REGENERATION AND PRESERVATION OF POULTRY BREEDS THROUGH TRANSPLANTATION PROCEDURES ON GONADAL TISSUES

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By

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ABSTRACT

Live poultry flocks are costly to maintain, especially for rare breeds in small populations or non-commercial circumstances. One strategy to reduce these costs could be cryopreservation of the chick gonadal tissues and subsequent transplantation into recipients, which would help to maintain the genetic resources of some heritage chicken breeds that are rare and threatened with extinction. Thus, the overall goal of the study was to produce offspring derived from donors of different chicken breeds, including heritage chicken breeds. In our studies, a vitrification-warming procedure was employed to preserve chick gonadal tissues; and a modified transplantation technique was used to regenerate chicken breeds by the production of donor-derived offspring. An assessment of the vitrification-warming procedure was also conducted by using a morphological scoring system and detecting apoptotic cells via caspase-3 immunofluorescence. Additionally, cryoprotective agent (CPA) diffusivity measurements were made to facilitate mathematical prediction of minimally damaging CPA treatments.

One significant aspect of this study focused on post-cryopreservation chick ovarian tissue transplantation to address the failures of this procedure in previous studies. The goal of this experiment was to demonstrate that vitrified/warmed one-day-old chick ovarian tissues could be transplanted and developed generally in the different recipient chicken breeds and can produce the donor-derived offspring; in addition, to evaluate the potential of different chicken breeds to receive a donor graft. In particular, two main experiments were conducted on different chicken lines. The first experiment was transplantation between chickens of similar genetic background of Lohmann White (Oldenhof et al.) and Barred Rock (BR). The second experiment was transplantation between chickens of different genetic background Brown Leghorn (BL), BR and BL/BR. The

results differed depending on chicken lines. In the first trial, 50% of ovarian tissues were taken from adult recipients derived from donors of fresh and vitrified-warmed BR ovaries. Moreover, one hen appeared to have a donor-derived graft, as well as two of the embryos from this bird showed alleles of the donor bird. In the second trial, BL and BL/BR background could sustain the development of a graft regardless of the genetic background of the donor. However, all these grafts were enclosed by a membrane and had a variety of growth, suggesting some level of rejection by the recipients.

The assessment of the vitrification-warming procedure was conducted on one to three-day-old chick testicular and ovarian tissues. Two methods were used to evaluate the damages of the tissues: Periodic Acid Schiff-Hematoxylin (PAS-H) and an immunohistochemistry technique via caspase-3 immunofluorescence. From the first method, the morphological damages were evaluated, based on the morphological grading system. The second method showed the detection of apoptotic cell execution on the gonadal tissues. Overall, the results show that the vitrification-warming procedures affect the cellular integrity of one to three-day-old chicken gonadal tissues through morphological alterations. While the process does not cause more apoptotic cells on testicular tissues, there are some challenges in ovarian tissues for detecting apoptosis. Taken together, these results suggest the need to optimize this preservation technique for long-term storage and the surgery transplantation, especially in chicken ovarian tissues. Finally, to explore possible relationships between CPA (cryoprotectant agent) equilibration and tissue type, experiments were performed to determine the rate of diffusion of three CPAs into chick testes and ovaries. In the future, this diffusion rate can be used with a mathematical model to create an optimal protocol minimizing the toxicity of CPAs and intracellular ice formation, including diminishing gonadal tissue damages.

In summary, the studies of this thesis confirmed the potential of the cryopreservation of chicken genetic material through vitrification and the successful subsequent transplantation of the vitrified-warmed gonadal tissues into the appropriate recipients. Additionally, limitations have been described, such as the effects of the vitrification-warming procedure, the rejections from immune responses of recipients, etc. Some solutions were provided, which could increase the efficiency of the strategy to be practical for poultry studies in diverse fields.

CARE AND USE OF ANIMALS

The University of Saskatchewan's, University Committee on Animal Care and Supply (UCACS), Animal Research Ethics Board (AREB) approved all protocols and procedures mentioned in this thesis. With the UCACS number for these projects between 2017-2020 being 20120110. An on-site veterinarian was present for a portion of the surgeries to be able to monitor the well-being of chicks and to make sure the approved protocols were followed. All birds were housed and maintained in the Animal Care Unit of the Western College of Veterinary Medicine, unless otherwise mentioned.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
BR	Barred (Plymouth) Rock
CPAs	cryoprotectant agents
DBL	Dark Brown Leghorn
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPBS	Dulbecco phosphate buffered saline
EG	Ethylene glycol
FBS	fetal bovine serum
FMD	Formamide
HM	Holding medium
IHC	immunohistochemistry
LN_2	Liquid nitrogen
LW	Lohmann White
MHC	major histocompatibility complex
PAS-H	Periodic acid Schiff – Hematoxylin
PFA	paraformaldehyde
PG	propylene glycol
RT	room temperature
RWTW	running warm tap water
SD	standard deviation
SPSS	statistical package for the social sciences
VS	vitrification solution
WL	White Leghorn

CHAPTER 1. INTRODUCTION

Biodiversity and species conservation are critical topics with only vague solutions affecting extinction risk. Many factors are related to this including inbreeding depression, environmental change and disasters, human actions in over-exploitation leading to habitat and genetic diversity loss. Besides wild species, domestic animal biodiversity is also threatened due to changes in farming systems such as feed shortage, economic and demographic effects etc. It is especially apparent that because of marketing, industry control of the farming system according to customer demands, there is significant breed selection pressure in traditional farming goals (Cunningham, 1995). Many strategies for species protection have been identified including *in-situ* conservation, *ex-situ* conservation and the use of DNA technology.

Cryopreservation is a popular method used in ex-situ species conservation. Towards this goal, spermatozoa were the first reproductive cells successfully cryopreserved (Polge et al., 1949a). But nowadays, cryopreservation has been applied to many different species and other cells, tissues and organs (F. G. Silversides et al., 2013a; Jianan Liu et al., 2013a; Fahy et al., 2004). Slow-freezing and vitrification are two conventional processes used for the conservation of animal germplasm (Saragusty and Arav, 2011; Dalman et al., 2017). These two strategies have both beneficial and negative effects applied for different kinds of cells, organs. The slow freezing method was demonstrated to be suitable for specific cell types than multicellular types (Jianan Liu et al., 2013a) because specific cellular characteristics require different cooling rates (Mazur, 1963; Fahy et al., 1987). However, avian embryos or oocytes cannot be used for cryopreservation because of the characteristics of the egg yolk such as its large size and high lipid level. Therefore,

avian semen cryopreservation could be the best way for ex-situ management (E Blesbois, 2007; Zaniboni et al., 2014). Although regular use of some livestock semen cryopreservation appears in dairy and beef cattle, poultry semen cryopreservation results in fertility rates lower than mammalian species (Long, 2006).

With these limitations, other strategies are needed to conserve poultry genetic resources. These methods can include the perpetuation of live animals, blastodisc cryopreservation, and primordial germ cell cryopreservation (J.M. Pisenti et al., 1999). However, live animals are costly to maintain, especially for rare breeds in small populations or non-commercial circumstances. Polge (1951) demonstrated that avian germplasm, including semen, blastodisc, primordial germ cells (PGC), reproductive tissues, can be cryopreserved. Besides the low fertility rate of cryopreserved sperm, sex determination in the chicken *(Gallus gallus domesticus)* and birds are different from mammals having an XX:XY sex chromosome system with XY (heterogametic sex) represented male. In contrast, chickens and birds have a ZZ:ZW sex chromosome system with ZZ (homogametic sex) represented male (Smith and Sinclair, 2004). Thus, cryopreserved sperm does not contain the "W" chromosome to conserve the entire genome of the breed (F. G. Silversides et al., 2012b). The feasibility of cryopreserved PGC has brought more benefits than cryopreserved sperm that would enable the conservation of the whole genetics of the line (Moore et al., 2006). Moreover, cryopreserved chicken PGCs could be applied to connect genetic conservation with the reproduction of live stocks during early development (Nakamura et al., 2010). However, during germ cell development, there are not many germ cells represented in the germinal crescent, an extraembryonic region, after cryopreservation (Petitte, 2006) that will impact to the efficacy of genome preservation. Because of these limitations, transplantation and cryopreservation techniques on mammalian gonads (Schlatt et al., 2002; Gunasena et al., 1997a) have been

optimized and standardized for use on some species such as Japanese quail (*Coturnix japonica*)(J. Liu et al., 2013c; Y. Song and Silversides, 2008b) and chickens (F. G. Silversides et al., 2013b; Y. Song and Silversides, 2007a) by using vitrified gonadal tissues. With these achievements, transplantation and cryopreservation of the gonadal tissues can be used widely and effectively for regeneration and preservation of heritage chicken breeds, especially ovarian tissue transplantation that would conserve the entire genome of the breed.

Our ancestors raised heritage breeds before industrial agriculture became dominant. These breeds were selected and bred over time to develop characteristics that made them well-adapted to different environments. With intense industrial agricultural development, the genetic diversity of heritage breeds has been reduced by the selective pressure aiming to meet consumer demand such as increased milk or egg production as well as increased weight gain. In addition to agricultural industrialization, several major livestock and poultry breeds have lost the genetic diversity necessary to resist to new diseases and parasites and other environmental conditions that threaten their existence (FAO, 2015). Silversides's (Agriculture & Agri-Food Canada) research group is considered the first to optimize and standardize mammalian cryopreserved gonad transplantation techniques and applied them to some avian species. Their approaches included orthotopic ovarian transplantation (the transplant of tissue in its position) in the chicken (Y. Song and Silversides, 2006); cryopreserved chicken testicular tissue procedures to produce offspring (Y. Song and Silversides, 2007a); ovary transplantation in Japanese quail (Y. Song and Silversides, 2008b); and a model for cryobanking female germplasm in Japanese quail (J. Liu et al., 2013b; 2015; J. Liu et al., 2010). The surgical transplantation of vitrified-warmed ovarian tissues of different chicken breeds is still not reliably achieved, and evaluation of the viability of chicken gonadal tissues after vitrification and warming by different histological methods has not been

considered meticulously. This thesis intends to address this missing information, especially with respect to heritage chicken breeds. Overall, I hypothesized that optimization of transfer procedures of vitrified-warmed gonad into an appropriate recipient breed can improve the regeneration and preservation of the genetic diversity of poultry breeds. Two main objectives were to optimize the gonadal tissue transfer technique into different chicken breeds and evaluate the potential of different chicken breeds to receive a graft; and to optimize conditions for CPAs equilibration to minimize the toxicity, osmotic stress and freezing injury of gonads.

CHAPTER 2. REVIEW OF THE LITERATURE

2.1. Stream of chicken genetic resources

2.1.1. The role of animal genetic diversity

Genetic diversity is the variation of genes between distinct individuals of a species that produce a variety of traits. Genetic diversity varies significantly from species to species. For example, there is about a 3% variation in the genome of a fruit fly (*Drosophila simulans*) versus 0.1% variation in humans (Ellegren and Galtier, 2016). Habitat loss and degradation are causing the loss of genetic diversity in a population of many species and reducing their population size (Lowe et al., 2005; Barnett and Kohn, 1991). Additionally, unique genes will be lost in the next generation and lead to the loss of other genes, eventually depleting the genetic variation (Lande, 1988). Genetic diversity allows a species to adapt to harsh environments, weather, or changes in the temperature and to resist different diseases. In short, genetic variation is crucial in the survival and development of species (Yamin Liu, 2018).

The loss of genetic diversity is related to the extinction risk of species caused by four combined factors: demographic stochasticity due to age, genotype or body size; environmental stochasticity such as habitat loss, diseases; natural catastrophes, such as floods and fires; and genetic stochasticity due to changes in gene frequencies caused by founder effect or inbreeding (Shaffer, 1981). Also, reduced biological fitness associated with inbreeding depression can play a crucial role in the extinction of species. Inbreeding causes detrimental effects on reproduction and survival, including sperm production, age at sexual maturity, etc. (R Frankham et al., 2002a; Richard Frankham, 2005). Darwin was the first to perform experiments to clarify this issue in the comparison between self and cross-fertilization in the progeny of plants. Seed production and

height were reduced by 41% and 13%, respectively, in self-fertilization (Darwin, 1876; Richard Frankham, 2005). In small populations, extinction risks will be dramatically increased by loss of genetic diversity, causing the reduced ability of species to adapt to changes in the environment and response to diseases.

Genes that are accountable for inbreeding depression can be revealed by scanning the genome for markers to recognize its signature. With this method, some harmful recessive genes have been associated with inbreeding depression in many species such as the Scandinavian wolf (*Canis lupus*), where two hundred and fifty-eight microsatellite markers were used (Hagenblad et al., 2009). In other species such as the Belgian Blue cattle and Italian Chianina cattle, the recessive deficiency was mapped by the highly effective single nucleotide polymorphism technique (SNP, a mutating of a single nucleotide in the genome at a specific position), (Charlier et al., 2008). In another fruit fly (*Drosophila melanogaster)* QTL (quantitative trait locus) mapping (to identify the locations of genes in the genome) was used to analyze the lethal effects of harmful genes related to inbreeding (Vermeulen et al., 2008).

Human impacts and inbreeding effects are accountable for the fast decline of endangered species (Richard Frankham et al., 2014). Genetic diversity is a crucial factor and priority for adaptation to cope with future challenges (Scherf and Pilling, 2015). Ellegren and Galtier (2016) claimed that if across-genome diversity was determined accurately in various ways, such as selective pressure, adaptation, or purification, remarkable progress could be gained related to genome evolution, a determinant of genetic diversity, as well as species conservation. The term gene flow is used to depict the movement of animal genetic resources around the world by many different methods from live animals to frozen semen and embryos. These methods can lead to the augmentation of the genetic diversity of species because gene flow has created the growth of a

broad range of breeds that can adapt to all kinds of environments or new diseases (Scherf and Pilling, 2015).

2.1.2. Genetic diversity in poultry

In poultry, chickens and turkeys (*Meleagris gallopavo*) are the most ancient domesticated types with different purposes that include providing eggs, meat, and breed shows (physical traits and ornamentation). Additionally, chickens can be used as model species for vertebrate biology in basic and applied agricultural research and even as human biomedical disease models. A number of avian characteristics play an essential key in biological research, including availability, a high rate of embryo production and considerable experimental flexibility during embryo development. Current commercial strains were developed from popular breeds to produce rapidly growing hybrid strains that carry on desirable features for market demand such as high egg and meat production. In comparison, heritage chicken breeds adhere to other attractive features such as natural mating, long, productive outdoor lifespan, or a slow growth rate (livestockconservancy.org). However, it is costly and time consuming to raise and grow pure strains to satisfy the market (Mathias and Mundy, 2005). Some chicken breeds were employed for the surgical transplantation such as Barred Rocks (BR), Brown Leghorns (BL) and Lohmann White (LW). According to a history of breeds provided by University of Illinois Extension (History of Breeds, n.d), Barred Rocks originated from America that were mixed with different blood lines (in particular, a Dominique-Black Cochin or Dominique-Black Java cross); however, the exact origin is still controversial. This breed was put on the Standard of Excellence of the American Poultry Association in 1874. Another chicken line is Lohmann White that originated from Germany under the name of the company that reproduced new breeds and is one of the branches

in Lohmann hybrids. While Brown Leghorns come from Italy that belongs to the Mediterranean class and then brought in America in 1853.

According to a conservation list of Rare Breeds Canada, chicken breeds were classified basing on their population such as critical (Brown Leghorn, Saskatchewan Plymouth, for instance), endangered (Shaver White Leghorn, New Hampshire Red, for instance), at risk (Barred Plymouth Rock).

In general, the decline of poultry breeds is happening at an alarming rate. The genetic diversity is threatened due to the success of commercial strains, farm development, and inbred lines that were associated with homozygous and homogeneous offspring (J.M. Pisenti et al., 1999). A vast number of heritage breeds are at risk, and limited facilities are available for maintaining them. Moreover, funding is inadequate for research to conserve poultry heritage breeds (FG Silversides et al., 2008). Some of these populations have unique characteristics, and the reduction of genetic diversity could lead to a loss of unique alleles represented by unique features. For example, body shape, strength, and disease resistance are some dominant characteristics that have improved generation by generation. Commercial strains do not absolutely possess these unique features such as adaptation to different environments, and stronger disease resistance that might cause the loss of genetic diversity in the population. There are some limitations to hybrid chicken strains, including decreased disease resistance, a high risk of growing too fast, and a short egg-production life span (J.M. Pisenti et al., 1999). Therefore, it is necessary to maintain unique genes from heritage chicken breeds to stabilize the stocks.

2.1.3. Genetic diversity conservation programs

Genetic diversity conservation programs can be divided into three major groups: *in situ* conservation, *ex-situ* in vivo conservation and *ex-situ in vitro* conservation. *In situ* conservation is a way to keep species breeding in their native populations. Some benefits can be found in this technique, such as the adaptation of breeds to the environment, development of strategies regarding the management and support. However, this method can face risks of diseases and potential environmental disasters (Scherf and Pilling, 2015).

Ex-situ in vivo conservation is where live species are kept in a distinct area from their native population (e.g. on a zoo, farm, etc.). This method can facilitate reproductive management, especially if the population is tiny, and there are not sufficient samples for cryopreservation. However, these separated areas might not be similar to their natural environment and thus might not sustain the entire genetic diversity of breeds (Scherf and Pilling, 2015).

Ex-situ in vitro conservation is defined as the way to preserve species using reproductive and preservation tools that include keeping cells or tissues at cryogenic conditions for long-term storage and then reconstituting these materials into a live population. This technique can decrease risk of a disease outbreak or environmental changes. Depending on their category that could include critical, endangered and at-risk breeds, each breed or species will need a specific conservation strategy to increase the population, maintain genetic diversity, develop productivity or preserve additional cryogenic materials (Scherf and Pilling, 2015). Cryopreservation is a technology that can fulfill some *ex-situ in vitro* conservation purposes. For instance, cryopreserved genetic resources can be used to reconstitute populations as needed to solve issues related to genetics. Also, cryopreserved samples can be used for research to develop new breeds or genomic

studies. However, in the chicken, germplasm is limited due to a lack of hatcheries that provide rare or heritage breeds (Blackburn, 2006).

2.2. Gonadal cryopreservation and application

2.2.1. Cryobiology background

2.2.1.1. Principles of cryobiology

Cryobiology is the study of life at low temperatures. Cryopreservation is the preservation of life using low temperatures to reduce or eliminate metabolism and all chemical reaction. It is one of the ways to preserve biological materials such as oocytes, embryos, sperm, cell, tissue, and organs. However, freezing is usually lethal to living organisms. Cryopreservation aims to protect cells or tissues from injury due to low temperature (Shaw and Jones, 2003). It is understood that cryobiology can preserve the integrity of cells or interrupt biochemical reactions of cells (Mazur, 1970). Because of these effects, there are many fields of biology related to freezing and low temperature, such as cell physiology, food sciences, cryosurgery, etc. In particular, understanding ice formation is an especially important aspect of living cell responses to low temperature and crystallization of water.

At low subzero temperatures, ice formation will cause physical and chemical changes to cells (Fuller et al., 2004). Water plays an essential role in cellular structure and a key role in cryobiology. Cell volumes are 60 to 85% water that will interact with other intracellular elements, including proteins, lipids, and others. The state of water in cells is a critical aspect of cryobiology and includes the movement of water into and throughout the cell. Ice is formed by combining water molecules with ice nuclei at 0 °C or below in the condition of pure water. Water can then continue bonding on the ice surface to increase its size to form ice crystals. During this process energy is

released by the transformation of water into a crystal that can temporarily increase the temperature to the solution's melting temperature (e.g. 0 °C for pure water) and remain at that point until ice formation is complete and water still can endure the liquid phase under the melting point. Moreover, a process of cooling and warming will be associated with the physical transition of water in solution (Muldrew et al., 2004). At each physical transition, the concentration of solutes and temperature are dependent on each other. As ice formation removes pure water from the system, the melting temperature is reduced in the solution as the solute concentration increases due to colligative properties such as freezing point depression, boiling point elevation and osmotic pressure.

Cells lose water once they are exposed to low temperatures because intracellular water moves out under the vapour pressure differential (Mazur, 1963). This response depends on the rate of cooling and the cellular permeability of water. Cells will be dehydrated at a slow cooling rate or with high permeability of water due to the exosmosis of intracellular water. Otherwise, if water cannot leave the cell, intracellular ice will likely be crystallized. In other words, the permeability of the cell membrane allows water to move out of the cell so that intracellular volume will be decreased.

In summary, cells under the influence of freezing cause dehydration and concentrate intracellular and extracellular solutes (Mazur, 1963). Moreover, Mazur (1963) reported that cell viability after exposure to low temperatures at a slow cooling rate was higher than a rapid cooling rate. This was hypothesized to be because damaging intracellular ice formation would be minimized because water could move from the cell accordingly, and protoplasm would be kept at its freezing point. Many factors influence this survival including temperature, cooling rate, membrane permeability or cellular surface area. For example, slowly cooled yeast cells were killed

below -20 °C (Mazur and Schmidt, 1968), or human red cells are further be damaged with the increases in cooling rates (Rapatz et al., 1968). Furthermore, during the freezing process, the physical and chemical mechanisms of cells are altered, and these can affect cell viability. The optimum cooling rates that are sufficiently fast to avoid “solute damage” but not too fast allow for intracellular ice formation (Mazur, 1970). Cells that can survive at a specific cooling rates require correct warming rates; therefore, the warming rate also contributes to the survival of cell (Woods et al., 2004).

Successful cryopreservation techniques depend on many factors. Evidence suggests that the warming rate and appropriate cryoprotectant solutions are among the most critical factors for the cryopreservation of biological materials (Shaw and Jones, 2003). In addition, in the absence of careful treatment, these living materials will be killed by very low temperature (e.g., in liquid nitrogen) due to intracellular and extracellular ice formation; thus, many methods have been used to minimize this damage including cryoprotectants, antifreeze proteins (O'neil et al., 1998) or ice blocking factors (Wowk et al., 2000). Cryoprotective agents (CPAs) increase the survival of cells during the freezing and warming process (Rajan and Matsumura, 2018), including human sperm (Gao et al., 1997), erythrocytes (Terwilliger and Solomon, 1981) or oocytes (Hunter et al., 1992).

The equilibration of high concentrations of CPAs is required, which contributes to the success of the cryopreservation (Elmoazzen et al., 2005). During cooling and warming, the colligative properties cause a reduction in melting temperature, and the resulting increased viscosity causes a reduction in the likelihood of intracellular ice formation (Mazur, 1984). However, osmotic effects can damage cells during the equilibration with CPAs, and CPAs can be toxic at a high concentration. At a high concentration of permeating CPAs, rapid dehydration occurs when adding CPAs that makes cells shrink, and rehydration happens quickly when

removing CPAs that makes cells swell. These cell responses to CPA equilibration protocols can cause damage or cell death (Mazur and Schneider, 1986). One possible way to avoid this damage is adding high concentrations of sugar as an impermeable solute to minimize osmotic effects during CPA removal after warming (Leibo, 1984).

The "critical concentration" of CPAs is the concentration required to prevent ice formation during cooling (Woods et al., 2004). Thus, CPAs play an essential role in maintaining cell survival post-cooling and warming, and CPAs are critical to successful cryopreservation, but it is well understood that CPAs are toxic, which is a critical barrier to cryopreservation of cells (Best, 2015; Fahy, 1986; Gilmore et al., 1997). Toxicity is also dependent upon temperature, concentration, and has varying mechanisms of action depending on which tissue or cells are used. CPA toxicity can manifest as the release of caspases, proteases, or kinases in cryopreservation of cells or tissues and results in apoptosis execution (Bissoyi et al., 2014). CPAs interrupt the hydrogen bonding of water in the solution to prevent ice formation, and these influences induce non-specific toxicity (Towey and Dougan, 2012). Some popular CPAs have been used, such as ethylene glycol (EG), propylene glycol (PG), dimethyl sulfoxide (DMSO), formamide (FMD). All of these are permeable CPAs that can pass the cell membrane.

It has been shown that toxicity can be reduced by combining different CPAs. One study showed that a combination of DMSO with PG or FMD could reduce toxicity compared to PG alone (Jomha et al., 2010). Moreover, non-permeable CPAs are often combined with permeable CPAs because of the ability to reduce extracellular ice formation and prevent intracellular ice formation (Meryman, 2007). One such example, sucrose, a non-permeable CPA, is often used as an extracellular CPA due to the limited ability to pass cell membranes. In addition, these non-permeable CPAs help to facilitate the absorption of permeating CPAs to cross cell membranes by

the prevention of excessive osmotic swelling; thus, contributing to the decrease in the toxicity of permeating CPAs. Also, adding sugar dehydrates cells contributing to the prevention of ice formation, and allows the regulation of osmotic pressure upon warming and removal of cryoprotectants (Leibo and Songsasen, 2002).

