63.1% pregnancy rates after the transfer of IVD biopsied embryos by the microblade technique.

According to the findings of ROSCHLAU et al. (1997), the pregnancy rate in sexed bovine IVD embryos that were transferred fresh was 45.6%, which was not significantly different from that of intact embryos (53.0%) while cryopreservation further decreased the pregnancy rate in biopsied embryos (44.4%). LOPES et al. (2001) concluded that the pregnancy rate achieved with fresh IVD biopsied embryos (50.0% – 60.0%) was similar to that of fresh intact embryos (55.0% – 61.0%).

3.2. Pregnancy rates after transfer of fresh or cryopreserved IVD biopsied embryos classified as grade 1 and grade 2

Due to the limited amount of data, we pooled the cryopreserved embryos together (VitTrans and Cryotop® groups) in order to determine whether the quality or the developmental stage of embryos influenced the pregnancy rate, when they were transferred into recipients either fresh or after cryopreservation.

Unlike the previous experiments, in which IVP embryos were used, less strict selection parameters for developmental stage and quality grade were applied in IVD embryos. Excellent or good I and fair II quality IVD embryos of compacted morula, early blastocyst, blastocyst, and expanded blastocyst stages were selected before the biopsy procedure. IVD embryos were biopsied at both morula and blastocyst stage using a microscalpel attached to a micromanipulator. After the 3 h period of culture post-biopsy, embryos were vitrified regardless of their morphological quality, compactness, or re-expansion.

In our results, there were no significant differences in pregnancy rates of IVD biopsied embryos classified as grade I (35.3%) and grade II (33.3%) in the cryopreserved group ($p = 0.592$). However, in embryos transferred fresh there was a tendency for a higher pregnancy rate in IVD biopsied embryos that were classified as grade I (60.0%) compared to those graded as quality II (45.5%) ($p = 0.108$).

HASLER et al. (2002) reported significantly lower pregnancy rates after the transfer of grade II IVD biopsied fresh embryos (49.0%) in comparison with corresponding grade I biopsied fresh embryos (61.9%). TAJIMI et al. (2018) graded the quality of bovine blastocysts obtained 7–8 days after the beginning of IVF as good (high-quality embryos with less than 10% degenerated blastomeres), fair or poor (low-quality embryos with less than 30% and 50% degenerated blastomeres respectively) and these were then vitrified or slow frozen. All vitrified embryos survived after MCV (minimum volume cooling) vitrification and warming in a 1-step CPA dilution, regardless of their quality rating. Survival rates of embryos frozen by slow freezing, on the other hand, were related to embryo quality, with survival rates decreasing as embryo quality decreased. When transferred fresh, HASLER et al. (2002) reported significantly lower pregnancy rates after the transfer of grade II IVD biopsied embryos (49.0%) when compared to grade I biopsied embryos (61.9%).

Also, no differences in pregnancy rates were observed in this study after biopsied embryos at different developmental stages were transferred fresh ($p = 0.720$) or cryopreserved ($p = 0.719$).

The developmental stage of the embryo at the time of vitrification, could be a key factor affecting pregnancy outcomes (TUCKER and LIEBERMANN, 2007; UCHIKURA et al., 2016). When IVD embryos are cryopreserved by slow freezing, morulae tend to yield higher pregnancy rates than blastocysts. This is due to the fact that blastocysts have a fluid-filled blastocoel, which may be inadequately dehydrated and cause damage by ice crystal formation during cryopreservation (TUCKER and LIEBERMANN, 2007; MOUSSA et al., 2014). However, after the biopsy procedure, the zona pellucida is disrupted and the embryo shrinks allowing a faster dehydration. In fact, several studies in human and equine reproductive medicine propose the forced collapse of the blastocoel cavity before cryopreservation (SON et al., 2003; KOVAČIČ et al., 2018; HERRERA, 2021; WILSHER et al., 2021). MIN et al. (2014) recorded that IVP bovine blastocysts

subjected to forced collapse of the blastocoel cavity before vitrification were found to have a lower apoptotic index than those intact.

Similar to our results, HASLER et al. (2002) reported no significant differences in pregnancy rates of biopsied IVD embryos at different stages of development following transfer to recipients. LACAZE et al. (2009) also reported pregnancy rates that ranged from 47.3% to 62.5% after transfer of frozen-thawed biopsied IVD embryos, without any significant effect of embryo stage.

3.3. Relationship between the sex of embryos and their potential to establish a pregnancy

When embryos were sorted for sex, female IVD biopsied embryos transferred fresh produced significantly higher pregnancy rates (70.6%) than their male counterparts (33.3%, $p = 0.027$). However, no relation between the sex of embryos and their pregnancy potential was found when IVD biopsied embryos were cryopreserved and transferred into recipients ($p = 0.588$).

In this experiment, the biopsied cells were analysed for sex identification using LAMP method, and the analysis was completed within 40 min. This allowed us to transfer the embryos of the desired sex without cryopreservation. In this regard, it should be emphasized that 17 out of a total of 20 freshly transferred embryos were females. On the other hand, among the cryopreserved embryos, 12 were females and 18 were males.

The success rate of sex identification LAMP-based in our study was 81%. Similarly, other field studies reported 88.9 - 94.4% of success rate using LAMP method and a pregnancy rate of 57.4% after the transfer of IVD embryos biopsied by the microblade technique (HIRAYAMA et al., 2004; KAGEYAMA and HIRAYAMA, 2012).

3.4. Pregnancy outcome of biopsied IVD embryos transferred after different time after warming, while remaining in the transferring gun

Previous research has shown that long-term exposure to high sucrose concentrations reduces embryo survival (KASAI et al., 1981; MAZUR and SCHNEIDER, 1986). While embryos cryopreserved using the Cryotop® method

were transferred in a medium lacking of sucrose, in-straw warmed embryos were transferred in a medium supplemented with 0.5M sucrose. Because of this, the effect of the time required from the placement of the straw into the transferring gun until transfer on the ability of the embryo to stablish pregnancy rate was recorded for each embryo.

Because warming was performed in the laboratory when the Cryotop® method was used and the straw ready for transfer was transported to the clients' farm with the embryo transporter TREO (WTA, Cravinhos, Brazil), the time interval between warming and placing the straws into the transferring gun and embryo transfer ranged from 5 and > 20 min (up to 45 min) with an average time of 20 min. However, embryos vitrified on VitTrans were both warmed and transferred directly at the client´s farm within 5 or 10 min, with an average time of 8 min. As far as we know, the effect of the time interval from warming until embryo transfer on pregnancy rate in IVD embryos cryopreserved by vitrification has not been directly tested. However, in previous research on frozen-thawed bovine IVP embryos, holding straws for up to 30 min at 26°C after thawing had no effect on survival rates, whereas 60 min of holding decreased it significantly when the holding time increased to 60 min (MATOBA S. et al., 2004). INABA et al. (2011) found that exposure to a 0.3 M sucrose dilution had no effect on in vitro survival rates of cryopreserved IVP bovine embryos until 30 min after warming. Thus, BO et al. (2007) found no difference in pregnancy rate after transferring frozen-thawed bovine IVD embryos at three different thawing to transfer intervals (<3 min; 3 to 6 min; and 6 to 16 min).

