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Stress Preconditioning of Bovine Embryos

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Abstract

Cryopreservation of bovine embryos is a biotechnological tool crucial in preserving and conserving genetically superior embryos. Nevertheless, the process is not without consequences, particularly impacting the viability of embryos. This review aims to investigate the preconditioning methods to improve survivability during cryopreservation. Preconditioning embryos employ sublethal stress to upregulate genes promoting RNA processing, transcription and regulating protein synthesis while downregulating genes responsible for cell death and apoptosis. Embryos are subject to osmotic pressure changes and oxidative stress under standard cryopreservation methods. By manipulating the osmotic or oxidative concentrations in culture conditions, embryos initiate a stress-induced response that augments their robustness and post-cryopreservation survivability. Alternatively, sublethal applications of high hydrostatic pressure initiate a comparable stress response but offer a distinct advantage through non-penetrating and high precision capabilities. The research highlights discrepancies in results by using preconditioning tools. These inconsistencies can be attributed to differences in preconditioning and cryopreservation protocols, culture conditions, embryo grading and developmental stage. The overall consensus among studies validates the implementation of preconditioning tools to improve embryo viability, suggesting its potential integration as a new strategy into mammalian embryology.

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List of Abbreviations

ART	Assisted Reproduction Technology
BSA	Bovine Serum Albumin
COC	Cumulus-oocyte Complexes
CSPs	Cold Shock Proteins
CVM	Cryologic Vitrification Method
DMSO	Dimethyl Sulfoxide
FCS	Foetal Calf Serum
GPX	Glutathione peroxidase
HES	Hydroxyethyl Starch
HHP	High Hydrostatic Pressure
HSP	Heat Shock Proteins
ICM	Inner Cell Mass
IETS	International Embryo Technology Society
IVP	In vitro Produced
MOET	Multiple Ovulation and Embryo Transfer
ROS	Reactive Oxygen Species
SCNT	Somatic Cell Nuclear Transfer
SOD	Superoxide Dismutase
SOF	Synthetic Oviducal Fluid
TE	Trophectoderm

1. Introduction

With an ever-growing world population, having the resources to feed the world continues to be a point of importance. The production of essential dietary components, especially protein derived from eggs, milk, and meat, is crucial for maintaining a healthy population. To meet the demands of this growth, the agricultural sector has incorporated innovative technology into the production processes. Assisted reproduction technologies (ART) represent a pivotal role, offering a platform to increase the number of domestic animals and maintain a high genetic standard. Improvements have occurred across many ART methods, such as estrus synchronisation, superovulation, artificial insemination, embryo recovery, transfer, *in vitro* fertilisation, cryopreservation, and transgenesis[1]. Embryo transfer is considered one of the most essential techniques because it can yield multiple offspring produced from genetically superior dams. Embryo transfer can be achieved through fresh embryo transfer, where the embryo is transferred directly from the donor dam to the recipient dam, or frozen embryo transfer, where the embryo is cryopreserved for future utilisation. The annual International Embryo Technology Society (IETS) statistics of 1997-2017 indicated that transferred embryos' fresh: frozen ratio decreased from 91% in 2012 to 73% in 2017, indicating a shift towards using frozen embryo transfer[2].

Cryopreservation of bovine embryos is a biotechnology technique used to preserve and store embryos that facilitates extensive distribution of genetically superior embryos. Embryos are frozen at temperatures well below zero, with some methods using liquid nitrogen, leading to the potential for long-term storage for future breeding and reproductive programs. Cryopreservation is a multifaceted subject with numerous factors contributing to successfully transferring genetically viable embryos. There are two primary cryopreservation techniques: slow-rate cryopreservation and vitrification. Slow-rate cryopreservation involves low concentrations of cryoprotectant and a gradual decrease in temperature, whilst vitrification involves solidifying the embryos into a glass-like structure using a rapid decrease in temperature and a higher concentration of cryoprotectant[3]. Cryoprotectants are any substances that improve the tolerance of embryos to the freezing and thawing processes.

Although the cryopreservation technique plays a significant role in embryo transfer, embryo quality is vital to ensure survival during freezing and thawing. Embryos produced *in vitro* have compromised physiology, gene expression, and development compared to those

derived via *in vivo* techniques[4]. Embryos are graded according to the number of viable cells within the embryo[3]. For the cryopreservation of embryos, the highest-graded embryos are required to achieve the best chance of survival during the cryopreservation and thawing process, resulting in viable offspring. Even the highest quality embryos can have a relatively low success rate following cryopreservation.

Recently, research has gone into the preconditioning of embryos for cryopreservation. Preconditioning of embryos involves applying sublethal stress to elicit a stress response. The stress response causes an improved tolerance to stress, morphological intactness, fertilising ability, and developmental competence[4]. Sublethal stress originated from the food industry in an attempt to control the microbial load in foodstuffs[5]. The procedure involved the application of multiple forms of mild stress to reduce microbial load whilst maintaining food quality. In a study by Wemekamo-Kamphuis *et al.* (2002), sequential sublethal stress applied to *Listeria monocytogenes* resulted in increased bacterial count[6]. *Listeria monocytogenes* can survive in cold temperatures, such as fridge temperatures, which is problematic in the food industry. The study attempted to apply a sequence of stressors to combat this problem. The study used cold shock, followed by high hydrostatic pressure (HHP) in food products, and assessed the *listeria monocytogenes* numbers. Following the cold shock, the bacteria responded by increasing the level of cold shock proteins (CSPs). *L. monocytogenes* expressed a similar response when exposed to HHP. When these treatments were used sequentially, the cold shock treatment preconditioned the bacteria to the harmful effects of the subsequent HHP treatment. Therefore, the result of the sequence of cold shock followed by HHP led to the proliferation of *L. monocytogenes*[6].

The study indicated that these treatments were not beneficial to eliminate *Listeria monocytogenes* in the food industry. However, the response of cells to sublethal stress leads to further investigation in other fields of science, such as the preconditioning of embryos.

1. Aim

This review investigates stress preconditioning techniques in bovine embryos to improve cryotolerance. The review focuses on strategies to induce sublethal stress using variations in osmotic levels, oxidative agents, and high hydrostatic pressure.

