IMPROVING CRYOPRESERVATION OF BOVINE OOCYTES AND EMBRYOS

Honors Thesis
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ABSTRACT

Cryopreservation is a cornerstone technique in assisted reproductive technology (ART). Vitrification is a cryopreservation method reported to produce better embryo and oocyte viability post-thaw than the other cryopreservation method, slow-cooling. Vitrification allows cells to dehydrate at room temperature and to be cooled rapidly, decreasing exposure to the ice-nucleation temperatures to avoid ice formation. Most bovine embryos are vitrified by the in-straw system with a cooling rate of ~2,550°C/min. In human ART, the Cryotop® system achieves a cooling rate of 23,000°C/min. However, vitrified cells still have compromised outcomes compared with fresh cells due to cryodamage, suggesting a demand for improvements in the rate of cooling.

Recently, a proprietary vitrification device, NANUQ™ (MiTeGen, Ithaca, NY) was developed with a cooling rate of 600,000°C/min but has not been tested on living cells. Our objective was to determine if this ultra-fast cooling rate can improve embryo and oocyte viability post-thaw. Our first set of experiments compared morphological features and developmental rates of embryos vitrified by NANUQ™ or the in-straw system. The second set of experiments compared developmental rates of embryos and oxidative stress of oocytes, vitrified by NANUQ™ or Cryotop®. Our results suggested generally better performances produced by NANUQ™ than the in-straw system or Cryotop® system. We concluded that the fastest-cooling-rate vitrification system showed superior post-thaw viability. Further studies are comparing performance of different cooling rates by NANUQ™, to determine if the improved outcomes are indeed from faster cooling rate. These studies contribute to the understanding of cryodamage mechanisms and improvement in ART technology.

Key words: Oocyte and Embryo Cryopreservation, Bovine Cryopreservation, Vitrification, Cryodamage, Assisted Reproductive Technology (ART)
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### ABBREVIATIONS

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<th>Abbreviation</th>
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<tr>
<td>ART</td>
<td>Assisted reproductive technology</td>
</tr>
<tr>
<td>BSC</td>
<td>Biological safety cabinet</td>
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<tr>
<td>COC</td>
<td>Cumulus-oocyte complex</td>
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<tr>
<td>CPA</td>
<td>Cryoprotective agent</td>
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<tr>
<td>CTCF</td>
<td>Corrected total cell fluorescence</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DPBS</td>
<td>Dulbecco's phosphate-buffered saline</td>
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<tr>
<td>EG</td>
<td>Ethylene glycol</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>IVD</td>
<td><em>In vivo</em> derived</td>
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<tr>
<td>IVF</td>
<td><em>In vitro</em> fertilization</td>
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<tr>
<td>IVP</td>
<td><em>In vitro</em> produced</td>
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<td>LN</td>
<td>Liquid nitrogen</td>
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<tr>
<td>MDS</td>
<td>Minimum drop size</td>
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<td>OPS</td>
<td>Open pulled straw</td>
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<tr>
<td>PVA</td>
<td>Polyvinyl alcohol</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>RT-qPCR</td>
<td>Quantitative reverse transcription polymerase chain reaction</td>
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<tr>
<td>SE</td>
<td>Standard error</td>
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<tr>
<td>TL</td>
<td>Tyrode's lactate</td>
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INTRODUCTION

Cryopreservation has been a revolutionary tool in the field of assisted reproductive technology (ART). Oocyte and embryo cryopreservation benefits the genetic gains in farm animals and the conservation of endangered genetics, and also provides options for women to preserve oocytes or embryos for future use. Traditionally, cryopreservation is performed using the slow-cooling technique by soaking samples in solutions containing cryoprotective agents (CPAs) and cooling slowly at about 1 °C/min, enabling ice to grow outside the cells and thus decreasing chilling injuries [1]. Recently, vitrification cryopreservation technique was developed and has dominated the oocyte and embryo cryopreservation industry. Vitrification, as a rapid cryopreservation method allowing a non-crystalline amorphous solid to form, has been demonstrated to prevent both intracellular and extracellular ice formation [2]. Currently, two prevailing vitrification devices are: 1) tubing techniques, including the conventional vitrification method, the in-straw system (Minitube, Verona, WI); and 2) surface techniques, including the vitrification industry leader, the Cryotop® system (Kitazato, Tokyo, Japan) [3]. The in-straw system is able to reach a cooling rate of 2,550 °C/min [4]. The current “Gold-standard” for human embryo vitrification, the Cryotop® system, can achieve a faster cooling rate of 23,000 °C/min [5]. The Cryotop® system minimizes the media volume surrounding the cell and directly exposes the sample to liquid nitrogen (LN), while the in-straw system prevents direct contact with LN using a layer of plastic [5]. Cryotop® with the faster cooling rate has been shown to result in superior post-thaw viability over other cryopreservation systems [3].

The decrease in post-thaw viability is due to cryodamage during the freezing and thawing process. Intracellular ice formation disrupting the microstructure of the cells by mechanical action is a major cause of cryodamage to oocytes and embryos [6]. The main strategy to prevent intracellular ice formation is to decrease the exposure to the temperature range where ice-nucleation occurs leading to formation of ice crystals form, a process which mainly depends on the concentration of CPA [6]. A higher concentration of CPA allows a smaller temperature range where ice nucleation occurs, but also results in overwhelming osmotic stress that can be detrimental to the cryopreserved cells [7, 8]. However, faster cooling rates can reduce the concentration of CPA required for vitrification as the droplet containing the cells and the
surrounding solution passes through the ice-nucleation temperature range more rapidly, avoiding ice crystal formation [3]. Therefore, optimizing cooling rates can reduce cryodamage by decreasing ice formation and by reducing osmotic stress from high CPA concentrations. Reducing cryodamage within oocytes and embryos should improve the post-thaw morphological features, developmental rates, and reduce oxidative stress leading to higher pregnancy rates.

The fastest vitrification cooling rate reported is from using the NANUQ™ (MiTeGen, Ithaca, NY) device. It was designed to maximize cooling rate for protein crystallography with the automated ultra-fast (<2ms) plunging and the removal of the cold gas layer above LN preventing pre-cooling before plunging into LN, leading to ultra-fast cooling rates of ~600,000°C/min, which is 26-235 times faster than the currently used methods in the market [9]. Such a high cooling rate can largely decrease the ice crystal formation and effectively prevent chilling injuries. The NANUQ™ vitrification system also allows small-volume micro-drops and individual freezing, thus reducing CPA’s toxicity and the chance of LN contamination. Due to the extensive automation of the NANUQ™ system, its cooling curves have high repeatability and human errors are minimized, which is critical for quality control and better outcomes. However, the NANUQ™ system has not been used with biological samples, and its validation in decreasing cryodamage in oocyte and embryo vitrification is needed.

The objective of this study was to test the NANUQ™ system with the fastest cooling rate on bovine oocytes and embryos compared with two currently popular vitrification methods in the market with slower cooling rates, namely the in-straw system and the Cryotop® system. We compared morphological features, developmental rates, and oxidative stress level as three indicators of cell cryodamage.
1.1 Oocyte and Embryo Cryopreservation

1.1.1 Current Applications of Oocyte and Embryo Cryopreservation

Cryopreservation, a preservation technology of living cells and tissues in extremely low temperatures, has been a revolutionary tool in the field of ART [6]. By preserving oocytes and embryos, this technology can benefit both the food and agriculture industries as well as human reproduction.

Cryopreservation enables assisted reproduction to accelerate genetic gains in farm animals, which can have tremendous effects on farm profitability. In the dairy industry, for instance, the widespread use of elite bull genetics has enabled the average milk production to increase by 141 kg/cow/year from 1980 to 2017 [10]. However, although both fresh and cryopreserved sperms from elite bulls contribute to half of the genetics, further improved genetic gains should be achieved by increasing the contribution of elite cows, whose reproduction ability is limited by the long gestation, slow recovery of uterine and ovarian function after calving[11, 12]. Recently, scientists have used techniques including in vitro fertilization (IVF) and embryo transfer to maximize the use of elite cow genetics. In 2018 alone, more than 1 million embryo transfers in cattle were reported worldwide, in which the number of in vitro produced (IVP) embryos accounted for about 60% [13]. However, the success rates of either IVF or embryo transfer highly rely on the accurate time management and coordination between each step, as major events involved in these techniques including oocyte maturation, fertilization, and blastocyst formation only allow a narrow window of time for management; on the other side, optimal seasons and temperature as well as transportation between different facilities require more flexibility of timing. Therefore, oocyte and embryo cryopreservation technology, providing much more timing and management flexibility required for mass adoption of IVF and embryo transfer technology, recently has been widely used in farm animals. From the same worldwide report on 2018 bovine embryo transfer mentioned above, frozen-thawed embryos accounted for 60.1% of the in vivo
derived (IVD) embryos transferred and 26.8% of the IVP embryos transferred, respectively, indicating the wide use of embryo cryopreservation technology.

Besides more options and increased profits brought by the accelerated genetic gains in farm animals, cryopreservation technology also benefits the work of animal conservation. As civilization and industrialization have been expanding to an invasive level that threatens the genetic diversity among many species, the vulnerability of such populations are drastically increased, which has an irreversible impact on our functional ecosystems, food supplies, and even human health [14]. In the few but important scenarios when ART and intensive genetic management is required for the conservation of endangered animals, cryopreservation of embryos and gametes not only enables the preservation of valuable genetic information but also allows more transportability and timing flexibility for some recipients whose donors may be in a different location or even deceased [14]. Similarly, gamete and embryo cryopreservation has also recently been utilized for the conservation of farm animal genetic resources, as the past few decades have witnessed an alarming decline of livestock genetic diversity due to the large market demand for a few specific breeds [15].

Another crucial application of oocyte and embryo cryopreservation is directly related to human reproduction. Although embryo cryopreservation was previously thought to produce a higher pregnancy rate compared with oocyte cryopreservation, recent research and technology improvement have established protocols for mature oocyte cryopreservation to achieve similar results, offering more options for women who desire to preserve their fertility by cryopreservation [16]. Many factors can lead to a woman’s decision to store her viable eggs for later use – from simply delaying child bearing to pursue a career, to preserving fertility prior to potential ovarian insufficiency, gonadotoxic cancer therapies, or surgeries with risk of ovarian damage [17, 18]. In all of these situations, oocyte cryopreservation plays an essential role and a high success rate is required, while safety concerns are also raised. Moreover, despite numerous ongoing ethical debates, egg donation programs have been launched with the help of cryopreservation technology, allowing one single donor to benefit multiple recipients [17].
1.1.2 A Historical Review of Oocyte and Embryo Cryopreservation Techniques

1.1.2.1 The start of Cryopreservation

In spite of the popularity it shares in various fields nowadays, embryo cryopreservation did not achieve real success until 1970s. In 1972, a paper by Whittingham et al. reporting a method of slow-cooling and slow-warming to avoid intracellular ice formation within the mouse early embryos published, producing 65% pregnancy rate and over 40% of these pregnancies proceeding to term [19]. Oocyte cryopreservation, however, took even more time to gain successful birth but with not yet satisfactory pregnancy and delivery rates. In 1997, Porcu et al. reported a successful birth from a newly-established slow freeze-rapid thaw oocyte freezing technique, which has been considered to be the first success in oocyte cryopreservation despite numerous attempts reported before then [20]. The difficulties in the attempts of embryo cryopreservation and oocyte cryopreservation are mainly due to the relatively large volume-to-surface ratio compared with other types of cells; for oocyte cryopreservation, the sensitivity of the miotic spindle to problems with disassembly and subsequent reassembly makes oocytes particularly vulnerable to chilling injury [17].