In this study, the interval between warming and placing the straws into the transferring gun and embryo transfer had an influence on pregnancy rates ($p = 0.003$), as well as the method used for warming (Cryotop or VitTrans, ($p = 0.002$)). Embryos vitrified on VitTrans and warmed in-straw resulted in significantly higher pregnancy rates when they were transferred immediately after warming (up to 10 min). However, pregnancy rates were higher when embryos vitrified using the Cryotop® method were transferred at 20 to 35 min after warming.

In our study, although embryos warmed using the VitTrans method were warmed in-straw under farm conditions, we examined each of the straws after warming under a stereomicroscope to confirm that there was indeed an embryo inside the straw before transfer. Moreover, no embryos were discarded after vitrification or

during the warming procedure and embryos were transferred regardless of the quality upon warming. Discarding of embryos would not have been a fair comparison since the Cryotop® method allows for visualization of the embryo at every warming step, whereas VitTrans just allows manipulation of the resulting transfer straw.

3.5. Other possible sources of variation

Straws of all groups were transferred by different veterinarians and technicians. Nevertheless, some studies showed that operators are not a significant source of variation in pregnancy rate after the transfer of embryos (VAN WAGTENDONK-DE LEEUW et al., 1997; BALABAN et al., 2007; INABA et al., 2011).

Moreover, the recipient cows and heifers that were used, were also from a wide range of parities and different barns. Our data model did not improve when we included the random effect of the different recipient animals located in different barns, meaning that the farm in our study had no significant effect.

4. Conclusions

Regarding the biopsy procedure, the use of the microblade technique is a practical biopsy method for bovine embryos that are transferred without cryopreservation. However, if vitrification methodologies are to be implemented into routine practice for biopsied bovine embryos, the efficacy and efficiency of the biopsy technique needs to be further evaluated.

Overall, the Cryotop® vitrification method can be effectively used for the vitrification of biopsied IVP and IVD bovine embryos, but it is not convenient to perform this method under field conditions because of the stepwise warming procedure and the necessity to use a stereomicroscope. On the other hand, the VitTrans method needs to be refined for the routine use in the field. The need to maintain the warming solution at 40 °C while flushing the embryo into the lumen of the 0.5 ml plastic straw requires a manipulation that may result in temperature variations. Variations of the temperature during warming as well as warming rates are very critical in terms of embryo cryosurvival (MORATO and MOGAS, 2014). As mentioned above, the major handicap of the VitTrans methodology is the risk of losing embryos while preparing the straw for transfer. To perform the warming

and transfer using the VitTrans method, a 0.5 ml transfer gun is required instead of the standard 0.25 ml embryo transfer gun, however this task can be easily solved by using an equine embryo transfer gun.

This is the first study to assess the new VitTrans in-straw warming method under field conditions and, not only pregnancy rates were recorded, but also live and healthy calves were born (GONZÁLEZ-RODRÍGUEZ et al., 2022). *In vivo* survival was estimated by a limited number of transfers of vitrified-warmed embryos and increasing the number of embryos transferred would help us to better estimate pregnancy rates and determine the successful application of this direct embryo transfer vitrification method under typical conditions in the field.

Based on the results obtained in this study, both vitrification technologies produced similar embryo survival rates for biopsied IVP and IVD bovine embryos, so the selection of the VitTrans vitrification method as being more practical compared to the Cryotop® is still subject to further consideration. However, in a recent report (OWEN et al., 2022) slow freezing also emerged as a viable option for cryopreservation of IVP bovine embryos that have been cultured in a high-quality medium to reduce cryoinjuries. Hence, slow freezing may facilitate the implementation of cryopreservation and transfer of biopsied bovine IVD or IVP embryos under field conditions since this technique is already widely used.

VI. SUMMARY

Comparison of two vitrification-systems, Cryotop® versus VitTrans, in biopsied bovine embryos

The assessment of genomic estimated breeding values and sex determination of embryos prior to transfer can be used to produce superior donor animals. Cells obtained by biopsy in embryos provide the material for early genetic evaluation; but the biopsied embryo must be cryopreserved until its corresponding biopsy is analysed. The biopsy procedure, in terms of embryo development stage and technique, influences the results of the subsequent embryo re-expansion after cryopreservation, the establishment of pregnancy and the reliability of the genetic analysis from the DNA obtained. Over the past years, vitrification has become more popular as an alternative method of choice for cryopreservation of human and bovine *in vitro* produced (IVP) embryos since there is a formation of a glass-like state in the rapid cooling process that prevents intracellular ice crystal formation. However, vitrified bovine embryos need to re-expand in several warming solutions before transfer to recipients, making the transfer procedure under field conditions a practical challenge. The overall objective of the present study was to assess the efficiency of a vitrification method suitable for direct in-straw warming of biopsied bovine embryos. This would reduce the costs of embryo transfer and help to increase the genetic improvement of cattle. Experiments were done to determine *in vitro* embryo viability of IVP biopsied bovine embryos and pregnancy rates of *in vivo* derived (IVD) biopsied bovine embryos, using two different vitrification methodologies. The VitTrans method, that enables direct embryo transfer to the uterus of the recipient cow, was compared to the Cryotop® method.

In the first two experiments, IVP biopsied bovine embryos were vitrified and warmed using the Cryotop® or the VitTrans method. *In vitro* embryo survival did not differ in experiment 1 and 2; 3-step warming using the Cryotop® resulted in 75.8% and 61.7% in intact and biopsied embryos respectively ($p = 0.367$), whereas it was higher 84.6% in intact compared to biopsied embryos ($p = 0.058$); 57.1% in the biopsied 0h and 62.8% in the biopsied vitrified after 3 h of culture group ($p = 0.632$), when embryos were warmed in-straw using the VitTrans method.

A third experiment was conducted to evaluate pregnancy rates of IVD bovine

embryos that were vitrified after 3 h of culture following biopsy and warmed by the two different vitrification methods. A total number of 61 *in vivo* derived embryos were biopsied using the microblade biopsy technique and randomly distributed between three groups: control, fresh biopsied embryos (n=26), VitTrans (n=15), and Cryotop® (n=20). The pregnancy outcome following transfer of biopsied IVD embryos was similar, 35.0% in the Cryotop®, 33.3% in the VitTrans and 53.8% in the fresh group ($p = 0.280$). Biopsies were carried out by dissociating 2 to 3 blastomeres at the morula stage or 10 to 15 cells from the blastocyst and resulting DNA material was used for determining the sex of the embryos. Female embryos produced higher pregnancy rates than their male counterparts when they were transferred fresh ($p = 0.027$). We measured the interval between warming and embryo transfer and found that embryos warmed with VitTrans resulted in significantly higher pregnancy rates when transferred immediately after warming (up to 10 min) compared with the Cryotop® method, which had a higher pregnancy outcome with a longer time interval (20 to 35 min) ($p < 0.003$).

According to the results of this study, both vitrification technologies produced comparable embryo survival rates for biopsied *in vitro* and IVD bovine embryos, so the selection of the VitTrans vitrification method as being more practical compared to the Cryotop® method would still warrant further investigations.