2. Brief History of Embryo Transfer

The first successful mammalian embryo transfer was achieved in 1890, using two four-cell angora rabbit embryos in an inseminated Belgian doe by Walter Heape[7]. After this, research in embryo transfer began to surge. However, it was not until 1949 that Umbaugh accomplished the first successful bovine embryo transfer. Umbaugh produced four bovine pregnancies using embryo transfer, although all pregnancies were terminated before parturition. Two years later, the first calf was born using embryo transfer. The embryo transfer practice transitioned from laboratory-based research to private veterinary practitioners and small commercial companies, which further developed the technology for on-site application[7]. In 1974, the International Embryo Transfer Society (IETS) was established and provided a platform to exchange scientific-based information, discuss embryo transfer, and develop new technologies. Currently, the IETS functions to further the science behind animal embryo technology, promoting research and the distribution of information.

Commercial embryo transfer in cattle established a means to rapidly increase animal populations, enhance genetic advancement, manage infectious disease transmission, and facilitate importing and exporting valuable genetic material[7]. The development of multiple ovulation and embryo transfer (MOET) led to increased selection intensity and reduced generation intervals, ultimately enhancing genetic advances. Genomic analysis technologies created a platform to produce sires by selecting high-producing cows and proven bulls[7]. Genomic analysis made it possible to reduce the time interval of genetic testing in bulls compared to traditional progeny testing. Additionally, valuable genetic material can be collected from premature individuals, individuals with anatomical or sub-fertile conditions or those that have surpassed their reproductive age[8].

The transmission of infectious diseases can be dramatically reduced with embryo transfer. Provided that embryos are handled correctly, undergo washing treatments, and microscopic examination of the zona pellucida, the risk of disease transmission is negligible[7]. During a disease outbreak, the genetic material from valuable livestock can be collected and preserved, ensuring genetic progress is retained in the case of catastrophic outbreaks. Additionally, by preventing disease transmission and the ability to store biological material for long periods, cryopreservation allows for biological material to be exported and imported

with negligible risks. Moreover, the cost of transferring animals internationally, quarantine periods, and transport risks can be reduced substantially.

One potential constraint associated with direct embryo transfer was the availability of suitable recipients. With the development of cryopreservation, biological material can be successfully stored until recipients are in the appropriate phase of their reproductive cycle. Freezing and thawing of biological material often cause damage and reduced viability. Furthermore, cryopreservation was a time-intensive process and required highly experienced technicians and specialised biological freezers. Vitrification in liquid nitrogen and the development of suitable cryoprotectants made it possible to overcome these obstacles.

Data from IETS in 2016 indicated that the number of transferable *in vivo*-derived embryos was surpassed by viable *in vitro*-produced (IVP) embryos [8]. IVP embryos are removed from the organism and allowed to develop and mature in controlled conditions. Oocytes are collected and fertilised in a suitable culture medium before reimplantation in a recipient dam. Using IVP, embryos from individuals with desirable genetic traits can undergo genomic analysis, develop a controlled embryo, and be preserved for the future. The rise in IVP embryos may be attributed to genomic selection and the use of sexed semen from high-quality bulls[8]. While a certain degree of reduced viability remains, ongoing research has demonstrated notable success in overcoming this challenge.

3. Embryos under stress

All living organisms are exposed to external environmental stress. Environmental alternations in temperature, osmotic changes, pH, inappropriate nutrition, oxidation, or light/irradiation are stressors to embryos[9]. When embryos are exposed to severe adverse stress factors, programmed cell death (apoptosis) will occur. Under mild adverse conditions, the embryos can adapt and overcome these factors and survive. The sublethal stress stimulates a stress reaction in the cell. The stress reaction involves detection, assessing, and counteracting stress-induced damage[10]. Embryos used for cryopreservation, culture, and *in vitro* maturation require meticulously controlled conditions to minimise damage incurred due to osmotic, oxidative, cold/heat shock, nutritional and mechanical stress[11].

Under adverse conditions, the embryos respond by stimulating stress-induced proteins. The response is controlled at a translational and transcriptional level through alterations in gene expression[4]. Specific genes are upregulated, and others are downregulated to combat adverse conditions. Upregulated genes result in the transcription of stress-related proteins in the chaperone family. These proteins contribute to various cell functions such as stabilisation and repair of proteins, DNA and chromatin, cell cycle control, regulation of redox reactions, management of energy, fatty acids and lipids metabolism, and removal of damaged proteins[4]. An essential stress-induced protein family are heat shock proteins (HSPs). All organisms respond to an increase in temperature by inactivating physiological protein synthesis and activating the synthesis of HSPs[12]. This process is accomplished by upregulating HSP genes, the most important being the Hsp70 gene[9]. HSPs are a set of highly conserved proteins that preserve cell survival under adverse environmental conditions. They prevent fatal injury and act by signalling danger within the embryo to elicit an immune response[9]. The primary function of HSPs is to mediate the folding and transport of intracellular proteins, assist in the maintenance of inactive proteins and prevent intracellular proteins from denaturing.

Although the initial hypothesis was centred around the activation of HSPs in response to heat stress, it became apparent that HSPs can be activated under various sudden environmental changes[4]. In addition to heat, it has been reported that HSPs are produced under HHP, cold stress, osmotic stress, pH changes and starvation[13]. Under adverse conditions that exceed the embryo's tolerance limit, HSPs stimulate programmed cell death

or apoptosis. Depending on the stress level, these proteins may either reduce or stimulate cellular apoptosis[4].

Embryos are exposed to reactive oxygen species (ROS) under typical environmental conditions. ROS are generated during the reduction of oxygen during the process of aerobic metabolism. The primary ROS produced are superoxide anion radicals (O_2^-), hydroxyl radicals (OH), and hydrogen peroxide (H_2O_2)[14]. Additionally, external factors may result in increased ROS production. Environmental factors such as oxygen concentration under atmospheric conditions, trace metallic cations in the culture medium (such as Fe and Cu), and UV irradiation from visible light may induce oxidative stress on embryos. Oxidative stress on embryos can lead to several types of embryo injuries. ROS induce lipid peroxidation (affecting the cell division and mitochondrial function), protein oxidation (leading to enzyme inactivation), DNA strand fragmentation (resulting in a halt in embryo development), ATP depletion (by competitive consumption of reduction equivalents), and potentially apoptosis (when ROS exceed antioxidant agents)[14]. *In vitro*-produced embryos tend to experience more significant structural and functional damage to DNA, lipids, proteins, suppression of cell division, and apoptosis due to ROS compared to *in vivo*-produced embryos because of oxidative factors present in the culture media[15].