2.2.1.2. Two methods of cryopreservation: slow freezing and vitrification

There are two main methods for cryopreservation: slow freezing and vitrification. Slow cooling allows sufficient time for water to move out of the cell during cooling as the osmotic pressure in the intracellular water equilibrates with extracellular water (Mazur, 1984). However, this method can be ineffective for tissues or organs, multicellular systems, because of the intracellular and extracellular ice formation that can damage tissues or organs. It means that multicellular systems are complex with different cell types requiring optimal cooling rates to prevent damages (Jianan Liu, 2013). Additionally, organ freezing has not been very successful because of the combination of different cells, different responses to freezing, and their large sizes with large volumes (Alink et al., 1977). Thus, the increase in the concentration of solutes, along with suitable cooling rates, would be considered to avoid ice formation and solution effect (Jianan Liu, 2013). Also, slow freezing leads to shrunken cells through osmosis, and cytoplasm volume decreases equally with the decrease of cell volume (Meryman, 1974). The harmful effects were reported in human red blood cells where viability decreased with slow freezing conditions, such as low temperature or cryoprotective agents. The viability of cells could be dependent upon three possible significant factors, including cooling rate, thawing rate, cryoprotective agents, and their concentrations (Mazur, 1984). Cryoprotective agents are used along with appropriate cooling rate depending upon cell types based on a programmable freezing device in a slow freezing procedure

(Kuleshova et al., 2007). In addition, the slow freezing technique can be costly and time-consuming, requiring expensive devices for controlling the cooling rate.

Vitrification is the process of the ice-free solidification of a liquid to form a glass-like substance. This solidification process is generally considered safe for cells. However, vitrification requires high concentrations of cryoprotective agents and typically a super-fast cooling rate (Fahy et al., 1984). At these cooling rates, the glassy state of water is formed when the viscosity increases enough that no ice crystals can form. This can lead to avoiding ice-related cell damage and has been applied to living biological systems. For example, the vitrification method was successfully applied to mouse embryos (Rall and Fahy, 1985). Mazur (1984) showed that human red blood cells had higher survival at a faster cooling rate with a rapid warming rate compared to a slower cooling rate with slow thawing. Another study found the same result on mammalian embryos (Whittingham et al., 1979). Vitrification comprises the combination of penetrating CPAs to help the formation of viscosity and non-penetrating CPAs to help dehydrate inside the cell (Kuleshova et al., 2007). To vitrify, it is necessary to satisfy some conditions, including very high cooling and warming rates, and adequate solute concentrations. The faster cooling and warming rates are essential so that a stable amorphous state can be achieved in a selected solution.

Cryopreservation through vitrification is becoming increasingly more widely used to preserve various biological materials for long term storage (J. Liu et al., 2012; Fahy et al., 1984). As opposed to slow freezing, vitrification has some advantages: directly plunging samples into liquid nitrogen is much simpler and faster than the use of controlled rate (slow) freezing. Additionally, vitrification is not dependent upon serum or proteins to culture cells that some slow freezing protocols use (Kuleshova et al., 2007). The addition of these sera or proteins may cause adverse effects for transplantation. Some reports have shown that the high contamination of mouse

protein used during cryopreservation penetrated embryonic stem cell (ESC) lines and caused the mutation of ESC due to the presence of sialic acid in mouse protein (Martin et al., 2005). Moreover, to protect embryo samples during the vitrification procedure, a specific strategy was designed during cryopreservation in liquid nitrogen by using a double straw to eliminate contamination (Kuleshova and Shaw, 2000). Besides those advantages, vitrification has also shown limitations. Using cryoprotective agents (CPAs) at high concentrations is one of the adverse effects due to their toxicity and osmotic damages that might be caused by the penetration of CPAs moving inside or outside the cell. However, these limitations may be reduced by the use of combinations of CPAs (Saha et al., 1996) and using stepwise dilution to prevent osmotic shock.

In general, measures of the efficiency of cryopreservation methods are based on the quality of samples post-thawing and survival compared to fresh samples. To evaluate slow freezing or vitrification and select the appropriate method, it is necessary to consider whether the same factors are employed in both methods, age, size, and supplementation. In a few special cases, vitrification is used instead of slow freezing due to its characteristics (Szeptycki and Bentov, 2016), but in general, cell therapy and cell culture cryopreservation are mostly slow freezing. Some reports have shown that vitrification has been preferentially employed for human oocytes and embryos with high survival, and fewer intracellular injuries (Edgar and Gook, 2012; Setti et al., 2014; Wang et al., 2012). Vitrification also has some benefits such as low cost, less time, and less liquid nitrogen required (Klocke et al., 2015). Vitrification has been applied for gonads and other organs on various species with the potential of the following transplantation (Arav et al., 2005). For human ovarian tissue, slow freezing is considered more advantageous because it allows adequate time for CPAs to penetrate into the tissue, but adoption is somewhat limited due to the

cost, long process with a programmable freezer, and the need for more liquid nitrogen (Klocke et al., 2015).

2.2.2. Applications of cryopreservation to genetic management

Cryopreservation is an indispensable technique in reproductive technology, and especially in the genetic fields where germ cells are cryopreserved for long-term storage and warmed as needed. This is particularly significant in application to rare endangered species to prevent extinction. Furthermore, the cost and labour for cryopreservation are reduced compared to keeping a live colony. Also, cryopreservation can be used to regenerate later generations that can avoid the effects of diseases, environmental changes, or genetic mutations in current populations (Rajan and Matsumura, 2018). In general, cryopreservation has become essential for preserving genetics that can be used in the future. Cryopreservation has been applied to several reproductive cells such as spermatozoa, embryos, and oocytes and in reproductive tissues, including ovarian tissues and testicular tissues (Woods et al., 2004). For example, semen is frequently cryopreserved to exchange genetic resources of superior animals or valuable endangered species. To wit, the mouse is used as a popular model for basic research with mammalian species, and mouse sperm was one of many materials that were cryopreserved to maintain the genetic resources (Ward et al., 2003). In 1949, Polge et al. successfully cryopreserved fowl semen (Polge et al., 1949b). This achievement made a significant impact on using artificial semen insemination (AI) broadly, especially on dairy and beef cattle (Karow and Critser, 1997). Spermatozoa cryopreservation is extremely important for cancer patients that preserved sperm before treatments and can be used to prevent disease transmission to females.

However, the sperm cryopreservation procedure depends on many factors, including transmembrane and trans-tissue CPA movement, cooling, and warming rates. These factors can be evaluated to minimize cellular freezing injury (GJ Morris et al., 1999). For example, human spermatozoa were successfully cryopreserved by some alterations of cooling rate, choice of CPA, and extenders (Gilmore et al., 1997). In particular, a selection and concentration of CPA can be made to prevent osmotic stress, or the optimization of the cooling rate could minimize intracellular ice formation (Woods et al., 2004). However, different species spermatozoa exhibit different biological characteristics, tolerance to temperature, and CPA toxicity, so an appropriate cryopreservation protocol is considered species-dependent (Phelps et al., 1999; Kumar et al., 2003). Vitrification has also been used for spermatozoa cryopreservation (Le et al., 2019), but many limitations have been shown in this method, such as disease transmission due to the direct contact of open straws with liquid nitrogen that could be a vector for disease transmission (Isachenko et al., 2019). In summary, these studies suggest that the improvement of the sperm cryopreservation technique depends on many factors, but that successful sperm cryopreservation has significant applications in many fields such as agriculture, medicine, and biology. In general, slow freezing is the preferred method for spermatozoa cryopreservation, but vitrification techniques for sperm freezing are being improved (Hidalgo et al., 2018).

Oocytes are defined as an immature ovum and are produced during female gametogenesis (Rajan and Matsumura, 2018). Cryopreservation of oocytes plays an important role in different species in assisted reproduction technologies. (Matsumura et al., 2018), yet low temperatures break up cytoskeletal elements, plasma membrane, and leads to low fertilization and cell injury (Vincent and Johnson, 1992). Because of these factors, oocytes tend to be sensitive to cell freezing injury, primarily intracellular and extracellular ice formation (Leibo et al., 1996). The permeability of

oocytes is low to water and CPAs due to the small surface area to volume ratio. This means that slow freezing approaches must be much slower than for other somatic cell types and sperm. Because of this, vitrification has been applied to human oocytes in several studies, but the success rate was low as measured by low survival, low fertilization or abnormal development of oocytes after cryopreservation (Matsumura et al., 2018). Vitrification requires high concentrations of CPAs that can cause osmotic stress and toxicity effects from CPAs (Rayos et al., 1994; Fahy, 1986). However, Kuleshova et al. (1999) were able to successfully vitrify mature oocytes with ethylene glycol, resulting in a baby born. Vitrification has been improved by modification of some factors such as CPAs, temperature, cooling, and warming rate to protect samples (Gutnisky et al., 2020; Mazur and Seki, 2011).

An embryo is an early growth stage of a multicellular organism after fertilization of an oocyte with a sperm. Cryopreservation of embryos can maintain endangered species and genetic resources. The first achievement was in mouse embryo cryopreservation, and the procedure started using broadly in many studies on different species such as a rat, rabbit, sheep through a slow freezing method (Willadsen et al., 1976; Whittingham, 1975; Whittingham and Adams, 1976). Moreover, mouse embryos that were vitrified with the combination of different CPAs retained high viability (Rall and Fahy, 1985) and opened the potential of embryo vitrification for humans (Trounson and Mohr, 1983; Li et al., 2007). With embryo cryopreservation, rodent genetic resources can be maintained at a low cost, preventing genetic loss due to their short life cycle (Woods et al., 2004). In the bovine industry, breeding programs have been significantly advanced by embryos cryopreservation, and these cryopreserved embryos can be provided to recipients as needed (Woods et al., 2004). Both slow and rapid freezing is used for embryo cryopreservation, depending on species, but in light of the long processing times and cost of slow freezing, rapid

cooling may be preferable as it has been improved and achieved success in avoiding intracellular ice formation, osmotic stress, and appropriate warming rate (Woods et al., 2004). With cryopreservation, embryos can be stored and used as needed. However, there are still many challenges related to this technique, such as cooling rate, temperature, and warming rate, impacting survival (Wittingham, 1972). These approaches are not valid for avian species because of the large egg yolk size and high lipid concentration.

Cryopreservation of testicular tissue is a technique that can be considered as a source of sperm for future uses (Hovatta, 2000; Honaramooz, 2012). This method allows the retrieval of advanced stage germ cells for further application in reproduction, but each species needs to have an optimal freezing procedure (Reš et al., 2000; Avarbock et al., 1996). In particular, some reports have shown that the male germline of species could be maintained by grafting testicular tissues of young pigs into mice (Honaramooz et al., 2002) or germ cell transplantation in fish (Okutsu et al., 2006). This means that cryopreservation becomes a critical reproductive tool due to the combination of those technologies in different growth stages of germ cells (Gosden and Nagano, 2002). Spermatozoa from testicular tissue cryopreservation were used to inject mouse oocytes for fertility to produce offspring as an example (Kimura and Yanagimachi, 1995). Moreover, immature species spermatozoa, which cannot be cryopreserved due to the absence of functional germ cells, would be replaced by testicular grafting in maintaining the growth of the testes. However, some issues can occur during the cryopreservation procedure of this tissue kind because of large size and the combination of different cell types such as spermatid, Leydig, Sertoli cells, among others. Because of the heterogeneity of cells, cryopreservation of testicular tissue requires a complicated protocol to satisfy all conditions include temperature, cooling and warming rate, CPA concentrations to prevent osmotic stress, injuries or damage to cells. The adverse effects of

cryopreservation protocols were shown in rat testicular biopsies that showed damage in seminiferous tubules, especially on the basal membrane of Sertoli cells (Jezek et al., 2001). In summary, testicular tissue cryopreservation is a "backup" method in case other methods for reproductive preservation are not appropriate or available, although there are some limitations in the procedure. Therefore, there is a need for species dependent cryopreservation protocol modification including cooling and warming rates, CPA concentrations, osmotic conditions to prevent damages for tissues.

Cryopreservation of ovarian tissue is an exciting method in a variety of fields, from agriculture to medicine, especially in the reproduction with animal models because after warming, cryopreserved ovarian grafts can be manipulated to stimulate the primordial follicles (Woods et al., 2004; Gunasena et al., 1997b; Oktay et al., 1998). In the ovarian tissue cryopreservation process, CPA concentration plays an essential role in the integrity of tissues. As an example, glycerol was employed in mouse ovarian graft cryopreservation that caused oocyte damages at a higher concentration, and grafts did not survive after warming (Parrott, 1960). Another report showed that glycerol was a slowly permeating CPA that caused osmotic stress during the addition or removal period (Paynter et al., 1999). To address this limitation, different CPAs were explored, such as ethylene glycol, propylene glycol, and it was found that slow freezing and a combination of CPAs can be successfully employed in ovarian tissue cryopreservation where success was demonstrated by follicular growth in mice (Gunasena et al., 1997b). In short, cryopreserved ovarian tissue transplantation is a potential approach to human infertility issues or maintenance of genetic resources for endangered species (Agca, 2000; Klocke et al., 2015; Dalman et al., 2017).

2.2.3. Application of cryopreservation to avian species

2.2.3.1. Cryopreservation of avian primordial germ cells

Primordial germ cells (PGCs) are undifferentiated primary gametes from which oocytes and spermatogonia are derived from through meiosis and can be differentiated for genetic alteration. They have applications in both research and industry because PGCs can be used as a cell-based system for modifications (Pérez Sáez et al., 2016). PGCs play an important role in transferring genetic information from one generation to another by gametogenesis (Nakamura, 2017). In chickens, there are 46 stages of development from fertilization until hatch (Hamburger and Hamilton, 1951). Avian PGCs are distinct from mammals because PGCs move through the blood circulation to the genital ridge (Nakamura, 2017); thus, PGCs can be collected from the bloodstream at stages 14 to 16 of embryonic development or stages 26 to 28 located in the germinal ridge (Petitte, 2006). Petitte (2006) also reported that culturing cells could be used to increase the amount of PGCs in, for example, endangered species before cryopreservation because of the small population. To achieve this transfer of genetic material from one individual to another, the first step is to remove endogenous PGCs from the recipient host before transferring donor PGC (Naito et al., 1994). Morphologically, avian PGCs consist of a nucleus with lipids in the cytoplasm. The amount of glycogen in the chicken PGC cytoplasm can be detected by periodic acid-Schiff staining (PAS) to distinguish among their development stages, but no glycogen is found in Japanese quail PGCs (Nakamura, 2017; Swartz and Domm, 1972). Additionally, it is worth noting that avian sex determination is at fertilization, and the sex chromosomes are combined to form male (ZZ) or female (ZW) zygotes. These biological characteristics help to establish the appropriate techniques for conservation the genetic resources (Nakamura, 2017).

The genetic resources of endangered or heritage chicken breeds play an essential role in the biodiversity of the species. Because contagious diseases may result in catastrophic mortality, it is necessary to develop cryopreservation methods and re-establish valuable avian genetic resources. Cryopreservation methods can maintain these genetic resources and enable them through crossbreeding as needed (Svoradová et al., 2019). However, preserving and regenerating these genes requires much effort due to biological properties, including the fact that ova are covered with a large yolk that makes cryopreservation a near impossibility. To overcome this and other challenges, PGC cryopreservation has been developed to maintain poultry genetics. This approach is particularly attractive because the migration route of avian PGCs is specific, and thus easy to access and can be applied for further studies (Molnár et al., 2019).

Slow freezing is a popular technique for chicken PGCs. Several studies have reported different results regarding PGC viability and apoptosis in using different CPAs, concentrations, cooling, and warming rates (Tonus et al., 2016; Sawicka et al., 2015). Using different commercial CPAs (marked as A, B, C & D) and slow cooling approaches, one paper was reported high rates of viability and recovery of PGCs from chicken embryos (Setioko et al., 2007). In addition to slow freezing protocols, vitrification has also been used for chicken PGCs and was reported more efficient than slow freezing in gonadal colonization rates (Tonus et al., 2017). However, Kohara et al. found that gonadal germ cell (GGC) viability was reduced using vitrification compared to slow freezing (Kohara et al., 2008).

With efficient PGC cryopreservation techniques, poultry gene banking has been employed to regenerate valuable genetics. The purpose is to conserve the precious genetic diversity of poultry stocks with efficient techniques at a reduced cost compared with maintaining live stocks. In this approach, PGCs, immature ova and semen are cryopreserved in liquid nitrogen and

transplanted to appropriate recipient hosts. These recipients are raised until maturity and artificially inseminated or mated to produce the new generation to maintain the genetic resources (Nakamura, 2017; Woodcock et al., 2019). Although this method has been improved to increase the success after cryopreservation, the whole process to produce offspring from PGCs cryopreservation may result in chimeric birds with a low level of germline transmission. Moreover, the long process may be costly, but the efficiency is not high in preserving genetic resources (Petitte, 2006).

2.2.3.2. Avian semen cryopreservation

Semen cryopreservation has been employed for more than fifty years (E Blesbois, 2007; Shaffner et al., 1941; Lake et al., 1981). Cryopreservation of avian semen is a common technique to maintain the genetic resources without negative effects for donors and recipient hosts. However, this technique cannot preserve the W chromosome in the heterogametic sex of the female due to the homogametic sex of the male (E Blesbois, 2007). Additionally, cryopreserved avian semen is stored in cryobanks for future needs (E Blesbois, 2007). One advantage of semen cryopreservation is that a large number of materials can be stored at the same time (Benesova and Trefil, 2016). In particular, avian spermatozoa show distinct biological characteristics, including a plasma membrane with a high surface area to volume ratio and cytoplasm at a small ratio in comparison, and mitochondria or condensed chromatin in the nucleus (Gliozzi et al., 2011; Etches, 1996). Thus, the plasma membrane and mitochondria may be mainly damaged and would have negative effects on fertility (E Blesbois, 2007). Moreover, the damage of the membrane may impact cell function due to the presence of carbohydrates in the plasma membranes of spermatozoa (Peláez and Long, 2007). Also, the CPA dependence of acrosome reaction that influences fertility after warming was demonstrated (Moce et al., 2010). Furthermore, limitations regarding CPAs, concentration, cooling, and warming rate conditions are required to obtain high survival spermatozoa after

warming (Massip et al., 2004). It was reported that the fertility rate of post warming chicken semen was remarkably lower than in mammalian species (Wishart, 1985). Several studies have been successfully reported regarding fowl sperm viability, post thawing, and the acceptable fertility rate (Chalah et al., 1999). The success rates of bird semen cryopreservation vary from species to species or even in the same breed (Alexander et al., 1993). Additionally, the success of the cryopreservation of rooster semen regarding the viability, recovery, and fertility depended upon the breed and age (Long et al., 2010).

Membrane fluidity plays a vital role in bird spermatozoa that the physiological state can be recovered post-cooling and warming. Chicken *(Gallus gallus domesticus)* have higher fluidity than guinea fowl and, therefore, when evaluated, demonstrate higher sperm viability and intact morphology (E Blesbois et al., 2005). These results were explained by reducing cholesterol/phospholipid ratios after cryopreservation on guinea fowl (E Blesbois et al., 2005). One paper showed that avian spermatozoa reacted differently, depending upon osmotic changes, equilibration time, cooling-warming rates, temperature and CPAs concentration (Juan M Blanco et al., 2000). In a comparison of avian species, slower freezing caused damage to turkey and guinea fowl spermatozoa, but this could be avoided through a fast freezing method that kept sperm at an acceptable fertility rate (Varadi et al., 2013). This was echoed by Liu (2013), who reported that vitrification was more suitable for avian spermatozoa than slow freezing. Vitrification was also employed on Japanese quail spermatozoa and showed that spermatozoa could tolerate conditions of cryopreservation that resulted in a normal fertility rate (Kang et al., 2016). Avian semen cryopreservation is one of the critical methods to maintain genetic resources.

2.2.3.3. Avian testicular tissue cryopreservation and transplantation

In birds, a pair of testes is located along the dorsal side of the abdominal cavity, and the left testis is usually larger than the right. The tunica albuginea, a connective tissue layer, covers the testes. Each testis is constituted of many convoluted seminiferous tubules that consist of spermatogonia, primary, secondary spermatocytes, spermatids, spermatozoa, and Sertoli cells. A very thin layer divides seminiferous tubules and contains a few interstitial cells (Bacha Jr and Bacha, 2012).

While avian semen cryopreservation has been employed to preserve species' genetic materials, several limitations such as variable fertility of cryopreserved semen and challenges in collecting semen and performing AI on some breeds are barriers to the success of the method (Elisabeth Blesbois, 2011; E Blesbois, 2007). In this situation, testicular tissue cryopreservation and transplantation strategies have been employed to conserve the male germlines of heritage or endangered chicken species (Juan Manuel Blanco et al., 2009; Jianan Liu, 2013). Moreover, an attractive feature of this approach is the high recovery rate of germlines that may be obtained from this method due to the presence of spermatogonia in the testes. There have been several reports (Y. Song and Silversides, 2007a; F. G. Silversides et al., 2013b) of successful cryopreservation of chicken testicular tissue using slow freezing where offspring were produced, and the results showed that spermatogenesis occurred in the seminiferous tubules. These reports indicated that germ cells could maintain viability during the cryopreservation process (Y Song and Silversides, 2007a). However, this technique also carries some limitations regarding cooling and warming rates since tissues with multicellular structures are more complicated and much larger than single cells.

A vitrification protocol was applied to Japanese quail testes preservation, and the efficiency of the method was evaluated by using the chicken CAM (the chorioallantoic membrane, a vascular membrane) for grafting. After warming and transplantation, vascular formation and the viability of the vitrified/warmed tissue were at an acceptable level when compared to fresh tissues (Jianan Liu, 2013). Alternatively, by using slow freezing for testicular tissues, slow cooling rates give adequate time for the tissue to equilibrate the interior and exterior cells that may facilitate the avoidance of damaging intracellular ice formation. Still, the efflux of water out of cells into tissues leads to extracellular ice formation that generally is not assumed to cause much damage to most cells (Woods et al., 2004). Slow warming causes recrystallization or devitrification during the process that leads to cell injuries (Mazur and Seki, 2011).

Presently, vitrification protocols are preferred for preserving testicular tissues (Jianan Liu, 2013). Post warming, transplantation has resulted in subsequent regeneration of the cryopreserved testicular tissues. The surgery is simple to transfer either whole donor testes or small testicular pieces into the position of the host testes that are removed. There are no manipulations of connection with the vas deferens for transporting sperm because of the tiny size of structures. To increase the success of cryopreservation and transplantation, pre-transfer castration of the recipient has contributed to the resumption of testicular tissue function measured by spermatogenesis (Y. Song and Silversides, 2007a). In chickens, a slow freezing testicular tissue cryopreservation method achieved a high rate of recovery and produced offspring derived from donors through orthotopic transplantation (Y. Song and Silversides, 2007a). Moreover, vitrification has been applied for chicken testicular tissues, that were then transferred to recipient hosts of similar or different genetic backgrounds (Liptoi et al., 2013; Liptoi et al., 2020). The success of allografting depends on breeds at a rate ranging from 20% to 100%. In summary,

testicular tissue cryopreservation and transplantation strategies have provided a solution to regenerate the genetic resources of male germlines that could cover some negative effects of previous techniques. However, these strategies depend upon many factors such as breed, cooling or warming rates, age and castration (Y. Song and Silversides, 2007a).

2.2.3.4. Avian ovarian tissue cryopreservation and transplantation

In most avian species, the left ovary is active with a normal function that depending on age. However, at hatch the right ovary is present but does not function and gradually degenerates or remains small and inactive during the life of the bird (Rothchild, 2003). In the chick, the ovary is dorsally located, and while initially very small, it obtains a more significant scale at sexual maturity. The outer and inner portions of the ovary consist of the cortex and vascular medulla respectively. In the cortex, the stroma contains the most follicles with different stages, including developing and large-yolk oocytes covering the spherical nucleus. Each oocyte is covered by four layers, including perivitelline, granulosa, internal theca, and external theca (Bacha Jr and Bacha, 2012). Moreover, mature oocytes and embryos are extremely difficult to cryopreserve because of the large yolk and massive quantity of lipids. Because of these limitations, the cryopreservation of ovarian tissues has not been considered as an alternate choice for the preservation of poultry genetic resources. A large number of primordial follicles in avian ovarian tissues could produce mature follicles that would be recovered after transplantation (Johnson and Woods, 2009).

Ovarian tissue cryopreservation was successfully demonstrated on Japanese quail, and a vitrification protocol was more successful compared to slow freezing (J. Liu et al., 2010). A vitrification procedure was shown to maintain intact tissues with a high recovery rate, and no negative effects of vitrification on the follicle formation were found (Y Song et al., 2007). Also,

the recovery of vitrified ovarian grafts was demonstrated by the viability and vascularization in the transplantation of these grafts into chicken CAM (Jianan Liu, 2013). To achieve repeatable high survival and high cooling rates, tissues were punctured by acupuncture needles, plunged into liquid nitrogen, and stored long term in cryovials in liquid nitrogen. These changes have contributed to the success of this method in Japanese quail ovaries (J. Liu et al., 2010).