VII. ZUSAMMENFASSUNG

Vergleich von zwei Vitrifizierungssystemen, Cryotop® versus VitTrans, mit biopsierten Rinderembryonen

Die genomische Zuchtwertschätzung und die Geschlechtsbestimmung von Embryonen vor dem Transfer können zur Produktion von ausgewählte Spendertieren genutzt werden. Zellen, die durch Biopsie von Embryonen gewonnen werden, stehen dabei als Material für eine frühzeitige genetische Untersuchung zur Verfügung. Bis zum Vorliegen des Analyseergerbnisses ist jedoch notwendig, dass der biopsierte Embryo kryokonserviert wird. Das Biopsieverfahren hat dabei, in Abhängigkeit vom Entwicklungsstadium des Embryos und der Kryokonservierungsmethode, Enflus auf die Re-Expansion des Embryos nach der Kryokonservierung, das Zustandekommen einer Trächtigkeit und die Zuverlässigkeit der genetischen Analyse der gewonnenen DNA. In den letzten Jahren hat die Popularität der Vitrifikation als alternative Methode für die Kryokonservierung von humanen und *in vitro* produzierten Embryonen vom Rind zugenommen. Durch, die sehr hohe Abkühlrate wird bei der Vitrifikation ein glas-ähnlicher Zustand erreicht, der die intrazelluläre Eiskristallbildung verhindert. Allerdings müssen vitrifizierte Embryonen vor der Übertragung auf Empfängertiere erst in verschiedenen Auftaulösungen re-expandieren, weshalb diese Vorgehensweise unter Feldbedingungen eine Herausforderung darstellt. Das Hauptanliegen dieser Studie war es, die Effizienz einer Vitrifikationsmethode zu untersuchen, die das direkte Auftauen des biopsierten Embryos in der Paillette erlaubt. Das würde die mit dem Embryotransfer verbundenen Kosten reduzieren und den Zuchtfortschritt beim Rind erhöhen. Es wurden mehrere Experimente zur Bestimmung der *in vitro* Lebensfähigkeit von *in vitro* produzierten Rindere Embryonen durchgeführt, und die Trächtigkeitsraten bei der Übertragung von in vivo gewonnen, biopsierten Rinderembryonen bestimmt. Dabei kamen zwei unterschiedliche Vitrifikationsmethoden zur Anwendung. Die VitTrans Methode, die den direkten Transfer des Embryos in den Uterus eines Empfängertieres ermöglicht, wurde mit der Cryotop®-Methode verglichen.

In den ersten beiden Experimenten wurden *in vitro* produzierte Embryonen nach der VitTrans- oder der Cryotop®-Methode vitrifiziert und erwärmt. Die *in vitro*-

Überlebensraten zeigte in beiden Experimenten keinen Unterschied. In der Cryotop® Gruppe lagen die Überlebensraten bei 75,8% bei Intakten und 61,7% bei biopsierten Embryonen (p = 0,367). In der VitTrans Gruppe mit direktem Erwämen in der Paillette lagen die Überlebensraten bei intakten im Vergleich zu biopsierten Embryonen bei 84,6% (p = 0,058); bei biopsierten und sofort Embryonen 57,1% und bei biopsierten und nach einer dreistündigen Kultur vitrifizierten Embryonen (p = 0,632).

Auf Basis der erhaltenen Resultate wurde ein drittes Experiment durchgeführt, um die Trächtigkeitsraten bei *in vivo* gewonnenen und biopsierten Rinderembryonen zu bestimmen, die nach dreistündiger Kultur mit den zwei unterschiedlichen Vitrifikationsmethoden kryokonserviert und wieder aufgetaut wurden. Insgesamt 61 *in vivo* gewonnene Embryonen unter Verwendung der Microblade-Biopsietechnik biopsiert und zufällig auf drei Gruppen verteilt: frisch biopsierte Embryonen, als Kontrolle (n = 26), VitTrans (n = 15) und Cryotop® (n = 20). Die erzielten Trächtigkeitsraten der in vivo produzierten und biopsierten Embryonen waren ähnlich. Sie lagen bei der Cryotop®-Gruppe 35,0%, bei der VitTrans-Gruppe in der 33,3% und bei der Kontrollgruppe bei-53,8% (p = 0,280). Im Morulastadium wurden bei der Biopsie 2 – 3 Blastomeren entnommen und im Blastozystenstadium 10 bis 15 Trophektodermzellen von der Blastozyste losgelöst. Das daraus gewonnene DNA-Material wurde für die Geschlechtsbestimmung der Embryonen verwendet.

Bei der Übertragung von frischen weiblichen Embryonen wurden höhere Trächtigkeitsraten als bei frischen männlichen Embryonen erzielt (p = 0,027). In Hinblick auf das Zeitintervall zwischen dem Auftauen- der Embryonen und dem Transfer wurden bei der VitTrans-Methode bessere Trächtigkeitsraten bei kürzerem Zeitintervall (bis 10 min) erzielt, während mit Trächtigkeitsraten bei der Cryotop®-Methode bei einem längeren Zeitintervall (bis zu 35 min) höher waren (p < 0.003).

Da im Rahmen dieser Studie beide Vitrifikationsmethoden sowohl in vitro als auch in vivo vergleichbare Ergebnisse lieferten, wäre es auf Grund der Praktikabilitätgerechtfertigt, die VitTrans-Methode für die praktische Anwendung im Feld einer weiteren Prüfung zu unterziehen.

VIII. REFERENCES

Abdalla H, Shimoda M, Hara H, Morita H, Kuwayama M, Hirabayashi M, Hochi S. *Vitrification of ICSI- and IVF-derived bovine blastocysts by minimum volume cooling procedure: effect of developmental stage and age*. Theriogenology 2010; 74: 1028-35.

Abdullah R, Nor-Ashikin MNK, Daud D, H R, Mohamed Z, Faizah S, S M. *The effects of biopsy and cryopreservation by vitrification on the in vitro development of eight-cell mouse embryos*. Biomedical Research 2006; 17: 61-66.

Abe H, Yamashita S, Satoh T, Hoshi H. *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 2002; 61: 57-66.

Adler A, Lee HL, McCulloh DH, Ampeloquio E, Clarke-Williams M, Wertz BH, Grifo J. *Blastocyst culture selects for euploid embryos: comparison of blastomere and trophectoderm biopsies*. Reproducion Biomedicine Online 2014; 28: 485-91.

Agca Y, Monson RL, Northey DL, Peschel DE, Schaefer DM, Rutledge JJ. *Normal calves from transfer of biopsied, sexed and vitrified IVP bovine embryos*. Theriogenology 1998; 50: 129-45.

Akiyama K, Kobayashi J, Sato Y, Sata R, Ohashi M, Sasaki E, Oda Y, Ogawa Y, Ueda S, Nabenishi H, Matoba S. *Calf production from vitrified bovine sexed embryos following in-straw dilution*. Journal of Animal Science 2010; 81: 461-6.

Ambrosini G, Andrisani A, Porcu E, Rebellato E, Revelli A, Caserta D, Cosmi E, Marci R, Moscarini M. *Oocytes cryopreservation: State of art*. Reproductive toxicology (Elmsford, N.Y.) 2006; 22: 250-62.

Arav A. *Vitrification of oocytes and embryos*. New Trends in Embryo Transfer. Portland Press, Cambridge 1992: 255–64.

Arav A, Zeron Y. *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 1997; 47:2.

Balaban B, Isiklar A, Ata B, Yakin K, Urman B. *The effect of the embryologist/technician on fertilization rate and embryo quality following ICSI and pregnancy rate following embryo transfer procedures*. Fertility and Sterility 2007; 88: S113.

Bermejo-Alvarez P, Pericuesta E, Miranda A, de Frutos C, Perez-Cerezales S, Lucio A, Rizos D, Gutierrez-Adan A. *New challenges in the analysis of gene transcription in bovine blastocysts*. Reproduction in Domestic Animals 2011; 46 (Suppl 3): 2-10.