Embryos employ two defence mechanisms against oxidative insult: enzymatic or non-enzymatic antioxidants. Non-enzymatic antioxidants can be produced naturally in embryos or, in the case of *in vitro*-produced embryos, supplemented in the culture media. Vitamin A, C, E, pyruvate, and sulphur-containing compounds, such as glutathione, taurine, hypotaurine and cysteamine, are important non-enzymatic antioxidant compounds[14]. Enzymatic antioxidants are regulated at a pre-transcriptional level due to the strong correlation of mRNA, proteins, and enzymatic activity[14]. The primary enzymatic antioxidants are superoxide dismutase (SOD) and glutathione peroxidase (GPX), which are produced by upregulating Sod2 and Gpx4 genes, respectively.

Embryos subject to stress upregulate specific genes for stress tolerance. These stress-induced genes result in the production of proteins and enzymes that act to strengthen the embryo. This 'eustress' can improve membrane stabilisation conformation of proteins and assist in maintaining cell function[11]. The protective nature of these products acts globally within the embryo, and a similar set of proteins and enzymes are produced under multiple types of stress. Pridneszky and Vajta (2011) indicated that antioxidant-related genes (Sod2 and

Gpx4) and HSP genes (Hsp70) were upregulated in embryos placed under HHP treatment[16]. As mentioned, if the stress level reaches a point that exceeds the defence of the embryo, apoptosis will occur. Under sublethal stress conditions, stress tolerance genes are upregulated, and a global protection mechanism is initiated. Due to the nature of general protection by these proteins and genes, sublethal stress can stimulate a temporary increase in the general robustness of the embryo to further insults, such as cryopreservation.

4. Cryopreservation of Embryos

Cryopreservation preserves biological material at extremely low temperatures, typically in subzero or liquid nitrogen (-196°C). At these temperatures, cellular metabolism and biochemical reactions are reduced to negligible rates, allowing for cells to be stored for an extended period, with the ability to be restored whenever necessary. There are two main methods of cryopreservation of embryos: Slow-rate cryopreservation and vitrification. With slow-rate cooling, the cooling rate is carefully controlled to 0.3-0.6°C/min to negative 30 to 32°C[17]. A low concentration of cryoprotectants induces ice crystal formation while osmotically drawing moisture out of the cell. With slow-rate cooling, embryo damage may occur due to ice crystal formation, osmotic changes, cryoprotectant toxicity, chilling harm, and embryo fracture[18].

Vitrification is a rapid cooling process that solidifies the liquid into a glass-like structure without ice crystal formation. High amounts of cryoprotectants and a rapid rate of cooling, 1000°C min or greater, are used to increase viscosity and suppress ice crystal formation[17, 18]. Vitrification eliminates the damaging factors caused by slow-rate cooling. Additionally, vitrification uses liquid nitrogen to freeze specimens instantly instead of expensive biological freezers that decrease temperatures at controlled increments. Vitrification also requires less technical skills and laboratory equipment.

During cryopreservation, the embryos are challenged osmotically (cell dehydration), mechanically (damage of the cell membrane due to ice formation), thermally (decrease in temperature), and toxically (from the cryoprotectants)[17][19]. These factors negatively affect embryo viability and pregnancy rates after embryo transfer.

4.1 Cryoprotectants

Cryoprotectants are substances used to improve the survival of the embryos throughout the cryopreservation process. They protect the embryos from the adverse effects of intracellular ice formation, lower the freezing point, reduce cellular dehydration, stabilise cell structures, and enhance thawing viability. Lipids in cell membranes undergo a phase transition during freezing that can result in chilling injury. The fluidity of the lipid bilayer changes to a more

solid gel phase during cryopreservation. Lowering the freezing point diminishes the risk of chilling injury on lipids in cell membranes[20].

The primary requirements for cryoprotectants are high solubility, low toxicity at high concentrations, and low molecular weight[21]. The toxicity depends on the concentration and type of cryoprotectant. Although cryoprotectants shield embryos from harm during freezing and thawing, they modify the osmolarity of the surrounding medium, resulting in osmotic stress.

Cryoprotectants are categorised based on their ability to permeate the cell. They are either penetrating or non-penetrating cryoprotectants. Penetrating cryoprotectants cross the cell membrane, acting intracellularly. These solutions are composed of molecules of lower molecular weights, allowing the solution to enter the cell. Once in the cell, these cryoprotectants act by reducing the freezing point of the intracellular fluid and reducing intracellular ice crystal formation. In addition, penetrating cryoprotectants stabilise cell membranes, proteins, and cytoskeleton, protecting cells against ice crystal formation. The most common penetrating cryoprotectants are dimethyl sulfoxide (DMSO), glycerol, ethylene glycol and propylene glycol.

Non-penetrating cryoprotectants act extracellularly, affecting the osmolarity of the freezing solution and improving membrane stability[3]. They prevent embryo damage by lowering the solution's freezing point and reducing ice crystal formation extracellularly. Initially, they were used to reduce the osmotic shock during cryopreservation[21]. In addition, non-penetrating cryoprotectants preserve cell shape and prevent cell dehydration, providing a more viable embryo post-thawing. Non-penetrating cryopreservation solutions are usually sugar-based, such as sucrose, maltose, galactose, or trehalose[3]. Alternatively, non-sugar-based solutions include hydroxyethyl starch (HES) and polyvinylpyrrolidone. The type and concentration of cryoprotectants depend on the cryopreservation protocol and the biological material being preserved. Usually, slow-rate cryopreservation employs two penetrating cryoprotectants, such as glycerol and ethylene glycol. In contrast, vitrification may include both penetrating and non-penetrating cryoprotectants.

4.2 Embryo Viability in Cryopreservation

Embryos intended for cryopreservation are subject to various factors that impact their viability following thawing. Factors that affect embryo quality include breeding animals (donor dam, semen quality, environmental conditions, species, breed, nutritional and health background), embryo development (culture conditions, quality and composition of culture medium, gas atmosphere), cryopreservation technique (slow rate or vitrification protocols, cryoprotectant type and quantity, thawing method, technical skills), and embryo quality[19].

Embryo quality considerably impacts survivability throughout the freezing and thawing process. The embryo quality correlates to successful embryo survival. Grading embryos is usually based on their morphological appearance where shape, colour, number of cells, perivitelline space size, presence of extruded cells within the perivitelline space, number of degenerated cells, trophectoderm (TE) to inner cell mass (ICM) ratio, degree of blastocoel expansion, number of vesicles and overall appearance[3, 17, 22]. Embryos are graded from 1–3, where grade 1 embryos represent the highest quality embryos and grade 3 represent the lowest quality. These parameters are individually and subjectively evaluated, which may lead to discrepancies. Cryopreservation decreases the number of viable cells within embryos; therefore, the highest-grade embryos should be used.