1.1.2.2 Two Major Cryopreservation Techniques: Slow-cooling and Vitrification

Currently, there are two major cryopreservation techniques - slow-cooling technique and vitrification technique. The slow-cooling technique (also called slow-freezing method) utilized by Whittingham et al. in 1972 and Porcu et al. in 1997 mentioned above allows gradual cell dehydration [1]. By soaking samples in solutions containing CPAs and cooling slowly at about 1 °C/min, the slow-cooling technique allows ice to grow outside the cells as CPAs replace the intracellular water before rapid submission into LN, decreasing the amount of intracellular ice formation and thus the level of chilling injury suffered by cryopreserved cells [1].
As the use of the slow-cooling method in oocyte and embryo cryopreservation had been reported to yield acceptable but less than desirable results, meanwhile, another cryopreservation technique called vitrification appeared. Unlike the slow-cooling technique, vitrification is a rapid cryopreservation method in which samples are processed by CPAs with much higher concentrations at room temperature, and immediately cooled at faster than 100 °C/min into LN, leading to the formation of a “glassy” layer, a non-crystalline amorphous solid, both inside and outside the cell [2]. Vitrification has been designed to enable the intracellular molecular and ionic distributions to remain the same as the original liquid state, but in a highly viscous supercooled liquid phase [21]. The theoretical comparison of these two major cryopreservation methods can be briefly summarized in Fig 1.1: slow-freezing results in extracellular ice crystal formation while vitrification circumvents both intracellular and extracellular ice formation, indicating superior yields from the vitrification method [22].
Fig 1.1 A comparison of the slow-freezing technique and the vitrification technique

In the slow-freezing technique (left), low CPA concentration is applied to the cell at room temperature; as the cell is slowly cooled down, the CPA slowly permeates into the cell, replacing the intracellular water; when most of the water has been pulled out of the cell that it is considered to be sufficiently dehydrated, the cell is rapidly cooled to allow a glass-like intracellular environment to form, while ice crystals grow outside the cell. In the vitrification technique (right), a series of solutes containing CPAs with increasing concentrations are applied to the cell at room temperature, allowing the cell to be sufficiently dehydrated before cooling down; the cell is then rapidly plunged into LN, enabling the glassy environment to form both intracellularly and extracellularly. As a result, the slow-cooling technique avoids intracellular ice formation by allowing extracellular ice formation, while the vitrification technique avoids the formation of both intracellular and extracellular ice crystals.
Until now, numerous studies have confirmed the superiority of the vitrification method over the slow-freezing method in both embryo cryopreservation and oocyte cryopreservation in different animal models and even humans. In bovine oocyte and embryo cryopreservation, for instance, evidence at the cellular level such as higher developmental rates as well as at molecular level including relative amounts of gene transcripts have revealed that the vitrification method can be more suitable than the slow-cooling method for freezing bovine embryos [23-25]; comparisons in morphology, meiotic spindle configuration, and DNA integrity between vitrified and slow-frozen bovine oocytes also suggested vitrification to be more promising [26]. In human clinics, a higher performance including higher survival rate and pregnancy rate has been shown in the patients whose oocytes were preserved using vitrification compared to slow-freezing, while another study reported that vitrified human oocytes performed as well as fresh oocytes in their clinical trial [27, 28]. Although there have been some successful attempts in cryopreservation on human and mouse oocytes using the slow-freezing method, there has been no meaningful success with bovine oocyte. So far, the vitrification method remains the only way to cryopreserve oocytes in a commercial setting. Overall, despite the first successes achieved by the slow-freezing technique, the recent achievements in mammalian oocyte and embryo cryopreservation have been in vitrification rather than slow-freezing [29].

1.1.2.3 Continuous Improvements of Vitrification

Many systems have been established to further improve the outcomes of vitrification and to adapt the industry for specific species and purposes. The conventional vitrification method, the in-straw system (Minitube, Verona, WI), achieved a cooling rate and a warming rate as fast as 2,550 °C/min and 2,438 °C/min, respectively [4]. The open pulled straw (OPS) system, first introduced to the industry in 1998 as a practical method specifically designed for bovine embryo and oocyte vitrification, achieved a cooling and warming rate of over 20,000 °C per minute and a CPA exposure time of less than 30 seconds, which efficiently prevents chilling injury and avoids the toxicity of CPAs [4]. The OPS system, the in-straw system, and other tubing techniques have dominated the vitrification industry for nearly 13 years and are still popular in the field of livestock ART due to easy and safe handling in a rather closed system [3, 17].
The Cryotop® system then appeared and has been commercially produced at Kitazato Co., Fujinomiya, Japan, using a film strip and a plastic holder to achieve much higher success rates in human oocyte and embryo vitrification than any other system at that time [30]. By cooling at up to 23,000 °C/min and warming at up to 42,000 °C/min using a droplet in a volume of less than 0.1 μL, the Cryotop® system and other surface techniques including minimum drop size (MDS) technique and CryoLoop™ (Hampton Research, Aliso Viejo, CA) circumvent chilling injury and CPA toxicity even more than the OPS system [3, 30, 31]. Although it has been widely employed commercially by embryologists nowadays yielding more healthy babies, it has been recognized that the Cryotop® method is demanding and difficult to manage due to the strict time constraints and the requirement for extensive technical skills, which has become one of the reasons some embryologists are reluctant to shift from the straw system (tubing techniques) to the Cryotop® system [32].

Recently, many attempts have been made to improve oocyte and embryo vitrification, aiming to have a performance better than the Cryotop® method. Some of them are trying to lower the costs, in which one example is replacing plastic containers with paper containers, achieving comparable, yet not superior results [33]; some are trying to increase the warming rates and handling, in which one example is the VitTrans method that has an inner channel for introducing the warming solutions to dilute the CPA within a straw and is easily managed by a regular syringe, resulting in comparable performance to the Cryotop® system [34]; some are trying to increase the cooling rates, in which one example is the Solid Surface Vitrification method that improves heat exchange by placing the micro-drop containing cells directly onto a cold dry metal surface, which leads to similar results to the Cryotop® system [35, 36]. Although it may be obvious that all of the techniques mentioned above suffer from the drawbacks of manual management including slow cooling and warming rates as well as demanding technical skills, it was not until recently that the cryopreservation industry started to realize the huge potential and importance of automation. One of the attempts in the automated vitrification system was the invention of the Sarah® (FertileSAFE, Ness Ziona, Israel) device. This machine guarantees the precision of timing and temperature and also removes the tedious manual procedures, as a vertical robotic handle automatically moves the straws between different temperature-controlled solutions at predetermined time intervals [37]. Although no comparisons have been drawn between the Sarah® system and the Cryotop® system, the automation of the vitrification process is in itself a revolutionary advancement leading to broad
possibilities to improve vitrification technology. However, despite these numerous attempts and dazzling inventions, it is worth noticing that so far none of them has been confirmed to result in a significantly better outcome compared with the Cryotop® system.

1.2 Mechanisms and Indicators of Cryodamage

1.2.1 Mechanisms and Prevention of Cryodamage

Cryodamage, the damage caused by or during cryopreservation, can be generally defined as a series of events due to compromised cell functions after cryopreservation, precisely the freeze-thaw cycle [38].

1.2.1.1 Freezing of water

One obvious and dominating effect of oocyte and embryo cryopreservation that can lead to severe cryodamage is the freezing of water, both within and outside the cells. As temperature declines to the ice-nucleation range (or sometimes called crystallization range) - between equilibrium freezing temperature and glass transition temperature of its internal solvent, the ice crystals nucleate and grow, which can pierce or tear apart the cells and destroy cellular structures simply by mechanical action [6].

Apart from mechanical damage, the freezing of water also causes solution effects at the same time. Ice crystal formation dramatically changes the composition of the liquid phase by concentrating solutes dissolved in it and precipitating the solutes that are oversaturated, resulting in cell osmotic dehydration [39]. Such composition changes and biomolecule aggregation occur both within the cell and outside the cell, but usually with different extents due to the inconsistency between intracellular and extracellular environment, while a homeostasis is being achieved by constant exchange of water and other molecules transported through cell membranes. Moreover, the thermal contraction and expansion of cell membranes and other structures differs from solutions within and surrounding them [40]. As a result of both osmotic and thermal stress, associated shrinkage and expansion of the cells during the freeze-thaw cycle can be too
overwhelming for them to maintain cellular structural integrity, resulting in irreversible puncture of cell membranes and disruption of vulnerable cytoskeleton including the meiotic spindle [38].

Interestingly, while the cooling process leads to the formation of ice, it is the warming process that controls the subsequent events that happened to the ice crystals already formed. When the sample is warmed above the glass transition temperature, the tiny nuclei formed during cooling start to grow and growth accelerates as temperature rises, leading to a large fraction of initially vitrified solvent to convert to ice that is harmful for the cell structures [41].

1.2.1.2 The Cryoprotection Mechanisms and Toxicities of CPAs

In order to prevent such freezing injuries mentioned above, cryoprotective agents, or cryoprotectants, abbreviated as CPAs, have been used to provide protection for cells and embryos before cooling. The first CPA was discovered in the 1940s, when the addition of glycerol prior to cryopreservation was reported to significantly lower the cryodamage level and improve survival rates[42]. Other compounds including dimethyl sulfoxide (DMSO), ethylene glycol (EG), propylene glycol, polyethylene glycol, sucrose, and proline then were confirmed to have similar properties and therefore have been frequently used as CPAs [7]. These properties are simple – CPAs should have the ability to be transported through cell membrane by penetration, and they must be able to increase the total concentration of all solutes with very limited toxicity [6]. High concentrations of CPAs allow the reduction of intracellular ice formation at any given temperature, which is especially important in vitrification because higher concentration is used to achieve a glass-like stage within and outside the cells compared with the slow-freezing technique [43]. In other words, a more concentrated CPA allows a higher glass transition temperature, leading to less ice nucleation and crystallization [3].