Besenfelder U, Havlicek V, Brem G. *Role of the Oviduct in Early Embryo Development*. Reproduction in Domestic Animals 2012; 47: 156-63.

Best BP. *Cryoprotectant Toxicity: Facts, Issues, and Questions*. Rejuvenation Res 2015; 18: 422-36.

Bo G, Mapletoft R. *Evaluation and classification of bovine embryos*. Animal Reproduction 2013; 10: 344-48.

Bo G, Peres L, Pincinato D, de la Rey M, Tribulo R. *Influence of the interval between thawing to transfer on pregnancy rates of frozen-thawed direct-transfer embryos*. Reproduction Fertility and Development 2007; 19.

Bredbacka K, Bredbacka P. *Glucose controls sex-related growth rate differences of bovine embryos produced in vitro*. Journal of Reproduction and Fertility 1996; 106: 169-72.

Caamano JN, Gomez E, Trigal B, Munoz M, Carrocera S, Martin D, Diez C. *Survival of vitrified in vitro-produced bovine embryos after a one-step warming in-straw cryoprotectant dilution procedure*. Theriogenology 2015; 83: 881-90.

Campos-Chillon LF, Walker DJ, de la Torre-Sanchez JF, Seidel GE, Jr. *In vitro assessment of a direct transfer vitrification procedure for bovine embryos*. Theriogenology 2006; 65: 1200-14.

Cenariu M, Pall E, Cernea C, Groza I. *Evaluation of bovine embryo biopsy techniques according to their ability to preserve embryo viability*. Journal of Biomedicine and Biotechnology 2012: 541384.

Charpigny G, Marquant-Le Guienne B, Richard C, Adenot P, Dubois O, Gélin V, Peynot N, Daniel N, Brochard V, Nuttinck F. *PGE2 Supplementation of Oocyte Culture Media Improves the Developmental and Cryotolerance Performance of Bovine Blastocysts Derived From a Serum-Free in vitro Production System, Mirroring the Inner Cell Mass Transcriptome*. Frontiers in Cell and Developmental Biology 2021; 9: 672948.

Chen H-H, Huang C-C, Cheng E-H, Lee T-H, Chien L-F, Lee M-S. *Optimal timing of blastocyst vitrification after trophectoderm biopsy for preimplantation genetic screening*. PLOS ONE 2017; 12: e0185747.

Chesné P, Heyman Y, Chupin D, Procureur R, Ménézo Y. *Freezing cattle demi-embyos: Influence of a period of culture between splitting and freezing on survival*. Theriogenology 1987; 27: 218.

Cockburn K, Rossant J. *Making the blastocyst: lessons from the mouse*. J Clin Invest 2010; 120: 995-1003.

Cuello C, Sanchez-Osorio J, Almiñana C, Gil M, Perals ML, Lucas X, Roca J, Vazquez J, Martinez E. *Effect of the cryoprotectant concentration on the in vitro embryo development and cell proliferation of OPS-vitrified porcine blastocysts*. Cryobiology 2008; 56: 189-94.

Dinnyés A, Dai Y, Jiang S, Yang X. *High developmental rates of vitrified bovine oocytes following parthenogenetic activation, in vitro fertilization, and somatic cell nuclear transfer*. Biology of Reproduction 2000; 63: 513-8.

Dinnyes A, Liu J, Nedambale TL. *Novel gamete storage*. Reproduction, fertility, and development 2007; 19: 719-31.

Do VH, Walton S, Aw T-R. *Benefits and Constraints of Vitrification Technologies for Cryopreservation of Bovine in vitro Fertilized Embryos*. Journal of Veterinary Science and Animal Husbandry 2014; 2(4): 401.

Do VH, Catt S, Kinder JE, Walton S, Taylor-Robinson AW. *Vitrification of in vitro-derived bovine embryos: targeting enhancement of quality by refining technology and standardising procedures*. Reproduction, fertility, and development 2019; 31: 837-46.

El-Danasouri I, Selman H. *Successful pregnancies and deliveries after a simple vitrification protocol for day 3 human embryos*. Fertility and Sterility 2001; 76: 400-02.

El-Sayed A, Hoelker M, Rings F, Salilew D, Jennen D, Tholen E, Sirard MA, Schellander K, Tesfaye D. *Large-scale transcriptional analysis of bovine embryo biopsies in relation to pregnancy success after transfer to recipients*. Physiological Genomics 2006; 28: 84-96.

Enright BP, Lonergan P, Dinnyes A, Fair T, Ward FA, Yang X, Boland MP. *Culture of in vitro produced bovine zygotes in vitro vs in vivo: implications for early embryo development and quality*. Theriogenology 2000; 54: 659-73.

Eroglu B, Szurek EA, Schall P, Latham KE, Eroglu A. *Probing lasting cryoinjuries to oocyte-embryo transcriptome*. PLOS ONE 2020; 15: e0231108.

Fahy GM, Wowk B, Wu J, Paynter S. *Improved vitrification solutions based on the predictability of vitrification solution toxicity*. Cryobiology 2004; 48: 22-35.

Fair T, Lonergan P, Dinnyes A, Cottell DC, Hyttel P, Ward FA, Boland MP. *Ultrastructure of bovine blastocysts following cryopreservation: Effect of method of blastocyst production*. Molecular Reproduction and Development 2001; 58: 186-95.

Fisher PJ, Hyndman DL, Bixley MJ, Oback FC, Popovic L, McGowan LT, Berg MC, Wells DN. *BRIEF COMMUNICATION: Potential for genomic selection of bovine embryos*. Proceedings of the New Zealand Society of Animal Production 2012; 72: 156–58.

Ghanem N, Ha AN, Fakruzzaman M, Bang JI, Lee SC, Kong IK. *Differential expression of selected candidate genes in bovine embryos produced in vitro and cultured with chemicals modulating lipid metabolism*. Theriogenology 2014; 82: 238-50.

Ghanem N, Salilew-Wondim D, Gad A, Tesfaye D, Phatsara C, Tholen E, Looft C, Schellander K, Hoelker M. *Bovine blastocysts with developmental competence to term share similar expression of developmentally important genes although derived from different culture environments*. Reproduction 2011; 142: 551-64.

González-Rodríguez N, Martínez-Rodero I, Scherzer J, Jung S, Reichenbach M, Zablotski Y, Otzdorff C, Zerbe H, Mogas T. *Vitrification and in-straw warming do not affect pregnancy rates of biopsied bovine embryos*. Theriogenology 2022; 191: 221-30.

González N, Scherzer J, Reichenbach M, Otzdorff C, Zerbe H. *Survival rates of vitrified biopsied bovine in vitro-produced blastocysts using the VitTrans device*. Reproduction, fertility, and development 2019; 31: 138.

Guignot F, Baril G, Dupont F, Cognie Y, Folch J, Alabart JL, Poulin N, Beckers JF, Bed'hom B, Babilliot JM, Mermillod P. *Determination of sex and scrapie resistance genotype in preimplantation ovine embryos*. Molecular Reproduction and Development 2009; 76: 183-90.

Hamawaki A, Kuwayama M, Hamano S. *Minimum volume cooling method for bovine blastocyst vitrification*. Theriogenology 1999; 51: 165.