Additionally, the thawing process affects the quality of embryos. Culture medium, additives (such as proteins, cytokines, antioxidants, hormones) and thawing procedure influence the viability of embryos post-thaw. Embryos are sensitive cells exposed to many variables that dictate their viability, especially when undergoing cryopreservation. With many external variables affecting the cells, research has gone into improving the robustness of the embryos before vitrification. This is achieved through stimulating a stress response, leading to a change in gene expression that stimulates proteins, such as HSPs, and enhanced cellular immunity. This stress response may lead to cross-protection that assists the cell by providing fortification against various stress factors, such as vitrification. Vitrification decreases the viability of embryos, so artificially stimulating the stress response can improve embryo survival and ultimately improve pregnancy rates. This phenomenon was demonstrated in the food industry, where *listeria monocytogenes* were more tolerant to cold shock after high hydrostatic pressure treatment[6].

5. Preconditioning embryos for Cryotolerance

Recent research has gone into applying sublethal stress to embryos to improve their robustness prior to cryopreservation. The aim is to stimulate gene expression that produces protective proteins when the embryo is exposed to a stressor. Cryopreservation is a tool that can preserve biological material for future use. This ART is essential in advancing genetic capacity and preserving genetic material where epidemiological diseases may lead to the mass culling of domestic animals. Additionally, cryopreserved genetic material, such as sperm, oocytes, and embryos, can be stored for an extended period and transported from donor animals that cannot transfer their genetic potential naturally. This technology enables the broader utilisation, global transportation, and extended storage of valuable genetic material from domestic animals. Although this valuable tool has many benefits, the cryopreservation process negatively affects the viability of embryos, leading to lower pregnancy rates and viable offspring. As mentioned previously, embryos are exposed to many stress factors during cryopreservation. Under laboratory conditions, it is possible to control the external stresses that act on embryos; however, they will always be subject to some stress. Preconditioning the embryos strengthens them for cryopreservation to increase their viability post-thaw.

5.1 High Hydrostatic Pressure

The food industry initially adapted the concept of utilising high hydrostatic pressure to enhance cell resilience. HHP was used to preserve and extend the shelf life of food products by reducing the microbial load while inflicting minimal adverse effects on the product itself. Although HHP treatment followed by cold shock or heat treatment decreased microbial load, *listeria monocytogenes* increased. Wiemekamp-Kamphuis *et al.* (2002) demonstrated that treating *listeria monocytogenes* with a cold shock treatment, followed by high hydrostatic treatment, improved bacterial survival instead of inhibition[6]. Although this did not prove helpful against *Listeria monocytogenes* in the food industry, it led to the investigating of sequential sublethal stress mechanisms in ARTs. High hydrostatic pressure is a valuable tool that can be controlled with high accuracy and does not penetrate the biological material. Embryos are typically exposed to 0.1MPa at normal atmospheric pressure. However, some authors have reported up to 90MPa without any visible morphological changes[23].

A study by Pribenszky *et al.* (2005) evaluated the effects of HHP on mouse blastocysts before their transfer into recipients and cryopreservation[21]. The blastocysts were loaded into 0.25ml plastic straws without air bubbles and heat-sealed straws. The embryos were separated into groups of 14-16 per group. The groups were exposed to different levels of HHPs at 10 MPa increments, ranging from 10 MPa to 150 MPa, for various periods ranging from 1s. to 300 min. The HHP treatment was performed at room temperature. It has been shown that levels of up to 90 MPa for 1s or 30 MPa for 2h cause no visible morphological damage to mouse blastocysts[23]. However, embryos can collapse under these HHP levels and duration. Collapsed blastocysts can return to normal morphology following a 4-5h *in vitro* culture. The experiment determined an inverse relationship between pressure and time[21]. With increased pressure levels, the duration should be decreased to result in viable blastocysts. When embryos are subject to high-pressure levels for an extended period, irreversible changes to blastocyst morphology occur.

Furthermore, blastocysts subject to sublethal pressure levels had improved *in vitro* development following vitrification. Blastocyst morphology was 98% identical to the non-pressurised control group after 6h following vitrification, and 95% fully hatched within 20h. Furthermore, non-pressurised control blastocyst re-expansion rates were significantly lower than those subjected to the pressure treatment (46% versus 98%, respectively).

The HHP induces transcriptional changes where genes for protecting the embryo are upregulated. Some genes increased immediately after the treatment, such as Sod2, Hsp70, antizyme inhibitor (Azin1), and growth arrest-specific 5 (Gas5) genes. Others, such as growth arrest and DNA damage-inducible 45 gamma (Gadd44g), increased after 120 min [4]. This demonstrates that the HHP treatment upregulates both short- and long-term genes related to embryo resistance.

In a study by Filho *et al.* (2011), the effect of HHP on post-thaw survival of 440 bovine *in vitro* produced (IVP) blastocysts was investigated. The study found that the HHP treatment significantly improved the post-warming developmental competence of vitrified bovine blastocysts[24]. In the treatment group, embryos were exposed to 60MPa for 1h, followed by vitrification at different times after the treatment (0h, 1h, and 2h). It was discovered that the HHP treatment improved blastocyst hatching rates compared to the non-treated vitrified groups, with significant improvements in re-expansion rates of the treatment groups followed by 0h and 1h equilibration prior to vitrification[24]. This demonstrates the

importance of an equilibration period, where the protective effect of HHP treatment depends on the time between the initial treatment and the subsequent insult. The equilibration period is beneficial for the embryos to develop an elevated tolerance from the initial stress, better preparing them for the subsequent stresses. During the equilibration period, the peak gene expression for protective proteins is achieved, promoting cross-protection within the embryo, leading to improved cryotolerance of embryos and increased *in vitro* survival and hatching rates. Pribenszky *et al.* (2010) found that bovine IVP embryos could be treated with 80MPa for 45 min, where the strongest cryotolerance was realised with embryos vitrified following a 1h equilibration period[4]. This is because time is needed for HHP-activated genes to synthesise the related RNA and proteins. In bovine blastocysts exposed to sublethal HHP levels responded by upregulating antioxidant stress-related genes such as Sod2 and Gpx4, along with lipid synthesis (Sc4mol) and heat shock-related genes (HSPA1A) [4][11].

In 2012, Trigel *et al.*(2012) analysed the effects of HHP on *in vitro* survival of IVP bovine embryos with the Cryologic Vitrification Method (CVM). The approach used differential staining to determine the level of trophectoderm (TE) cells and inner cell mass (ICM) as indicators for the developmental competence of the embryos. A certain minimum number of TE and ICM cells is assumed to be essential for obtaining pregnancy[25]. Following vitrification and warming, the HHP treatment groups displayed increased ICM, indicating that HHP and the culture period after warming may result in the proliferation of these cells. A Western blot was used in the same study to assess HSP (Hsp70) levels. In this case, the Hsp70 levels were not affected by the HHP treatment. Trigal *et al.* (2012) indicated that the HHP treatment did not improve the survival rates of bovine embryos to vitrification.