After warming, ovarian tissue can be transferred into the selected breeds to regenerate the valuable genetic resources that contribute to the genetic diversity of species. The first transplantation of the fresh ovarian tissues was conducted on chicken from 24-30 days old, but no ovarian grafts were formed at maturity (Grossman and Siegel, 1966; George Hall, 2015). Another study showed that the orthotopic transplantation of chicken fresh ovarian tissues from 30-45 day-old chickens obtained low breed-dependent fertilization rates (Kosenko, 2007). These failures could be explained by a high immune rejection intensively occurring in older chicks (Mast and Goddeeris, 1999). Alternatively, one to three-day-old chicks have an improved ability to receive a donor graft until maturity (Y. Song and Silversides, 2006; 2007b). For example, the transplantation of ovarian tissue of Muscovy ducks (*Cairina moshcata*) into Pekin ducks (*Anas platyrhynchos*) was confirmed by the presence of donor grafts that produced offspring (Y Song et al., 2012). Furthermore, another study showed that week-old Japanese quails could tolerate the transplantation of adult ovarian tissues of different genetic backgrounds (J. Liu et al., 2015). These studies could indicate that each species may respond differently to donor grafts, and challenges still exist after transplantation because of the immune response of the host to the donor. Immunosuppressive drugs have been applied to address this issue. However, in several studies, no significant differences were found between the group using the drug and the group without using the drug (Y. Song and Silversides, 2007b; 2008b).

Taken together, transplantation of cryopreserved ovarian tissue can be used to regenerate previous genetic resources of heritage or endangered avian species. As discussed above, this strategy was applied to ovarian tissues of Japanese quail, where both slow freezing and vitrification methods produced offspring derived from donors (J. Liu et al., 2010). In summary, heritage or endangered avian ovarian tissues could be cryopreserved and transplanted successfully for some species such as quail, chicken, or duck. The valuable genetic resources could be maintained from generation to another. Vitrification has shown to be more efficient than slow freezing regarding viability, vascularization, morphological integrity, and the recovery ability after transplantation. To obtain improved recovery, many factors in the procedure require modifications, include CPA type, concentration, cooling and warming rates, that would prevent negative effects of intra or extracellular ice formation and minimize CPA toxicity. Regarding the post cryopreservation transplantation, the combination between donor and recipient breeds plays an essential role in the success of the strategy that produces offspring derived from donors. Many studies have reported that the success rates were different depending upon the breed (Liptoi et al., 2020; Liptoi et al., 2013).

2.3. Avian immune system in transplantation

In general, the immune system consists of the innate and adaptive systems. The innate system helps an organism to react quickly after the invasion of foreign agents. The reaction can result in cellular inflammation that can last longer than the initial invasion and have a significant impact on tissues. In the meantime, the adaptive immune system is also triggered by the innate system, but changes depending upon the kind of foreign antigens or infections experienced during the life of the organism. T cells and B cells are the main components of the adaptive immune reaction (Farrar et al., 2013). These responses, including the inflammation response occurs after the transplantation of tissues or organs because heterologous grafts are considered foreign agents and rejected by the host's immune system. To avoid rejection, many factors must be assessed, including the use of immunosuppressive drugs and the selection of the compatibility of species in transplantation (Jianan Liu, 2013).

2.3.1. The importance of the avian immune system in transplant rejection

The chicken immune system is one of the most critical systems of avian species to study because of their economic contribution. In general, avian immune responses are similar to mammalian species and include innate and adaptive immune systems. Cell-mediated and humoral immunity are two main response mechanisms that involve T cells and B cells, respectively. In birds, a variety of cells and organs are associated with the immune system and include lymphoid tissues, bursa of Fabricius, spleen, bone marrow, Meckel's diverticulum, and the Harderian gland. Among them, lymphocytes play a critical role in the prevention of the invasion of foreign antigens (Davison, 2014), and as such, an essential part in tumour graft rejection, also in fighting infections (Silverstein, 2001). In addition, Silverstein found that graft failure was related to the lymphocytes that the rejection was considered as a consequence. One possible explanation was that grafting led

to the interference of vascularization, and cellular immune responses caused the rejection. The bursa of Fabricius also plays an integral part in the adaptive immune response. This bursa is located near the cloaca under a sac-like structure and is considered a primary lymphoid organ (Kaspers and Schat, 2012). For decades researchers have speculated on the specific functions of this bursa. For example, one report showed that antibody production was related to the bursa of Fabricius (Glick et al., 1956). The chicken antibody is found in the late period of embryos and after hatching in a short time. At young ages, B cells go through a process to fully form antibodies until 5-7 weeks, and the bursa is mature at this period (Kaspers and Schat, 2012). Because of these features, any foreign antigens have negative impacts on this bursa and cause negative effects on the antibody level. When chickens reach sexual maturity, the bursa of Fabricius regresses, and B cells are generated from the bone marrow.

Newly hatched chicks have an immune system that is not fully developed and requires assistance from hens (Speer, 2015). Serum immunoglobulins are provided to chicks at a very early period of egg formation from hen's serum. At the beginning stage, egg yolk contains IgY from blood, and then IgM and IgA are added from the oviduct after the fertilization. The chicks are assisted by the maternal antibodies maintained in the yolk sac within a few days after hatching, and chicks will be able to form and develop their complete immune system (Speer, 2015). The avian immune response depends upon the genes and relates to innate and adaptive immunity that detects foreign agents or antigens and submits them to T and B cells. Several cellular components such as macrophages, thrombocytes, natural killer cells, and granulocytes (Kaspers and Schat, 2012) help clear pathogens. The innate response is crucial at the first stage of the invasion and minimizes the infected areas. In the next step, the adaptive response appears to clear the pathogens or foreign antigens. With the broad receptors, this system can recognize a large number of

pathogens and react quickly, but the differentiation of pathogens is not specific. The adaptive immune response, represented by B and T cells, is efficient and activated for particular memory cells (Kaspers and Schat, 2012).

In relation to ovarian tissue transplantation, two major issues were identified after transferring the donor graft to the recipient host: the ischemic injury that occurs during transplantation (Farrar et al., 2006) and acute post-transplantation rejection (Pratt et al., 2000). Markers of damaged tissues could be detected by the innate response through cell receptors (Medzhitov and Janeway, 2002). The inflammatory responses are induced and stimulate the adaptive responses to those tissues (Murphy et al., 2011). Murphy et al. (2011) also showed that most rejections were caused by anti-graft effector T cells, whereas regulatory T cells were promoted by a specific treatment that led to graft tolerance. Moreover, natural killer cells or dendritic cells from the innate immune system could be triggered by inflammation at the first stage to activate T cell response defending the donor grafts. Thus, graft tolerance strategies may be applied by treatment directed at T cells (Taylor et al., 2005). Although the innate immune responses contribute to the graft rejections, they have also served as a transplantation tolerance. The explanation was that the mechanisms of immune suppression were induced by a variety of mediators such as mast cells through differentiation of cytokine production (de Vries and Noelle, 2010).

2.3.2. The role and application of chicken MHC

The major histocompatibility complex (MHC) is a group of immune response genes. The chicken MHC is found on chromosome 16 (JE Fulton et al., 2016). The MHC is divided into MHC class I and II molecules that belong to the adaptive immune system (Miller and Taylor Jr, 2016).

The MHC class I and II molecules are detected through CD8-bearing T cells and CD4-bearing T cells, respectively. In particular, those T cells have contributed to the immune response through the induction of antibodies (Kaspers and Schat, 2012). Moreover, the chicken MHC is polymorphic and situated on both regions B and Y of the same chromosome. In addition, the B locus corresponds with BF/BL regions, and Y locus corresponds with Rfp. MHC-B haplotypes are mainly focused on specificity related to diseases resistance (Bacon et al., 2000), allograft rejection (Pazderka et al., 1975). The MHC genes were described in the relationship with transplantation rejections (Pharr et al., 1996). Some observations showed that the rejection occurred in the case where the donor and recipient host are unrelated (Pazderka et al., 1975). These observations were also demonstrated for skin allografts where mismatched genotypes received higher rejection rates than matched recipients (Taylor Jr et al., 2016).

In tissue or organ transplantations, the adaptive immune system is known to be the main factor in response to the MHC molecules located at the donor cellular surface. Moreover, the host's immune system recognizes the donor tissue and starts a process of an alloimmune response that is the same process for pathogens and transplantation. This response can be avoided: graft tolerance was described in the transplantation between matched donor and recipient (Ayala García et al., 2012). A variety of cellular surface molecules cause the stimulation of the immune system including MHC molecules and blood group. This recognition is called allorecognition that triggers T cells of the host. The naïve T cells and memory T cells are activated. As the immune response results, most memory T cells show up at the transplantation site in recipients. At this point, antibody production is produced through B cells with the help of T cells. The antibody influences red blood cells, donor leukocyte antigens, and other cells to participate in the rejection (Wood and Goto, 2012). In summary, the MHC molecule is considered to be one of many factors involving

rejection. The immune system is complicated in response to pathogens, foreign antigens, and tissue transplantations that require the reactions of different components, including antibodies, cytokines, immune cells (Ayala García et al., 2012). However, immune responses alone are not the sole mechanism of graft failure. Additional factors that might damage or destroy the graft could be considered, including ischemic injury, the locate of transplanted tissues or organs, and age at the time of transplantation (Wood and Goto, 2012).

2.3.3. Prevention of transplant rejection by immunosuppressive drug (mycophenolate mofetil)

As rejection is the main reason for transplantation failure through the innate and adaptive immune responses from the host, immunosuppressive treatments play a crucial part in transplantation to facilitate long term transplantation success (Wood and Goto, 2012; Taylor et al., 2005). Immunosuppressive drugs are one of the few effective ways to solve the rejection of a graft. Their main purpose is to inhibit the recipient's immune responses of T cells from the adaptive immune system because of their major role in the rejection. A variety of immunosuppressants have been employed depending upon type and location of transplanted tissues or organs, species, and age, and each drug shows benefits and negative effects (Post et al., 2005). Some immunosuppressive agents are not specific for a particular tissue or organ, e.g. prednisolone, azathioprine, mycophenolate mofetil (MMF), cyclosporine, sirolimus. These non-specific drugs dramatically increase the risk of infection (Taylor et al., 2005). However, prednisolone and azathioprine were successfully employed for the renal transplantation, but not for the heart or liver, and cyclosporine A was used for skin graft (Borel et al., 1994).

Most immunosuppressants have been developed and employed for humans (Taylor et al., 2005) undergoing kidney transplantations and skin grafts. Recently, some of these drugs have been applied to different species. Among them, mycophenolate mofetil (MMF) has been used for avian gonad transplantation and has facilitated the successful recovery of tissues in recipients (Y Song et al., 2012; Frederick G Silversides and Liu, 2012). Although this drug is directed for humans, it is effective in Japanese quail (Y. Song and Silversides, 2008b) and chickens (Y. Song and Silversides, 2007b) through empirically administered doses. MMF belongs to an anti-proliferative class and a compound of mycophenolic acid. The enzyme, named inosine monophosphate dehydrogenase (IMPDH), is the target that the mycophenolic acid tends to impact (Taylor et al., 2005). This enzyme has a vital role in the biosynthesis of purine nucleotides (Shu and Nair, 2008). It is understood to be the rate-limiting step at the first synthesis stage of guanine nucleotides, as guanine nucleotides play a significant part in receptor systems through their regulation. Also, IMPDH is considered to be a candidate for the exploration of anticancer drugs and immunosuppressive treatments (Shu and Nair, 2008). Thus, the administration of MMF post-transplantation inhibits the guanine nucleotide levels, resulting in cytotoxicity due to the disruption of DNA or RNA synthesis (Shu and Nair, 2008). Furthermore, guanine nucleotides can be biosynthesized through two pathways, including salvage and de novo (or IMPDH) pathways, to synthesize the ribonucleotides. Additionally, IMPDH is related to lymphocyte proliferation (Jonsson and Carlsten, 2001), thus MMF treatment inhibits IMPDH and leads to the inhibition of lymphocyte proliferation (B and T lymphocyte) as well. The IMPDH enzyme is encoded by isoform I and isoform II (Jain et al., 2004). Among them, type II plays a crucial role in proliferating B and T lymphocytes (Zimmermann et al., 1998). Taken together, MMF treatment targeting IMPDH can suppress B and T lymphocytes and help to maintain the allograft in transplantation.

Beside benefits, MMF treatment was also associated with several negative effects such as diarrhea and vomiting in human. These side effects are not significant and MMF has been applied for renal transplantation or liver transplantation. The results showed that MMF helped to prevent the rejection and regenerate the graft (Taylor et al., 2005).

CHAPTER 3. OBJECTIVES AND HYPOTHESES

3.1. Hypotheses

Optimization of transfer procedures of vitrified-warmed gonad into an appropriate recipient breed can improve the regeneration and preservation of the genetic diversity of poultry breeds.

3.2. Objectives

- To optimize the gonadal tissue transfer technique into different chicken breeds and evaluate the potential of different chicken breeds to receive a graft.
- To optimize conditions for CPAs equilibration to minimize the toxicity, osmotic stress and freezing injury of gonads.

CHAPTER 4. OPTIMIZE THE GONADAL TISSUES TRANSFER TECHNIQUE INTO DIFFERENT CHICKEN BREED

4.1. Abstract

The preservation of the avian genetic material, especially from endangered or heritage avian species, can be achieved via various techniques, but each technique has both advantages and disadvantages. Among the available techniques, the gonadal tissue transfer technique has been considered as one of the best methods to preserve the variability in the genetic material. Gonadal transplantation can contribute to the progress of the genetic preservation by combining with vitrification-warming procedures that maintain the gonadal tissues for the long term before transplantation. In this experiment, the technique was adapted to chickens and was tested on different chicken breeds. The goal of this study was to demonstrate that one-day-old chick ovarian tissue can be stored and transplanted through the vitrification-warming procedure and that it can develop when implanted in a different breed of chicken, producing donor-derived offspring. Two experiments were conducted using four different chicken lines. In the first experiment, ovarian tissue was transplanted to chicks of similar genetic backgrounds, either Lohmann White (Oldenhof et al.) or Barred Rock (BR) and the second experiment, transplantation was similar or different genetic background hens (Brown Leghorn (BL), BR and BL/BR). Recipient birds were given a daily dose of the immunosuppressive drug (mycophenolate mofetil) until maturity. The rate of success was assessed by genotyping donor-derived ovaries (either gonadal tissues or embryos). In the first experiment, there were 16 recipients. The results showed that donor-derived ovarian tissues were not detected in any of the LW hens. However, they were detected in 50% of BR hens for the transplantation between BR recipients with fresh/vitrified-warmed BR ovaries. Also, one hen appeared to have a donor-derived graft, and two of the embryos from this bird had alleles of the donor bird. In the second experiment, among 40 recipients that survived and grew to maturity

for each group of the donor, 11 recipients laid eggs. Genotyping of produced embryos from these hens revealed that they were derived from the remnant recipient ovarian tissue, not from the donor ovaries. Necropsy results of 16 hens determined three hens with the presence of a graft. A total of 9 grafts were recovered from recipients having BL and BL/BR genetic background. Among three recipient breeds, BL and BL/BR background could sustain the development of a graft regardless of the genetic background of the donor. However, all these grafts were enclosed by a membrane and had a variety of tissue growth, suggesting some level of rejection by the recipients. Overall, the study partly sustained the hypothesis that one to three-day-old chick gonadal tissues could be transplanted and develop and could produce the donor-derived offspring.

4.2. Introduction

Heritage chicken breeds have been raised for a long time by selecting and breeding to develop characteristics that incorporated unique genes which considered as dominant trends. Due to their genetic diversity, heritage breeds are well-adapted to diverse circumstances such as harsh environments and a number of diseases. Unfortunately, the number of heritage chicken breeds has been reduced alarmingly because of the industrialization of chickens, producing commercial breeds to suit the high demand of consumers in terms of chicken meat, eggs and pleasure (Janet E Fulton and Delany, 2003; Pisenti et al., 1999). Moreover, these breeds do not have the tremendous genetic diversity necessary to resist the appearance of new diseases and parasites or any other environmental conditions that might threaten their existence (FAO, 2015). In Canada, research activities and maintenance of heritage chicken lines have declined at Canadian research institutions and Universities due to limited financial resources to maintain and regenerate their genetic diversity and reduce disease outbreak risks (FG Silversides et al., 2008). There are a variety of methods to maintain heritage chicken breeds, but each method has brought up both advantages and

disadvantages. For instance, *ex-situ* preservation programs such as gene banks, captive breeding, gametes and embryos cryopreservation are being implemented in several industrial countries to prevent future erosion of genetic diversity because maintaining the live heritage chicken breeds is costly and time-consuming (FG Silversides et al., 2012a). Cryopreservation of poultry semen can be achieved, but frozen-thawed sperm cells partially lose their ability to fertilize an egg during artificial insemination (Holt, 2000; Medeiros et al., 2002; Watson, 2000). Importantly, roosters can produce only homogametic germ cells, limiting the ability to preserve the entire genome of a poultry breed. Alternative strategies, such as the preservation of gonadal tissues, could solve the issues related to the conservation of poultry sperm (Y. Song and Silversides, 2007a; Yonghong Song and Silversides, 2007b; Y. Song and Silversides, 2006).

Gonadal tissue transplantation was first applied by a former researcher of Agriculture and Agri-Food Canada and his research group (Y. Song and Silversides, 2006). The results showed that it is possible to transfer chicken ovarian tissues from one breed to another (Y. Song and Silversides, 2006). Another group tried to replicate this preservation strategy, and a successful gonadal graft was obtained when donor and recipient lines had a similar genetic background when testicular tissue was transplanted, but not when ovarian tissue was transplanted (Liptoi et al., 2013). To our knowledge, only our lab has successfully replicated this technique, which is currently considered as the gold standard technique to regenerate the genetic diversity of poultry breeds. The first part of this technique is a vitrification-warming procedure that preserves the gonadal tissues for long term storage. The vitrification procedure has been previously used to store and preserve gonadal tissues harvested from a chick (Fahy et al., 1984; Pegg, 2007; J. Liu et al., 2012). With this procedure, gonadal tissues are stored at very low temperature in liquid nitrogen (-196 °C), combined with cryoprotectant agents (CPAs) to prevent degeneration and death of the

tissue due to intracellular ice formation (Mazur and Seki, 2011; Hopkins et al., 2012; Fahy et al., 1984). The second part of the technique involves a surgical procedure that regenerates heritage chicken breeds. This procedure will help gonadal tissues to grow in recipient hosts until maturity to produce their offspring. Theoretically, under the right conditions, testis or ovary can be indefinitely stored in cryogenic conditions. Thus, when regeneration of a particular gene is required, a vitrified-warmed gonad can be transferred into a recipient chicken that had had its gonads removed surgically (Y. Song and Silversides, 2006). Finally, the third and last part of the gonadal tissue regeneration technique involves immunosuppressive treatments applied to the recipient chicken to decrease graft rejection and allow the graft to grow and become fully functional. Silversides' research group was able to successfully demonstrate the growth of gonadal grafts in Japanese quail (J. Liu et al., 2010; Y. Song and Silversides, 2008b; J. Liu et al., 2013c), chicken (Y. Song and Silversides, 2007b; 2007a; 2006), and Muscovy duck (Y Song et al., 2012).

Based on our experiences with the gonadal tissue regeneration technique, we have introduced some modifications and assessed its efficacy on different chicken breeds. In our study, we hypothesized that one to three-day-old chick gonadal tissues through a vitrification-warming procedure can be transplanted and developed normally in the different recipient chicken breeds; and can produce the donor-derived offspring. The main goal of the project was to confirm that one to three-day-old chick ovarian tissues, with the use of the vitrification-warming procedure, could be successfully transplanted to a different recipient chicken breed, and to evaluate the potential of the ovarian graft to sustain tissue development with follicular activity in the recipient chicken, to produce donor-derived offspring.

4.3. Materials and methods

4.3.1. Chick breed preparation and incubation procedures

Barred Plymouth Rock (BR) and Lohmann White (LM) fertilized eggs were obtained from the Poultry Research and Teaching Unit at the University of Saskatchewan. Dark Brown Leghorn (BL) fertilized eggs were purchased from the Poultry Research Center at the University of Alberta. The surgical transplantation protocol was approved by the Animal Research Ethics Board (UCACS, 2012). Hybrids between BL and BR were produced using BR semen to artificially inseminate the BL hens (Animal Care Unit, University of Saskatchewan). All fertilized eggs were incubated in an incubator (Digital 1502 Sportsman) at around 99.9 °F and 60% relative humidity (RH) for 18 days, and then transferred to a hatcher (Digital 1550 Sportsman) at around 98 °F and 70% RH of the last three days of 21 days incubation period.

4.3.2. Collection of fresh and vitrified-warm ovarian tissue for transplantation

For the collection of fresh ovaries, one to three-day-old chicks from each breed were humanely euthanized by cervical dislocation, and ovaries were harvested from the abdominal cavity, and any remnant of connective tissue or blood clots were removed. The collected ovaries were placed in a holding media (HM) prepared with Dulbecco's Phosphate Buffered Saline Solution (DPBS) and 20% Fetal Bovine Serum (FBS) and maintained on ice to store before the procedure. All chemicals and reagents were purchased from Life Technologies Inc. (Burlington, ON, Canada) unless otherwise stated.

For the vitrified-warmed ovary, an adapted and modified version of the vitrification warming protocol described by Liu and colleagues (2012) was followed. The harvested and cleaned ovaries were gently placed on acupuncture needles (0.20 mm x 30 mm, Dong Bang

Acupuncture Inc., Chungnam, Korea). For the male chick, one needle contained two testicular tissues and another needle contained one ovarian tissue regarding female chick, then submerged in the first equilibrium solution held on ice (7.5% dimethyl sulfoxide (DMSO), 7.5% ethylene glycol (EG) mixed in HM) for 15 minutes. In the second step, the needles were transferred to the second equilibrium solution (15% DMSO, 15% EG, 0.5M sucrose mixed in HM) for 3 minutes, also on ice. Following this step, the needles and tissues were blotted on gauze to remove excess moisture around the tissues and immediately plunged into a tray holding liquid nitrogen (LN_2). The frozen tissues were transferred to cryovials (one needle in each cryovial) and transferred immediately to a dewar with LN_2 for at least five days before the warming process.

For the warming procedure, the frozen cryovials containing the ovarian tissue were removed from the LN_2 storage tank, and the needles with the gonadal tissue were removed from the cryovial and placed into a weigh boat filled with LN_2 to facilitate the process. Immediately, the needles were transferred into HM with added 1 M sucrose at room temperature (RT) for 5 minutes. The needles and tissue were then transferred to a 0.5 M sucrose solution in HM at RT for 5 minutes and finally moved to 0.25 M sucrose in HM at RT for 5 minutes. In the last step, the needles were placed in a petri dish containing DMEM (Dulbecco's Modified Eagle Medium with 20% FBS) on ice, and the tissues were removed from the needle until they were grafted. The time to store the tissues post-warming is at a maximum of 3 hours to prevent damages.

4.3.3. Experimental design

Two experiments were conducted using orthotopic transplantation, in which the donor tissue or organ was removed and transferred to the recipient host in the same position. Then, chicks were raised until maturity and artificially inseminated to produce fertile eggs.

In the first experiment, both fresh and vitrified-warmed ovarian tissue from one to three-day-old BR and LW were transplanted to recipients of the same breed and age. The sample sizes of LW/LW and BR/BR were 16 (LW/LW: n=10; BR/BR: n=6). In the meantime, the number of donor ovaries was 16 (7 fresh LW ovaries, 3 vitrified-warmed LW ovaries, 4 fresh BR ovaries and 2 vitrified-warmed BR ovaries). The control group included chicks without surgery (n=3). The intent of this trial was to replicate the technique described by Silversides et al. (Y. Song and Silversides, 2007a; F. G. Silversides et al., 2013a).

In the second experiment, a 3x3 experiment was designed with 9 groups in total (n=40). BR, BL and hybrids between BL and BR were used as both donors and one to three-day-old recipients. Group 1 was BR/BR with BR/BR (n=4); group 2 was BR/BR with BL/BL (n=5), group 3 was BR/BR with BL/BR (n=4); group 4 was BL/BL with BR/BR (n=3), group 5 was BL/BL with BL/BL (n=5), group 6 was BL/BL with BL/BR (n=5), group 7 was BL/BR with BR/BR (n=4), group 8 was BL/BR with BL/BL (n=5) and group 9 was BL/BR with BL/BR (n=5). The control group included chicks without surgery (n=3). The aim of the second experiment was to evaluate the potential of different recipient chicken breeds through the transplantation of ovarian tissue donors, to assess the role of MHC (major histocompatibility complex) in tissue transplantation, and to determine whether these combinations were able to minimize the rejection of graft tissues.

In addition, each experiment had a control group with three chicks. These chicks were the same age as the surgical groups and were raised under the same conditions until maturity. Moreover, the control group included chicks without surgery that simply hatched and raised. The goal was to compare with the surgical groups during the time regarding weight, development and egg laying.