Han YM, Yamashina H, Koyama N, Lee KK, Fukui Y. *Effects of quality and developmental stage on the survival of IVF-derived bovine blastocysts cultured in vitro after freezing and thawing*. Theriogenology 1994; 42: 645-54.

Hansen PJ. *The incompletely fulfilled promise of embryo transfer in cattle-why aren't pregnancy rates greater and what can we do about it?* J Anim Sci 2020; 98

Hasler JF. *Factors affecting frozen and fresh embryo transfer pregnancy rates in cattle*. Theriogenology 2001; 56: 1401-15.

Hasler JF, Cardey E, Stokes JE, Bredbacka P. *Nonelectrophoretic PCR-sexing of bovine embryos in a commercial environment*. Theriogenology 2002; 58: 1457-69.

Herrera C, (2021). *Vitrification of Equine In Vivo-Derived Embryos After Blastocoel Aspiration*. In: Wolkers WF, Oldenhof H (Eds.), Cryopreservation and Freeze-Drying Protocols. Humana, New York, NY, 517-22.

Hirayama H, Kageyama S, Moriyasu S, Sawai K, Onoe S, Takahashi Y, Katagiri S, Toen K, Watanabe K, Notomi T, Yamashina H, Matsuzaki S, Minamihashi A. *Rapid sexing of bovine preimplantation embryos using loop-mediated isothermal amplification*. Theriogenology 2004; 62: 887-96.

Hochi S, Akiyama M, Minagawa G, Kimura K, Hanada A. *Effects of cooling and warming rates during vitrification on fertilization of in vitro-matured bovine oocytes*. Cryobiology 2001; 42: 69-73.

Inaba Y, Aikawa Y, Hirai T, Hashiyada Y, Yamanouchi T, Misumi K, Ohtake M, Somfai T, Kobayashi S, Saito N, Matoba S, Konishi K, Imai K. *In-straw cryoprotectant dilution for bovine embryos vitrified using Cryotop*. Journal of Reproduction and Development 2011; 57: 437-43.

Inaba Y, Miyashita S, Somfai T, Geshi M, Matoba S, Dochi O, Nagai T. *Cryopreservation method affects DNA fragmentation in trophectoderm and the speed of re-expansion in bovine blastocysts*. Cryobiology 2016; 72: 86-92.

Ito K, Sekimoto A, Hirabayashi M, Hochi S, Kimura K, Ueda M, Nagao Y. *Effect of Time Interval between Biopsy and Vitrification on Survival of In Vitro-Produced Bovine Blastocysts*. Journal of Reproduction and Development 1999; 45: 351-55.

Jain JK, Paulson RJ. *Oocyte cryopreservation*. Fertility and Sterility 2006; 86: 1037-46.

Jin B, Kusanagi K, Ueda M, Seki S, Valdez DM, Jr., Edashige K, Kasai M. *Formation of extracellular and intracellular ice during warming of vitrified mouse morulae and its effect on embryo survival*. Cryobiology 2008; 56: 233-40.

Jung S, Reichenbach M, Fries R, Wolf E, Gschoederer C, Scherzer J, Grupp T, Reichenbach H-D. *Genomic evaluation of bovine embryos within 24 hours*. Reproduction, fertility, and development 2014; 27: 247-47.

Kageyama S, Hirayama H. *Sexing of Bovine Preimplantation Embryos using Loop-Mediated Isothermal Amplification (LAMP)*. Journal of Mammalian Ova Research 2012; 29: 113-18.

Kasai M. *Advances in the cryopreservation of mammalian oocytes and embryos: Development of ultrarapid vitrification*. Reproductive Medicine and Biology 2002; 1: 1-9.

Kasai M, Niwa K, Iritani A. *Effects of various cryoprotective agents on the survival of unfrozen and frozen mouse embryos*. Journal of Reproduction and Fertility 1981; 63: 175-80.

Kelly J, Kleemann D, Kuwayama M, Walker SK. *Vitrification of in vitro- produced bovine and ovine embryos using the minimum volume cooling cryotop method*. Reproduction, fertility, and development 2003; 16: 172-73.

Khurana NK, Niemann H. *Energy metabolism in preimplantation bovine embryos derived in vitro or in vivo*. Biology of Reproduction 2000; 62: 847-56.

Kim YM, Uhm SJ, Gupta MK, Yang JS, Lim JG, Das ZC, Heo YT, Chung HJ, Kong IK, Kim NH, Lee HT, Ko DH. *Successful vitrification of bovine blastocysts on paper container*. Theriogenology 2012; 78: 1085-93.

Kong IK, Lee SI, Cho SG, Cho SK, Park CS. *Comparison of open pulled straw (ops) vs glass micropipette (gmp) vitrification in mouse blastocysts*. Theriogenology 2000; 53: 1817-26.

Kovačič B, Taborin M, Vlaisavljević V. *Artificial blastocoel collapse of human blastocysts before vitrification and its effect on re-expansion after warming – a prospective observational study using time-lapse microscopy*. Reproductive BioMedicine Online 2018; 36: 121-29.

Kruse S, 2012. *Vitrification of in vitro- and in vivo- produced bovine embryos for direct transfer*. Department of Biomedical Sciences. Colorado State University, Fort Collings, Colorado.

Kuleshova LL, MacFarlane DR, Trounson AO, Shaw JM. *Sugars Exert a Major Influence on the Vitrification Properties of Ethylene Glycol-Based Solutions and Have Low Toxicity to Embryos and Oocytes*. Cryobiology 1999; 38: 119-30.

Kuwayama M. *Highly efficient vitrification for cryopreservation of human oocytes and embryos: The Cryotop method*. Theriogenology 2007; 67: 73-80.

Kuwayama M, Hamano S, Nagai T. *Vitrification of bovine blastocysts obtained by in vitro culture of oocytes matured and fertilized in vitro*. Journal of Reproduction and Fertility 1992; 96: 187.

Kuwayama M, Vajta G, Kato O, Leibo SP. *Highly efficient vitrification method for cryopreservation of human oocytes*. Reproductive Biomedicine Online 2005; 11: 300-08.

Lacaze S, Lesclaux J, Coupet H. *The sexing of bovine embryos in the south-west of France. Part I. Efficiency, accuracy and pregnancy rates after 3 years activity*. Proceedings of the 12th Annual Meeting of AETE 1996; 156.

Lacaze S, Ponsart C, Humblot P. *Influence of embryo stage on pregnancy rates following transfer of bovine biopsied embryos under on-farm conditions*. Proceedings of the 25th AETE 2009: 208.

Lane M, Schoolcraft WB, Gardner DK. *Vitrification of mouse and human blastocysts using a novel cryoloop container-less technique*. Fertility and Sterility 1999; 72: 1073-8.

Lauri A, Lazzari G, Galli C, Lagutina I, Genzini E, Braga F, Mariani P, Williams JL. *Assessment of MDA efficiency for genotyping using cloned embryo biopsies*. Genomics 2013; 101: 24-9.

Leoni G, Ledda S, Bogliolo L, Naitana S. *Novel approach to cell sampling from preimplantation ovine embryos and its potential use in embryonic genome analysis*. Journal of reproduction and fertility 2000; 119: 309-14.

Liebermann J, Nawroth F, Isachenko V, Isachenko E, Rahimi G, Tucker M. *Potential Importance of Vitrification in Reproductive Medicine*. Biology of reproduction 2003; 67: 1671-80.