Additionally, the recovery time (1 vs 2h) did not affect survival rates following vitrification. This difference between the results of this study and those presented by Filho *et al.* (2011) may be attributed to the use of different culture and vitrification systems. Filho *et al.* (2011) used synthetic oviducal fluid (SOF) supplemented with amino acids, citrate, myoinositol and 5 % foetal calf serum (FCS) as a culture medium. Trigal *et al.* (2012) utilised SOF supplemented with amino acids, citrate, myoinositol and 6g/L bovine serum albumin (BSA) as a culture medium. Additionally, there were differences in the vitrification protocols used. These factors may be the reasons for the discrepancies when comparing results.

The following year, Popovic *et al.* (2013) published an article utilising HHP before vitrification of grade 1 and 2 quality embryos utilising the CVM. Before vitrification, bovine

blastocysts received 60 MPa for 1h, with an equilibration period of 1h following the HHP treatment [22]. After the thawing procedure, representative samples from the control, control vitrified, and the HHP and vitrified groups were transferred into the uterine horns of synchronised recipient females. The study evaluated the *in vivo* survival of IVP bovine blastocysts subject to HHP prior to vitrification, using ultrasonography to analyse fetal heartbeats from day 35 to day 65. Following Day 65, the potentially viable fetuses were terminated.

Additionally, differential staining was used to determine the live vs necrotic cell numbers in blastocysts following vitrification. The study found that within the grade 1 groups, there was no significant difference between the HHP and vitrified groups and the control vitrification groups. This suggests that grade 1 blastocysts tolerate the insult of cryopreservation, and HHP treatment may not provide additional benefit. However, a higher proportion of grade 2 bovine blastocysts, subject to HHP prior to vitrification, remained of transferable quality compared to the grade 2 vitrified control group. Of the grade 2 vitrified control group, a more significant proportion of blastocysts deteriorated to non-transferrable grade 3 quality. Using differential staining, grade 2 blastocysts exposed to HHP and vitrification had more live cells after warming than the controlled vitrified group. This supports the theory that vitrification causes a decrease in embryo quality. Moreover, it supports the theory that embryos subject to sublethal levels of HHP prior to vitrification may provide resistance to the insult of cryopreservation. Regarding *in vivo* survival of IVP bovine blastocysts, there was no significant difference between the HHP-treated and vitrified groups compared to the control vitrified groups for grade 1 and grade 2 blastocysts at day 65 of gestation.

A study published by Jiang *et al.* (2016) examined the effects of HHP on the expression of profiles of IVP bovine blastocysts[11]. They used three different pressure levels (40, 60, and 80MPa) along with three varying equilibration periods (0, 1, and 2h) after HHP treatment. The re-expansion rates of bovine blastocysts were significantly higher in the 40 MPa and 60 MPa groups compared to the control groups. In contrast, the 80 MPa treatment groups showed significantly lower re-expansion rates than the control groups[11]. Using hierarchical clustering of expression profiles, it was shown that the 40 and 60 MPa groups had overall higher gene expression in comparison to the control and 80 MPa treatment groups. These results correlate to the re-expansion rates of blastocysts and indicate that pressure plays a role in gene expression changes. To better understand the molecular background of improved resistance following HHP treatment, the effects of HHP and

equilibration periods on gene expression were analysed. For the 40 and 60 MPa treatment groups, genes involved with cell death and apoptosis were downregulated, while genes promoting RNA processing and transcription and regulation of protein synthesis, ultimately promoting embryo survival, were upregulated. Conversely, the 80 MPa treatment group showed downregulation for protein folding and cell cycle genes and the upregulation of genes associated with cell death, apoptosis, and chromatin assembly/disassembly[11].

The equilibration period is essential for HHP-related gene expression as it allows the cellular metabolism to synthesise RNA and proteins. For the 1h equilibration group, apoptosis, proteolysis, and phosphate metabolic process-related genes were downregulated, with cell growth and proliferation, cell morphology and cell function and maintenance-related genes upregulated. In the 2h equilibration group, protein folding, cell cycle and cell death genes were downregulated, whilst cellular growth and proliferation, DNA replication and mitotic cell cycle-related genes were upregulated. Notably, the study showed a drastic difference in gene expression when comparing the 0 vs 2h and 0 vs 1h, indicating the 2h of equilibration allows for additional gene expression changes. With this in mind, the 2h equilibration period did not promote better re-expansion rates compared to 1h. The study suggests that the further gene expression realised in the 2h equilibration period may have corrected the changes occurring during the first hour, therefore cancelling the changes required to withstand the insult of vitrification.

5.2 Osmotic pressure.

Osmotic pressure plays a crucial role in embryos destined for cryopreservation. With embryos produced by IVP, the conditions are carefully controlled to provide a stable environment for embryo development. Among others, osmotic pressure is an external factor that may stress the embryo. For cryopreservation to be successful, cryoprotective agents are used to improve the survivability of the embryos. These cryoprotectants pose an osmotic challenge to the embryo. However, when used in the correct concentrations, cryoprotectants aid the embryos during freezing. Cryoprotectants create an osmotic gradient that drives water out of the embryo, causing dehydration. It has been suggested that stress-related genes, such as HSPs, are stimulated when embryos undergo osmotic pressure[13], similar to embryos preconditioned with HHP. In the vitrification procedure, cryoprotectants expose embryos to

various osmotic gradients. Thus, using osmotic pressure as a preconditioning tool may optimise embryo cryotolerance.

Lin Lin *et al.* (2009) investigated elevated NaCl concentrations to improve the cryotolerance of porcine oocytes[13]. NaCl is commonly used in embryo culture mediums to act as a buffer and maintain osmotic balance. The initial step in the study was to determine the optimal concentration of NaCl, leading to survival rates comparable to the controls. The optimal osmotic levels were 288-593 and 1073-1306 mOsmol. Between 593 – 1073 mOsmol, a dramatic decline in survival rates was found. The exact mechanism for this is unknown, but the study hypothesises that it may be comparable to the 'danger zone' experience when applying cold shock treatment in embryos. Following the 1st phase of the study, the optimal osmotic pressure levels were used prior to vitrification to evaluate oocyte survival. The treatment of 593 mOsmol on oocytes produced the best developmental rates. An equilibration period is essential for transcribing stress-induced genes into RNA and proteins. The study analysed two different recovery times (1 and 2 h). This study indicated no significant differences when comparing cleavage rates of the control group (0h) and the treatment group (1 and 2h). However, there were significantly higher blastocysts rates when comparing the treatment and control groups. The results indicate that osmotic pressure may play a role in improving the cryotolerance of oocytes. It should be noted that attributes other than osmotic pressure by the NaCl treatment may be present. Mechanisms such as elevated intracellular NaCl concentrations, NaCl's ionic effect, and the osmotic pressure caused by cryoprotectants may cause alterations in the results.