4.3.4. Transplantation procedure

Newly hatched chicks were genotyped to determine the sex of each individual. Surgical procedures with small modifications were performed as described in the protocol according to Silversides et al. (J Liu et al., 2013d; Y Song et al., 2012; Y. Song and Silversides, 2008b; 2007b). Briefly, the recipient chicks were weighed and administrated 0.5 mg/kg orally of Meloxicam to provide pre-emptive analgesia (Metacam, Boehringer Ingelheim Ltd., Burlington, ON, Canada) and then anesthetized by Isoflurane 99.9% (Fresenius Kabi Animal Health, Richmond Hill, ON, Canada). Once anesthetized, a modified mask was attached to the chick's head to facilitate the nasal inhalation of anesthetic. The surgical area was shaved and cleaned with Germi-Stat Gel 4% (Germiphene Corporation, Branford, ON, Canada). The chicks were then placed on a heated pad at dorsal recumbency, and an incision was made to open the skin and the muscle, exposing the abdominal cavity. The yolk sac located at the bottom of the body cavity was removed from the abdomen by cutting and suturing. The abdominal viscera were moved to allow exposure and collection of the ovaries. Donor ovarian tissues were orthotopically implanted into recipient chickens without suture or glue. If a chicks' breathing rate were too high, a catheter that was connected to isoflurane vaporizer placing into their chest after nicking an air sac membrane until the close of the last muscle suture. This could prevent chicks from waking up during surgery. The incision was closed using (Monocryl; Ethicon US). Prior to recovery from anesthesia, 0.1-0.3 ml of subcutaneous saline was administrated to replace body fluid loss and maintain blood pressure. After surgery, chicks were placed in a recovery cage with water and food, equipped with a heating lamp, and chicks were given an antibiotic and an immunosuppressant, 0.2 mg ceftiofur (Excenel, Zoetis Canada Inc., Kirkland, QB, Canada) and 100 mg/kg of mycophenolate mofetil (Cellcept, Hoffmann-LaRoche Ltd., Mississauga, Ontario, Canada). The immunosuppressive drug was orally

administered daily until maturity to minimize the chances of tissue rejection. At maturity (5-6 months age), artificial insemination (AI) was performed on the laying recipient, and fertilized eggs were incubated until day 10 to 15 to check the feather colour of the fetus (in case of different lines with different feather colours). These eggs were cooled for an hour at -20 °C to euthanize the fetus, and DNA was extracted from the fetus for genotyping.

4.3.5. DNA extraction and genotyping of grafted tissues

The four steps of the microsatellite polymerase chain reaction (PCR) are listed below, and fluorescence was used for genotyping labelling on locus-specific primers for analysis. The first step involved the extraction of genomic DNA from the collected tissues using the DNeasy Blood and Tissue kit (Qiagen Inc., Burlington, Ontario, Canada). The second step involved making copies of the DNA using the PCR technique. Microsatellite loci were chosen based on their sensitivity to detect donor alleles and polymorphic content. In total, 11 microsatellite loci were genotyped (ADL0278, ADL0268, LEI0094, MCW0248, MCW0216, MCW0081, MCW0034, MCW0069, MCW0016, LEI0166, LEI258) by PCR using 1 μl of DNA template and a commercially available kit and protocols (Qiagen Inc., Burlington, Ontario, Canada). The third step was running with PCR fragments on an ABI 3500xL (Applied Biosystems) and Genotyping DNA fragments after diluting the PCR product, equipped with a 50 cm array and filled with POP7 polymer. Genotypes were determined using GeneMapper® version 5.0 software (Applied Biosystems). The last step was genotyping of recipient and donor chickens, and comparing these results to that of resected gonadal tissues to determine the origin and subsequent positive or negative graft acceptance by the recipient chicken (Nordskog and Ghostley).

4.3.6. Histology examination

Harvested tissues were cleaned to remove the blood and connective tissue artifacts and immediately fixed in Bouin's solution 24 hours at 4 °C. After fixation, tissues were removed from Bouin's solution by washing three times in 70% ethanol; and stored in 70% ethanol (tissue must be immersed) at 4 °C in the fridge. Fixed samples were loaded into biopsy embedding cassettes and placed into the tissue processor (Leica ASP300S, Leica Biosystems Nussloch GmbH, Nussloch, Germany).

Processed tissues were mounted using wax and sectioned using a microtome (Rankin 17040, MI, USA) into 4 µm sections, that were transferred onto slides. After mounting, the slides were dried on a warmer plate overnight to evaporate any potentially trapped water under the tissue section. Slides were stained using Hematoxylin and Eosin (H&E) protocol. The sections were viewed and photographed using a conventional light microscope at various magnifications.

In this histological examination, six to eight-month-old hens were humanely euthanized to collect ovarian tissues by isoflurane vaporizer and then cervical dislocation. Five grafts that were identified derived from donors were taken to evaluate by the H&E staining technique compared to two control ovaries (without surgery) that were collected from three control hens. Donor-derived ovarian tissues were collected to evaluate their histological morphology. The evaluation was based on the differences of structures between control ovarian tissues and donor grafts in the cortex and the medulla of the ovary. With this technique, intracellular structures regarding morphological histology were evaluated by immune cells showing inflammatory responses.

4.4. Results

Experiment 1. Transplantation on recipients of similar genetic background

Donor-derived ovarian tissue from the same strain of hen was not detected in any of the LW hens (Table 4.1). However, donor-derived ovarian tissue was detected in 50% of BR hens for both fresh and vitrified-warmed tissues. Moreover, one hen's ovarian tissue was derived from fresh donor BR ovary laid eggs in a total of six BR recipients. DNA extraction from the resulting chick embryos confirmed that tissues were derived from the donor's ovary. In comparison between LW and BR, BR recipients were able to sustain BR donor grafts until maturity, while LW recipients could not tolerate LW donor grafts.

Table 4.1. Transplantation to recipients of similar genetic background

Number of grafts present in recipients of similar genetic background at maturity of Lohmann White and Barred Rock laying hens after transplantation into one to three-day-old chicks (LW: n=10; BR: n=6). LW: Lohmann White, BR: Barred Rock.

Recipient	Donor	Number of surgical chicks	Number of ovarian grafts derived from donor
LW	Fresh LW ovary	7	0/7
LW	Vitrified-warmed LW ovary	3	0/3
BR	Fresh BR ovary	4	(2/4) 50%
BR	Vitrified-warmed BR ovary	2	(1/2) 50%

Experiment 2. Transplantation on recipients of similar or different genetic background

No donor-derived tissue was detected in adult BR recipients (Table 4.2). In addition, eggs that were laid by the adult hens proved to be derived from the recipient's remnant ovary, instead of the donor's tissue. Regarding BL recipients, the number of donor-derived ovarian tissues at maturity were obtained by transferring of BR, BL and BL/BR donors were 40%, 20% and 20%, respectively. Two layers, that had received BR or BL donor ovarian tissues, successfully laid eggs that derived from the donors' tissues. Finally, regarding BL/BR recipients, the number of donor-derived ovarian tissues detected at maturity from BR, BL and BL/BR donors were 0%, 40% and 60%, respectively. Eight of the BL/BR recipient hens laid eggs. However, it was confirmed that all of the embryos were derived from the recipient's remnant ovaries, meaning that the ovary itself was still growing after transplantation although being removed. Moreover, ovarian tissues of 11 laying hens were harvested to determine the presence of donors' grafts. However, genotyping results indicated that ovarian tissue was derived from the remnant recipient ovary, not from donors. In a comparison of BR, BL and BL/BR recipients, BL and BL/BR recipients were able to sustain the development of donor's grafts until maturity of similar or different genetic backgrounds. Moreover, BL/BR recipients could recover well their ovarian tissues to function as normal and lay eggs at maturity (8/14 hens).

Table 4.2. Transplantation to recipients of similar or different genetic backgrounds.

Detection of graft in mature recipients of similar or different genetic backgrounds. BR: Barred Plymouth Rock, BL: Brown Leghorn, BL/BR: Brown Leghorn mixed Barred Plymouth Rock, layer: egg laid hens.

Donor	**BR/BR**		**BL/BL**		**BL/BR**	
Recipient	**Number of grafts present**	**Layer**	**Number of grafts present**	**Layer**	**Number of grafts present**	**Layer**
BR/BR	0/4	1/4	0/3	0/3	0/4	0/4
BL/BL	2/5	1/5	1/5	1/5	1/5	0/5
BL/BR	0/4	2/4	2/5	2/5	3/5	4/5

Necropsy assessment of adult hens

A total of 40 chicks survived until maturity after the surgical procedures. Of these, nine hens (Table 4.2) showed donor-derived ovarian tissues, but they did not lay eggs compared to other control hens without surgical procedures. We euthanized them at the same age (7-8 months) to dissect their ovarian tissues. At collection, ovarian tissues showed a variety of sizes and shapes, which varied from hen to hen (ranging 0.5 – 4 cm). Some hens (6 out of 9 hens) had ovaries that were covered by a membrane filled with fluid or small fluid sacs (Figure 4.1 - A, B, C and D). In the remaining three hens, grafts were located at the bottom of the remnant of the recipient's ovaries. A thin membrane had formed to separate the grafts and recipient's ovaries. These grafts were smaller than the recipient's ovaries and appeared to have no biological function (Figure 4.1 – E and F). In contrast, the recipient's ovaries had recovered and developed normally, producing a hierarchy of follicles. These recipients produced eggs at the same time the control hens did.

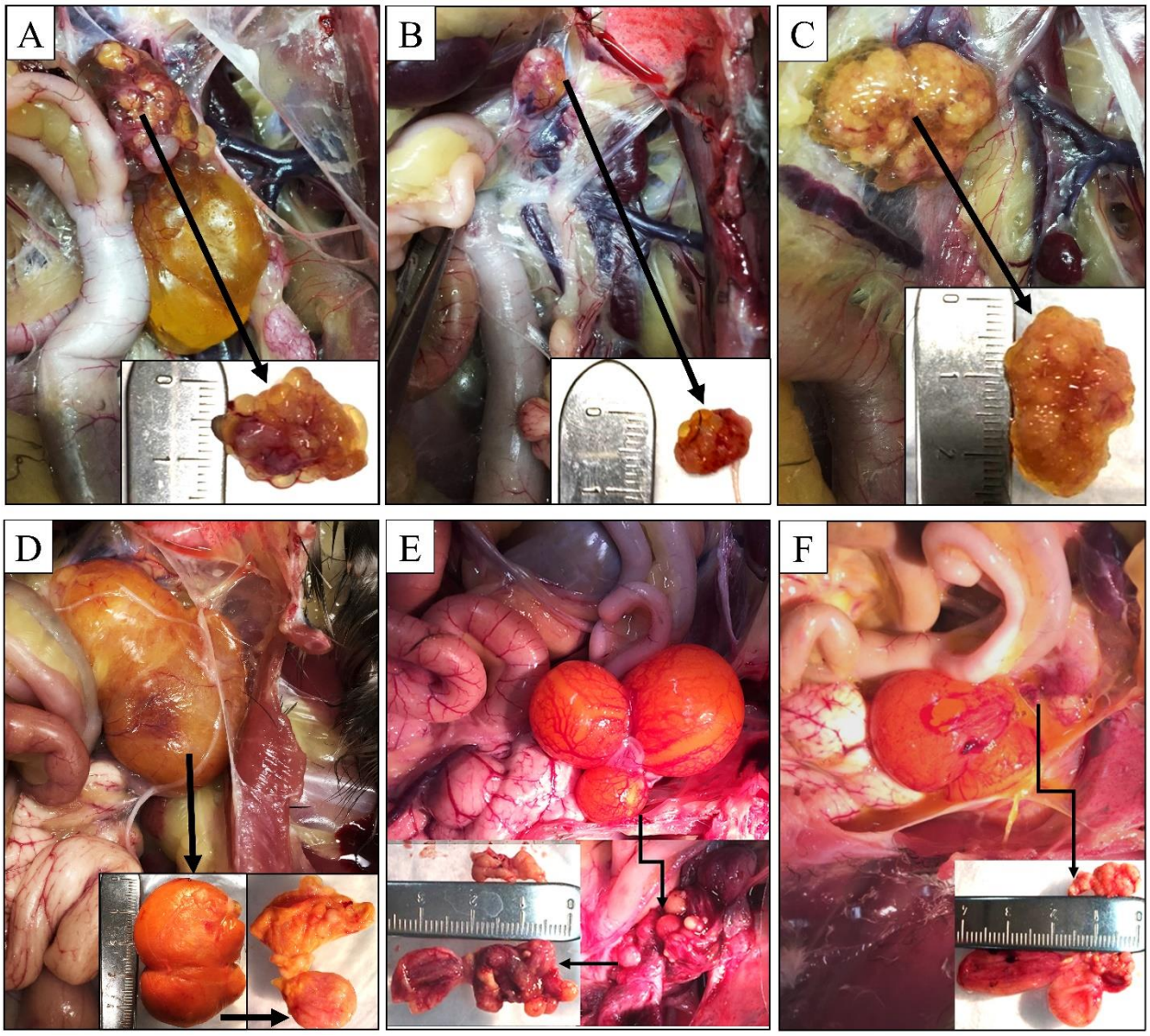

Figure 4.1. Necropsy assessment of adult hens (7-8 months old).

Hens were euthanized to dissect their ovarian tissue grafts. Grafts were covered with membranes filled with fluid (A, B, C, D) or located at the bottom of the recipient's ovaries (E, F) showed inside the opened abdomen cavity and detached membranes with fluid (bottom corner of pictures). The size and shape of ovarian tissues varied from hen to hen (ranging from 0.5 – 4 cm).

Histological assessment

Histological inspection of ovarian tissue from control hens clearly showed cortex cells stained with hematoxylin (dark blue) and medulla in eosin dye (bright pink), with the vascular medulla enveloped by the outer cortex (Fig 4.2A). In comparison, the cortex and medulla of donor-derived ovarian tissues were not readily distinguishable (Figure 4.2-B, C, D). Immune cells within ovaries from control birds were scant in the cortex and the medulla of the histological section of the control ovaries (Figure 4.2A), while donor-derived ovarian tissues showed infiltrates of immune cells in both areas by histological evidence of inflammatory responses (Figure 4.2B, C). Heterophil infiltrates (H) were detected in the ovarian cortex (Figure 4.2B), while infiltrates of lymphocytes (L) were observed in the cortex and the medulla (Figure 4.2C). Golden-brown hemosiderin was contained in macrophages were observed (M) (Figure 4.2D). In summary, histological sections of donor ovaries showed clusters of immune cells, distributed over the cortex and the medulla but did not show in the control.

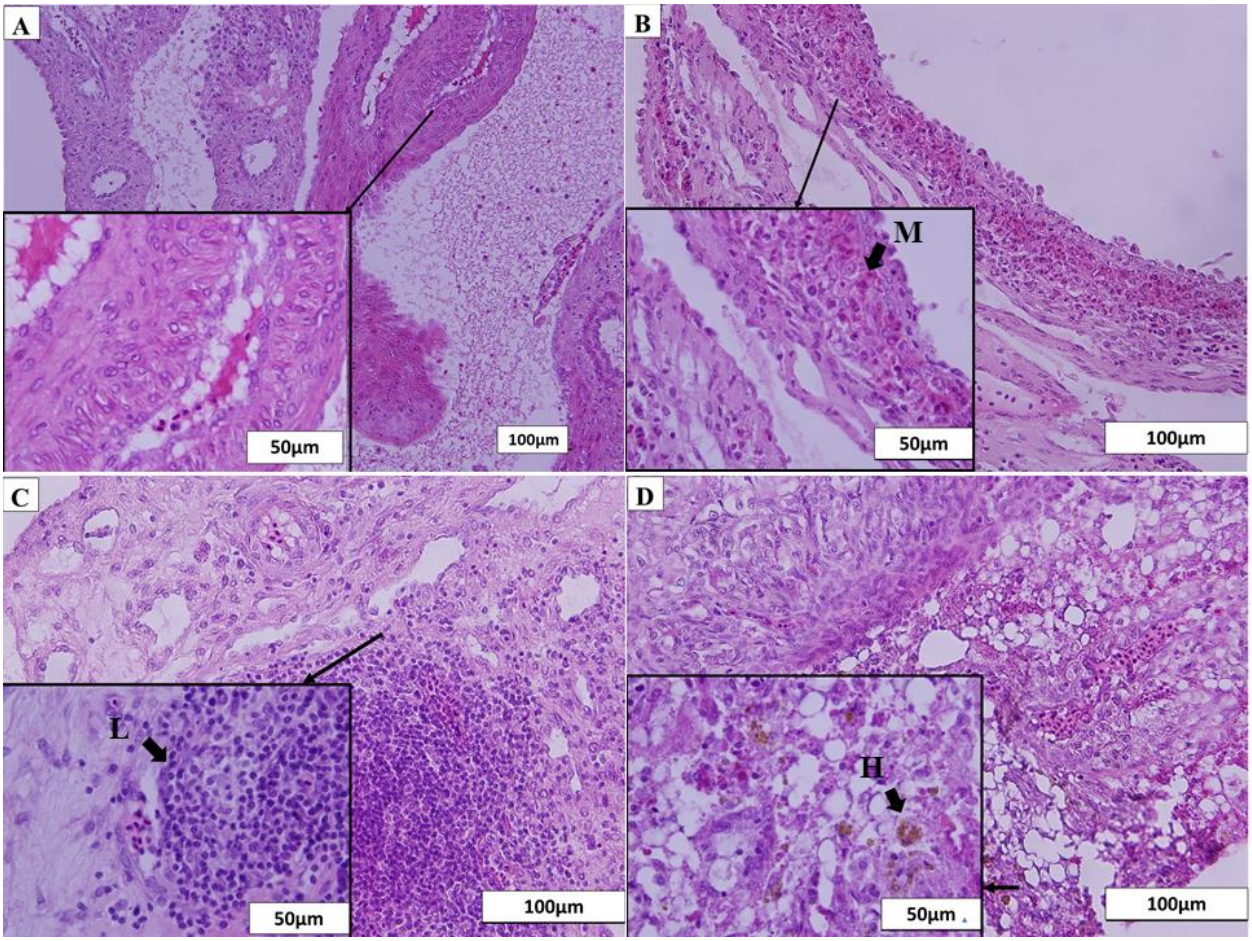

Figure 4.2. Hematoxylin and eosin stained hen ovarian grafts

H&E stained ovarian grafts showed a sign of inflammatory response with macrophages (M), lymphocytes (L) and heterophils (H) at the left corner with arrows (1000X magnification) in images (B), (C) and (D), respectively at 400X magnification. Normal histological morphology of the control ovary showed in the image (A) at 200X magnification.

4.5. Discussion

Our current study partially supported the hypothesis that the vitrification-warming procedures and transplantation strategies could be applied to preserve and transplant ovarian tissues between chicken breeds. The overall goal was to produce offspring derived from donors of different chicken lines including heritage chicken breeds. We were able to demonstrate that we could transplant ovarian grafts that are capable of sustained development in different recipient lines (Table 4.1&2). However, follicular activity was not observed (Figure 4.1A-D). Previous studies demonstrated that recipient hens could maintain ovarian donor grafts until maturity, and even produce offspring from donor ovaries through orthotopic transplantation strategies (Y. Song and Silversides, 2007b; 2007a; 2006). Moreover, these strategies have been successfully applied for different species such as the Japanese quail (J. Liu et al., 2010; Y. Song and Silversides, 2008b; J. Liu et al., 2013c) and Muscovy duck (Y Song et al., 2012). However, our study indicates that there still exist limitations in follicular activities to produce offspring in the implanted chickens in comparison with the control one. Another important finding was that donors and recipients used for the surgery transplantation with different combinations of similar (Table 4.1) or different genetic backgrounds (Table 4.2), and recipients could sustain the development of ovarian donor grafts regardless of similar or different genetic background.

The current results provide further support for the hypothesis regarding using similar or different genetic background for the surgical transplantation. Our findings broadly support the work of other studies (Y. Song and Silversides, 2008a; 2006; J Liu et al., 2013d) in using a variety of chicken breeds including WL, BR, BL, BL/BR chicks of similar or different genetic backgrounds for the surgical transplantation to evaluate the compatibility between breeds. The previous studies mainly focused on WL and BR chicks that were used as recipients and donors,

respectively (Y. Song and Silversides, 2007b; 2007a; 2006). To be stable and consistent for the surgery, “closed flocks” were used for the experiment obtained from the Poultry Research and Teaching Unit at the University of Saskatchewan (WL, BR breeds) and the Poultry Research Center at the University of Alberta (BL breed). These facilities have been breeding for many years and have no new genetic material brought in from outside sources. However, our findings are contrary to another study which has described that the ovarian grafts were not obtained in both donor and recipient lines of similar or different genetic backgrounds (Liptoi et al., 2013). This failure could be associated with using busulfan treatment on eggs to impede the growth of the recipient’s ovarian tissue. As far as we know, no lab has been able to completely successfully replicate these techniques in females.

Another interesting finding was that, after using both fresh and vitrified-warmed donor ovarian tissues for the transplantation, recipient chicks could maintain the growth of ovarian donor grafts in both fresh and vitrified donor ovarian tissues. This is in accord with previous observations using fresh ovarian tissues (Y. Song and Silversides, 2007b; 2007a; 2006) or vitrified-warmed ovarian tissues (Jianan Liu, 2013) for the transplantation. The importance of the age of the chicks used for transplantation seems to be important as reflected by the higher transplant success rate obtained in the current study as compared to previous publications. One to three-day-old chicks were used for the transplantation because the success rate was expected to be higher than with older chicks (Y. Song and Silversides, 2006). However, another study has applied orthotopic ovarian transplantation of similar or different genetic backgrounds on 30-45 days of age chicken (Kosenko, 2007). Their findings showed that donor grafts could remain and grow until chickens were 7 to 12 months old. However, most donor grafts were infertile, degenerated, and functional grafts achieved at a low success rate (Kosenko, 2007). Our results support the hypothesis that

ovarian tissues could be preserved, transplanted and developed in chickens of different genetic backgrounds by using the vitrification-warming and transplantation techniques employed in the current study. However, the results also indicate that still exist limitations in follicular activities to produce viable offspring.

Contrary to our expectations, it has been challenging for recipient chickens to successfully grow ovarian donor grafts until maturity. Among the possible causes are differences among chicken breeds and loss of viability during the vitrification-warming procedure or immunosuppressive treatment. Our findings demonstrated that different chicken breeds had different abilities to reject, receive, sustain or grow the grafts. These differences were noted during the pathological examination, including no graft present, ovarian grafts located aside of the recipient's ovary (Figure 4.1E-F), and grafts enclosed by a membrane (Figure 4.1A-D), regardless of the genetic background of the donor and recipient. Another study has shown that the compatibility between donors and recipients was important for the success of the transplant (Liptoi et al., 2013). A possible explanation for this might be that the compatibility between breeds is necessary to prevent rejection or abnormal development of ovarian donor grafts in recipients. The first successful ovarian transplantation was attained between BR and WL chicks from pure lines, used as donors and recipients, respectively (Y. Song and Silversides, 2007b; 2006). This suggests that this could be one of the right combinations leading to a successful transplant. These results are in line with those of another study that has demonstrated that the success rate of surgery transplantation depended on the breed (Kosenko, 2007). In that study, a variety of breeds received a different percentage of donor grafts. Among the used breeds, WL and New Hampshire breeds seemed more suitable than other breeds (Kosenko, 2007). Moreover, each chick showed adaptation to surgical transplantation differently due to their body size as well as vascular structures. For

instance, it was extremely challenging to perform surgeries on newly hatched BR chicks. They had a high degree of vascularization around the yolk sac, and severe bleeding might occur after its removal. This has just happened to a lesser degree on any of the other breeds utilized in our studies. Severe bleeding may result in surgery failure, as the mechanism of revascularization would be hindered and cause an ischemia-reperfusion injury. However, some of the endogenous processes occurring in the recipient chicken can help the remaining follicle pool, so the recovery of a donor graft was still effective (J. Liu et al., 2010).

The next challenge of our study concerned the vitrification-warming procedures that were used to preserve and transplant ovarian tissues. Although vitrification has been applied for embryos, oocytes, ovarian tissues of mammals, including humans (Dattena et al., 2004; Gunasena et al., 1997a; Mazur and Seki, 2011), it is yet under development in avian species, especially in the chicken. The vitrification-warming procedures of chick gonadal tissues were modified from a previous study on quail that demonstrated the effectiveness of this technique in preserving quail's ovarian tissues (J. Liu et al., 2010). However, the vitrification-warming procedure itself is necessary to be considered whether it would cause any damage in chick ovarian tissues that could result in unexpected failures in the surgery transplantation. Besides, as the procedure was firstly applied for research on mammals, there are vast differences between species that would require the procedure to be modified accordingly. Some of these are the kinds of cryoprotectants (CPAs) used and their correct concentrations as well as the time of exposure in CPAs, and the best way of storing tissues in LN_2. Regarding this issue, another study on turkeys has applied the vitrification-warming procedures for turkey gonadal tissues (George Hall, 2015). This study compared the vitrification-warming procedure using different times of exposure to CPAs and found the standard protocol resulted in fewer damage tissues than others (George Hall, 2015). However, it still caused

some negative influences on the transplanted tissues. Another issue regarding cryopreservation is CPAs toxicity. Although CPAs can reduce intracellular ice formation, it can become toxic at high concentrations, leading to unforeseen injuries to the tissue (Best, 2015).