Liebermann J, Tucker MJ, Graham JR, Han T, Davis A, Levy MJ. *Blastocyst development after vitrification of multipronuclear zygotes using the Flexipet denuding pipette*. Reproductive Biomedicine Online 2002; 4: 146-50.

Lindemans W, Sangalli L, Kick A, Earl CR, Fry RC. *Vitrification of bovine embryos using the CLV method*. Reproduction, Fertility and Development 2004; 16: 174-74.

Lonergan P, Evans AC, Boland E, Rizos D, Fair T, Duffy P, Sung LY, Du F, Chaubal S, Xu J, Yang X, Tian XC. *Pregnancy and fetal characteristics after transfer of vitrified in vivo and cloned bovine embryos*. Theriogenology 2007; 68: 1128-37.

Lonergan P, Rizos D, Gutierrez-Adan A, Fair T, Boland MP. *Effect of culture environment on embryo quality and gene expression - experience from animal studies*. Reproductive Biomedicine Online 2003; 7: 657-63.

Lopes RF, Forell F, Oliveira AT, Rodrigues JL. *Splitting and biopsy for bovine embryo sexing under field conditions*. Theriogenology 2001; 56: 1383-92.

Loutradi KE, Kolibianakis EM, Venetis CA, Papanikolaou EG, Pados G, Bontis I, Tarlatzis BC. *Cryopreservation of human embryos by vitrification or slow freezing: a systematic review and meta-analysis*. Fertility and Sterility 2008; 90: 186-93.

Marc S, Godja G, Milovanov C, Tulcan C, Cernescu H, Otava G, Bonca G, Ciobota A, Hutu I, Mircu C, 2014. *Morphological aspects of cumulus–oocyte complexes in different species*. Medicina Veterinaria. 3-4.

Martinez-Rodero I, Garcia-Martinez T, Ordonez-Leon EA, Vendrell-Flotats M, Olegario Hidalgo C, Esmoris J, Mendibil X, Azcarate S, Lopez-Bejar M, Yeste M, Mogas T. *A Shorter Equilibration Period Improves Post-Warming Outcomes after Vitrification and in Straw Dilution of In Vitro-Produced Bovine Embryos*. Biology (Basel) 2021; 10.

Martino A, Songsasen N, Leibo SP. *Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling*. Biology of Reproduction 1996; 54: 1059-69.

Massip A. *Cryopreservation of Embryos of Farm Animals*. Reproduction in Domestic Animals 2001; 36: 49-55.

Massip A, Mermillod P, Van Langendonckt A, Touze JL, Dessy F. *Survival and viability of fresh and frozen-thawed in vitro bovine blastocysts*. Reproduction, Nutrition, Development 1995; 35: 3-10.

Massip AZ, P. van der Ectors, F. *Recent progress in cryopreservation of cattle embryos preserved by vitrification*. Cryo Letters. 1987: 270-73.

Matoba S., Imai K., Mimaki Y., Narita M., Tagawa M., Dochi O., Saito N. *Toxicity of ethylene glycol on frozen and thawed in vitro bovine embryos in direct transfer*. Reproduction Fertility and Development 2004; 16: 175-76.

Matsunari H, Maehara M, Nakano K, Ikezawa Y, Hagiwara Y, Sasayama N, Shirasu A, Ohta H, Takahashi M, Nagashima H. *Hollow fiber vitrification: a novel method for vitrifying multiple embryos in a single device*. Journal of Reproduction and Development 2012; 58: 599-608.

Mazur P. *A biologist's view of the relevance of thermodynamics and physical chemistry to cryobiology*. Cryobiology 2010; 60: 4-10.

Mazur P, Schneider U. *Osmotic responses of preimplantation mouse and bovine embryos and their cryobiological implications*. Cell Biophysics 1986; 8: 259-85.

Mazur P, Seki S. *Survival of mouse oocytes after being cooled in a vitrification solution to -196°C at 95° to 70,000°C/min and warmed at 610° to 118,000°C/min: A new paradigm for cryopreservation by vitrification*. Cryobiology 2011; 62: 1-7.

Mazur P, Seki S, Pinn IL, Kleinhans FW, Edashige K. *Extra- and intracellular ice formation in mouse oocytes*. Cryobiology 2005; 51: 29-53.

Min S-H, Jeong HJ, Koo D-B. *Efficient Cryopreservation of Porcine Blastocysts produced by in vitro Fertilization*. Journal of Embryo Transfer 2016; 31: 39-46.

Min SH, Kim JW, Lee YH, Park SY, Jeong PS, Yeon JY, Park H, Chang KT, Koo DB. *Forced collapse of the blastocoel cavity improves developmental potential in cryopreserved bovine blastocysts by slow-rate freezing and vitrification*. Reproduction in Domestic Animals 2014; 49: 684-92.

Mogas T. *Update on the vitrification of bovine oocytes and in vitro produced embryos*. Reproduction, fertility, and development 2019 Jan; 31(1):105-117.

Morato R, Izquierdo D, Paramio MT, Mogas T. *Survival and apoptosis rates after vitrification in cryotop devices of in vitro-produced calf and cow blastocysts at different developmental stages*. Reproduction, fertility, and development 2010; 22: 1141-7.

Morato R, Mogas T. *New device for the vitrification and in-straw warming of in vitro produced bovine embryos*. Cryobiology 2014; 68: 288-93.

Mori M, Otoi T, Suzuki T. *Correlation between the cell number and diameter in bovine embryos produced in vitro*. Reproduction in Domestic Animals 2002; 37: 181-4.

Moussa ME, Shu J, Zhang X, Zeng F. *Cryopreservation of mammalian oocytes and embryos: current problems and future perspectives*. Science China Life Sciences 2014; 57: 903-14.

Mukaida T, Nakamura S, Tomiyama T, Wada S, Kasai M, Takahashi K. *Successful birth after transfer of vitrified human blastocysts with use of a cryoloop containerless technique*. Fertility and Sterility 2001; 76: 618-20.

Mullaart E, (2002). *Biopsying and Genotyping Cattle Embryos*. In: Van Soom A, Boerjan M (Eds.), Assessment of Mammalian Embryo Quality. Springer, Dordrecht, 178-94.

Naitana S, Loi P, Ledda S, Cappai P, Dattena M, Bogliolo L, Leoni G. *Effect of biopsy and vitrification on in vitro survival of ovine embryos at different stages of development*. Theriogenology 1996; 46: 813-24.

Najafzadeh V, Bojsen-Møller Secher J, Pihl M, Ærenlund A, Jørgensen N, Jensen KK, Jensen MT, Fenner MF, Strøbech L, Hyttel P. *Vitrification yields higher cryo-survival rate than slow freezing in biopsied bovine in vitro produced blastocysts*. Theriogenology 2021; 171: 44-54.

Nedambale TL, Dinnyés A, Groen W, Dobrinsky JR, Tian XC, Yang X. *Comparison on in vitro fertilized bovine embryos cultured in KSOM or SOF and cryopreserved by slow freezing or vitrification*. Theriogenology 2004; 62: 437-49.

Nel-Themaat L, Nagy ZP. *A review of the promises and pitfalls of oocyte and embryo metabolomics*. Placenta 2011; 32: S257-S63.

Oliveira CS, Romano Quintao CC, de Freitas C, Dos Reis Camargo AJ, Serapiao RV, de Almeida Camargo LS. *Post implantation development reveals that biopsy procedure can segregate "healthy" from "unhealthy" bovine embryos and prevent miscarriages*. Animal Reproduction Science 2017; 184: 51-58.