Expanding on the use of NaCl as an osmotic agent, similar authors evaluated the use of non-permeable agents (trehalose and sucrose) compared to NaCl and their effects on oocyte cryotolerance and developmental competence following somatic cell nuclear transfer (SCNT). Trehalose and sucrose are commonly used as non-permeable cryoprotectants to cause dehydration in embryos before vitrification or slow-rate freezing. Compared to the untreated control group, oocytes pretreated with 588 mOsmol NaCl, trehalose or sucrose solutions for 1h, followed by a 1h equilibration period, demonstrated markedly increased rates of cleavage (on day 2) and blastocysts formation (on day 7), following the processes of vitrification, warming and parthenogenic activation[20]. Osmotic pressure improves cryotolerance by facilitating HSP production and reducing the melting temperature, improving lipid stability during phase transition of cell membranes. As mentioned previously, chilling injury results from the phase transition of lipids in the cell membrane.

Decreasing the chilling point reduces chilling injury by lipid phase transition. It should be noted that although the use of these agents improved oocyte cryotolerance, studies of these effects on embryos have not been performed.

5.3 Oxidative stress

As mentioned previously, ROS is a product of aerobic metabolism and environmental conditions. ROS induces lipid peroxidation in cell membranes, injuring cell membranes and disrupting cell division and mitochondrial function. Moreover, ROS oxidises proteins, thereby inactivating enzymes, fragmenting DNA and inhibiting embryo development. Oxidative stress is more prevalent in *in vitro*-derived embryos than *in vivo*-derived counterparts, as embryo culture conditions cannot match the environment created in the oviduct and uterus.

In order to test the effects of oxidation, Vandele *et al.* (2010) investigated the use of hydrogen peroxide (H_2O_2) at different concentrations on mature cumulus-oocyte complexes (COC) and the effects on embryo development and apoptosis. Short-term exposure of COC to high H_2O_2 (50-100μmol/L H_2O_2) improved embryo development and did not affect apoptosis in blastocysts[26]. While under low and medium levels of H_2O_2, blastocyst apoptosis levels were increased, indicating an inverse relationship between H_2O_2 levels and apoptosis. The mechanism for this is unknown, but it is hypothesised that exposure to H_2O_2 may elevate antioxidant levels in COC, leading to improved embryo development. The study proposes that exposure to high levels of H_2O_2 stimulates transient stress resistance, leading to enhanced embryo development and increased defence against embryonic apoptosis. Oxidative stress alters gene expression, an influential group being HSPs. Additionally, hypoxia-inducible factor 1 (HIF1) plays a role in COC resistance to stress by controlling gene expression and regulating the adaptive response to fluctuations in oxygen levels[26]. The study indicated that the increase in GPX content and embryo fertilisation or penetration was not facilitated by H_2O_2 treatment.

6. Discussion

The primary goal of cryopreservation is to store biological material for extended periods while maintaining viability and functionality for future use. The freezing of biological material has both detrimental and beneficial characteristics. Ongoing research focusing on maximising the beneficial attributes while mitigating the detrimental ones is essential to optimise cryopreservation as a tool for biological preservation. Research focused on refining cryoprotectant solutions led to reduced stress and mechanical damage caused by ice crystal formation during cryopreservation. Moreover, freezing and thawing protocols have advanced to improve overall recovery and viability, accentuating the beneficial effects of cryopreservation. Concentrating on the biological material itself, efforts to explore and understand the consequences of cryopreservation have been conducted. At almost all levels of life, exposure to sublethal stress triggers a temporary increase in general resistance[4]. Wemekamo-Kamphuis *et al.* (2002) discovered this occurs in bacteria when assessing sequential stress treatments on food products to reduce microbial load. *Listeria monocytogenes* showed increased tolerance to HHP following a cold shock treatment, indicating that his phenomenon also manifests at a cellular level. Like humans or animals receiving a vaccine to boost immunity, sublethal stress at a cellular level can improve resistance to manipulations such as cryopreservation. The process involves detecting, evaluating, and mitigating the damage incurred due to the stress.

Embryos have been shown to respond to stress by upregulating genes that enhance membrane stabilisation and protein conformation and maintain cellular function. In response to different forms of sublethal stress, an analogous set of genes is upregulated to promote a more robust cell or, in this case, embryo. Under cryopreservation conditions, embryos are exposed to numerous detrimental factors. Osmotic pressure poses a threat by disturbing the normal water balance. Cryoprotectants increase osmolarity, dehydrating to improve freezing and thawing viability. Although useful for cryopreservation, it acts as a stress on the embryo.

Furthermore, cryoprotective agents pose a toxicity threat. Lin Lin *et al*. (2009) investigated the effects of osmotic pressure as a tool to precondition embryos for cryopreservation. Using NaCl to exert osmotic pressure, the study proved that in the range of 228-593 and 1073-1306mOsmol, embryos stimulate a stress response that improves tolerance to cryopreservation.

A further study by the same authors proved that non-permeating solutions such as trehalose and sucrose demonstrated improved cleavage rates of blastocyst formation[20]. In both studies, exposing embryos at a concentration of 588mOsmol for 1h, followed by a 1h recovery period, significantly improved performance compared to non-treated control groups. NaCl outperformed the non-permeable counterparts as the latter two reduced cell numbers in blastocysts following SCNT[20]. The exact mechanism of the preconditioning method is unknown. The study suggests that HSPs are stimulated. However, it is understood that HSPs are produced under various stresses, and it is not clear that they are produced solely due to osmotic pressure. NaCl treatment may impact the embryos in more ways than osmotic pressure alone. In NaCl solutions, intracellular NaCl concentrations are elevated, posing an ionic effect that may elicit the HSP response. Additionally, osmotic agents stabilise lipids in cell membranes, thereby reducing the chilling injury occurring during freezing. Nevertheless, the improved viability following the treatment is noteworthy and warrants further research into the underlying mechanisms.