However, as their concentration increases, the toxicity of CPAs becomes unacceptable. In fact, CPA toxicities have been listed as one of the major causes of cryopreservation-related damage [44]. Although it may be helpful to maintain the stability of membranes and intracellular organelles, the exposure to extremely viscous CPAs for improper duration of time can cause osmotic injury that is similar to the detrimental osmotic stress suffered by cryopreserved cells and embryos during ice formation, which is exactly what the usage of CPAs aims to avoid [7].
Potential damage from CPAs can be due to the molecular and pharmacological toxicities of the CPAs themselves. Despite the diversity of CPAs ranging from alcohols and sugars to amines and polymers, all of them have one thing in common – they must be able to permeate into cells and many intracellular compartments. Such permeation makes it possible for a fraction of CPAs to remain within the cells and their organelles even after post-thaw dilution, exposing vulnerable cellular structures to CPAs for longer time. Whether this exposure will cause long-term and irreversible cell damage is then dependent on the concentration, exposure time, the type of cells that are cryopreserved, and the chemical and physical properties of CPAs themselves [7].

Due to the complexity of mechanisms for CPA toxicity, the consequent damage to cryopreserved cells and embryos can range beyond osmotic stress and can vary a lot. For example, scientists have identified disruptive protein interaction and subsequent destabilization of the enzymes caused by the possible hydrogen bonding between CPA polar groups and H2O, resulting in interruption of a series of important biochemical processes [45]. The mitochondrial integrity damage and the alterations in membrane potential, oxidative stress, and apoptosis have also been observed as results of CPA-specific toxicities [46, 47]. Moreover, recent studies have suggested a relationship between the use of CPAs and changes in the epigenetic profile of cryopreserved cells including gametes and embryos, indicating potential further developmental effects, although a direct causal relationship is not yet well-defined [48, 49].

1.2.1.3 LN Contamination

Apart from CPAs in the solutes surrounding the cell, cryopreserved samples also contact LN during cooling in many vitrification systems. Therefore, potential contamination due to numerous microorganisms alive or dormant within LN has been a concern. Although in oocyte and embryo cryopreservation specifically, no clinical records of pathogenic contamination caused by contaminated LN have been reported, there is still a risk of disease transmitted from infectious agents present in the same LN container [8]. Thus, the closed vitrification systems have the advantage of avoiding the potential LN contamination that could possibly be presented in the open systems. However, it has been challenging to design a closed vitrification system with a similar cooling rate to the open systems in order to prevent LN contamination without compromising outcomes. Previously, a study demonstrated that the embryo survival, pregnancy, and delivery
rates of a closed vitrification method, CryoTip, did not differ from the open system Cryotop [31]. Another closed vitrification system, Rapid-i™, has been reported to achieve over 90% survival rates and similar clinical outcomes for in vitro mature oocytes and blastocysts compared with fresh cells [50].

1.2.1.4 Three Key Factors for Cryodamage Prevention

In order to mitigate cryopreservation-related damage briefly summarized above, it is suggested that we should take three key factors into consideration: the cooling and warming rates, the viscosity of the medium containing CPAs, and the total volume of the cryopreserved sample [3].

First of all, the increased cooling rate and warming rate both allow a short exposure to the ice-nucleation temperature range, thus decreasing the amount of ice crystals formed during cooling and also avoiding further growth of the ice during warming, preventing both physical damage and osmotic stress caused by ice formation [6]. To increase the cooling rate, a simple way is to increase the speed of plunging the sample into the LN. Apart from plunging, previous studies have also reported the importance of minimizing the cold gas layer above LN where samples are slowly pre-cooled before direct exposure to LN [51].

High viscosity of the medium, or high concentrations of CPAs and other additives in the solutes, as illustrated in detail above, narrows the ice-nucleation temperature range by raising the glass transition temperature, which allows a decline in ice crystal nucleation and crystallization [3].

Finally, volumes greatly affect the rate of heat transfer, thus the decreased volumes have the potential of achieving faster cooling and thawing, mitigating associated cryodamage. Since the volume of oocytes and embryos is large and fixed, it is crucial to minimize the volume of the cryopreserved droplet composed of oocytes or embryos and the surrounding solution [52]. Based on this theory, the MDS technology has been established and confirmed to be effective in improving vitrification outcome, which has been employed in many vitrification systems today [53].

Scientists have formulated a general equation shown below (1-1) to predict the probability of vitrification and to improve the vitrification technology accordingly [3]:

\[
\text{Probability of Vitrification} = \frac{1}{1 + e^{-\left(\frac{V - V_c}{V_d}\right)}}
\]
According to this equation, it is worth noticing that raising cooling and warming rates enables the CPA concentrations (i.e. viscosity) to be lower without compromising the final vitrification outcome, which would efficiently prevent the effects caused by the toxicities of CPAs.

The limitation of oocyte and embryo cryopreservation can also be indicated from this equation. Many experimental practices have demonstrated that oocyte and embryo cryopreservation is generally more difficult than other cells, due to the significantly larger volume of mammalian oocytes and embryos [54]. Moreover, the low viscosity of some stages in early embryonic development, especially the blastocyst stage, can further increase the difficulty of vitrification. In addition, a major obstacle faced by current oocyte and embryo cryopreservation industry is the high sensitivity of oocytes and early embryos to cryodamage as extremely active meiosis and mitosis are ongoing during these stages [43]. As the chances of cytoplasm degeneration and subsequent chromatin degeneration are increased in these sensitive cells leading to apoptosis, any damage to the vulnerable organelles and intracellular structures can be accumulated in the surviving cells and affect later embryonic development and fetal growth [38, 55].

1.2.2 Common Cryodamage Indicators

Due to the complexity of cryodamage mechanisms, many factors ranging from the molecular level to the cellular level can be considered as cryodamage indicators. We summarize several indicators of cryodamage commonly evaluated in previous studies within the scope of mammalian oocyte and embryo cryopreservation and within the interest of this thesis.

1.2.2.1 Morphological Features

Morphological features can be one of the direct indicators of severe cryodamage. Osmotic stress and ice formation during cooling and thawing can cause serious fracturing of the cell membrane and zona pellucida, while the tearing of zona pellucida is detrimental to the survival
and development of any early embryo [38]. Such rupture can be easily identified under the microscope.

1.2.2.2 Developmental Rates

Developmental rates are the quantifications of the cell behaviors post-thaw, and have been widely used to reveal the developmental potential of cryopreserved cells in many studies [56]. To clarify, the survival rate has been usually referred to the proportion of intact oocytes or embryos after cryopreservation, as cell structural integrity is the prerequisite for further development. Beside survival rate, the percentages of the embryos continuing to develop to the next few stages can be powerful to evaluate the impact of cryodamage on developmental potential in general. These developmental rates for early embryos can include morula rate, blastocyst rate, and hatching rate, depending on the interest of each experiment [3]. For blastocysts, whether they are able to continue re-expanding after cryopreservation, indicated by re-expansion rate, reveals their viability post-thaw and is essential for them to proceed to hatching. Massive studies have also been done in evaluating pregnancy rate, delivery rate and other clinical performance after attempting to implant cryopreserved and developed embryos, providing valuable information to evaluate the damage accumulated through embryonic development and fetal growth post-thaw [31, 57].

1.2.2.3 Oxidative Stress

Apart from the overall evaluation of cell behaviors, scientists also focus on the level of oxidative stress suffered due to cryopreservation. During cooling and thawing, oxidative stress occurs when increased oxidative metabolism or/and osmotic stress cause an imbalance between the reactive oxygen species (ROS) level and the detoxification capability of cells [58]. Although ROS is universally present as a by-product of mitochondrial phosphorylation and glycolysis within the normal and healthy cells, excessive accumulation of ROS can lead to damages to other molecules including DNA, proteins, and lipids, as well as cell membranes [59]. Such damages can therefore further result in the loss of mitochondrial function and decreased viability of reproductive cells.
It is worth mentioning that similar to oxidative stress, evaluations of intracellular organelle functions and cellular structural integrity are also used as cryodamage indicators, including evaluation of mitochondrial membrane potential and meiotic spindle behaviors.

1.2.2.4 Relative Gene Expression

The cryodamage can be also revealed at the molecular level by evaluating relative gene expression. As cryopreservation causes oxidative stress, endoplasmic reticulum (ER) stress, and apoptosis, along with interruptions of ultrastructural protein interactions within the nucleus and nuclear envelope, suboptimal DNA replication and transcription may occur, compromising later development of the oocytes and embryos [38]. Many cell damage markers including molecular-based cell death, oxidative stress, and ER stress have been identified, which were considered to be more sensitive cryodamage indicators compared with developmental rates in many studies [25].

1.3 NANUQ™ Vitrification

In order to achieve better vitrification outcomes, the NANUQ™ vitrification method, a proprietary vitrification system equipped with an automated hyperquenching cryocooling device, was developed to reduce cryodamage. The most important advantage of the NANUQ™ system is the unprecedentedly fast cooling rate. Currently, the major vitrification methods including the in-straw system and the Cryotop® system use hand-plunging, which is slow and troublesome. However, the automated plunging of the NANUQ™ system allows the sample to be rapidly plunged into LN at 2 m/s, which is unachievable by hand-plunging [9]. Compared with the cooling rate of hand-plunging that is normally up to 4,600 K/s, NANUQ™ plunge is able to achieve a cooling rate up to 51,000 K/s (Fig 1.2) [9]. Moreover, its patented gas-layer removal technology efficiently reduces the thickness of the cold gas layer above LN from typically more than 1-2 cm to less than 100 µm [60]. In previous vitrification methods where gas layer removal is not available, cooling occurs primarily in this cold vapor layer above LN for samples with a volume below 1 µL, at a rate determined by the transit time through the gas layer rather than in the liquid nitrogen, as the heat transfer in the gas is much slower than in the liquid nitrogen [60]. Therefore, minimizing the thickness of this gas layer by the NANUQ™ system can increase plunge cooling rates in LN
for cell-size samples from ~30,000 °C/min to ~900,000 °C/min [60, 61]. Such rapid cooling rate allows less time for the nucleation and growth of ice, and thus helps maintain the intracellular composition and the integrity of cell structures. As the cooling rate increases, the required CPA concentrations are also reduced to achieve the same outcome, thus minimizing the toxicities of CPAs to cryopreserved cells (Fig 1.3).
Fig 1.2 Comparison of the cooling rate between NANUQ™ plunge and hand plunge
NANUQ™ plunge can achieve a cooling rate more than 10 times faster than hand plunge due to its automated plunging and cold gas removal. This ultra-rapid cooling rate reduces the time for crystalline ice nucleation and crystal growth by more than 90%. Figure from MiTeGen, 2020

Fig 1.3 Comparison of the minimum CPA concentration to vitrify 100 µm drops in LN between NANUQ™ plunge and hand plunge
NANUQ™ plunge enables a reduction in the required concentrations of multiple CPAs compared with hand plunge by 50%~75%, which greatly mitigate the toxicities of CPA to cryopreserved cells. Figure from MiTeGen, 2020
Apart from accelerated cooling rates resulting from the rapid automated plunge and gas-layer removal technology, another advantage of the NANUQ™ system is the ability to minimize the cryopreserved sample volume. By using the MicroMeshes™ with 400 μm mesh filled apertures (in diameters) and 25 μm mesh openings, small samples (<30 μm) are able to be sieved out of solution while being supported continuously by the delicate meshes (Fig 1.4 left). Compared with the 0.4 mm wide film strip used in the Cryotop® system capable of minimizing the sample volume to less than 0.1 μL (Fig 1.4 middle), the MicroMesh™ used for cell capturing prior to cryopreservation achieves at least similarly small volume of the CPA solution surrounding the cryopreserved cells. Compared with the in-straw method, as a tubing technique, in which about 7 μL of solution surrounding the cell is used (Fig 1.4 right), both of the NANUQ™ system and the Cryotop® system, as surface techniques, allow rapid heat exchange and prevent the harmful effects of CPA toxicities by greatly reducing the sample volume [3, 7].