Ordóñez-León EA, Martínez-Rodero I, García-Martínez T, López-Béjar M, Yeste M, Mercade E, Mogas T. *Exopolysaccharide ID1 Improves Post-Warming Outcomes after Vitrification of In Vitro-Produced Bovine Embryos*. International Journal of Molecular Sciences 2022; 23.

Owen CM, Johnson MA, Rhodes-Long KA, Gumber DJ, Barceló-Fimbres M, Altermatt JL, Campos-Chillon LF. *Novel Synthetic oviductal fluid for Conventional Freezing 1 (SCF1) culture medium improves development and cryotolerance of in vitro produced Holstein embryos*. Journal of Animal Science 2022; 100.

Ozmen B, Safaa A-H. *Techniques for Ovarian Tissue, Whole Ovary, Oocyte and Embryo Cryopreservation*. Journal of reproduction & infertility 2010; 11: 3-15.

Pedersen LD, Kargo M, Berg P, Voergaard J, Buch LH, Sørensen AC. *Genomic selection strategies in dairy cattle breeding programmes: Sexed semen cannot replace multiple ovulation and embryo transfer as superior reproductive technology*. Journal of Animal Breeding and Genetics 2012; 129: 152-63.

Pereira R, Marques C. *Animal oocyte and embryo cryopreservation*. Cell and tissue banking 2008; 9: 267-77.

Perez O, Hannalie A, Ku L, Barnett B. *Trophectoderm biopsy. a simplified mechanical method*. Fertility and Sterility 2012; 98: S140.

Polge C, Willadsen SM. *Freezing eggs and embryos of farm animals*. Cryobiology 1978; 15: 370-73.

Pollard JW, Leibo SP. *Comparative cryobiology of in vitro and in vivo derived bovine embryos*. Theriogenology 1993; 39: 287.

Ponsart C, Le Bourhis D, Knijn H, Fritz S, Guyader-Joly C, Otter T, Lacaze S, Charreaux F, Schibler L, Dupassieux D, Mullaart E. *Reproductive technologies and genomic selection in dairy cattle*. Reproduction, fertility and development 2013; 26: 12-21.

Pugh PA, Tervit HR, Niemann H. *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 2000; 58: 9-22.

Rall WF, Meyer TK. *Zona fracture damage and its avoidance during the cryopreservation of mammalian embryos*. Theriogenology 1989; 31: 683-92.

Richardson A, Brearley S, Ahitan S, Chamberlain S, Davey T, Zujovic L, Hopkisson J, Campbell B, Raine-Fenning N. *A clinically useful simplified blastocyst grading system*. Reproduction Biomedicine Online 2015; 31: 523-30.

Rizos D, Lonergan P, Boland MP, Arroyo-García R, Pintado B, Fuente Jdl, Gutiérrez-Adán A. *Analysis of Differential Messenger RNA Expression Between Bovine Blastocysts Produced in Different Culture Systems: Implications for Blastocyst Quality1*. Biology of Reproduction 2002a; 66: 589-95.

Rizos D, Ward F, Duffy P, Boland MP, Lonergan P. *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 2002b; 61: 234-48.

Rocha JC, Passalia F, Matos FD, Maserati MP, Jr., Alves MF, Almeida TGd, Cardoso BL, Basso AC, Nogueira MFG. *Methods for assessing the quality of mammalian embryos: How far we are from the gold standard?* JBRA Assist Reprod 2016; 20: 150-58.

Rodriguez-Villamil P, Ongaratto FL, Fernandez Taranco M, Bo GA. *Solid-surface vitrification and in-straw dilution after warming of in vitro-produced bovine embryos*. Reproducion in Domestic Animals 2014; 49: 79-84.

Rodriguez Villamil P, Lozano D, Oviedo J, Ongaratto F, Bo G. *Developmental rates of in vivo and in vitro produced bovine embryos cryopreserved in ethylene glycol based solutions by slow freezing or solid surface vitrification*. Animal Reproduction 2012; 9: 86-92.

Roschlau K, Roschlau D, Roselius RR, Dexne U, Michaelis U, Strehl R, Unicki P, Rink N. *Over 5 years experience in sexing of bovine morulae and blastocysts during routine embryo transfer*. Theriogenology 1997; 47: 273.

Sanches BV, Marinho LSR, Filho BDO, Pontes JHF, Basso AC, Meirinhos MLG, Silva-Santos KC, Ferreira CR, Seneda MM. *Cryosurvival and pregnancy rates after exposure of IVF-derived Bos indicus embryos to forskolin before vitrification*. Theriogenology 2013; 80: 372-77.

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

Seki S, Jin B, Mazur P. *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 2014; 68: 71-78.

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

Skrzyszowska M, Smorag Z, Katska L. *Demi-embryo production from hatching of zona-drilled bovine and rabbit blastocysts*. Theriogenology 1997; 48: 551-7.

Slimane W, Heyman Y, Lavergne Y, Humblot P, Renard JP. *Assessing chromosomal abnormalities in two-cell bovine in vitro-fertilized embryos by using fluorescent in situ hybridization with three different cloned probes*. Biology of Reproduction 2000; 62: 628-35.

Son WY, Yoon SH, Yoon HJ, Lee SM, Lim JH. *Pregnancy outcome following transfer of human blastocysts vitrified on electron microscopy grids after induced collapse of the blastocoele*. Human Reproduction 2003; 18: 137-9.

Tajimi H, Yamazaki T, Oike S, Yoshida T, Okada K, Kuwayama M, Ushijima H. *Vitrification for bovine embryos with low-quality grade*. Journal of Animal Science 2018; 89: 1194-200.

Taskin A, Akkoc T, Sagirkaya H, Bagis H, Arat S. *Comparison of the development of mouse embryos manipulated with different biopsy techniques*. Turkish Journal of Veterinary and Animal Sciences 2016; 40: 157-62.

Teixeira MG, 2018. *Use of a synthetic substitute to animal products for rabbit and ovine embryo cryopreservation*. Biotechnologies de la Reproduction. Université Claude Bernard Lyon, Lyon.

Thibier M, Nibart M. *The sexing of bovine embryos in the field*. Theriogenology 1995; 43: 71-80.

Tominaga K, Hamada Y. *Gel-Loading Tip As Container for Vitrification of In Vitro-Produced Bovine Embryos*. Journal of Reproduction and Development 2001; 47: 267-73.

Tucker MJ, Liebermann J. "*Vitrification in Assisted Reproduction: A User´s Manual and Trouble-Shooting Guide",* Reproductive Medicine and Assisted Reproductive Techniques*,* edited by Michael Tucker, Juergen Liebermann, CRC Press, 2007, 120-71.

Uchikura A, Matsunari H, Nakano K, Hatae S, Nagashima H. *Application of hollow fiber vitrification for cryopreservation of bovine early cleavage stage embryos and porcine morula-blastomeres*. Journal of Reproduction and Development 2016; 62: 219-23.

Vajta G, Holm P, Kuwayama M, Booth PJ, Jacobsen H, Greve T, Callesen H. *Open Pulled Straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos*. Molecular Reproduction and Development 1998a; 51: 53-8.

Vajta G, Kuwayama M. *Improving cryopreservation systems*. Theriogenology 2006; 65: 236-44.