Another factor that could cause limitations in the success of the procedure was the immunosuppressive treatment after surgical transplantation. Mycophenolate mofetil was used as an immunosuppressant drug in our experiment. This drug has been previously used in the gonadal transfer of some species such as chicken (Y. Song and Silversides, 2007b; 2007a; 2006), quail (J. Liu et al., 2010; Y. Song and Silversides, 2008b), and duck (Y Song et al., 2012). For example, the rate of successful offspring produced from the donor ovarian tissues in the immunosuppressed group was higher than that of the non-immunosuppressive group in a study conducted by Song and Silversides (2007). During our study, a question was brought up regarding whether the dose or kind of immunosuppressant drug would be factors for the achievement of successful transplants in different chicken breeds. Although the immunosuppressive protocol was modified by orally administering a daily dose until maturity, many recipients could not sustain the normal growth of donor-derived ovarian grafts until maturity. The effect of the drug and its side-effects must be considered to determine if the drug would last long enough to block the immune system of any species at any age after transplantation. Also, unexpected side effects of the drug might happen, so it is necessary to test the efficiency of different immunosuppressive drugs and apply accordingly (Diehl et al., 2017). Donor ovarian grafts were histologically analyzed to evaluate their morphology. The results have demonstrated that inflammatory reactions occurred in the ovarian grafts, as reflected by clusters of immune cells throughout the tissues (Figure 4.2B-D). With these results, the use of any immunosuppressive drug needs to be considered along with different drugs

to evaluate the efficiency to apply and replace the current treatment so that ovarian donor graft could sustain and develop in recipients.

Another additional factor that might have affected our result is the experimental design. The number of chicks utilized must be evaluated to reach statistical significance. A good replication number is necessary for the result to be valid and reliable. In our trials, the number of surgical chicks was unbalanced between fresh groups and vitrified groups. Also, the small sample size might be one of the reasons for our results to be limited. A more significant number of chicks used for the surgical transplantation would have likely increased our chances of achieving successful donor-transplanted tissue engraftment and maturation, particularly when using BR recipient chicks. Furthermore, the genotype of BR chickens is very similar among individuals, creating several challenges to differentiate birds from one another. In addition, MHC, which is known as effective control of disease resistance, was considered a factor for transplantation. It was expected that chicken breeds with the same MHC could minimize the tissue rejection rate from recipients (JE Fulton et al., 2016). However, the results did not show what we were expecting when the BL breed was selected in the second trial because it has one MHC haplotype to test the possibilities of transplanting tissue from one individual to another so that they have the same genetic background. Although the ovarian tissues of the similar BL genetic background were used for the surgical transplantation, but one ovarian graft was derived from the donor. With this result, more understanding about MHC needs to dig deeper and to recognize the reality about the role of MHC in the gonadal transplantation.

In conclusion, our study demonstrated that it is possible to preserve gonadal tissue by the vitrification procedure for an extended period of time. Moreover, the cryopreserved tissue can be transfer into a recipient of similar or different genetic background, but without follicular activities.

This indicates that the procedure requires an optimization of the immunosuppressive treatment to allow an ovarian graft to develop to maturity with folliculogenesis activity.

CHAPTER 5. ASSESS THE VITRIFICATION-WARMING PROCEDURE ON ONE TO THREE-DAY OLD CHICK GONADS

5.1. Abstract

Vitrification can be used to preserve gonadal tissue from one to three-day-old chicks. Vitrified-warmed gonads can be transferred into recipients of the same age to sustain their growth and maturation, which could preserve the genetic material of heritage chicken breeds. The first aim of this study was to evaluate the damage of vitrification-warming procedure on one to three-day-old chick gonadal tissues through histological morphology and apoptosis execution via caspase-3 immunofluorescence. An additional aim of this study was to establish a mathematical model that can be used to optimize the procedure for improved preservation of gonadal tissue if the current vitrification-warming procedure causes damage. Gonadal tissues from chicks that were one to three days post-hatching were collected for fresh and cryopreserved evaluation. Vitrified-warmed and fresh testes were fixed for histological analysis, which was performed based on the grading system of the quality of germ cells such as cell nuclei, peripheral breakdown of the cortex, and formation of gaps, also based on apoptosis execution through immune-staining with the caspase-3 antibody. To identify the morphological damages, the tissue was scored blindly, and a non-parametric test (Mann-Whitney U test) was performed to determine the significance ($P<0.05$). Our study revealed that vitrification-warming procedures significantly affected the integrity of the testis cells; specifically, cellular distinctions between Sertoli cells and germ cells were difficult to visualize; increased observations of pyknotic nuclei and detachment of intratubular cells from the myoid cells were noticed, and gaps or shrinkage of cells were observed ($P<0.001$). Regarding ovarian tissues, our results demonstrated that vitrified tissues showed significant damages compared to fresh tissue with regards to nuclei, peripheral breakdown, and a higher number of

gaps. To determine whether apoptosis would induce the post-vitrification-warming process, tissues were immuno-stained with the caspase-3 antibody by an immunohistochemical technique where each testicular or ovarian tissue piece was cut into three sections in one microscope slide with peripheral and central areas. Then apoptotic cells were counted in five fields per area to calculate according to a percentage. The experiment was performed in three independent biological replicates, and data were analyzed using independent samples t-test to compare the means between two groups by SPSS. No significant difference was found for apoptosis between the fresh and vitrified testicular groups for both the peripheral and center area. Surprisingly, the fresh ovarian group showed more apoptosis than the vitrified ovarian group ($P<0.05$), but apoptosis appeared significantly higher for vitrified ovaries that were incubated two hours post-warming compared to the fresh ovarian group.

In summary, the vitrification-warming procedures affect the cellular integrity of one to three-day-old chick gonadal tissues through morphological alterations. Also, the procedure does not cause more apoptotic cells on testicular tissues, but there are some challenges in ovarian tissues for detecting apoptosis execution. Taken together, these results suggest the need to optimize this preservation technique for long-term storage and the surgery transplantation, especially on chicken ovarian tissues, to maintain the genetic diversity of species. A mathematical model was established to create an optimal protocol minimizing the toxicity of CPAs and intracellular ice formation including diminishing gonadal tissue damages. However, there are still some factors that need to be detected for future modelling research.

5.2. Introduction

Cryopreservation has been a practical method for genetic resource preservation in different fields and species including small animals, livestock, poultry and humans (Jianan Liu, 2013; Woods et al., 2004; Picton et al., 2000; Milazzo et al., 2008; F. G. Silversides et al., 2013a; Jiang et al., 2020). Genetic material preserved through germplasm cryopreservation plays an important role in the protection of the genetic diversity of heritage chicken breeds. This technique is unique in the genetic materials that are preserved at a low temperature in order to protect the integrity of cells and tissues. However, there are many factors that can affect the quality of the cells or tissues such as the type of cryoprotectant solutions used and the cooling and warming rate utilized (Pegg, 2007). Without careful control, changes in temperature have been demonstrated to be lethal for the tissue because of several factors, including intracellular and extracellular ice formation (Mazur, 1963). For example, cryopreservation caused adverse effects on rat bone marrow and dental pulp, reducing the viability of cells (Davies et al., 2014).

Cryopreservation methods, including slow freezing and vitrification techniques, are applied for many different purposes. Vitrification is the process of solidification of a liquid to form a glass-like substance without crystallization due to the use of cryoprotectant agents (Mazur, 1984). The selection of either slow freezing or vitrification method depends on the cells or tissues to be preserved. In human ovaries, no difference was seen between the slow freezing and vitrification methods (Klocke et al., 2015). Vitrification has been widely applied to preserve testicular tissue in mammalian species such as swine (Abrishami et al., 2010), mouse (Curaba et al., 2011b), and human (Curaba et al., 2011a) or fish species such as zebrafish (Bono-Mestre et al., 2009). Poultry gametes are a challenge to preserve as the large yolks prevent successful cryopreservation, but avian ovarian tissues can be vitrified as an alternative (Janet E Fulton and

Delany, 2003). However, the vitrification method might be associated with the contamination of pathogens because cells were directly plunged into liquid nitrogen (Tedder et al., 1995). Besides its benefits, cryopreservation causes some adverse effects that can damage cells and tissue, and thus improvement is necessary to increase practical application.

Whether the vitrification-warming procedures have any impact on the quality of chick gonadal tissues used for transplantation surgery is still unknown. Many studies have evaluated chick gonad vitrification-warming procedures and found alterations in morphology, biochemistry, function and apoptosis execution of cells after warming (Gutnisky et al., 2020). The effects of vitrification showed differently on ovarian tissues between human and animal models (Gandolfi et al., 2006). In human ovarian tissues, follicular morphology and function were normal after vitrification, and cells could recover rapidly after the procedure (Yuzhakov et al., 2018). Additionally, apoptosis was also considered as one of the variables used to evaluate the occurrence of any alteration and damages on cells or tissues caused by the vitrification-warming procedures (Yuzhakov et al., 2018).

Apoptosis is a form of cell death that occurs in multicellular organisms (Rager, 2015). Apoptosis maintains the balance of cells in the body and plays a critical key in metabolism, physiology and pathology. Apoptotic cells are determined by their shape alteration including blebbing, cell shrinkage, chromatin condensation, and chromosomal DNA fragmentation (Duan et al., 2003). The detection of apoptosis based on tissue sections was applied in some studies through histological morphology, TUNEL assay, and *in situ* end labelling to detect DNA strand breaks (Barrett et al., 2001; PA Hall, 1999). In the apoptotic molecular pathway, caspase-3 plays an essential role as the primary executioner for the cleavage of proteins and other processes (Slee et al., 2001). Because of its importance and specificity, caspase-3 can be utilized to facilitate

apoptotic detection in tissues. Moreover, caspase-3 is the main effector enzyme of apoptosis and helps the stimulation of endogenous endonucleases. Because of these characteristics, the early period of apoptosis can be determined by caspase-3 (Martinez-Madrid et al., 2007). Activated caspase-3 immunohistochemistry or cleaved CK18 immunohistochemistry has been demonstrated to be an easy, sensitive and reliable way for the identification and quantification of apoptotic cells compared with the TUNEL assay (Duan et al., 2003). Another study found that TUNEL assay could be the best application for necrosis in a tumor (Kraupp et al., 1995), but could not be used independently to detect apoptosis and necrosis (Levin et al., 1999). Furthermore, apoptosis regulating factors or anti-apoptotic, pro-apoptotic proteins, including BCL-2 and BAX, play an essential role in increasing apoptotic cells in the mouse embryo oocytes (De Felici et al., 1999). Apoptosis caused by cryopreservation was reported in mammalian species' gonadal tissues, including human ovarian tissue with DNA fragmentation (Keros et al., 2009), and mouse primordial ovarian follicles (Lee et al., 2000) using an immunohistochemistry method and histological morphology (Merdassi et al., 2011). This is also in accord with another report which showed an increase in the expression of apoptosis-related genes in ovarian tissues post warming (Dalman et al., 2017). During the growth of the mouse fetal ovary, primordial germ cell, and oocyte could be degenerated by apoptosis (De Felici et al., 1999). Another report indicated the negative effects of cryopreservation on the granulosa cells and oocytes of vitrified human ovarian tissues, and deterioration of secondary follicles (Dalman et al., 2017).

Cryoprotectants (CPAs) play an essential role in cryopreservation. CPAs are classified into two forms: permeating and nonpermeating (Muldrew et al., 2004). Permeating CPAs that easily cross the cellular membrane have the following typical characteristics: low molecular weight, non-ionic molecules, water solubility at cooling and equilibrium in protoplasm. Popular

permeating CPAs include glycerol, dimethyl sulphoxide (DMSO), formamide (FMD), propylene glycol (PG), ethylene glycol (EG). Nonpermeating CPAs are macromolecules and sugars, such as sucrose, that have a high solubility in water as well as restriction of crossing cell membranes. Cell dehydration occurs at low concentration because of high osmotic coefficients resulting in a reduction of the extent of crystallization (Muldrew et al., 2004). During cryopreservation, equilibration with permeating CPAs is challenging because of toxicity and osmotic stresses. This toxicity can be modulated by many different factors including CPA concentration, temperature, types of CPA, exposure time, and cell membrane condition (breached or damaged) that can lead to chemical or osmotic damage that in turn leads to, directly or indirectly, oxidative stress (Muldrew et al., 2004). In vitrified tissues, high cooling rates and high CPA concentrations are combined to prevent intracellular ice formation (Mazur, 1984). Because of this toxicity and osmotic stress, CPA equilibration plays a critical role in minimizing damage related to CPAs selection and concentration (Elmoazzen et al., 2005). One way to rationally deal with the interplay of osmotic and chemical toxicity is through mathematical modeling (Benson et al., 2018).

The goal of this study was to evaluate the standard vitrification-warming procedure through histological morphology and apoptosis appearance on gonadal tissues. To facilitate this analysis, a biophysical study of the diffusion of cryoprotective agents in gonadal tissues was undertaken, where the diffusivity D of the tissue was evaluated, and an estimate of α was made for both ovarian and testicular tissue. This information was then combined to facilitate predictions of improved protocols that would result in minimized CPA equilibration damage. In this study, we hypothesized that the vitrification-warming procedure induces damage and increase apoptotic cells in one to three-day-old chick testes and ovaries. Two main objective were that the integrity of testicular and ovarian tissues could be evaluated by using a morphological scoring system to assess

the vitrification-warming procedure and to test for the difference of apoptosis in fresh and vitrified-warmed testes and ovaries, evaluated via caspase-3 immunofluorescence.

5.3. Materials and methods

5.3.1. Gonad preparation

Lohmann White (LM) fertilized eggs were obtained from the Poultry Research and Teaching Unit (Department of Animal and Poultry Science, College of Agriculture and Bioresources, University of Saskatchewan), and Dark Brown Leghorn (BL) fertilized eggs were purchased from the University of Alberta. All fertilized eggs were incubated in an incubator (Digital 1502 Sportsman) at 99.9 °F and 60% RH (related humidity) and then transferred to a hatcher (Digital 1550 Sportsman) at 98 °F and 70% RH of the last three days of 21 days incubation period.

To collect fresh ovaries, one to three-day-old chicks of all breeds above were humanely euthanized by cervical dislocation, and the left ovaries were harvested from the abdominal cavity and removed of all remnant connective tissues or blood clots. The collected ovaries were placed in holding media (HM), Dulbecco's Phosphate Buffered Saline Solution (DPBS) with 20% Fetal Bovine Serum (FBS), on ice until the vitrification-warming process. All chemicals and reagents were purchased from Life Technologies Inc. (Burlington, ON, Canada) unless otherwise stated.

The vitrification warming protocol was adapted from the procedure described by Liu et al. (2012). Briefly, the ovaries were gently placed on acupuncture needles (0.20 mm x 30 mm, Dong Bang Acupuncture Inc., Chungnam, Korea). The needles were then submerged in the first equilibrium solution (7.5% dimethyl sulfoxide (DMSO), 7.5% ethylene glycol (EG) mixed in HM) for 15 minutes on ice. In the second step, the needles were transferred to the second equilibrium

solution (15% DMSO, 15% EG, 0.5M sucrose mixed in HM) for 3 minutes on ice. Following this step, the needles were carefully blotted on gauze to remove excess moisture around the tissues and immediately plunge into a tray holding liquid nitrogen (LN_2). The frozen tissues were then transferred to the cryovials and kept in the LN_2 tank for at least 5 days before the warming process.

For the warming procedure, the frozen cryovials containing the ovarian tissue were removed from the LN_2 storage tank, and the needles containing the gonadal tissue were removed from the cryovial and placed into a weigh boat filled with LN_2. Following this step, the needles were immediately transferred into a 1 M sucrose thawing media at room temperature (RT) for 5 minutes. The needles were then transferred to a 0.5 M sucrose solution with HM at RT for another 5 minutes and finally moved to a 0.25 M sucrose with HM at RT for 5 minutes. The needles were then placed in a petri dish containing DMEM (Dulbecco's Modified Eagle Medium with 20% FBS) on ice and the tissues were removed from the needle to be processed.

5.3.2. Periodic Acid Schiff-Hematoxylin (PAS-H)

Harvested tissues (4 fresh testes, 3 vitrified testes, 3 fresh ovaries and 3 vitrified ovaries) were immediately fixed in Bouin's solution for 24 hours at 4 °C following the vitrification-warming procedure. After fixation, tissues were removed from Bouin's solution by washing three times in 70% ethanol and stored in 70% ethanol (tissue was immersed) at 4 °C in the fridge. Fixed samples were loaded into biopsy embedding cassettes and placed into the tissue processor (Leica ASP300S, Leica Biosystems Nussloch GmbH, Nussloch, Germany).

Tissues were then mounted and sectioned by a microtome (Rankin 17040, MI, USA), and 4 µm sections were transferred onto slides. After mounting, the slides were dried on a warmer plate overnight and stained using a PAS-H (Periodic Acid Schiff counter stain Hematoxylin)

protocol: 2 minutes in xylene (2 times); 2 minutes in 100% alcohol (2 times); 2 minutes in 90% alcohol; 2 minutes in 70% alcohol; running warm tap water (RWTW) for 1 minute; 15 minutes in Leica Schiff's reagent; RWTW for 10 minutes; 2 minutes in Leica Hematoxylin 560; RWTW for 1 minute; 15 seconds in 0.15% acid alcohol; RWTW for 1 minute; 1 minute in Leica Blue Buffer 8; RWTW for 3 minutes; 15 seconds for each 90% alcohol and 3 times of 100% alcohol; 1 minute in xylene and keep the slides in the last xylene. The last step was adding the coverslip to cover the sections and drying overnight. The sections were viewed and photographed using a conventional light microscope at various magnifications. On testicular tissues, five observations were done at both the peripheral and center area of each section, three sections were seen on each slide. On ovarian tissues, five observations were evaluated in the cortex area. These slides were marked by coded numbers by one person, and then the evaluation was done by two other people to retain the objectivity.

5.3.3. Gonad assessment based on the morphological grading system

In this experiment, there were 4 fresh and 3 vitrified testes; 3 fresh and 3 vitrified ovaries were distributed on the same number of the slides for the observation. The quality of the gonads was assessed following a modified version of the grading system through the quantitative histology used by Milazzo et al. (2010; Table 1).

Table 5.1. Quantitative histology grading system

Tissue	Feature	Scores
Testes	*Sertoli cell distinction from spermatogonia*	0: easy; 1: difficult; 2: indistinguishable.
	Observation of the nuclei	0: easy; 1: indistinguishable
	Nuclei condensation	0: no appearance; 1: <40% of cells; 2: 40% of cells
	Intratubular cell detachment from the basement membrane	0: no appearance; 1: 50%; 2: 100%
	Gap formation or shrinkage	0: no appearance; 1: less; 2: a lot
Ovary	*Primordial germ cell distinction from immature granulosa cell*	0: easy; 1: difficult; 2: indistinguishable.
	Observation of the nuclei	0: easy; 1: indistinguishable
	Nuclei condensation	0: no appearance; 1: <40% of cells; 2: 40% of cells
	Peripheral breakdown of cortex	0: no appearance; 1: <25%; 2:>25%
	Cortex cell gap formation or shrinkage	0: no appearance; 1: less; 2: a lot

5.3.4. Immunohistochemistry technique via caspase-3 immunofluorescence

5.3.4.1. Apoptosis appearance between fresh and vitrified-warmed gonadal tissues

In this experiment, the tissues were collected as described above. There were 3 fresh and 3 vitrified testes (n=3); 3 fresh and 3 vitrified ovaries (n=3) distributed on the same number of the slides for the observation. After dissection, tissues were rinsed with 0.1% saline to remove the blood and connective tissue artifacts and then immediately fixed in ice-cold 4% paraformaldehyde (PFA) and left in 4 °C in the fridge for 20 hours. Then, tissues were removed from PFA by washing three times in 70% ethanol; and left those tissues in 10 mL of 70% ethanol (tissue must be immersed) at 4 °C in the fridge. Fixed samples were loaded into biopsy embedding cassettes and placed into the tissue processor (Leica ASP300S, Leica Biosystems Nussloch GmbH, Nussloch, Germany). A 6-hour cycle was run (3 times in 70% ethanol for 30 mins; 80% ethanol for 30 mins; 3 times in 100% ethanol for 30 mins; 2 times in 100% xylene for 30 mins; lastly, 2 times in paraffin for 30 mins at 60 °C).

Gonads were then mounted and sectioned using a microtome (Rankin 17040, MI, USA), and 4µm sections were fixed on slides. After mounting, the slides were dried on a warmer plate. Those sections were immune-stained with caspase-3 antibody (Anti-Caspase-3 antibody, ab90437, Abcam Inc., Toronto, ON, CA); including the following steps: deparaffinization and hydration of slides (5 minutes in xylene (2 times), 2 minutes in 100% ethanol (2 times), 2 minutes in 95% ethanol, 2 minutes in 70% ethanol, 2 minutes in 50% ethanol and 10 minutes in distilled water); quenching endogenous peroxidase activity and background fluorescence with H_2O_2 for 30 minutes at room temperature; washing, blocking and probing with the primary antibody at 1:200 dilution for overnight; washing and probing with the second antibody (Goat Anti-Rabbit IgG H&L Alexa

Fluor 488, ab150077, Abcam Inc., Toronto, ON, CA) at 1:500 dilution for 60 minutes at 37 °C in an incubator; washing and mounting slides with cytoseal 60 around the edge of the coverslip (applied Vectashield with Dapi) and storing in opaque slide box in the fridge. The sections were viewed and photographed using a fluorescence microscope at various magnifications. Five observations were done at both the peripheral and center area of each section; three sections were evaluated on each slide.

5.3.4.2. Apoptosis evaluation between fresh and vitrified-warmed gonadal tissues at different time points

Apoptosis was assessed via an anti-caspase-3 immunofluorescence technique, as described in the previous section. The experiment was running in five groups including the group of fresh tissues at 0 h (tissues used right after collection – fresh 0h), the tissues kept in holding media 2 h after the collection (fresh 2 h), the tissue exposed to CPAs 2 h after collection and fixed (CPA 2 h), the vitrified-warmed tissues at 0 h (tissues used right after warming – vitri 0 h), and the tissues after vitrification kept in holding media 2 hours (vitri 2 h). Among groups, two groups including fresh 0h and vitri 0h were described in 5.3.4.1. All tissues were incubated at 37 °C for 2h. In this experiment, five groups of tissues were compared together to evaluate apoptosis.

5.4. Biophysical modelling of CPA equilibration to model the toxicity, osmotic stress and freezing injury of gonads

The experiment was designed to evaluate CPA diffusivity at three different temperatures of CPAs (37 °C, 22 °C and 0 °C) along with three types of CPAs (30% PG, 30% DMSO and 30% Formamide) and 6-time points (3, 5, 10, 20, 40 & 80 minutes). Tissues were obtained from chicks and placed in DPBS, holding media until processing. In this experiment, whole testes (n=53) and

ovaries (n=29) were harvested from one to three-day-old Dark Brown Leghorn (BL) chicks. Those samples were replicated three or more times, depending on the amount of sample, at 37 °C, 22 °C and 0 °C with 6 times points (3, 5, 10, 20, 40 & 80 minutes).

At each chosen temperature and CPA (30% PG, 30% DMSO and 30% Formamide), tissues were distributed to all three media for 120 min to fully equilibrate. After this incubation, the tissues were placed into tubes with 150 µl distilled water. At each time point (3, 5, 10, 20, 40 & 80 minutes), an aspirate of 20 µl of solution (now distilled water plus the diffusate from the tissue) was withdrawn from the tissue sample tube (6 times while keeping the tissue under solution until the last withdrawal) for the osmometer (Wescor 5520 Vapor Pressure Osmometer, Wescor, INC, Utah, USA) to measure the solution osmolality. The resulting osmolalities were then fit to equation (2) and (3), where the initial concentration of $C(x,0)$ is defined as 30% CPA and the $C_{ext}(t)$ is assumed to be 0 for the first withdrawal, and adjusted for the measured concentration at each subsequent step.