![Fig 1.4 A comparison of the cell capturing tools in indicated groups](image)

The NANUQ™ system (left) uses a MicroMesh™ with mesh openings as small as 25 μm to sieve the solution surrounding the cell. The Cryotop® system (middle) uses a 0.4 mm wide film strip to load the cryopreserved cells and requires removal of the remaining solution around the cells manually by pipette. These two surface techniques both allow less than 1 μL sample volume. However, the cryopreserved sample for the in-straw system (right) using a 140 mm, 0.5 μL straw can reach a volume of 7 μL. The first two images were captured in our lab under the microscope.

According to all vitrification protocols, cells are always exposed to highly concentrated CPA solution for a short time and immediately loaded into the device while being dehydrated before immediate immersion into LN, which requires fast cell handling and transfer in order to avoid inappropriate dehydration and slow cooling. In the in-straw system (Fig 1.5 right), such handling process relies on manually sucking a small amount of media containing the cells into the straw, heat-sealing the open end of the straw, and then hand-plunging into LN. In the Cryotop®
system (Fig 1.5 middle), the sample droplet is manually loaded onto a film strip connected to a simple plastic holder and the solution surrounding the cells removed by controlling a pipette before hand-plunging into LN. However, the NANOQ™ system (Fig 1.5 left) waives these tedious and slow steps by using a magnetic wand and a goniometer base connected to the MicroMesh™ containing the sample, which were designed to easily and securely manage and transfer the samples, improving the efficiency and manageability and thus resulting in faster process. In addition, the automatic and individual puck loading also enables minimized management and human errors, which is critical for the repeatability of the cooling curves and the better quality-control. Unlike the Cryotop® system allowing up to 4 cells to be processed at a time, the NANOQ™ system only processes one cell at a time, but this individual cryopreservation effectively may be able to limit the chance of contamination in theory, although more studies are needed. Overall, the designs of the plunging, cell capturing, transferring, and storage in the NANOQ™ system ensure a faster and more secure vitrification process.

The invention of the NANOQ™ system has made a lot of great improvements and presented the possibility for faster cooling and process. Although it has been demonstrated to reduce cryodamage in protein crystal samples, it has not been tested on live cells [9]. The objective of this study, therefore, is to determine if the ultra-fast cooling rate (produced by the NANOQ™ system), can improve viability post-thaw on bovine oocytes and embryos, compared with lower
cooling rates (produced by two currently prevailing vitrification methods in the market, the in-straw system and the Cryotop® system).
MATERIALS AND METHODS

2.1 Overview of Experimental Design

The objective of this study is to test the performance of different vitrification systems with different cooling rates on post-thaw embryo and oocyte viability. IVP bovine cumulus-oocyte complexes (COCs) and embryos were randomly assigned to one of the treatment or control groups. There were 2 sets of experiments evaluating the cryopreservation systems in 3 different developmental stages: 1) Metaphase II oocytes after \textit{in vitro} maturation but before \textit{in vitro} fertilization, 2) cleaved embryos at day 4 post fertilization, and 3) early blastocyst stage (Fig 2.1).

![Experimental Design Diagram]

In the first set of experiments, cleaved embryos at day 4 post fertilization were randomly assigned to one of the three treatment groups: 1) NO-FREEZE control (vehicle control group), in which embryos were exposed to the BO-VitriCool media (IVF Bioscience, Falmouth, UK) according to the vitrification and warming protocol, but without cryopreservation, 2) NANUQ...
group, in which embryos were exposed to the BO-VitriCool media and vitrified using the NANUQ™ vitrification method with the fastest cooling rate of 600,000°C/min, and 3) STRAW group, in which embryos were exposed to the BO-VitriCool media and vitrified using the straws (Minitube, Verona, WI) with a cooling rate of approximately 2,550°C/min. All embryos were then treated with the BO-VitriWarm media (IVF Bioscience, Falmouth, UK) according to manufacturer’s recommendation, in order to thaw or/and rehydrate them. The morphological features and development rates of the treated embryos were then evaluated and compared across all of the three groups.

The second set of experiments is composed of two parts based on the different stages of the cryopreserved cells. One part is based on the metaphase II stage bovine oocytes derived from slaughterhouse ovaries, while the other part is based on the embryos on the blastocyst stage 7–9 days after in-vitro fertilization. In both parts, oocytes or blastocysts were randomly assigned to one of the four groups: 1) NORMAL control (negative control group), in which cells were cultured with no exposure to pre-cooling media and not cryopreserved, 2) NO-FREEZE control (vehicle control group), in which cells were exposed to the pre-cooling media (recipe shown in Table 2.1 and Table 2.2), but without cryopreservation, 3) NANUQ group, in which cells were exposed to the pre-cooling media and vitrified using the NANUQ™ vitrification method with the fastest cooling rate of 600,000°C/min, and 4) CRYOTOP group, in which cells were exposed to the pre-cooling media and vitrified using the Cryotop® US device (Kitazato, Tokyo, Japan) with a cooling rate of 23,000 °C/min. All cells in the No-Freeze control group, the Nanuq group, and the Cryotop group were then treated with post-thaw media (recipe shown in Table 2.3) while no treatment for cells in the Normal group. The developmental rates of the blastocysts and the oxidative stress of the metaphase II oocytes were then evaluated and compared across all of the four groups.

2.2 In Vitro Maturation of Bovine Oocytes, Fertilization, and Culture

2.2.1 Collection and selection of Bovine COCs

Fresh bovine ovaries were collected from Cargill slaughterhouse in Wyalusing, PA and transported to the lab immediately. Harvested ovaries were strained with 0.9% saline and massaged to minimize residual blood in the ovaries. Strained ovaries were then kept in 0.9% saline at 37°C
for aspiration. Aspiration was done using an 18-gauge needle attached to an aspiration pump, and aspirated follicular fluids containing bovine oocytes were stored in a 50 mL conical collecting tube. Only follicles between 2 and 8 mm in diameter were aspirated, while dark colored follicles and follicles near corpus luteum or cysts were avoided in order to minimize the amount of blood in the collected follicle fluids.

The collected follicle fluids were filtered to minimize the interruption of debris during COC searching. The filter was washed with holding media (recipe shown in Table 2.4) using a syringe immediately after filtering. The holding media containing COCs was placed into a scored disposable 150 mm petri dish for COC searching.

A mouth suction pipette tip attached to a pulled glass pasteur pipette was used to search for and move the COCs. The COCs with full coverage of cumulus cells surrounding the oocyte and homogeneous colored nucleus were selected as good-quality COCs. Selected COCs were moved to a 35 mm petri dish containing holding media by mouth pipette. Searching was done more than twice for each petri dish in order to collect most of the COCs.

### 2.2.2 In Vitro Maturation of Bovine COCs

The collected COCs were washed with BO-IVM media (IVF Bioscience, Falmouth, UK) to avoid the contamination from remaining debris and holding media. Washed COCs were placed to a five-well plate containing BO-IVM media using a mouth pipette. The COCs were equally assigned into five wells to prevent an over-crowded environment. Each well contained 0.4 mL BO-IVM media and no more than 30 COCs. The five-well plate containing COCs was then incubated at 38.5 °C with 5% CO₂ in air for 20–24 hours. After this procedure, the COCs were mostly matured (reaching metaphase II) and could be used in the experiment for evaluation of oocyte oxidative stress.

### 2.2.3 Bovine Semen Preparation and In Vitro Fertilization

Two straws of bovine semen were taken out from a liquid nitrogen tank and immediately thawed in a 37° C water bath. All following steps involved with semen were completed in the biological safety cabinet (BSC) with clean micropipettes while avoiding light. Bovine semen
preserved in the two straws were pulled out into a 15 mL conical tube containing 2 mL BO-SemenPrep (IVF Bioscience, Falmouth, UK) that had been incubated with 5% CO₂ at 38.5 °C. The tube was then centrifuged at 350g and 37°C for 5 minutes. The supernatant was removed, and the pellet was gently resuspended with 1 mL of BO-SemenPrep in a new clean 15 mL conical tube. The tube was centrifuged with the same setting, and most of the supernatant was removed again with about 200 µL fluid remaining in the tube. The sperm pellet was gently mixed by flicking the tube, and a total of 10 µL mixed pellet was immediately placed into a 1.5 mL centrifuge tube containing 500 µL of S100 Detergent (Chemometec, Copenhagen, Denmark) for evaluation of semen concentration using Nucleocounter SP-100 (Chemometec, Copenhagen, Denmark). Meanwhile, another 5 µL of sperm sample was placed on a pre-warmed microscope slide mounting with a coverslip for motility evaluation under the microscope. The calculated concentration and estimated motility of the semen sample were recorded. The remaining semen sample was incubated with 5% CO₂ at 38.5 °C for about 15 minutes.

Before fertilization, matured bovine COCs were removed from the 5-well plate containing BO-IVM and carefully washed in a well containing BO-IVF media (IVF Bioscience, Falmouth, UK) using a glass pulled pipette. The washed COCs were placed into another 5-well plate containing 400 µL BO-IVF for in vitro fertilization.

As soon as the semen sample and matured COCs were ready, a calculated amount of semen sample was added to the corner of each well for a final semen concentration of 2 million sperm/mL using a micropipette. The 5-well plate containing matured COCs and sperms was then incubated at 38.5 °C with 5% CO₂ for 18~20 hours.

2.2.4. Denuding and In Vitro Culture of Putative Embryos

After incubating for 18~20 hours for fertilization, the putative zygotes were transferred into a 35 mm petri dish containing 2 mL holding media with 0.2 mg/mL hyaluronidase using a pulled pipette. The putative zygotes along with about 50 µL attached media were then transferred into a 1.5 mL centrifuge tube for a 20-second vortex in order to remove the cumulus cells. After vortex, the zygotes were transferred into a 35 mm petri dish containing 2 mL holding media for observation, and the ones with cumulus cells surrounding them were moved to the dish containing holding media with hyaluronidase again and the vortex steps would be repeated.
As all putative zygotes were completely denuded, they were washed three times in BO-IVC media (IVF Bioscience, Falmouth, UK) micro-drops to eliminate the remaining holding media. The washed putative zygotes were placed into a five-well plate containing 400 µL/well BO-IVC media layered with 500 µL/well BO-Oil (IVF Bioscience, Falmouth, UK) on top of each well. The plate was incubated at 38.5 °C with 5% CO₂, 5% O₂, and 90% N₂ for 10 days, as observation and processing for evaluation of morphological features and developmental rates were made during this time period.