Like osmotic pressure, embryos risk oxidative stress when subject to cryopreservation conditions. Much like all levels of life, ROS are a product of aerobic respiration and play an essential role in cell signalling and regular physiological processes. Conversely, excessive levels of ROS cause damage to DNA, protein, and lipid conformation, disrupting cell function and can lead to apoptosis. The primary ROS produced in embryos are superoxide anion radicals (O_2^-), Hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH). To combat oxidative stress, non-enzymatic and enzymatic antioxidants are employed to neutralise excessive ROS levels. Upregulation of genes for antioxidant enzymes such as SOD and GPX when embryos are exposed to oxidative stress[14].

Under short-term exposure to high levels of H_2O_2 to COC, embryo development was improved, and the treatment did not affect the apoptosis rates[26]. The apoptosis rate and the level of H_2O_2 as a preconditioning agent showed an inverse relationship. This study by Vandele *et al*. (2010) indicated that the stress response is mediated by HSP and HIF1 gene expression following H_2O_2 treatment on COCs[26]. Guerin *et al.* (2001) suggest that the sensitivity of embryos to oxidative stress changes with the developmental stages[14]. As the oocyte matures or is fertilised, resulting in an embryo, the gene expression of oxidative stress could be altered, explaining the difference in gene expression. Considering that antioxidants can be non-enzymatic, culture conditions must be considered. Embryos or COCs in different

culture mediums may have varying access to non-enzymatic antioxidants, which could activate antioxidant genes that are otherwise deficient to counteract the oxidative insult.

Additionally, oxygen concentration, co-culture systems and light can disrupt ROS in culture conditions[14]. More investigation is required to comprehend the mechanisms involved. Numerous environmental factors can cause oxidative stress, and embryos have the potential to respond and counteract the oxidative insult. Further investigation into applying alternative methods to control oxidative stress precisely may yield more standardised results and better prepare embryos for cryopreservation.

Under typical embryo culture conditions or in the context of natural reproduction, elevated pressure is seldom a cause for stress for embryos. Pressure is a unique method compared to other treatments mentioned in this paper because it can act uniformly over the entire embryo surface and with extreme precision. Furthermore, pressure does not penetrate the sample, lowering the potential for undesirable damage. HHP, as a preconditioning tool for cryopreservation, depends on two significant variables: pressure level and duration of HHP exposure. A balance between pressure level and duration is essential to maximise the stress response within embryos. Excessive HHP level negatively affects gene expression to promote embryo survival,

Additionally, the equilibration period following the pressure treatment is essential to allow embryos to produce proteins following the treatment. An insufficient brief equilibration period does not allow embryos an adequate opportunity to respond to the treatment. Excessively long equilibration periods have demonstrated additional gene expression that adversely impacts protective proteins produced during the initial stages of equilibration[11, 24]. Protective proteins are produced by upregulating specific genes following exposure to sublethal stress. Along with HSP, SOD and GPX genes, as mentioned above, HHP causes the upregulation of Azin1, Sc4mol, Gas5, and Gadd44g[4][11]. This indicates that pressure upregulates numerous genes promoting cell growth, proliferation, morphology, and function.

The challenge of comparing findings from studies regarding HHP and a preconditioning tool for cryopreservation arises due to the inherent variability associated with cryopreservation protocols, specific culture medium employed, the developmental stage of embryos or oocytes, and even the species under examination. Additionally, researchers use different parameters to compare the control and treatment groups to estimate the likelihood of

improved viability. In light of these considerations, the research findings indicate that HHP can benefit embryos, enhancing their ability to withstand the challenges associated with cryopreservation. One study indicated that HHP does not improve survival rates of IVP bovine embryo survival to vitrification[25]. However, similar studies on IVP bovine embryos found significantly improved cryotolerance following HHP[4, 11, 22, 24]. The variability in results may be attributed to differences in cryopreservation protocols and culture conditions. Popovic *et al.* (2013) followed similar cryopreservation protocols and found significant improvements in the viability of bovine blastocysts following HHP treatment. Additionally, the study indicated that embryo grade can cause discrepancies in results. Embryos of grade 1 quality subjected to HHP treatment were not significantly different from those in the control group, suggesting that grade 1 embryos may possess the inherent ability to withstand the challenges associated with cryopreservation[22]. Nevertheless, the study found that bovine embryo viability improved significantly when comparing grade 2 embryos in the treatment versus control groups, indicating that HHP stimulates resistance to cryopreservation.

7. Conclusion

Cryopreservation has proven essential in preserving biological material for future use and can effectively safeguard genetic material during catastrophic disease outbreaks. Furthermore, it is a secure and reliable means for international transport of genetic material without risk of disease transmission. However, the procedure comes with challenges that harm the survivability of biological material following the freezing and thawing process. Improvements in freezing protocols and culture conditions have proven to reduce the adverse elements associated with cryopreservation, but challenges will always remain. Ongoing research is imperative to harness cryopreservation's full potential as a preservation tool. Studies have explored the physiological response of embryos to sublethal stressors, revealing their ability to upregulate genes, promote cellular resilience and boost the capacity of embryos to withstand the challenges posed by cryopreservation.

Embryos encounter osmotic and oxidative stress due to standard cryopreservation protocols; therefore, using these stressors as a preconditioning tool holds the potential for enhanced resilience. High hydrostatic pressure has been shown to improve the viability of biological material in a range of species and embryo quality. While a level of variability is present in the research due to cryopreservation protocols, culture conditions, grading of embryos and level of HHP treatment and duration, the overall consensus suggests that it holds potential for improved survival during cryopreservation.

Cryopreservation remains a dynamic and evolving field in ART for animals and humans. Research into understanding the intricacies of this procedure proceeds to optimise the process with the ultimate goal of achieving higher success rates and preserving biological material with greater efficiency. Standardised protocols are needed to understand the physiological mechanisms of preconditioning methods fully. As the field advances, a consistent approach is crucial to reveal comparable results and further our knowledge of how to best protect our biological material for preservation.