The use of mathematical modeling to predict cell and tissue response to CPA equilibration is common in the field of cryobiology (Anderson et al., 2014). In short, models are used to quantify both the mechanical damage due to excessive osmotically induced volume changes, the completion of CPA equilibration processes, and the accumulation of CPA induced chemical toxicity (Benson, 2015). These models couple three ingredients. First is a model of cell damage due to high concentrations of CPA. Towards this, Benson et al. defined a toxicity cost function, $J_{\mathrm{tox}}(p)$, that accounts for the concentration and time-of-exposure dependence during CPA equilibration (Benson et al., 2018):

$$J_{\mathrm{tox}}(p) = \int \int_0^{t_f} C^{\alpha}(t,x)dt\ dx,$$

where C is the CPA concentration of a cell or a tissue at point x at time t, α is a rate parameter, t_f is the final time when the CPA equilibration protocol p (e.g. 1 M for 1 min, 2 M for 2 min, etc.) is complete. This model of damage is coupled with a model that predicts what the concentration C is at every space and time during the protocol p. Among many choices, for tissues, the standard choice is the diffusion model used by Benson et al. (Benson et al., 2018).

$$\frac{dC}{dt} = D\Delta C, \qquad (2)$$

$$C(t, x \text{ on the tissue boundary}) = C_{\text{ext}}(p), \qquad (3)$$

where D is the CPA diffusivity, and $C_{ext}(p)$ is the concentration on the exterior of the tissue as a function of protocol p.

Finally, this model is coupled with a model of the mechanical damage done by the osmotically induced volume changes. The choice of the best model is still not clear and beyond the scope of this work, but here we follow Benson et al. (Benson et al., 2018) and use the volume response of the cells on the exterior of the tissue as a proxy for the osmotically induced damage during equilibration. In short, if the protocol is such that it should not cause mechanical damage to exterior cells, then we assume that this protocol should be "safe" for all cells and structures in the tissues. Here we follow the choices of Benson et al. (2018) by using an endothelial cell model for transport and mechanical sensitivity. Briefly, a two-parameter model of water and CPA transport across the cell membrane is used to predict cell volume responses to CPA equilibration protocols (Benson, 2015). This model is coupled with estimates for the cellular osmotic tolerance limits, limits to which the cells can shrink and swell with minimal damage. All parameters for this mechanical damage model are defined in Benson et al (2018).

5.5. Statistical analyses

For the morphological analysis with PAS-H staining, the global scores were also compared between the fresh tissue group and the vitrified-warmed tissue group and each characteristic of each group was compared with each other. On testicular tissues, five observations were done at both the peripheral and center area of each section, three sections were seen on each slide (including 4 fresh testes & 3 vitrified testes). On ovarian tissues, one observation was evaluated at the cortex area because the primordial germ cells are often present in this area and three sections of each slide were seen (including 3 fresh ovaries & 3 vitrified ovaries). A nonparametric test (Mann-Whitney U test) was performed to compare the level of damage observed between fresh and vitrified tissues, ($P<0.05$). SPSS software (IBM SPSS Statistics, version 25, Armonk, NY, US) was used to analyze all data.

For apoptosis assessment with immunofluorescence staining via caspase-3 antibody, five observations were evaluated at both the peripheral and center area of each section. The experiment was performed in three independent biological replicates, and data were analyzed using independent samples t-test to compare the means of the apoptosis of the fresh and vitrified-warmed gonadal tissues by SPSS (significance was set at $P<0.05$).

For osmolality value, the data were analyzed by a multi-way ANOVAs test to ascertain if there were any effects of CPAs, different time points and different temperatures on the value of osmolality. Significance was set at $P<0.05$, and a post hoc Tukey test was used to test the interaction between them.

5.6. Results

5.6.1. Morphological histology of control and vitrified gonadal tissues by PAS-H

Overall, vitrified tissues resulted in higher damage compared to control tissue (Figures 5.1 and 5.2). The alterations included Sertoli cell distinction from spermatogonia, observation of the nuclei, nuclei condensation, intratubular cell detachment from the basement membrane and gap formation of shrinkage. The total scores in the vitrified group were significantly higher than that of the control group ($P<0.001$; Figure 5.1). In the control group, consistent form of seminiferous cords located around testis; Sertoli cells lined on the seminiferous tubules continuously; and spermatogonia with nuclei were close to the basal lamina of the seminiferous cords (Figure 5.3). In comparison, the vitrified group showed the non-uniform structure of the seminiferous cords in the testis; cords were altered across testis; Sertoli cells and spermatogonia were indistinguishable; and gap formation and shrinkage often appeared between the intratubular cell and the basement membrane. Moreover, the abnormal structures were seen in the center of the testis (Figure 5.4).

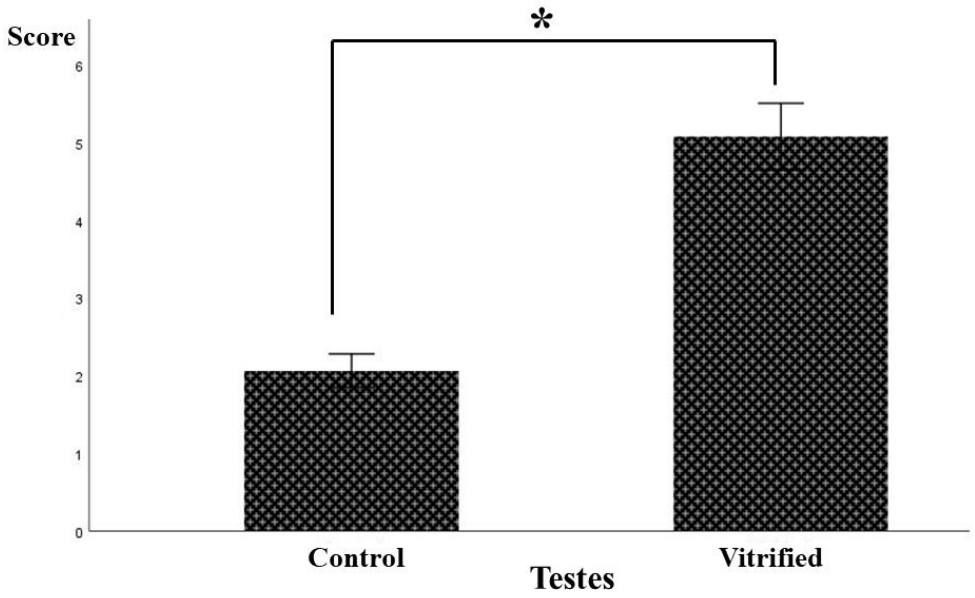

Figure 5.1. Total scores for histological morphology of control (n=4) and vitrified (n=3) one to three-day-old chick testes.

In testes, data are shown as mean ± standard error, and the * symbol indicates significant differences via the Mann-Whitney U test ($P<0.05$).

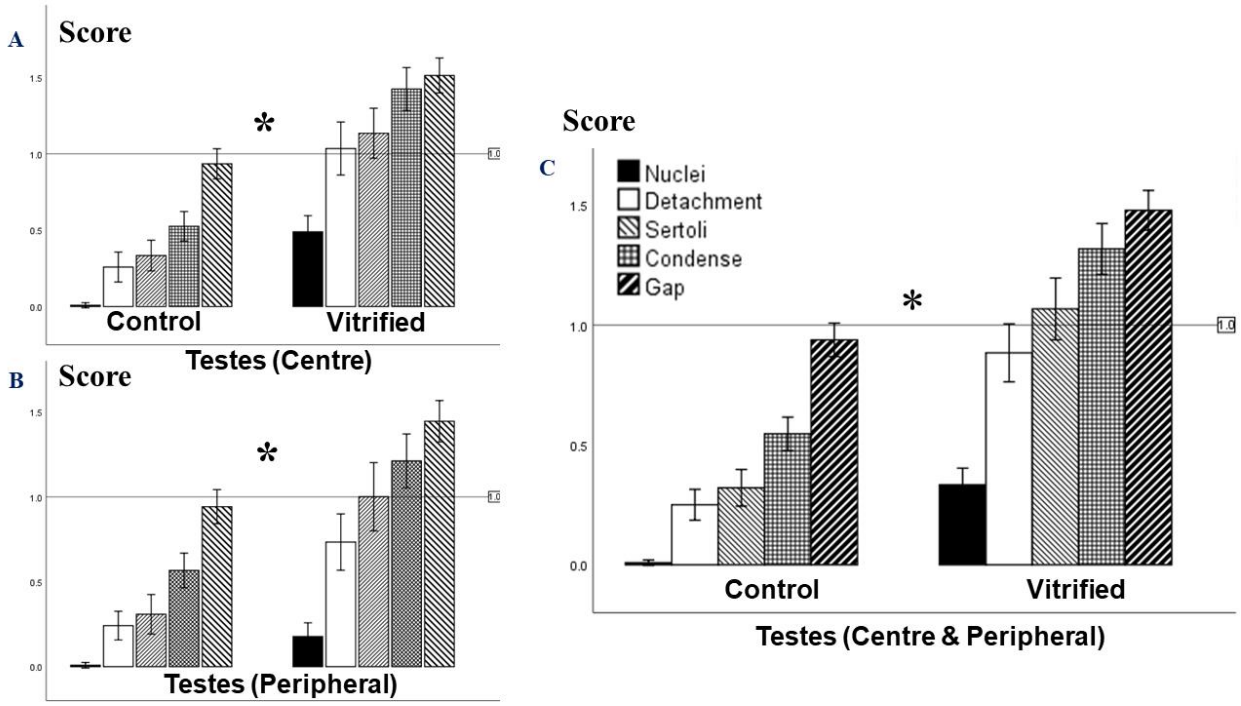

Figure 5.2. Scores for histological morphology of control (n=4) and vitrified (n=3) one to three-day-old chick testes.

Morphological scores for seminiferous cords at the centre (A), peripheral (B) and whole testes (C) are shown for five damage categories. Significant damage between fresh controls and vitrified testes was demonstrated with the Mann-Whitney U test ($P < 0.001$); scores ≤ 1 are the target. Data are shown as mean ± standard error, and the * symbol indicates significant differences.

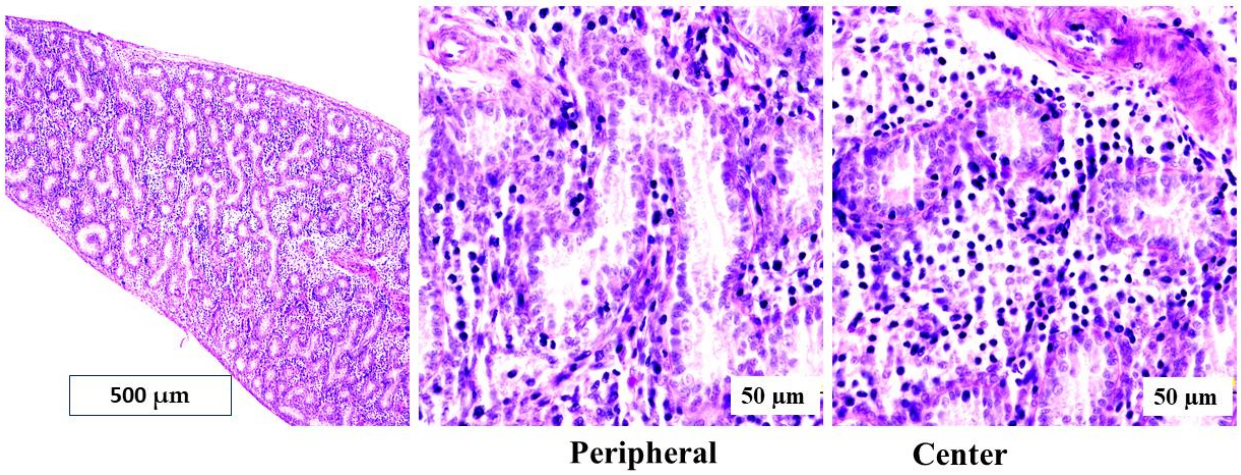

Figure 5.3. Morphological histology with PAS-H staining of control testes without vitrification. The normal structure of seminiferous cords is demonstrated. Scale bars 500 µm and 50 µm.

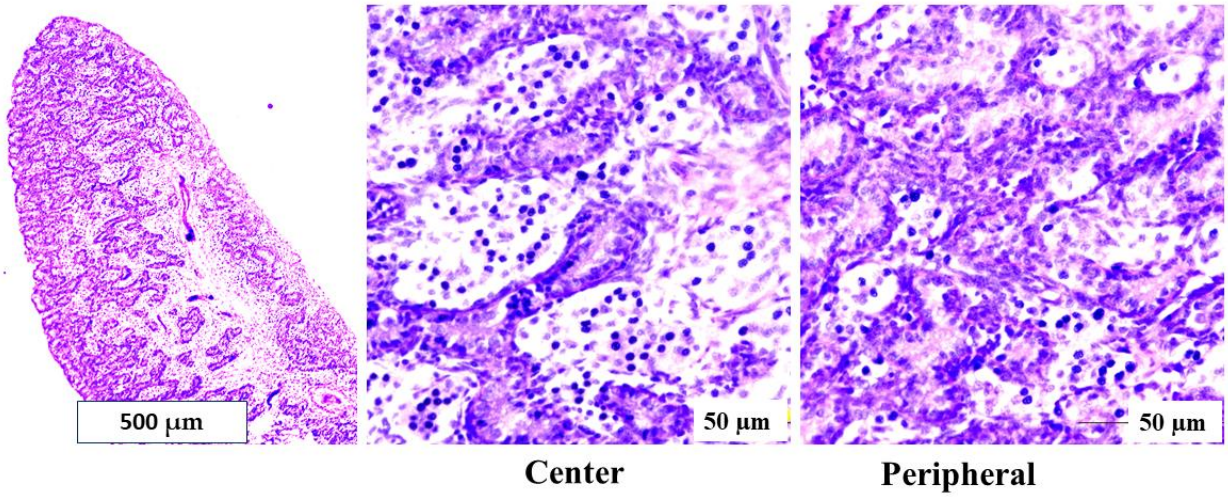

Figure 5.4. Morphological histology with PAS-H staining of vitrified-warmed testes demonstrating damaged seminiferous cords and cortex cell gap formation. Scale bars 500 µm and 50 µm.

Overall, total scores differed between the control and vitrified ovaries (P=0.027). Histological analysis of the fresh ovarian tissues showed less gap formation and shrinkage as well as less nucleic condensation compared to vitrified tissues (Fig 5.7). In the vitrified ovarian tissues (Fig 5.8), the nuclei were pyknotic and condensed. Gap formation and shrinkage were observed in a peripheral breakdown of the cortex. Two factors, nuclei and cortex cell gap formation and shrinkage, were significantly higher in the vitrified group than showed in the control group (P=0.028 & 0.006) (Fig 5.6).

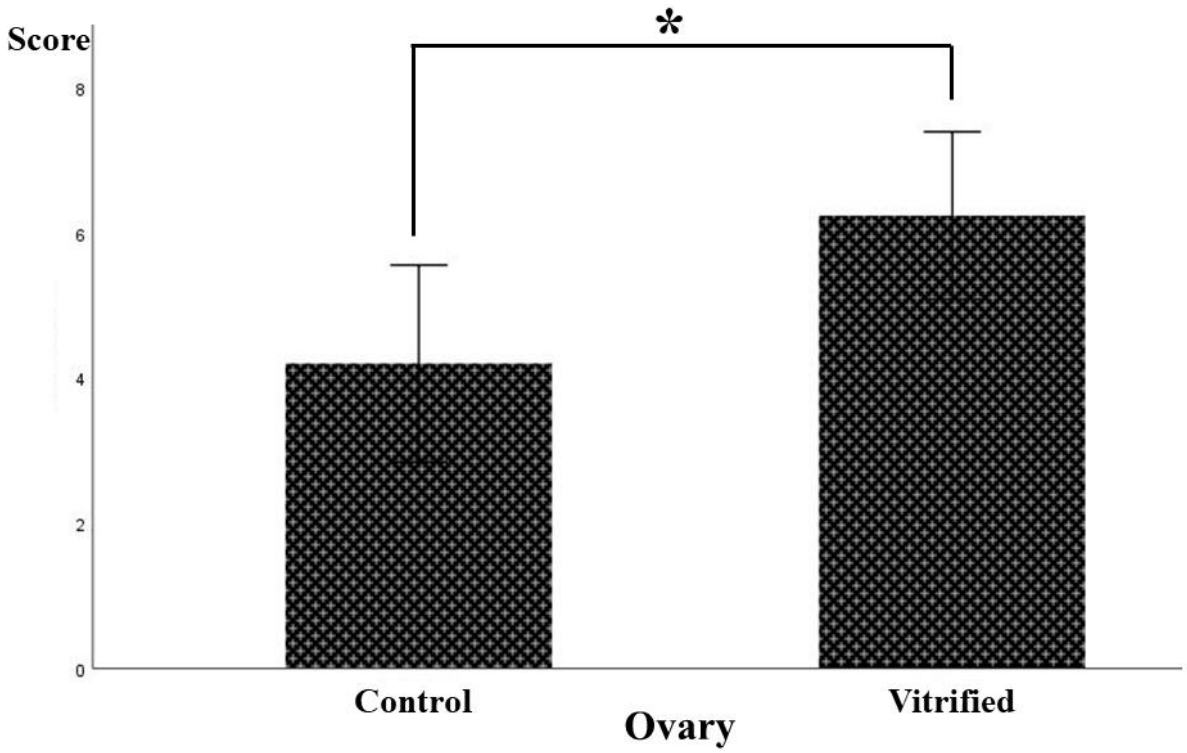

Figure 5.5. Total scores for histological morphology of control (n=3) and vitrified (n=3) one to three-day-old chick ovaries. Data are shown as mean ± standard error, and the * symbol indicates significant differences via the Mann-Whitney U test ($P<0.05$).

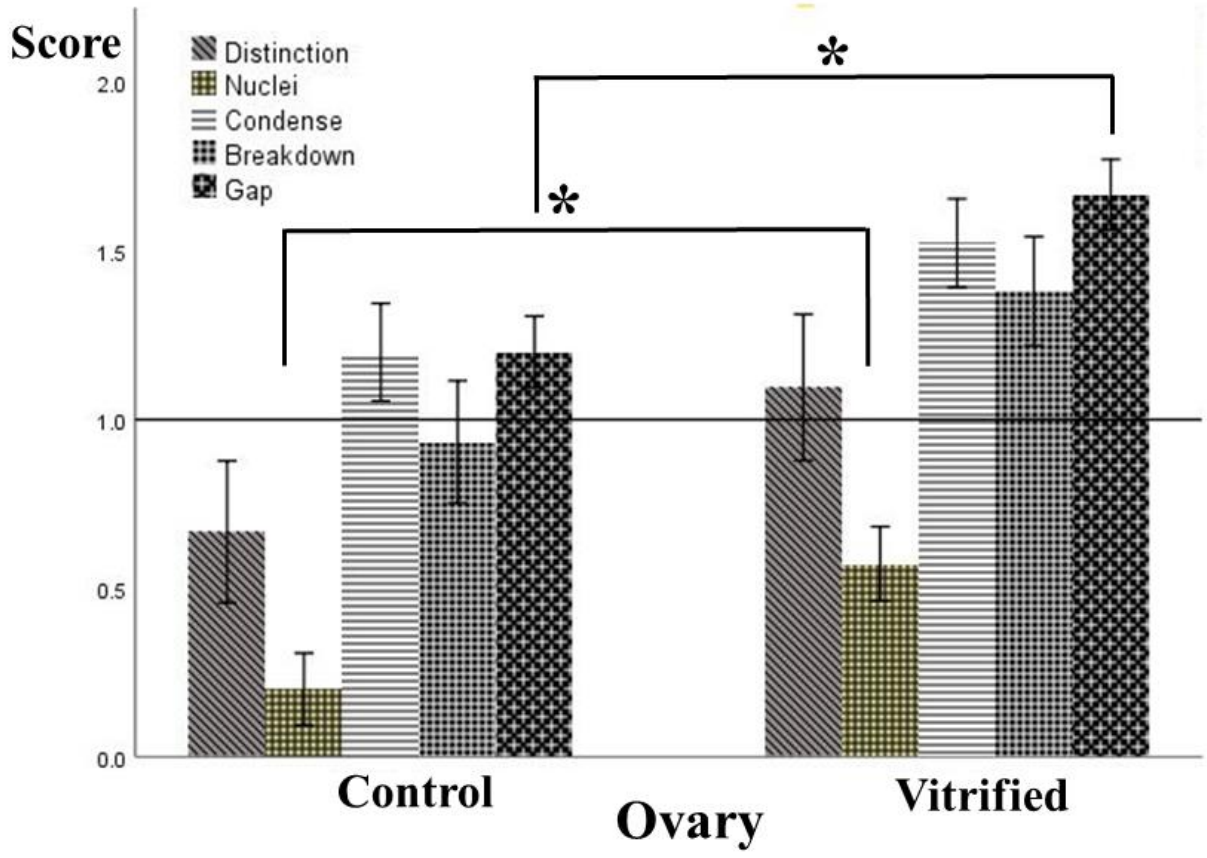

Figure 5.6. Scores for histological morphology of control (n=3) and vitrified (n=3) one to three-day-old chick ovaries. Data are shown as mean ± standard error, and the * symbol indicates significant differences via the Mann-Whitney U test ($P<0.05$).

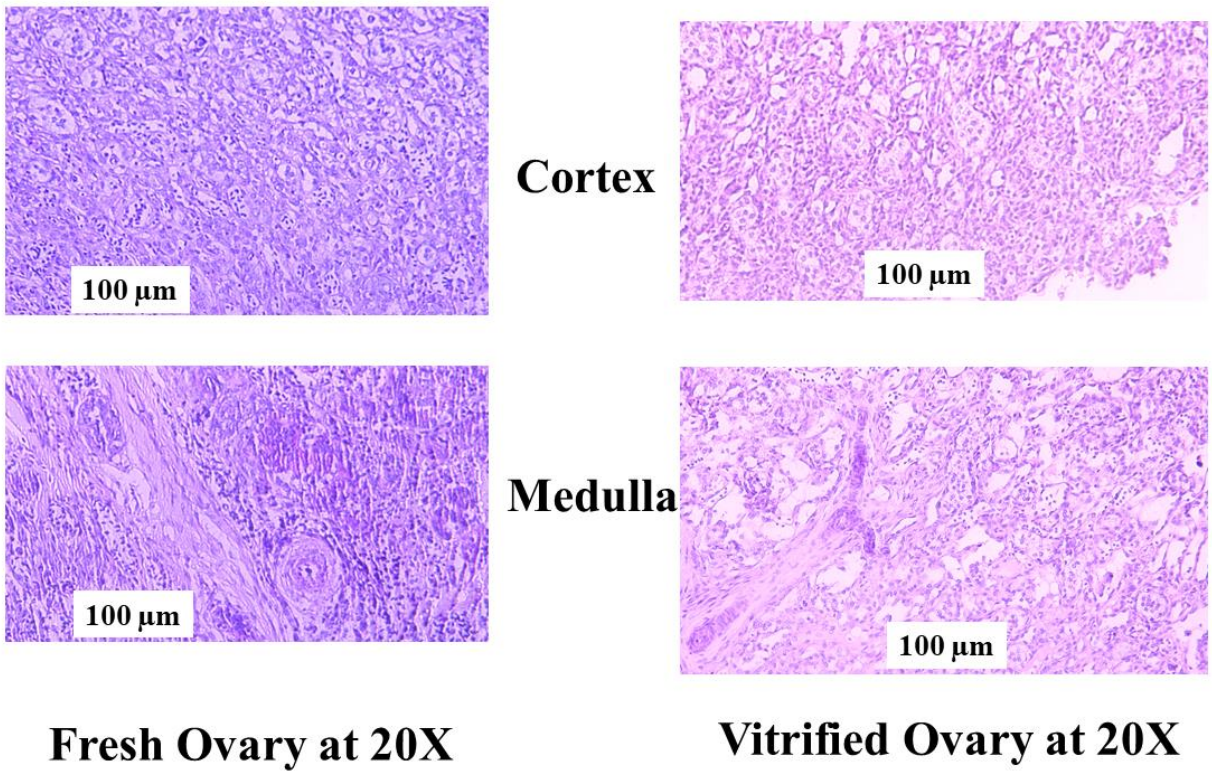

Figure 5.7. Morphological histology with PAS-H staining of ovaries demonstrating normal structure that can be distinguishable between the cortex and medulla area versus vitrified ovary showing abnormal structures (20x)

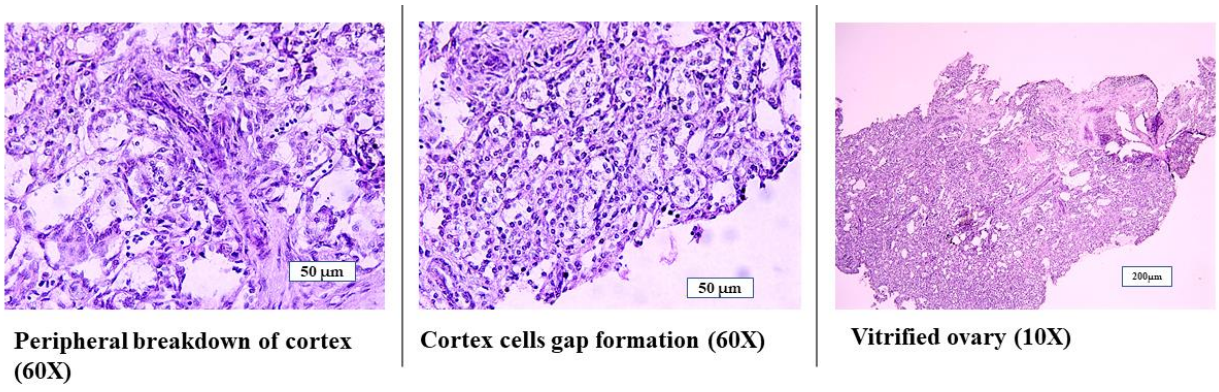

Figure 5.8. Morphological histology with PAS-H staining of vitrified-warmed ovary demonstrating gap formation and pyknotic and peripheral breakdown of the cortex.