2.3 Pre-Cooling and Post-Thawing Media Treatments

Pre-cooling and post-thawing media treatments are essential for oocyte and embryo vitrification. The same pre-cooling and post-thawing media treatments were completed for all groups except the Normal group.

In the first set of experiments, BO-VitriCool media and BO-VitriWarm media (IVF Bioscience, Falmouth, UK) were used for pre-cooling and post-thawing media treatments according to the manufacturer’s protocol. In the second set of experiments, the pre-cooling and post-thawing media were prepared as described below.

Before vitrification, cells were processed by pre-cooling media including the equilibrium solution and the vitrification solution (recipes shown in Table 2.1 and Table 2.2). Both solutions were warmed to 38.5 °C. Each group of cells were washed and placed into the equilibrium solution for 5 minutes, and then placed into the vitrification solution for 1 minute. During each transfer, the media attached with the cells was minimized. After each transfer, the cells were swirled several times using a pulled pipette to allow the surrounding media to fully penetrate into the cells. The cells in the Straw group, the Cryotop group, and the NANUQ group were immediately vitrified using corresponding vitrification method after pre-cooling media treatments, while the cells in the No-Freeze group were directly exposed to the warming solutions described below.

During thawing, the cells were treated with three warming solutions in order - warming solution I, warming solution II, and warming solution III (recipes shown in Table 2.3). All solutions were warmed to 38.5 °C. The cryopreserved cells were taken out of LN and immediately placed into the warming solution I for 1 minute. The cells were then transferred into the warming solution II for 2 minutes, and into the warming solution III for another 2 minutes. During each
transfer, the media attached with the cells was minimized. After each transfer, the cells were swirled several times using a pulled pipette to allow the surrounding media to fully penetrate into the cells.

For the Straw group and the Cryotop group, 35 mm petri dishes containing 2 mL of each solution were used, except that the volume of the warming solution I was 3 mL.

For the NANUQ group, during the pre-cooling media treatment, two 100 µL micro-drops of the equilibration solution were used for washing and staying, respectively. Similarly, a 100 µL micro-drop of the vitrification solution was used for washing before the cells were moved to a 10 µL micro-drop of the vitrification solution on a 96-well crystallization intelliplate (Art Robbins Instruments, Sunnyvale, CA). During post-thawing media treatment, 35 mm petri dishes were used to contain 3 mL of the warming solution I, 2 mL of the warming solution II, and 2 mL of the warming solution III, respectively, the same as the in-straw group and the Cryotop group.

2.4 In-Straw Vitrification of Bovine Embryos

The bovine cleaved embryos within the Straw group were vitrified using 0.5 mL, 140 mm long straws (Minitube, Verona, WI) according to the manufacturer’s protocol. Briefly, after the pre-cooling media treatment, the straw was loaded with about 40 mm warming solution I followed by 10 mm air, and then up to four embryos along with around 14 mm vitrification solution surrounding them were loaded into the straw. Another 10 mm air and 40 mm warming solution I were loaded into the straw before the straw was heat-sealed. The straw was immediately hand-plunged into LN for cooling and storage.

For thawing embryos, the straw was taken out of the LN tank and the contents were mixed to allow embryos to rehydrate by in-straw-dilution. The sealed end of the straw was then cut to empty all contents within it into a petri dish containing pre-warmed warming solution I. The following post-thawing media treatment was completed as described previously.
2.5 Cryotop® Vitrification of Bovine Oocytes and Embryos

Cryotop® US (Kitazato, Tokyo, Japan) was the device used for vitrification of the oocytes and embryos within the Cryotop group. The usage of this device in this study followed the manufacturer’s protocol and is summarized below.

After the pre-cooling media treatment, up to four oocytes or embryos were loaded onto the film strip of the Cryotop® US device using a glass pulled pipette under the microscope, with a minimum amount of vitrification solution surrounding each cell. The Cryotop® US device was then immediately inserted into LN and covered with the straw cap tightly several seconds later. After the cell loading process was completed, the Cryotop® US device was transferred into the LN tank for storage.

When thawing the oocytes or embryos, the Cryotop® US device was taken out of LN and immediately inserted into a petri dish containing pre-warmed warming solution I while swirling for several times, allowing the film strip to be completely immersed with solution. After all cells were unloaded from the film strip, the Cryotop® US device was set aside. The following post-thawing media treatment was completed as described above.

2.6 NANUQ™ Vitrification of Bovine Oocytes and Embryos

The oocytes and embryos within the NANUQ group were vitrified using the NANUQ™ device (MiTeGen, Ithaca, NY). The oocytes or embryos were transported from the lab to the NANUQ access facility in 1 mL holding media at 28~38°C and atmospheric CO2. The cells were added to 1 mL holding media in a 35 mm petri dish for a final volume of 2 mL at 38.5°C while awaiting transfer to the vitrification protocol. One cell was processed at a time. The cell was removed from holding media and washed in a 100 µL micro-drop of equilibration solution, followed by the pre-cooling media treatment as described previously. After the pre-cooling media treatment, the cell was removed from the micro-drop of vitrification solution using a 400/25 µm micro-mesh mounted on a magnetic base attached to a wand (MiTeGen, Ithaca, NY). The wand was transferred to the NANUQ™ and the sample was automatically plunged into LN upon closure of the door. The samples were held in a magnetic storage puck (MiTeGen, Ithaca, NY) at -196°C.
During the thawing process, the cells were retrieved one at a time from a storage puck submerged in liquid nitrogen. A magnetic wand was securely placed onto the goniometer base and the sample was removed from the puck to 3 mL of the warming solution I in a 35mm petri dish. The embryo was washed off from the micromesh mount using a swirling motion. The following post-thawing media treatment was completed as described previously.

2.7 Evaluation of Morphological Feature of Bovine Embryos

Cleaved embryos were produced by in vitro fertilization and four-day in vitro culture. The embryos that had achieved cleavage stage were selected and randomly assigned to the No-Freeze group, the NANOQ group, and the Straw group. Embryos in the NANOQ group and the Straw group underwent vitrification and thawing, while embryos in the No-Freeze group underwent media treatments without vitrification. All three groups of embryos were cultured for 1 hour in petri dishes containing BO-IVC media at 38.5 °C with 5% CO₂, 5% O₂, and 90% N₂. The embryos of each group were then taken out of the incubator and observed under the microscope while the images were captured, respectively.

2.8 Evaluation of Developmental Rates of Bovine Cleaved Embryos

Cleaved embryos were produced by in vitro fertilization and four-day in vitro culture. The embryos that had achieved cleavage stage were selected and randomly assigned to the No-Freeze group, the NANOQ group, and the Straw group. Embryos in the NANOQ group and the Straw group underwent vitrification and thawing, while embryos in the No-Freeze group underwent media treatments without vitrification. In order to record the numbers of the surviving embryos and calculate survival rates, all three groups of embryos were cultured for 1 hour in petri dishes containing BO-IVC media at 38.5 °C with 5% CO₂, 5% O₂, and 90% N₂, followed by observation under the microscope. The embryos with intact zona pellucida and without lysis were counted as surviving embryos post-thaw. The survival rates were based on the number of intact embryos over the total number of the embryos within each group. After observation, the three petri dishes containing three groups of embryos in BO-IVC media were continued being cultured at 38.5 °C with 5% CO₂, 5% O₂, and 90% N₂.
In order to record the morula rates, blastocyst rates, and hatching rates, microscopic observation was continued to perform for all groups until the 11th day after fertilization. The dishes were immediately returned to the incubator after observation to prevent negative impacts caused by the changing environmental conditions. The rates were calculated based on the numbers of the embryos that had achieved morula stage, blastocyst stage, or hatching, divided by the total number of the embryos within each group.

### 2.9 Evaluation of Developmental Rates of Bovine Blastocysts

Blastocysts were produced by *in vitro* fertilization and *in vitro* culture for 7–9 days. The healthy blastocysts were selected and randomly assigned to the Cryotop group, the NANUQ group, the No-Freeze group, and the Normal group. Blastocysts in the Cryotop group and the NANUQ group underwent vitrification; blastocysts in the No-Freeze group underwent media treatments without vitrification; and blastocysts in the Normal group were continued to be cultured in BO-IVC media. In order to record the numbers of surviving embryos and calculate survival rates, all four groups of embryos were cultured for 1 hour in petri dishes containing BO-IVC media at 38.5 °C with 5% CO₂, 5% O₂, and 90% N₂, followed by observation under the microscope. The embryos with intact zona pellucida and without lysis were counted as blastocysts survived post-thaw. The survival rates were based on the number of intact blastocysts and the total number of the blastocysts within each group. After observation, the four petri dishes containing four groups of embryos in BO-IVC media respectively were continued being cultured at 38.5 °C with 5% CO₂, 5% O₂, and 90% N₂.

In order to record the re-expansion rates and hatching rates, microscopic observation was continued to perform for all groups until the 11th day after fertilization. The dishes were immediately returned to the incubator after observation to prevent negative impacts caused by the changing environmental conditions. The re-expansion rates were calculated based on the numbers of the blastocysts that continued re-expanding in 24 hours post-thaw divided by the total number of the embryos within each group, while the hatching rates were calculated based on the numbers of the blastocysts that had accomplished hatching in 48 hours post-thaw divided by the total number of the embryos within each group.
2.10 Evaluation of Oxidative Stress Bovine Oocytes

Bovine matured oocytes were produced by in vitro maturation for 22 hours. The healthy bovine matured COCs were selected and partially denuded by being placed in a 35 mm petri dish containing 2 mL holding media with 0.2 mg/mL hyaluronidase for no more than one minute. A swirling motion to remove most of the surrounding cumulus cells using a pulled pipette was applied if necessary. The partially denuded COCs were randomly assigned to the Cryotop group, the NANUQ group, the No-Freeze group, and the Normal group. The COCs in the Cryotop group and the NANUQ group were vitrified and thawed using indicated methods, while the COCs in the No-Freeze group went through media treatments. No treatment was done to COCs in the Normal group. After vitrification, media treatment, or no treatment, COCs in each group were completely denuded, by being placed in a 35 mm petri dish containing 2 mL holding media with 0.2 mg/mL hyaluronidase, following vortex for 20 seconds along with 50 µL hyaluronidase-holding media solution. Vortex was repeated to completely eliminate the cumulus cells if necessary. The metaphase II oocytes within each group without cumulus cells surrounding them were then cultured for 1 hour in petri dishes containing holding media in the atmospheric incubation.