8. References

1. Jelani G, Kalwar Q, Kaka A, Channo A, Ahmed A, Soomro MH, Soomro H, Bhuptani DK, Jalbani YM (2022) Historical Background and Significance of Embryo Transfer Technology in Cattle with its Relevant Applications. Pak J Zool 55:959–973

2. Luis B Ferré, Michael E Kjelland, Ahmed M Taiyeb, Fernando Campos-Chillon, Pablo J Ross (2020) Recent progress in bovine in vitro-derived embryo cryotolerance: Impact of in vitro culture systems, advances in cryopreservation and future considerations. Reprod Dom Anim 55:649–676. https://doi.org/10.1111/rda.13667

3. Kenneth Bondioli (2015) Cryopreservation of Bovine Embryos. In: Richard M H (ed) Bovine Reproduction, First. John Wiley and Son inc., pp 718–722

4. Pribenszky C, Vajta G, Molnar M, Du Y, Lin L, Bolund L, Yovich J (2010) Stress for stress tolerance? A fundamentally new approach in mammalian embryology. Biol Reprod 83:690–697

5. Pribenszky C, Lin L, Du Y, Losonczi E, Dinnyes A, Vajta G (2012) Controlled stress improves oocyte performance - cell preconditioning in assisted reproduction. Reproduction in Domestic Animals 47:197–206. https://doi.org/10.1111/j.1439-0531.2012.02076.x

6. Wemekamp-Kamphuis HH, Karatzas AK, Wouters JA, Abee T (2002) Enhanced levels of cold shock proteins in Listeria monocytogenes LO28 upon exposure to low temperature and high hydrostatic pressure. Appl Environ Microbiol 68:456–463. https://doi.org/10.1128/AEM.68.2.456-463.2002

7. Mapletoft RJ (2013) History and perspectives on bovine embryo transfer

8. Ferré LB, Kjelland ME, Strøbech LB, Hyttel P, Mermillod P, Ross PJ (2020) Review: Recent advances in bovine in vitro embryo production: reproductive biotechnology history and methods. Animal 14:991–1004

9. Frey B, Janko C, Ebel N, Meister S, Schlücker E, Meyer-Pittroff R, Fietkau R, Herrmann M, Gaipl US (2008) Cells Under Pressure-Treatment of Eukaryotic Cells with High Hydro-static Pressure, from Physiologic Aspects to Pressure Induced Cell Death

10. Kültz D (2005) DNA damage signals facilitate osmotic stress adaptation. Am J Physiol Renal Physiol 289

11. Jiang Z, Harrington P, Zhang M, Marjani SL, Park J, Kuo L, Pribenszky C, Tian X (2016) Effects of High Hydrostatic Pressure on Expression Profiles of in Vitro Produced Vitrified Bovine Blastocysts. Sci Rep 6:. https://doi.org/10.1038/srep21215

12. Neuer A, Spandorfer SD, Giraldo P, Dieterle S, Rosenwaks Z, Witkin SS (2000) The role of heat shock proteins in reproduction

13. Lin Lin, Yutao Du, Ying Liu, Peter M Kragh, Juan Li, Stig Purup, Masashige Kuwayama, Xiuqing Zhang, Huanming Yang, Lars Bolund, Gábor Vajta Elevated NaCl concentration improves cryotolerance and developmental competence of porcine oocytes.

14. Guerin P, Mouatassim S El, Me Y, Âzo Â Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings

15. Combelles CMH, Hennet ML (2012) Media composition: Antioxidants/chelators and cellular function. Methods in Molecular Biology 912:129–159. https://doi.org/10.1007/978-1-61779-971-6_9

16. Pribenszky C, Vajta G (2011) Cells under pressure: How sublethal hydrostatic pressure stress treatment increases gametes' and embryos' performance? In: Reproduction, Fertility and Development. pp 48–55

17. Lee Jones Cryopreservation of Bovine Embryos

18. Vajta G (2000) Vitrification of the oocytes and embryos of domestic animals

19. Valente RS, Marsico TV, Sudano MJ (2022) Basic and applied features in the cryopreservation progress of bovine embryos. Anim Reprod Sci 239

20. Lin L, Kragh PM, Purup S, Kuwayama M, Du Y, Zhang X, Yang H, Bolund L, Callesen H, Vajta G (2009) Osmotic stress induced by sodium chloride, sucrose or trehalose improves cryotolerance and developmental competence of porcine oocytes. Reprod Fertil Dev 21:338–344. https://doi.org/10.1071/RD08145

21. Pribenszky Csaba (2004) Cryopreservation of late preimplantation stage mouse embryos, with a special emphasis on introducing hydrostatic pressure into the freezing protocol. Budapest

22. Popovic L, Berg DK, Pribenszky C, Henderson H V, Wells DN (2013) BRIEF COMMUNICATION: High hydrostatic pressure treatment increases cryo-tolerance of in vitro produced bovine embryos

23. Pribenszky C, Molnár M, Cseh S, Solti L (2005) Improving post-thaw survival of cryopreserved mouse blastocysts by hydrostatic pressure challenge. Anim Reprod Sci 87:143–150. https://doi.org/10.1016/j.anireprosci.2004.09.007

24. Filho ES, Caixeta ES, Pribenszky C, Molnar M, Horvath A, Harnos A, Franco MM, Rumpf R (2011) Vitrification of bovine blastocysts pretreated with sublethal hydrostatic pressure stress: Evaluation of post-thaw in vitro development and gene expression. Reprod Fertil Dev 23:. https://doi.org/10.1071/RD10203

25. Trigal B, Muñoz M, Gómez E, Caamaño JN, Martin D, Carrocera S, Casais R, Diez C (2013) Cell counts and survival to vitrification of bovine in vitro produced blastocysts subjected to sublethal high hydrostatic pressure. Reproduction in Domestic Animals 48:200–206. https://doi.org/10.1111/j.1439-0531.2012.02131.x

26. Vandaele L, Thys M, Bijttebier J, Van Langendonckt A, Donnay I, Maes D, Meyer E, Van Soom A (2010) Short-term exposure to hydrogen peroxide during oocyte maturation improves bovine embryo development. Reproduction 139:505–511. https://doi.org/10.1530/REP-09-0430

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Consultation – 1st semester

	Timing			Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2022	02	09	Discussion of relevant thesis topics, electronic	Prbenszky Csaba
2.	2022	02	22	In person meeting to go through literature and find the focus	Prbenszky Csaba
3.	2022	05	10	Further discussion about literature data - electronic	Prbenszky Csaba
4.	2022	09	21	Thesis anouncement signed	Prbenszky Csaba

Grade achieved at the end of the first semester: 5

Consultation – 2nd semester

	Timing			Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2023	10	30	First thesis draft sent for review - electronic	Prbenszky Csaba
2.	2023	11	02	Proposed final version sent for review- electronic	Prbenszky Csaba

Grade achieved at the end of the second semester: .....

The thesis meets the requirements of the Study and Examination Rules of the University and the Guide to Thesis Writing.

I accept the thesis and found suitable to defence,

Pribenszky Csaba
……...
signature of the supervisor

Signature of the student: ……

Signature of the secretary of the department: ……..……

Date of handing the thesis in…17 November 2023……..