5.6.2. Apoptosis determination on both fresh and vitrified chick gonadal tissues

In testicular tissues, apoptotic cells were distributed in the center area and peripheral area of the fresh and vitrified-warmed testes (Fig 5.9 left). These distributions were equal in both center, and peripheral areas (P=0.516 & 0.247) was found between groups (Fig 5.9 right). In general, there was no significant difference between fresh and vitrified-warmed testes when comparing regions within the testes, including both central and peripheral areas (Fig 5.9 left). The similarity was shown in Figure 5.10-5.11 that IHC image (PFA-fixed paraffin-embedded section) of anti-caspase 3 on fresh testes (Figure 5.10) versus vitrified-warmed testes (Figure 5.11). The images show apoptosis by bright green colour spots at the cytoplasm of cells covering around the nuclei (blue colour spots) in merged images in the center and peripheral areas. Moreover, the apoptotic appearance was seen without nucleic (in the middle of the images), or nuclei without apoptosis were shown in both areas.

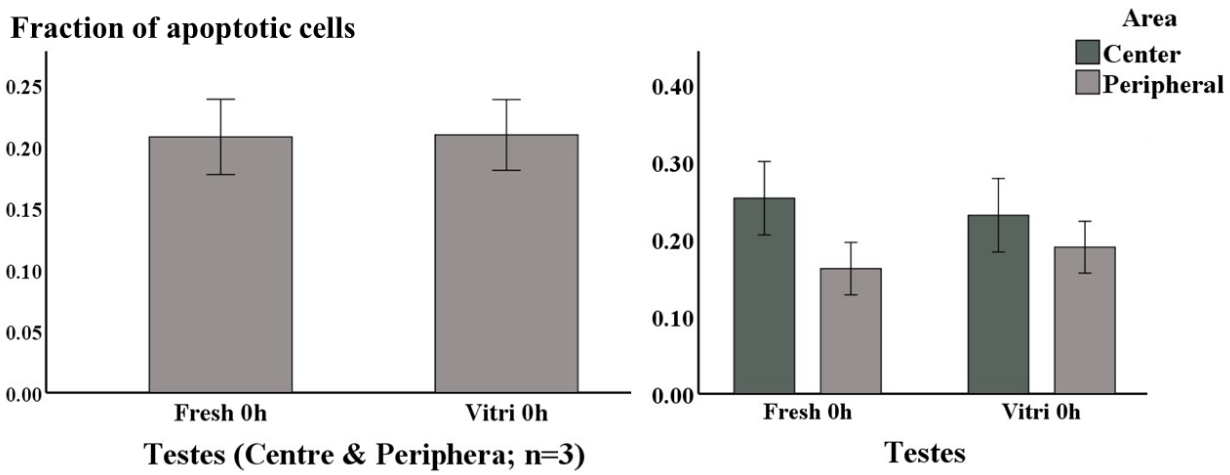

Figure 5.9. Fraction of apoptotic cells in fresh and vitrified chick testicular tissues (n=3 each group) from both in general and at the center vs peripheral of the tissue. No significant differences among treatments were found (P>0.05).

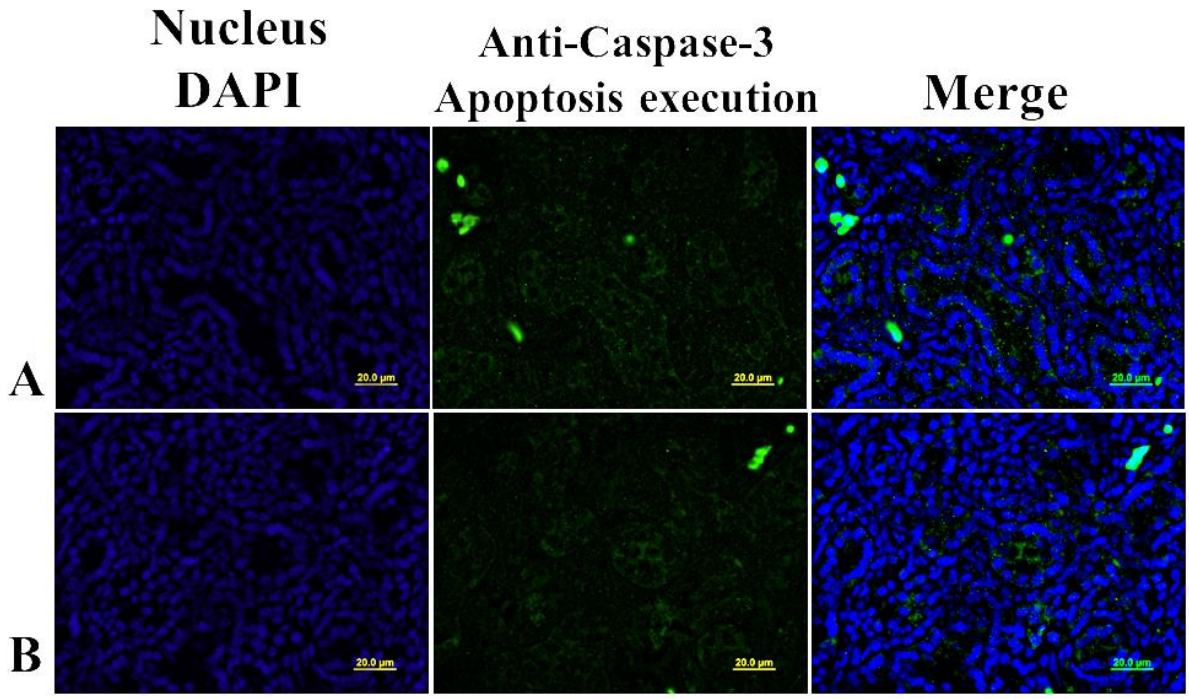

Figure 5.10. Immunohistochemistry image (PFA-fixed paraffin-embedded section) of anti-caspase 3 in fresh chick testes (A: center; B: peripheral)

The images show apoptosis by bright green colour spots at the cytoplasm of cells covering around the nuclei (blue colour spots) in merged images in the center and peripheral areas.

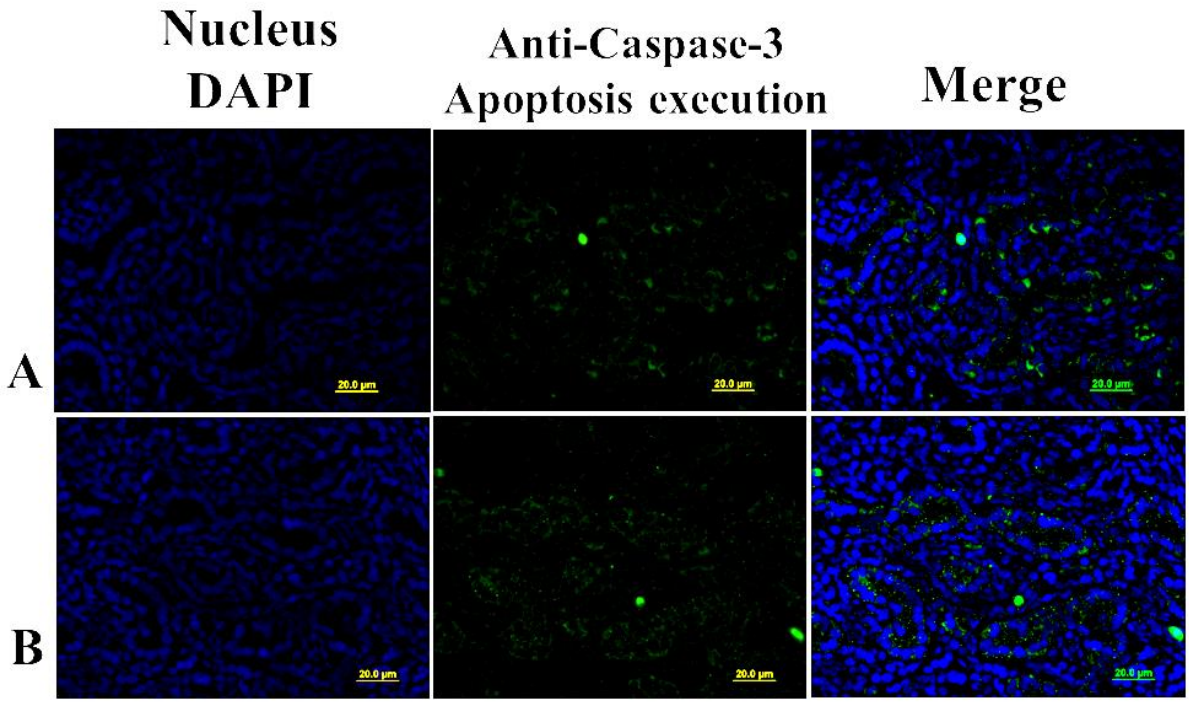

Figure 5.11. Immunohistochemistry image (PFA-fixed paraffin-embedded section) of anti-caspase 3 in vitrified-warmed chick testes (A: center; B: peripheral)

The images show apoptosis by bright green colour spots at the cytoplasm of cells covering around the nuclei (blue colour spots) in merged images in the center and peripheral areas.

Apoptosis appeared equally in both center and peripheral areas of both groups (Fig 5.12 right); however, fresh ovaries showed more apoptosis than vitrified-warmed ovaries (Fig 12 left). These differences were shown in figure 5.13-5.14, IHC image (PFA-fixed paraffin-embedded section) of anti-caspase 3 on fresh ovary (Figure 5.13) versus vitrified-warmed ovary (Figure 5.14). Apoptosis clusters were found in the cytoplasm of cells and around nuclei (blue colour spots with bright green colour clusters around). The fresh ovaries of images in both center and peripheral areas had more apoptosis appearance than the vitrified-warmed ovaries in comparison.

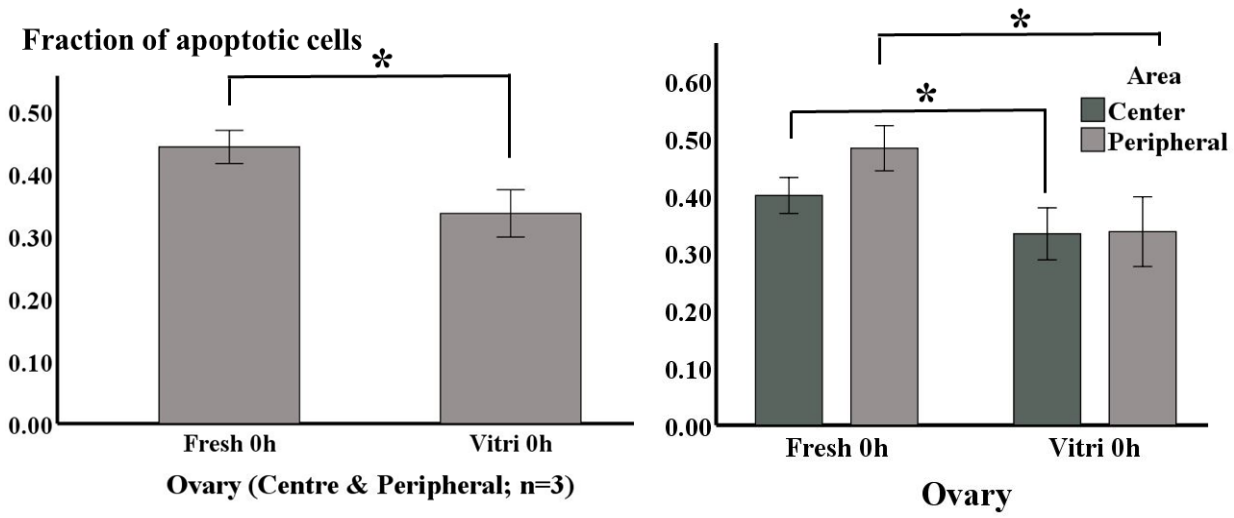

Figure 5.12. Comparison of apoptosis in fresh and vitrified chick ovaries (n=3 each group) both center and peripheral areas for apoptosis execution. Data are shown as mean ± SE as a fraction of apoptotic cells, and the * symbol indicates significant differences via a one-way ANOVA test ($P<0.05$).

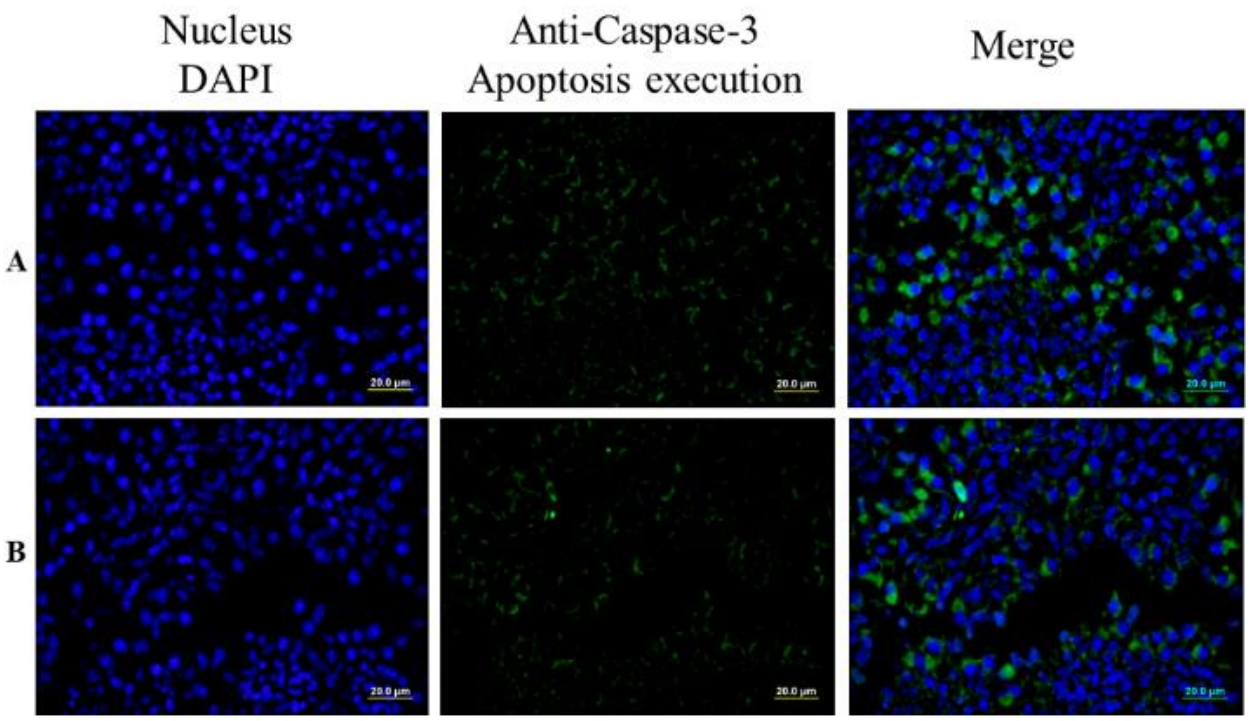

Figure 5.13. Immunohistochemistry image (PFA-fixed paraffin-embedded section) of anti-caspase 3 in fresh chick ovary (A: center; B: peripheral)

Apoptosis clusters were found in the cytoplasm of cells and around nuclei (blue colour spots with bright green colour clusters).

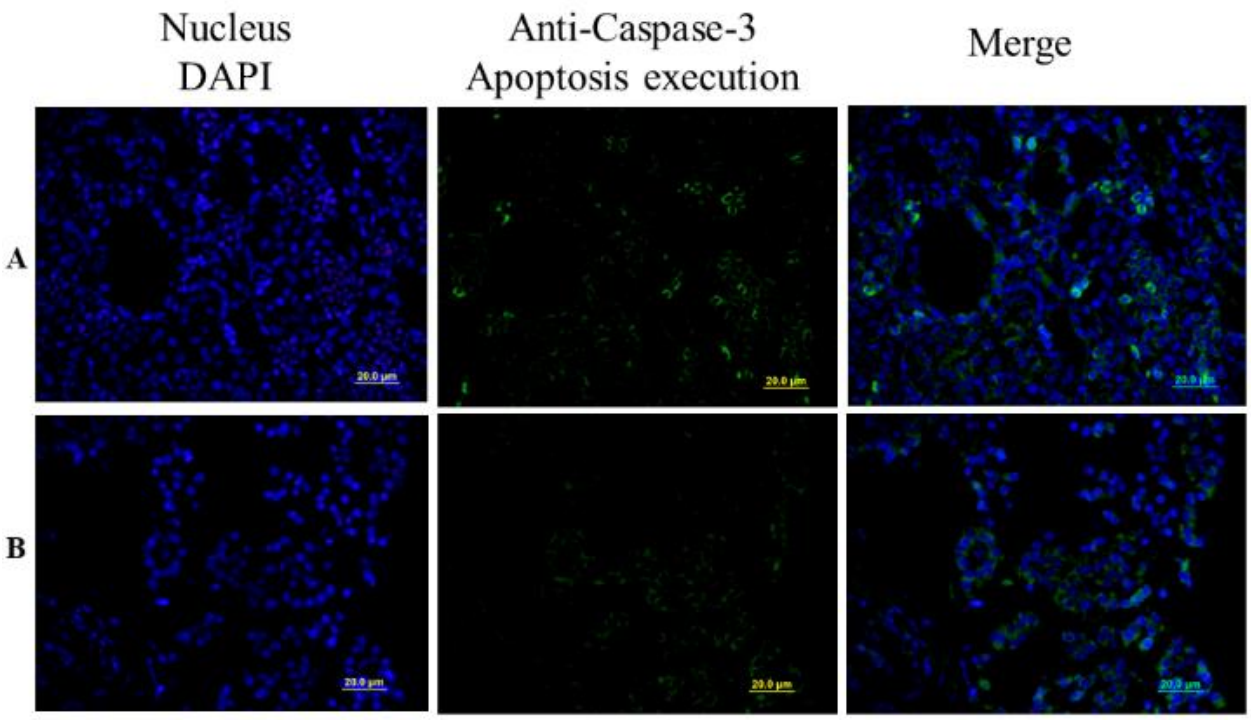

Figure 5.14. IHC image (PFA-fixed paraffin-embedded section) of anti-caspase 3 in vitrified-warmed chick ovary (A: center; B: peripheral)

Apoptosis clusters were found in the cytoplasm of cells and around nuclei (blue colour spots with bright green colour clusters around).

5.6.3. Apoptosis determination on five groups of chick gonadal tissues

There was no difference in apoptosis appearance between the Fresh 0h and Vitri 0h testes (Figure 5.15) ($P>0.05$). However, apoptosis appeared more in Fresh 2h, CPA 2h and Vitri 2h testes ($P<0.0001$). Furthermore, no significant difference was found among Fresh 2h, CPA 2h and Vitri 2h ($P>0.05$).

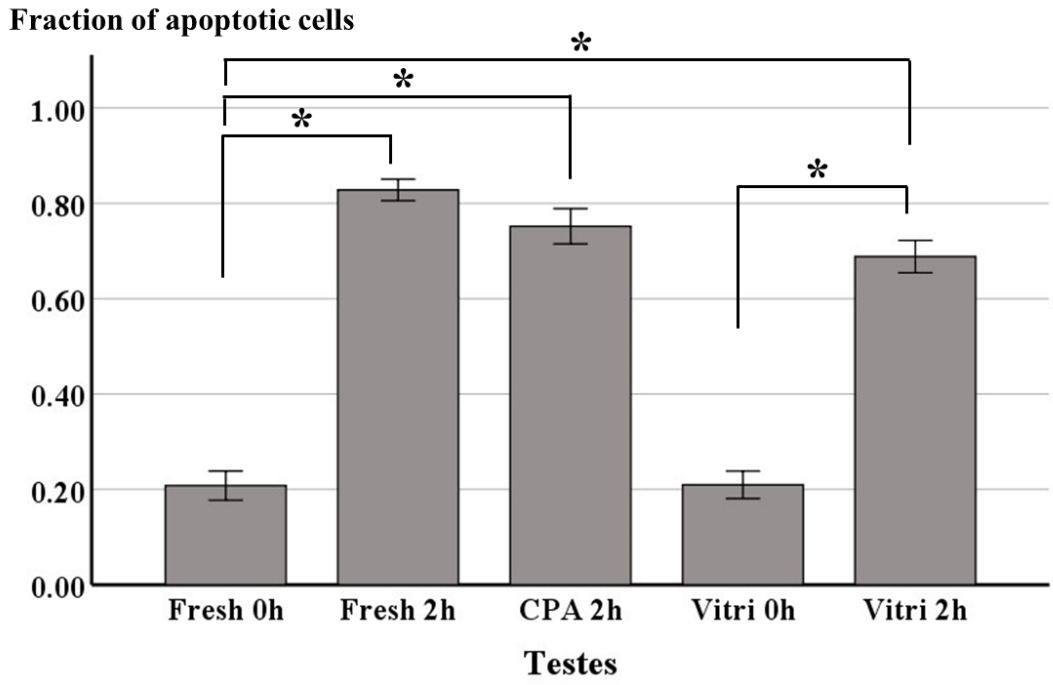

Figure 5.15. Testes apoptotic cells exposed to CPAs at different time points. Data are shown as mean ± SE of the fraction of apoptotic cells, and the * symbol indicates significant differences via a one-way ANOVA test ($P<0.05$).

For chick ovary, apoptosis execution was significantly different between Fresh 0h and Vitri 0h ovaries ($P<0.0001$). The most striking result to emerge from the data was that apoptosis appeared less in Vitri 0h group compared to Fresh 0h. The Fresh 2h, CPA 2h and Vitri 2h groups, were significantly higher than the Fresh 0h and Vitri 0h ovaries ($P<0.0001$). Furthermore, no significant difference was found among Fresh 2h, CPA 2h and Vitri 2h ($P>0.05$).

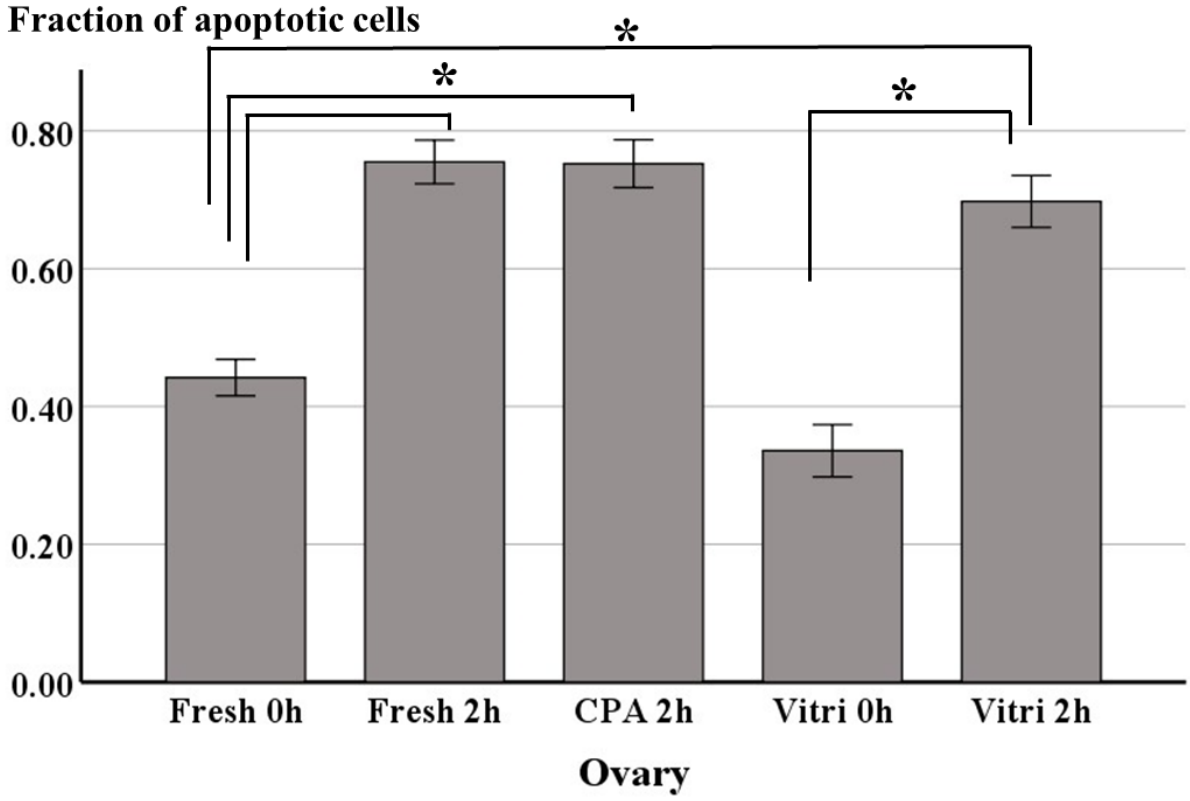

Figure 5.16. Ovary apoptotic cells are exposed to CPAs at different time points. Data are shown as mean ± SE of the fraction of apoptotic cells, and the * symbol indicates significant differences via a one-way ANOVA test ($P<0.05$).