In the meantime, the solution of the green fluorescent general oxidative stress indicator CM-H₂DCFDA (Thermo Fisher Scientific, Waltham, MA) was prepared in the dark in the BSC. The preparation was conducted following the manufacturer’s instructions. CM-H₂DCFDA is a green fluorescent general oxidative stress indicator – stronger green fluorescent level indicates higher ROS level. A tube of 50 µg CM-H₂DCFDA was dissolved by 17.3 µL DMSO to reach a concentration of 5 mM. A total of 155.7 µL Dulbecco's phosphate-buffered saline (DPBS) (Corning, NY) was then added to the tube to reach a concentration of 500 µM. Based on the final volume needed for the oocytes’ fluorescent staining, a calculated amount of mixed solution was added to a 5-well plate containing specific amount of Tyrode's lactate-HEPES-polyvinyl alcohol (TL-HEPES-PVA) (recipe shown in Table 2.5), reaching a final concentration of 5 µM. The prepared CM-H₂DCFDA solution was then incubated for several minutes in the atmospheric incubation at 38.5 °C.

After one hour of incubation and prior to fluorescent staining, oocytes within each group were washed twice in 50 µL micro-drops of TL-HEPES-PVA. The washed oocytes were moved into the 5-well plate containing 400 µL/well prepared CM-H₂DCFDA solution. About 10 oocytes
were allowed per well. The 5-well plate was then incubated for 30 minutes at atmospheric conditions.

Stained oocytes were washed three times in 50 µL micro-drops of DPBS-PVA, transferred into another 50 µL micro-drop of DPBS-PVA and images captured under the white light microscope followed by fluorescent microscopy. Images of all oocytes were analyzed using FIJI-ImageJ. The corrected total cell fluorescence (CTCF) level of each oocyte was measured as an indicator of the relative oxidative stress level within cells.

2.11 Statistical Analysis

The developmental rates, means, and standard errors were calculated by Excel (Microsoft, Redmond, WA). Differences in the means were analyzed on R Studio (RStudio, Boston, MA) using ANOVA followed by the Tukey’s test. For the experiment of oocyte oxidative stress, the comparisons in average CTCF levels were adjusted across weeks. The tables and graphs were made using Excel, and the data were presented as mean ± the standard error (SE) in the graphs. Significant differences were marked in the graphs accordingly, as ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

2.12 Media Recipes

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Chemical Name</th>
<th>Stock Conc.</th>
<th>Total Volume (100mL)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gibco</td>
<td>Medium 199 / “Holding Media Base”</td>
<td>100%</td>
<td>85 mL</td>
<td>85%</td>
</tr>
<tr>
<td>Spectrum</td>
<td>Ethylene Glycol, Reagent</td>
<td>99.0%</td>
<td>7.5 mL</td>
<td>7.5%</td>
</tr>
<tr>
<td>Sigma</td>
<td>Dimethyl Sulfoxide</td>
<td>99.9%</td>
<td>7.5 mL</td>
<td>7.5%</td>
</tr>
</tbody>
</table>

Combine in sterile beaker/container. Store at 4°C
### Table 2.2 Vitrification Solution Recipe

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Chemical Name</th>
<th>Stock Conc.</th>
<th>Total Volume (100mL)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gibco</td>
<td>Medium 199 / “Holding Media Base”</td>
<td>100%</td>
<td>70 mL</td>
<td>70%</td>
</tr>
<tr>
<td>Spectrum</td>
<td>Ethylene Glycol, Reagent</td>
<td>99.0%</td>
<td>15 mL</td>
<td>15%</td>
</tr>
<tr>
<td>Sigma</td>
<td>Dimethyl Sulfoxide</td>
<td>99.9%</td>
<td>15 mL</td>
<td>15%</td>
</tr>
</tbody>
</table>

Combine in sterile beaker/container. Store at 4°C

### Table 2.3 Recipes for Warming Solution I, Warming Solution II, and Warming Solution III

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Chemical Name</th>
<th>Stock Conc.</th>
<th>Warming Solution I</th>
<th>Warming Solution II</th>
<th>Warming Solution III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gibco</td>
<td>Medium 199 / “Holding Media Base”</td>
<td>100%</td>
<td>100 mL</td>
<td>100 mL</td>
<td>100 mL</td>
</tr>
<tr>
<td>Sigma</td>
<td>Sucrose</td>
<td>-</td>
<td>34.23 g (1 M)</td>
<td>17.12 g (0.5 M)</td>
<td>8.56 g (0.25 M)</td>
</tr>
</tbody>
</table>

Combine in sterile beaker/container. Store at 4°C

### Table 2.4 Holding Media Recipe

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Chemical Name</th>
<th>Stock Conc.</th>
<th>Total Volume (100mL)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gibco</td>
<td>Medium 199 / “Holding Media Base”</td>
<td>100%</td>
<td>87.750 mL</td>
<td>87.75%</td>
</tr>
<tr>
<td>Corning</td>
<td>Fetal Bovine Serum, Heat Inactivated</td>
<td>100%</td>
<td>10 mL</td>
<td>10%</td>
</tr>
<tr>
<td>Sigma</td>
<td>Sodium Pyruvate</td>
<td>20 mM</td>
<td>1 mL</td>
<td>1%</td>
</tr>
<tr>
<td>Sigma</td>
<td>Heparin for Holding Media</td>
<td>500 µg/mL</td>
<td>1 mL</td>
<td>1%</td>
</tr>
<tr>
<td>Sigma or Gibco</td>
<td>Gentamycin</td>
<td>10 mg/mL</td>
<td>250 µL</td>
<td>.25%</td>
</tr>
</tbody>
</table>

Combine in sterile beaker/container. Add stir bar & heat to 25°C. Adjust pH to 7.3-7.4 with HCl if too basic or NaOH if too acidic. Sterile filter through 22 µm porous syringe filter or vacuum filter system into a sterile tube or syringe. Label as “Filtered HM” with pH, expiration date, and initials. Store at 4°C. Expires in 1 month
<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Chemical Name</th>
<th>Total Weight (100mL)</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sigma</td>
<td>Sodium Chloride</td>
<td>0.6428 g</td>
<td>110 mM</td>
</tr>
<tr>
<td>Sigma</td>
<td>Potassium Chloride</td>
<td>19.9798 mg</td>
<td>2.68 mM</td>
</tr>
<tr>
<td>Fisher</td>
<td>Sodium Phosphate Monobasic Monohydrate</td>
<td>4.9672 mg</td>
<td>0.36 mM</td>
</tr>
<tr>
<td>Sigma</td>
<td>Sodium Bicarbonate</td>
<td>0.21 g</td>
<td>25 mM</td>
</tr>
<tr>
<td>Sigma</td>
<td>Magnesium Chloride Hexahydrate</td>
<td>9.9573 mg</td>
<td>0.49 mM</td>
</tr>
<tr>
<td>Sigma</td>
<td>Calcium Chloride Dihydrate</td>
<td>35.2752 mg</td>
<td>2.4 mM</td>
</tr>
<tr>
<td>Sigma</td>
<td>HEPES Sodium Salt</td>
<td>0.5957 g</td>
<td>25 mM</td>
</tr>
<tr>
<td>Sigma</td>
<td>D-(+)-Glucose, &gt;=99.5%</td>
<td>100 mg</td>
<td>5.56 mM</td>
</tr>
<tr>
<td>Sigma or Gibco</td>
<td>Gentamycin</td>
<td>60 µL</td>
<td>0.01% w/v</td>
</tr>
<tr>
<td>Sigma</td>
<td>Polyvinyl Alcohol</td>
<td>0.01 g</td>
<td>0.01% w/v</td>
</tr>
</tbody>
</table>

Dissolve in diH₂O and combine in sterile tubes. Store at 4 °C.
RESULTS

3.1 Part 1: Evaluation of Morphological Features and Developmental Rates of Bovine Cleaved Embryos

3.1.1 Evaluation of Morphological Features of Bovine Cleaved Embryos

After *in vitro* fertilization and four-day *in vitro* culture, embryos that had achieved cleavage stage were selected and randomly assigned to the No-Freeze group, the NANUQ group, and the Straw group. Embryos in the NANUQ group and the Straw group underwent vitrification while embryos in the No-Freeze group underwent media treatments without vitrification, followed by microscopic observation of each group of embryos. Images are shown in Fig 3.1. No apparent difference was observed on the morphological features between the NANUQ group (Fig 3.1 A) and the No-Freeze group (Fig 3.1 B), and all of the cleaved embryos in both groups appeared to have intact zona pellucida. However, the image of cleaved embryos in the Straw group (Fig 3.1 C) showed several obvious rupture of zona pellucida that impacted 4 out of 17 embryos. Such serious integrity loss of zona pellucida was not seen in other two groups.
Fig 3.1 Representative photographs of cleaved embryos within indicated groups
A) NANUQ group, B) No-Freeze group, and C) Straw group. Images were captured under the microscope and embryos were cultured in separate dishes containing BO-IVC media. Zona pellucida rupture is marked by red circle. No zona pellucida rupture was observed in the NANUQ group and the No-Freeze group, while five clefts had been observed on 4 cleaved embryos in the Straw group.
3.1.2 Evaluation of Developmental Rates of Bovine Cleaved Embryos

A total of 337 cleaved embryos were produced from 7 independent weeks (7 independent replicates), in which 125 embryos were in the No-freeze group, 116 embryos in the NANUQ group, and 96 embryos in the Straw group. For each week, right after the vitrification or media treatment procedure, we performed microscopic observation and searched for intact embryos to record survival rates, shown in Table 3.1. All embryos within the No-Freeze group survived and only 1 lysed embryo was observed within the NANUQ group, while only 87 out of 96 embryos were intact within the Straw group. After survival evaluation, embryos of all 3 groups were in vitro cultured for further observation until 11 days after fertilization, and the numbers of the embryos that had achieved morula stage, blastocyst stage, and hatching were recorded, shown in Table 3.1. It is worth noticing that no embryo in the Straw group had proceeded to morula stage, while there were several embryos proceeded to hatched blastocyst stage in both the No-Freeze group and the NANUQ group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total</th>
<th>Survival</th>
<th>Morula</th>
<th>Blastocyst</th>
<th>Hatching</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Num.</td>
<td>Rate (%)</td>
<td>Num.</td>
<td>Rate (%)</td>
</tr>
<tr>
<td>No-Freeze</td>
<td>125</td>
<td>125</td>
<td>100.00</td>
<td>18</td>
<td>14.4</td>
</tr>
<tr>
<td>NANUQ</td>
<td>116</td>
<td>115</td>
<td>99.14</td>
<td>5</td>
<td>4.31</td>
</tr>
<tr>
<td>Straw</td>
<td>96</td>
<td>87</td>
<td>90.63</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3.1 Bovine Cleaved Embryo Developmental Rates

Numbers and rates of survival, morula stage, blastocyst stage, and hatching of embryos within the No-freeze group, the NANUQ group, and the Straw group. Data were from 7 independent weeks.