Vajta G, Lewis I, Kuwayama M, Greve T, Callesen H. *Sterile application of the Open Pulled Straw (OPS) vitrification method*. Cryo Letters 1998b; 19.

Vajta G, Nagy Z. *"Are programmable freezers still needed in the embryo laboratory? Review on vitrification"*. Reproductive Biomedicine Online 2006; 12: 779-96.

Van Wagtendonk-de Leeuw AM, den Daas JH, Rall WF. *Field trial to compare pregnancy rates of bovine embryo cryopreservation methods: vitrification and one-step dilution versus slow freezing and three-step dilution*. Theriogenology 1997; 48: 1071-84.

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

Vanderzwalmen P, Bertin G, Debauche C, Standaert V, van Roosendaal E, Vandervorst M, Bollen N, Zech H, Mukaida T, Takahashi K, Schoysman R. *Births after vitrification at morula and blastocyst stages: effect of artificial reduction of the blastocoelic cavity before vitrification*. Human Reproduction 2002; 17: 744-51.

Viana JH, 2021. *2020 Statistics of embryo production and transfer in domestic farm animals.* Embryo Technology Newsletter, 24-36.

Vieira AD, Forell F, Feltrin C, Rodrigues JL. *In-straw cryoprotectant dilution of IVP bovine blastocysts vitrified in hand-pulled glass micropipettes*. Animal Reproduction Science 2007; 99: 377-83.

Wilsher S, Rigali F, Kovacsy S, Allen W. *Successful vitrification of manually punctured equine embryos*. Equine Veterinary Journal 2021; 53: 1227-33.

Wrenzycki C, Herrmann D, Lucas-Hahn A, Korsawe K, Lemme E, Niemann H. *Messenger RNA expression patterns in bovine embryos derived from in vitro procedures and their implications for development*. Reproduction, fertility and development 2005; 17: 23-35.

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

Youngs CR. *Cryopreservation of Preimplantation Embryos of Cattle, Sheep, and Goats*. Journal of Visualized Experiments 2011: e2764.

Yu XL, Deng W, Liu FJ, Li YH, Li XX, Zhang YL, Zan LS. *Closed pulled straw vitrification of in vitro-produced and in vivo-produced bovine embryos*. Theriogenology 2010; 73: 474-9.

Zayadan BK, Dzhansugurova LB, Turasheva SK. *Basics of biotechnology*. In University A-FKN 2020; 210.

Zenzes MT, Bielecki R, Casper RF, Leibo SP. *Effects of chilling to 0°C on the morphology of meiotic spindles in human metaphase II oocytes*. Fertility and Sterility 2001; 75: 769-77.

Zhang Y, Fu X, Chen L, Feng C, Bi J, Mo X, Cheng K, Zhang R, Li S, Zhu S. *A Simple and Efficient Vitrification Method for in-Straw Dilution and Direct Transfer of Bovine Embryos*. Cryo Letters 2015; 36: 392-8.

IX. APPENDIX

1. Media and solutions

1.1. Chemicals and Suppliers

Unless stated differently, chemicals and reagents that were used for in vitro production of embryos, were purchased from Sigma-Aldrich (Taufkirchen, Germany).

Chemical name	Supplier
Bovines Serum Albumin (BSA)	Sigma Aldrich
Basic amino acid solution (BME)	Sigma Aldrich
Non-essential amino acids (MEM)	Sigma Aldrich
Heparin	Sigma Aldrich
Pyruvic acid	Sigma Aldrich
Magnesium Clorid, $MgCl_2$	Sigma Aldrich
NaCl solution 0.9%	B.Braun, Melsungen, Germany
Polyvinylpyrrolidone	Sigma Aldrich
Sucrose	Sigma Aldrich
Foetal Calf Serum (FCS)	Sigma Aldrich
Ethylenglycol	Sigma Aldrich
Dimethylsufoxid	Sigma Aldrich
HEPES-buffered TCM199	Sigma Aldrich
Denaturation Buffer	Eiken Chemical Co., Tokyo, Japan
Tris-EDTA-Buffer	Eiken Chemical Co., Tokyo, Japan
EmXcell	IMV, l'Aigle, France

1.2. Bovine embryo production media

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 199 100.0 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% Oestrus calf serum (OCS) 50.0 µl (0.025 UI/ml maturation medium) Follicle-stimulating Hormone (FSH) 50.0 µl (0.0125 UIml maturation medium) Luteinizing Hormone (LH)

Swim-up medium for sperm capacitation	**Sperm TALP (500 ml)**
	2900.0 mg NaCl 1045.0 mg NaHCO3 20.0 mg NaH_2PO_2H2O 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_2PO4H_2O 32.5 mg Penicillin 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
	Supplementation (10 ml)
	400 µl BME amino acid solution 100 µl MEM aminoacid solution 5.0% OCS

Hormones and Stock Solutions	FSH 50 Units Sioux LH 25 Units Sioux Pyruvate stock Heparin stock Gentamycin stock

1.3. Vitrification media

Solution	Formulation
Holding medium (HM) (4 ml)	3200 µl 20 mM Hepes 199 Earle's Biochrom AG 800 µl FCS
Equilibration solution (ES) (4 ml)	2600 µl 20 mM Hepes 199 Earle's Biochrom AG 800 µl FCS 300 µl EG 300 µl DMSO
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
Dilution Solution 1 (DS1) (4 ml)	3840 µl 20 mM Hepes 199 Earle's Biochrom AG 950 µl FCS 1632 mg Sucrose
Dilution Solution 2 (DS2) (4 ml)	3400 µl 20 mM Hepes 199 Earle's Biochrom AG 850 µl FCS 722.5mg Sucrose

1.4. Biopsy medium

Solution	Formulation
Biopsy Medium	100 ml NaCl 150 PVP

2. Consumables and laboratory equipment

4-Well dish, Nunc, Germany
Petridish (35 mm diameter), Nunc, Germany
Petridish (60 mm diameter), Nunc, Germany
Centrifuge tubes (10 ml), Nunc, Germany
Pipette tips (0.1-1000 µl), Eppendorf, Germany
Pipette tips (0.1-10 µl) Biohit, Sigma-Aldrich, Taufkirchen, Germany
SafetySpace Filter Tip, Sigma-Aldrich, Taufkirchen, Germany
Hypodermic needle, Medical Planning Corporation, Japan
BD Syringe Luer-LokTM Tip 10 ml, BD, USA
Millex® GP Filter Unit 0,22 µm, Merck Millipore Ltd, Ireland
Ophtalmic blade Microfeather K-715 15° microblade FEATHER Safety Razor Co., Ltd., Osaka, Japan
Micromanipulator 5171 and Transjector 5246, Eppendorf, Hamburg, Germany
Galaxy® 48 R CO_2 incubator, Eppendorf, Germany
Galaxy® 48 R CO_2 - O_2 incubator Eppendorf, Germany
Motic SMZ171-TLED trino, Motic, Germany
Moticam 1080, Motic, Germany
Moticam X3 Plus, Motic, Germany
Nikon SMZ 745T, Nikon Europe, Amsterdam, The Netherlands

3. Drugs

Folltropin 700 IE, Vetoquinol, S.A., Lure, France
Dinolytic® 5 mg/ml, Zoetis, Berlin, Germany
Procamidor 20 mg/ml, WDT, Garbsen, Germany
CIDR intravaginal, Zoetis, Berlin, Germany

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