Based on the data acquired above, the results of statistical analysis are shown in Table 3.2 below. A final graph comparing developmental rates with significance levels is presented in Fig 3.2. It is worth noticing that the survival rate of the Straw group was significantly lower than both the NANUQ group and the No-Freeze group ($P < 0.001$), while no significant difference was shown between the No-Freeze group and the NANUQ group. No significant difference between the NANUQ group and the Straw group was shown in the morula rate, the blastocyst rate, and the hatching rate.
Table 3.2 P-values of Comparisons Between Bovine Cleaved Embryo Developmental Rates

<table>
<thead>
<tr>
<th>Group</th>
<th>Survival</th>
<th>Morula</th>
<th>Blastocyst</th>
<th>Hatching</th>
</tr>
</thead>
<tbody>
<tr>
<td>No-Freeze - NANUQ</td>
<td>0.913974</td>
<td>0.0045351**</td>
<td>0.0003264***</td>
<td>0.1270482</td>
</tr>
<tr>
<td>Straw - NANUQ</td>
<td>0.0006616***</td>
<td>0.4128521</td>
<td>0.7032124</td>
<td>0.5646204</td>
</tr>
<tr>
<td>Straw - No-Freeze</td>
<td>0.0001129***</td>
<td>0.0000623***</td>
<td>0.0000245***</td>
<td>0.0114905*</td>
</tr>
</tbody>
</table>

Data were from 7 independent weeks. Total sample size is 337, with 125 for the No-Freeze group, 116 for the NNUQ group, and 96 for the Straw group. Data analysis was completed on R Studio using ANOVA followed by the Tukey’s test (***P <0.001, **P <0.01, *P <0.05)

Fig 3.2 Developmental rates of cleaved embryos within indicated groups

Data were from 7 independent weeks. Total sample size is 337, with 125 for the No-Freeze group, 116 for the NNUQ group, and 96 for the Straw group. Data analysis was completed on R Studio using ANOVA followed by the Tukey’s test (***P <0.001, **P <0.01, *P <0.05)

3.2 Part 2: Evaluation of Developmental Rates of Bovine Blastocysts and Oxidative Stress of Bovine Oocytes

3.2.1 Evaluation of Developmental Rates of Bovine Blastocysts

After in vitro fertilization and in vitro culture for 7~9 days, healthy blastocysts were randomly assigned to the Cryotop group, the NNUQ group, the No-Freeze group, and the Normal
group. Blastocysts in the Cryotop group and the NANUQ group underwent vitrification; blastocysts in the No-Freeze group underwent media treatments without vitrification; and blastocysts in the Normal group were continued to be cultured in BO-IVC media. A total of 106 blastocysts were produced from 10 independent weeks (10 independent replicates), in which 40 blastocysts were from the Cryotop group, 31 from the NANUQ group, 13 from the No-Freeze group, and 22 from the Normal group.

For each week, right after the vitrification or media treatment procedure, we performed microscopic observation and searched for intact blastocysts to record the survival rates; for the Normal group, as no treatment was done, all blastocysts were healthy and intact, meaning that survival rate of the Normal group was always considered to be 100.00%. As is shown in Table 3.3, only 34 out of 40 blastocysts in the Cryotop group survived, while all blastocysts in the NANUQ group and the No-Freeze group survived. After survival evaluation, blastocysts of all four groups were in vitro cultured for further observation until the 11th day after fertilization, and the numbers of the blastocysts that continued re-expanding in 24 hours post-thaw and accomplished hatching in 48 hours post-thaw were recorded, shown in Table 3.3. It is worth noticing that the NANUQ group had similar re-expansion rate and hatching rate to the No-Freeze group and the Normal group, while the lowest re-expansion rate and hatching rate were produced by the Cryotop group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total</th>
<th>Survival</th>
<th></th>
<th>Re-Expansion</th>
<th>Hatching</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Num.</td>
<td>Rate (%)</td>
<td>Num.</td>
<td>Rate (%)</td>
<td>Num.</td>
</tr>
<tr>
<td>Cryotop</td>
<td>40</td>
<td>34</td>
<td>24</td>
<td>60.00</td>
<td>9</td>
</tr>
<tr>
<td>NANUQ</td>
<td>31</td>
<td>31</td>
<td>28</td>
<td>90.32</td>
<td>16</td>
</tr>
<tr>
<td>No-Freeze</td>
<td>13</td>
<td>13</td>
<td>13</td>
<td>100.00</td>
<td>8</td>
</tr>
<tr>
<td>Normal</td>
<td>22</td>
<td>22</td>
<td>22</td>
<td>100.00</td>
<td>13</td>
</tr>
</tbody>
</table>

Numbers and rates of survival, re-expansion and hatching of embryos within the Cryotop group, the NANUQ group, the No-freeze group, and the Normal group. Data were from 10 independent weeks.

Based on the data acquired above, the results of statistical analysis are shown in Table 3.4 below. A final graph comparing developmental rates with significance levels is presented in Fig 3.3. It is worth noticing that both the survival rate and the re-expansion rate of the Cryotop group were significantly lower than the NANUQ group ($P<0.05$, $P<0.01$, respectively).
Table 3.4 *P*-values of Comparisons Between Bovine Blastocyst Developmental Rates

<table>
<thead>
<tr>
<th>Group</th>
<th>Survival</th>
<th>Re-Expansion</th>
<th>Hatching</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryotop – No-Freeze</td>
<td>0.1596591</td>
<td>0.0026779**</td>
<td>0.0556808</td>
</tr>
<tr>
<td>NANUQ – No-Freeze</td>
<td>1.0000000</td>
<td>0.8337514</td>
<td>0.9218162</td>
</tr>
<tr>
<td>Normal – No-Freeze</td>
<td>1.0000000</td>
<td>1.0000000</td>
<td>0.9988607</td>
</tr>
<tr>
<td>NANUQ – Cryotop</td>
<td>0.0303490*</td>
<td>0.0023338**</td>
<td>0.0574886</td>
</tr>
<tr>
<td>Normal – Cryotop</td>
<td>0.0616767</td>
<td>0.0001964***</td>
<td>0.0235641*</td>
</tr>
<tr>
<td>Normal – NANUQ</td>
<td>1.0000000</td>
<td>0.7501324</td>
<td>0.9426183</td>
</tr>
</tbody>
</table>

Data were from 10 independent weeks. Total sample size is 106, with 40 for the Cryotop group, 31 for the NANUQ group, 13 for the No-Freeze group, and 22 for the Normal group. Data analysis was completed on R Studio using ANOVA followed by the Tukey’s test (**P <0.01, *P <0.05)

Fig 3.3 Developmental rates of blastocysts within indicated groups

Data were from 10 independent weeks. Total sample size is 106, with 40 for the Cryotop group, 31 for the NANUQ group, 13 for the No-Freeze group, and 22 for the Normal group. Data analysis was completed on R Studio using ANOVA followed by the Tukey’s test (**P <0.01, *P <0.05)
3.2.2 Evaluation of Oxidative Stress Bovine Oocytes

After *in vitro* maturation for 22 hours, healthy partially denuded bovine COCs randomly assigned to the Cryotop group, the NANUQ group, the No-Freeze group, and the Normal group. The COCs in the Cryotop group and the NANUQ group were vitrified and thawed using indicated methods, while the COCs in the No-Freeze group went through media treatments. No treatment was done to the COCs in the Normal group. Complete elimination of cumulus cells and fluorescent staining using CM-H₂DCFDA were then accomplished for all oocytes in four groups. Representative photographs are shown in Fig 3.4. A total of 281 oocytes were produced from 5 independent weeks (5 independent replicates), in which 65 were from the Cryotop group, 46 from the NANUQ group, 93 from the No-Freeze group, 77 from the Normal group.
Fig 3.4 Representative photographs of oocytes with white-light microscopy (A, C, E, G) and blue fluorescent microscopy (B, D, F, H) treated by CM-H$_2$DCFDA within indicated groups.
Images were analyzed for the CTCF level of each oocyte as an indicator of the relative oxidative stress level within cells. The average numbers and standard errors of all four groups acquired from 5 experimental weeks are shown in Table 3.5. The Cryotop group presented the highest average number of relative oxidative stress, followed by the NANUQ group.

### Table 3.5 Relative ROS Averages of Bovine Oocytes Within Indicated Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Total</th>
<th>Relative Oxidative Stress Average ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryotop</td>
<td>65</td>
<td>35.30506308 ± 1.69741739</td>
</tr>
<tr>
<td>NANUQ</td>
<td>46</td>
<td>26.45391677 ± 1.83732373</td>
</tr>
<tr>
<td>No-Freeze</td>
<td>93</td>
<td>9.80138495 ± 1.13174260</td>
</tr>
<tr>
<td>Normal</td>
<td>77</td>
<td>7.23364935 ± 1.02647176</td>
</tr>
</tbody>
</table>

Averages and standard errors of the relative ROS level within the Cryotop group, the NANUQ group, the No-Freeze group, and the Normal group. Data were from 5 independent weeks

Based on the data acquired above, the results of statistical analysis are shown in Table 3.6 below. A final graph comparing developmental rates with significance levels is presented in Fig 3.5. The oocytes in the NANUQ group showed significantly lower oxidative stress levels compared with the Cryotop group \((P<0.001)\). Significant difference in oxidative stress was also seen between the two control groups and the two vitrification groups \((P<0.001)\). No significant difference was observed between the No-Freeze group and the Normal group.

### Table 3.6 P-values of Comparisons Between Oocyte Relative ROS Level

<table>
<thead>
<tr>
<th>Group</th>
<th>Cryotop</th>
<th>NANUQ</th>
<th>No-Freeze</th>
</tr>
</thead>
<tbody>
<tr>
<td>NANUQ</td>
<td>0.0002106***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No-Freeze</td>
<td>0.0000000***</td>
<td>0.0000000***</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0.0000000***</td>
<td>0.0000000***</td>
<td>0.4246387</td>
</tr>
</tbody>
</table>

Data are from 5 independent weeks. Total sample size is 65 for the Cryotop group, 46 for the NANUQ group, 93 for the No-Freeze group, 77 for the Normal group. Data analysis was completed on R Studio using ANOVA followed by the Tukey’s test \((***P<0.001)\)
**Fig 3.5 Quantification of relative ROS level by measuring CTCF within indicated groups**

Data are from 5 independent weeks. Total sample size is 65 for the Cryotop group, 46 for the NANUQ group, 93 for the No-Freeze group, 77 for the Normal group, and values are means ± SEM. Data analysis was completed on R Studio using ANOVA followed by the Tukey’s test (**P <0.001**).
DISCUSSION

4.1 Part 1: Evaluation of Morphological Features and Developmental Rates of Bovine Cleaved Embryos

Previous studies have reported the fracturing of zona pellucida on cryopreserved embryos, as a severe cryopreservation damage, which has significantly impacted on subsequent blastomere survival [63]. It has been suggested that slow cooling rates and warming rates are responsible for the increased ice crystal formation, and thus cause zona pellucida fracturing by ice crystals’ mechanical piercing [38]. Moreover, dramatic osmotic stress due to slow cooling and warming rates can also result in overwhelming shrinkage and expansion of the cryopreserved cells, which eventually leads to the loss of embryo structure integrity [38].

Similar to morphological features, many previous studies have also used developmental rates to evaluate the cryodamage extent and the embryonic developmental capacity, as they are a series of important cryodamage indicators that can be easy to assess under the microscope throughout the whole embryonic developmental stage [56]. For cleaved embryos, these indicators can include survival rates, morula rates, blastocyst rates, and hatching rates [3].

In the evaluation of morphological feature, the fracturing of zona pellucida had been largely observed on cleaved embryos within the Straw group, but not the NANOQ group or the No-Freeze group. Such difference indicates the superiority of the NANOQ™ vitrification system over the in-straw vitrification system in the capability of avoiding the cryopreservation-related severe structure integrity loss.

In the evaluation of developmental rates, our results were consistent with our hypothesis and complemented previous morphological feature observations. A significantly higher survival rate ($P<0.001$) was achieved by NANOQ group embryos compared with the Straw group, confirming the ability of the NANOQ™ system in avoiding zona pellucida fracturing or lysis that we previously observed in the evaluation of morphological feature. As for later development, although a statistic significant difference ($P>0.5$) was not achieved between the NANOQ group and the Straw group in morula rate, blastocyst rate, and hatching rate, the embryos within the
NANUQ group still yielded high means across these stages while no embryo within the Straw group proceeded to next stage. Considering the already low morula/blastocyst/hatching rates and the extremely small numbers of embryos proceeded into morula stage and hatching (as 18 and 9, respectively) presented in the No-Freeze group, we can conclude that a larger sample size may be needed in order to more accurately and scientifically evaluate the differences in development potential across these three groups. Nevertheless, generally, the No-Freeze group still showed the highest developmental rates ($P<0.05$) among three groups, suggesting that the damage caused by vitrification may not be completely eliminated by the improvement of vitrification technology.

The cooling rate of the in-straw vitrification system as 2,550 °C/min, is not comparable to the cooling rate of the NANUQ™ system, achieving over 600,000 °C/min [4, 9]. It is plausible that the cleaved embryos vitrified with a 200 times faster cooling rate had a greatly shortened exposure time to the ice-nucleation temperature, forming less ice crystals, thus effectively avoiding chilling injuries to the embryos [6]. Such a fast cooling rate may have allowed mild to no zona pellucida damage that it even appeared similar to the embryos that were not vitrified under the microscope [38]. Apart from zona pellucida fracturing, the intracellular ice nucleation growth and nucleation together with the dramatically changed osmotic stress during cooling and warming have also been reported to damage essential intracellular organelles and disrupt intracellular microstructure, leading to delay or even failure in later embryonic development [38]. Although not eliminating the cumulative damage within the cleaved embryos that had shown in the later development, such a fast cooling rate had reduced cryodamage and allowed the cleaved embryos to proceed to next stages, while the slow cooling rate of in-straw system did not appear to enable the development of cleaved embryos in our study.

However, although the major difference between the NANUQ™ method and the in-straw method is considered to be the cooling rates, part of the differences in the compromising result produced by the in-straw method could be possibly explained by the larger sample volume used in the in-straw method. A reduced sample volume used in the NANUQ™ method enabled a better heat exchange efficiency and also prevented the toxicities of CPA. Despite its invisibility under the microscope right after the cooling-thawing cycle, the accumulation of CPA toxicities and possible LN contamination within embryos can also seriously impact on embryonic development later [7, 8].
4.2 Part 2: Evaluation of Developmental Rates of Bovine Blastocysts and Oxidative Stress Bovine Oocytes

The Cryotop® system, as a successful example in the recent decade, was developed to prevent cryodamage by achieving 22,800 °C/min cooling rate, which is closer to the 600,000 °C/min cooling rate of the NANUQ™ system than the in-straw system [9, 30]. The Cryotop® system also limits the sample droplet volume to less than 0.1 µL, which is similar to the NANUQ™ system [9, 30]. It is worth mentioning that until now, in spite of many ongoing attempts, no vitrification method had successfully demonstrated significantly better outcomes compared with the Cryotop® system. Previous inventions including the VitTrans and Solid Surface Vitrification method had achieved comparable but not superior results [34-36]. Therefore, it is of significance to compare the Cryotop® system and the NANUQ™ system in order to determine how increased cooling rate can impact on the oocyte and embryo viability post-thaw.

As for the evaluation of blastocyst developmental rates, our study suggested a superior performance produced by the NANUQ™ vitrification method over the leading commercial vitrification method, Cryotop® system. Using the NANUQ™ system was confirmed to significantly increase the survival rate and the re-expansion rate \( P<0.05 \), and \( P<0.01 \), respectively), both of which were comparable to the blastocysts without vitrification \( P>0.05 \). Although no significant result \( P>0.05 \) was shown in the hatching rate between the NANUQ group and the Cryotop group, the mean value of the NANUQ group was much higher than the Cryotop group; considering the already low hatching rate and the small numbers of blastocysts successfully hatched within the No-Freeze group and the Normal group, this lack of statistical significance in hatching rates can be hopefully addressed if a larger sample size was used. In fact, it is worth noticing that the sample size in this experiment was relatively small, especially for the No-Freeze group and the Normal group, which well explained why the 100% survival rates of both the No-Freeze group and the Normal group did not achieve statistical significance compared with the Cryotop group. Previous studies investigating different performances of bovine embryo cryopreservation techniques have reported significant results using several hundreds of bovine embryos [64]. However, in our study, a sample size as small as no more than 40 per group still allowed the NANUQ™ system to achieve significantly better performances regarding blastocyst survival and re-expansion compared with the Cryotop® system.
Apart from evaluations on developmental rates, previous studies on cryodamage also investigated essential biochemical alteration within the cells to decide the viability of cryopreserved cells, one of which is oxidative stress. It has been reported that cryopreservation is associated with altered redox status and increased ROS levels in post-thaw oocytes, which may result in important organelle damage and apoptosis [58, 65]. Therefore, relative ROS level, or oxidative stress, has been considered to be a quantifiable and sensitive indicator of cryodamage. In this study, the matured oocytes vitrified using the NANUQ¹⁰⁰ system presented significantly lower ROS level than those using the Cryotop® system (\(P<0.001\)), while a significantly increased ROS level (\(P<0.001\)) was confirmed in vitrified oocytes compared with un-vitrified ones. Our study indicated the capability of the fastest cooling vitrification method, the NANUQ¹⁰⁰ system, in effectively decreasing but not eliminating the cryodamage to female gametes.

Overall, our study has confirmed the ability of the NANUQ¹⁰⁰ system to achieve significant higher embryo developmental rates than the Cryotop® system. Such achievement is encouraging, but not surprising. Equipped with a cooling rate about 25x faster than the Cryotop, the NANUQ¹⁰⁰ vitrification method is able to more efficiently prevent cryodamage including chilling injuries and the toxicities caused by CPAs. Moreover, although the warming rate is similar between the Cryotop® and the NANUQ¹⁰⁰ system, the increased cooling rate in the NANUQ¹⁰⁰ system can greatly reduce the amount of ice crystals formed during cooling, thus limiting the growth of ice crystals during warming.

Besides, achievements in oocyte cryopreservation are greatly encouraging, as oocytes have been reported to be more difficult to vitrify than later embryos, due to their sensitivity and vulnerability to cryodamage during extremely active meiosis [43]. Yet, the demand for oocyte cryopreservation has been exponentially raised in recent years, especially driven by women’s wishes to store eggs out of diverse reasons [17]. Therefore, it is of great significance that the fastest cooling rate produced by the NANUQ¹⁰⁰ method is able to mitigate the imbalance between the excessive ROS level and the cryopreserved oocytes’ detoxification ability, thus avoiding damage to DNA, proteins, and lipids, and eventually preventing membrane fracture and mitochondria function loss [59].
4.3 Future Directions

Although some of the embryo developmental rates have initially demonstrated the superiority of the NANUQ™ method over both the in-straw method and the Cryotop® system, a larger sample size is in demand to further determine how much benefit the fastest cooling rate produced by the NANUQ™ method can bring to embryo cryopreservation. Besides, future directions in embryo developmental rates can expand beyond the early embryonic developmental stages, investigating the pregnancy rate and delivery rate, for example.

As our study has reported a decreased ROS level in oocytes cryopreserved by the NANUQ™ system compared with the leading commercial vitrification method, the Cryotop® system, it has been suggested in many studies that oxidative stress is associated tightly with the disruption of mitochondrial function [59]. Therefore, further investigation in mitochondrial function alteration within bovine oocytes cryopreserved by different techniques would complement our evaluation of oxidative stress. A red-orange fluorescent dye, tetramethylrhodamine ethyl ester (TMRE), along with a green fluorescent dye, MitoTracker™ Green, has been widely used in previous studies to evaluate the mitochondrial activity within oocytes and embryos[66]. This combination of TMRE-MTG can be a valid option for our future investigation in the mitochondrial function changes of cryopreserved oocytes.

Besides, to further evaluate the mechanisms of cryodamage and how the fast cooling rate could potentially decrease such cryodamage, one of our next steps would be focusing on the relative gene expression. Previous research has suggested that indicators at molecular level can be more sensitive and reveal significant differences that may appear to be non-significant in the evaluation of developmental rates [25]. Many studies have also suggested that cryopreservation can result in DNA integrity and fragmentation in both embryos and oocytes, and cause epigenetic alterations that can greatly impact on later fetal growth [48, 67]. On the other side, multiple markers have been identified to be associated with oxidative stress, ER stress, or apoptosis, which can be easily tested by quantitative reverse transcription polymerase chain reaction (RT-qPCR) or RNA sequencing [24, 59]. Thus, future directions could be the relative gene expression comparison using mRNA extraction to conduct RNA sequencing or reverse transcription and RT-qPCR to evaluate whether and how the fastest vitrification cooling rate reduces cryodamage.
Lastly, as our study initially confirmed the superior outcomes of the fastest cooling rate produced by the NANUQ™ vitrification method over the in-straw method and the Cryotop® system, more investigations using different plunging rates by the NANUQ™ system need to be done in order to further confirm the effects of cooling rate on the improvement of oocyte and embryo viability post-thaw.
CONCLUSION

The cryopreservation system with the fastest cooling rate had generally better post-thaw outcomes compared with cryopreservation systems with slower cooling rates. The NANUQ™ vitrification method has potential to be used for cryopreservation of livestock embryos and potentially oocytes. Further experiments are necessary to confirm that the improved outcomes are indeed from the faster cooling rate ideally using the NANUQ™ system with slower plunging rate, increasing the sample sizes, investigating mitochondrial function alterations and relative gene expressions, and performing embryo transfer experiments to determine if the improved post-thaw outcomes observed correlates with higher pregnancy rates.
REFERENCES