5.6.4. The CPA diffusivities

Figures 5.17 and 5.18 show the measured osmolality values that were then fit to equation (2) and (3) for the diffusivity *D* with regressions for all data. In addition, data are also summarized in Table 5.2. The diffusion experiments were fit for diffusivity using custom software (Mathematica, Wolfram, Inc). For both testes and ovaries, significant effects of CPA and temperature were found, with FMD significantly higher diffusivity at all temperatures compared with PG and DMSO.

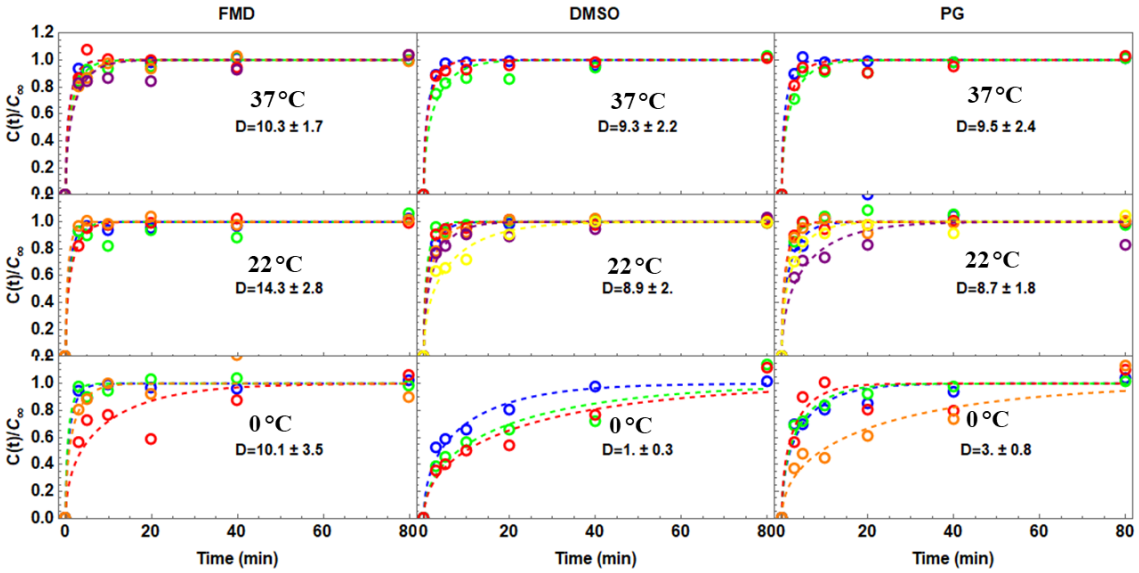

Figure 5.17. Testicular tissue with best fit regressions for three CPA types

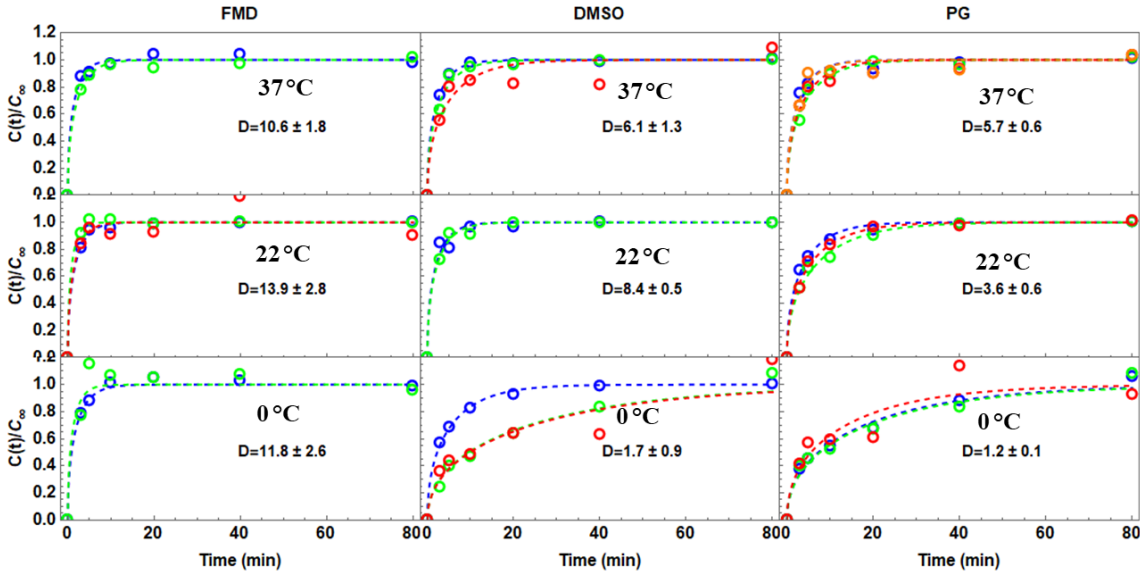

Figure 5.18. Ovarian tissue with best fit regressions for three CPA types

Table 5.2. Best fit (mean ± SEM) parameters for testes and ovaries

Tissue type	Temperature	CPA type		
		FMD	**DMSO**	**PG**
Testes	**37 °C**	$10.3 \times 10^{-6} \pm 1.7$	$9.3 \times 10^{-6} \pm 2.2$	$9.5 \times 10^{-6} \pm 2.4$
	22 °C	$14.3 \times 10^{-6} \pm 2.8$	$8.9 \times 10^{-6} \pm 2.$	$8.7 \times 10^{-6} \pm 1.8$
	0 °C	$10.1 \times 10^{-6} \pm 3.5$	$1. \times 10^{-6} \pm 0.3$	$3. \times 10^{-6} \pm 0.8$
Ovary	**37 °C**	$10.6 \times 10^{-6} \pm 1.8$	$6.1 \times 10^{-6} \pm 1.3$	$5.7 \times 10^{-6} \pm 0.6$
	22 °C	$13.9 \times 10^{-6} \pm 2.8$	$8.4 \times 10^{-6} \pm 0.5$	$3.6 \times 10^{-6} \pm 0.6$
	0 °C	$11.8 \times 10^{-6} \pm 2.6$	$1.7 \times 10^{-6} \pm 0.9$	$1.2 \times 10^{-6} \pm 0.1$

5.7. Discussion

The cryopreservation approach of using vitrification and rapid warming has been applied to tissues from different mammalian species such as mouse, cow, ewe, quail and human. In the current study, evidence of the impact of this procedure on chick gonadal tissues were studied with several damage measurement outcomes evaluated. Our results support the hypothesis that the vitrification-warming procedure induced damages on one to three days old chick testes and ovaries differently and increased apoptotic cells on these gonadal tissues. The overall goal was to evaluate how much damage occurred post-procedure by histological morphology and immunostaining. Because of this, we identified the parameters needed for a mathematical model of CPA equilibration damage used to determine potentially more optimal CPA equilibration protocols. These protocols can be tested in future studies. Overall, an optimal vitrification-warming procedure could preserve the genetic diversity of heritage chicken breeds, including their gonadal tissues.

The most prominent finding in this study is that there is significant damage associated with vitrification compared to fresh gonadal tissues, supported by a morphological scoring system to assess the vitrification-warming procedure (Table 5.1). These adverse effects appeared in the entire testes (Figure 5.1-5.2) and ovaries (Figure 5.5-5.6) and could be observed in the structure, nuclei, cell membrane and germ cells as well. One possible explanation of these results may be that gonadal tissues from very young chicks are vulnerable to low temperatures or changes from cooling to warming process. Other studies have demonstrated the differences between fresh and cryopreserved chick ovarian tissues by measuring the diameter of oocyte and follicle (Jianan Liu et al., 2017). Oocyte and follicle diameters in fresh tissues were comparatively larger than those in vitrified-warmed tissues. However, this study was confounded slightly by chick age. They

assessed tissues on different days, specifically day 7 in fresh and day 5 in vitrified tissues, leading to smaller diameters in vitrified tissues (Jianan Liu et al., 2017). Liu et al. (2007) suggest that because of their relatively small size, oogonia and primordial follicles at young ages might be more tolerant of cryopreservation, especially in one to three-day-old. In another study, Svoradová et al. (2019) confirmed that frozen/thawed day-old chick PGCs showed much lower viability than fresh day-old chick PGCs by using trypan blue exclusion (TBE) or propidium iodide viability assay (PIVA). Moreover, vitrified PGC damage was detected through swollen cells or mitochondria by using transmission electron microscopy, leading to a detailed evaluation in alterations of cell ultrastructure (Svoradová et al., 2019). Regarding testicular tissues, our results showed that damage occurred on vitrified-warmed tissues, but the viability and recovery of tissues after the surgery transplantation were acceptable. This finding is consistent with that of Liu et al. (2012), who also found that one-week-old chick vitrified-warmed testes showed typical structure and vascularization of the donor testicular grafts, and additionally was well established after implanting into a chicken embryo. Song and Silversides (2007a) found similar results, demonstrating regeneration of functional seminiferous tubules to maturity in newly hatched chick vitrified testes after transplantation. From these collective findings, the vitrification and transplantation of testicular tissues seem to be more successful than ovarian tissues. Although in this study, we found there were some damages due to the vitrification-warming procedure, the recovered viability was higher in comparison with ovarian tissues.

Our results showed that apoptosis in vitrified testicular tissues was similar to fresh testicular tissue, meaning that the procedure did not induce more apoptotic cells post-warming. In contrast, and surprisingly, vitrified ovarian tissues showed reduced apoptosis compared with the fresh group. Different mechanisms could possibly explain this latter result in inducing apoptosis

between the fresh group and the vitrified group leading to this contradiction. Liu et al. found that the percentage of apoptosis in cryopreserved tissues was less than the one in the fresh tissues of chicks at 1-3 days old, where apoptotic cells were identified by TUNNEL assay (Jianan Liu et al., 2017). Liu et al. (2017) suggested that the mechanisms that induce apoptosis in vitrification-warming procedures might be different compared to fresh tissues and that there could be differences in staining that impact detecting and calculating apoptosis from images. In agreement with this study, another finding demonstrated that apoptosis appeared more at day-old chicks and less after (Yoshimura and Nishikori, 2004). The increase in apoptotic cells could be related to the control of oocyte quality and the removal of abnormal oocytes in mammalian species (Tilly and Kolesnick, 2002; Yoshimura and Nishikori, 2004). In contrast to earlier findings, however, no significant difference was observed between fresh and vitrified mouse ovarian tissues concerning follicle viability (Mazoochi et al., 2008).

With respect to gonadal tissue incubation, apoptosis increased significantly after two hours of incubation at 37 °C. This echoes results from another finding (Milazzo et al., 2008) that demonstrated that after warming on immature mice, testicular tissue apoptosis appears after a brief culture period when identified using the TUNEL assay as opposed to the Annexin V marker that can be used at an early stage. Similarly, another article showed time-delayed evidence of apoptosis in the form of rounding, blebbing cells to lysis of cells, and necrotic cells could be named for dead cells because of the similarity of the DNA fragmentation process (Collins et al., 1997). These results reflect those of another report (Vanhulle et al., 2006), which showed that cytochrome c release that was related to caspase-3 activation occurred after 1 h incubation, which would lead to the increasing of apoptosis after incubation of vitrified tissues. In sum, the present work found at least two major differences between fresh and vitrified gonadal tissue, including histological

morphology and apoptosis evaluation. Although apoptosis was not detected post-warming on ovarian tissue, there were significant morphological damages. Regarding surgical transplantation from the previous chapter, some challenges still occurred in the surgical transplantation of donor ovarian tissue to recipient. It can thus be suggested that a modified vitrification-warming procedure is necessary to cryopreserve the chick gonadal tissue.

These findings have important implications for adjusting the vitrification-warming procedures related to many factors such as CPAs, concentration, temperature and time points. Because of the challenging interactions of all conditions, one approach to determining an improved vitrification-warming procedure is to establish a mathematical model (Benson, 2015). The modified procedure would be based on conditions of CPAs equilibration, including the specific CPA, concentration, time points for equilibration protocols and temperature. The overall goal of this study was to minimize damage for gonadal tissues post-procedure, including minimizing the toxicity, osmotic stress and freezing injury of chicken gonads. One of the overarching hypotheses of this study was that the vitrification protocol would induce significantly increase apoptosis. This increase, then, could be quantified using the damage model (Eq 1) developed by Benson et al. (2018) when coupled with the CPA diffusion model (Eq 2). However, we did not find increased apoptosis as a function of vitrification. As such, the model in Eq. 1 intended to be used to further optimize vitrification protocols for chick gonadal tissue could not be informed.

In contrast, this study found significant changes in histological features. This damage mechanism is not captured by the toxicity cost function described in Eq 1 and suggests that there may be important mechanical damage mechanisms that should be accounted for in future models of cryopreservation damage. This is an area for future modelling research. However, there is still utility in determining CPA diffusivity, *D*, as a function of temperature (Table 5.2). In particular,

this study showed that FMD is transported through both ovarian and testicular tissues much more rapidly than DMSO or PG. These results reflect those of Benson et al. (2018), who used PG measured in the study of diffusivities in human skin, myometrium, and fibroid that the diffusivity of PG was lowest on skin. We can use these data to design protocols where we equilibrate first with the slowest diffusing CPA, PG, e.g. for 2 min, then the second slowest DMSO for 2 min, then the fastest diffusing CPA, FMD, for 5 min to achieve complete equilibration of all three. This strategy will minimize the total exposure of FMD, which has a relatively high toxicity compared with other CPAs. This suggests that shorter CPA equilibration protocols could be developed to reduce total exposure time and its associated accumulation of toxicity. In future studies, these values will be used to predict more optimal and shorter CPA equilibration protocols that, for example, might not need direct-plunge vitrification techniques that require piercing tissues on a needle. It has been reported that PG caused fewer chromosome damages for ovary cell lines DMSO or EG, but more substantial chromosome damage than DMSO or EG (Best, 2015). Optimal equilibration prevents tissues from the intracellular formation that is associated with other factors. Moreover, low permeability of CPAs also causes negative impacts to cells or tissues as compared to high permeability, including osmotic stress, injury (Best, 2015).

In conclusion, the vitrification-warming procedures affect the cellular integrity of one to three-day-old chick gonadal tissues through morphological alterations. In addition, the procedure does not cause more apoptotic cells on testicular tissues, but it significantly reduces apoptosis on vitrified-warmed ovaries. The mathematical modelling was used to predict tissue response to CPA equilibration for both chemical damage and toxicity. However, there are still some factors that need to be detected for future modelling research.

CHAPTER 6. DISCUSSION

The main goal of this thesis was to optimize the conditions for successful preservation and regeneration of poultry gonads to protect the genetic diversity of poultry breeds effectively. It is possible to preserve poultry gonads, transfer them into a recipient of similar or different genetic backgrounds, and sustain growth into the recipient. These strategies might help to maintain precious genetic resources for the need for species in the future. Moreover, this thesis also showed the evaluation of the vitrification-warming procedure through histological morphology and produced the modified procedure based on a mathematical model. These improvements would allow an increase in the efficiency of cryopreservation and transplantation for the regeneration of the genetic resources of chicken breeds. Also, the poultry industry can apply these optimal strategies to maintain the unique genetic resources that preserve the value features to prevent the loss of species due to diseases or harsh environmental factors. From chapter 4, surgical transplantation was done on chicken breeds of similar or different genetic backgrounds from donor's fresh and vitrified-warmed ovarian tissues. The study partially supported the hypothesis that the strategies of cryopreservation and transplantation of ovarian grafts would be able to sustain the growth in different recipient lines. However, follicular activities were not found as expected that were associated with clusters of immune cells shown in histological sections of donor-derived ovarian tissues. These results suggested that many related issues need to be considered, such as the immune response of recipient, immunosuppressants, vitrification-warming procedure, etc. In chapter 5, a more mechanistic study was undertaken to evaluate the vitrified gonadal tissues that were employed in transferring. Two methods were applied for assessment of the procedure, including histological morphology and apoptosis execution via caspase-3 immunofluorescence. The results have shown that the gonadal tissues could not tolerate the impact of the procedure.

These adverse effects were confirmed by the presence of damage as well as the increase of apoptotic cells post-warming, suggesting an improvement of the procedure. It was hypothesized that a mathematical model could be developed that prescribes optimal protocols to define CPAs, concentration, temperature, and time points to minimize the toxicity of CPAs, cellular injury, or cellular ice formation. All these modifications aimed to protect gonadal tissues from damage. Because no significant apoptotic damage was found, the damage would not be useful for the analysis. I did identify diffusion parameters for each tissue type and CPA to facilitate the optimization of protocols using a different damage model. In the case of successful cryopreservation, the potential of the subsequent transplantation will help to regenerate the genetic resources contributing to the genetic diversity of species.

Two main objectives of this thesis included optimization of the gonadal tissue transfer technique into different chicken breeds and optimization of the conditions for CPAs equilibration to minimize the toxicity, osmotic stress, and freezing injury of gonads. For the first objective, the optimization of the technique was based on the achievement of the transplantation by the adjustment accordingly. In addition, the potential of different chicken breeds to receive a graft was evaluated that was associated with the development normally until maturity. In the second objective, it turned out that the major mechanism of damage we identified after vitrification and warming, mechanical disruption of tissues instead of programmed cell death, was not able to be captured by the existing model of CPA equilibration induced damage.

In this study, vitrification was the chosen procedure used to cryopreserve the chicken gonadal tissues. However, the vitrification procedure was modified from Japanese quail (J. Liu et al., 2010), and the following transplantation was operated that confirmed by high cell viability, integrity, the ability to produce offspring on this species. Thus, an appropriate assessment for the

strategy would be helpful in applying for chicken breeds. The application of those strategies is associated with chicken or turkey and requires more effort, troubleshooting, and fine-turning (George Hall, 2015; Y. Song and Silversides, 2006). Based on the evaluation of vitrified-warmed gonadal tissues, the intense impacts of the procedure caused damage for those tissues, both in morphology and the increased prevalence of apoptotic cells. These results suggested that chick gonadal tissues were quite sensitive to the related parameters in the procedure, such as cooling, warming rate, CPA toxicity, etc. In particular, these effects were shown in cryopreserved ovarian tissues that primary and secondary follicles were more tolerant than primordial follicles (Gandolfi et al., 2006).

It is clear then that there are two main aspects of damage. First is that the chick gonadal tissues could be especially sensitive to removal and transplantation. In this case, it is useful to determine optimal protocols to collect, store and process before and after cryopreservation and transplant that will minimize the physical injury and protect the viability and integrity of the tissue. The improvement of the related parameters in the vitrification-warming procedure might help to maintain the normal structure and functions. In fact, a specific species' cellular cryotolerance depends on specific biophysical characteristics. Comizzoli et al. (2012) identified four following factors that influence cell viability post-warming, including CPA transport (e.g. membrane permeability in the case of individual cells, or CPA diffusivity in the case of tissues), CPAs toxicity, osmotic tolerance, and cooling sensitivity. In addition, freezing injury can happen due to the exposure to cooling below physiologic temperatures associated with cold shock. Thus, the exposure time and cooling rate are assessed to protect cells or tissues (Mazur et al., 2008). From these determinations, it is evident that the CPA transport and chilling sensitivity could be considered traits that might not change. However, CPAs toxicity and cooling or warming rates

could be adjusted and changed depending on the cellular membrane. The evaluation of CPAs toxicity is needed to select appropriate CPAs that could minimize the toxicity. Also, the adjustment of cooling-warming rates needs to be assessed to diminish cellular damages. However, the vitrification can occur by the combination of high cooling-warming rate and high CPAs concentration that will challenge the high risk of damages due to osmotic stress and CPA toxicity (Davidson et al., 2014). Also, high CPA concentrations can help to minimize cellular ice formation in the process of cryopreservation (Best, 2015). Besides, volumetric damages could be occurred due to the differential permeability of the cellular membrane to CPAs and water (Davidson et al., 2014). Thus, the selection of CPAs types, including penetrating and non-penetrating CPAs, are essential to reduce the toxicity. Besides, the appropriate combination of CPAs also helps to lower the concentration but still maintain efficiency. Some universal CPAs have been employed, such as dimethyl sulfoxide (DMSO), formamide (FMD), propylene glycol (PG), ethylene glycol (EG), etc., along with non-permeable cryoprotectants, sucrose, glucose, fructose, etc., for instance (Best, 2015). Among them, some kinds could show specific toxicity, while others showed non-specific toxicity; EG as an example due to the toxicity to the liver or kidney. Another report was shown with FMD molecules that their moment of a dipole was two times higher than water molecular; thus, FMD should be combined with other CPAs to vitrify the biological materials (Brent, 2001; Richardi et al., 1997). CPAs showed their toxicity differently to diverse cells, tissues, and organs on different species, leading to the distinct mechanisms for the individual. The CPA permeability causes an influence on the cellular membrane that the low permeability will cause more damages that the high one; also, the molecule size impacts this permeability (Gilmore et al., 1997). One possible solution was the establishment of an appropriate CPA equilibration procedure through mathematical modelling based on the related parameters including CPAs osmolality, temperature,

CPA concentration, and time points. Different CPAs were employed at a high concentration (30%) for the chick gonadal tissues to measure the CPAs osmolality; these parameters were used to design CPAs equilibration procedure that can minimize the toxicity. These methods have been applied to the skin, fibroid, and myometrium that minimized toxicity and damages to cells (Benson et al., 2018). With these strategies, high CPA concentration was used to identify the critical concentration for the gonadal tissues, and then the final concentration would be advised for the distribution into the tissue. In the meantime, the toxicity accumulation would be evaluated at a high concentration (Benson et al., 2018). Through the process, the CPA equilibration procedure determined that CPA toxicity could be quantified. Thus, the optimal equilibration procedure was predicted to satisfy vitrification conditions that prevent the tissues from CPA toxicity.

These results from the vitrification and subsequent transplantation have shown that the immune response of the recipient would be an extreme issue to be considered carefully. In chapter 3, the results showed differences between chicken lines, confirmed by the presence of donor grafts in diverse sizes, or the absence of donor grafts at maturity. Besides, immune cells were found in donor-derived ovarian tissues collected from the mature hen through light microscopy, suggesting that immune responses have occurred on recipients. However, these immune responses have unknown at a certain period or during the time post-surgery until maturity. From chapter 2, it is evident that the immune responses happened to recipients regardless of fresh or vitrified-warmed tissues. These results confirmed that chicken showed a strong immune response to donor graft through transplantation, corroborating the findings of the previous works in the surgery transplantation. In general, success has not completely been achieved in several chicken breeds (Liptoi et al., 2013; Y. Song and Silversides, 2008a). Besides, chickens possess two distinct central lymphoid organs, including the thymus and the bursa (Hammer, 1974), which T and B

lymphocytes are derived from, respectively. The strong immune system might be one of many challenges that prevented the success of the transplantation, which was associate with the rejection of donor grafts. Immunosuppressant treatment (Mycophenolate Mofetil – MMF) has been employed to solve this issue. MMF has been demonstrated to inhibit the proliferation of T cells and B cells (RE Morris et al., 1990), suggested targeting ovarian transplantation (Y. Song and Silversides, 2007b). However, no significant difference was found between the treatment group and the group without treatment on chicken breeds in general (Y. Song and Silversides, 2007b; Liptoi et al., 2013). In the meantime, it is difficult to evaluate whether the immunosuppressant treatment could be effective because the use of this drug was empirical; thus, the need is an appropriate assessment regarding the use of immunosuppressants within species to complete the goal. The selection of appropriate drugs, along with a suitable dose, would allow recipients to receive donor grafts. Although MMF was challenged when employed for transplants among breeds of chickens, it was useful when employed with Japanese quail to produce offspring derived from donors (Y. Song and Silversides, 2008b). To master the effects of MMF on chicken, it needs to be tested to provide an appropriate treatment regarding dosing, aging, etc. Also, other immunosuppressants could be used by individuals or a combination of a different drug, which might help to prevent rejection. Cyclosporin A (CsA) is considered as an immunosuppressant for chicken transplants through its inhibition to T cell proliferation, failing the cytokine encoding, which is essential for the immune system (Bucy et al., 1990; Schreiber and Crabtree, 1992). In addition, the combination of immunosuppressants has been demonstrated by graft survival in renal transplants (Lindholm et al., 1992). Both dual therapy (cyclosporine & prednisolone) and triple therapy (cyclosporine, azathioprine, and prednisolone) were found conclusively to prevent acute rejection. Besides using immunosuppressive drugs to avoid rejection, a variety of methods have

been employed to increase the survival and growth of donor grafts. A method was described to prevent the organ transplant rejection, which was the modification of the immune system based on T cells (Thorp et al., 2015). The authors explained that modified or impaired T cells could not reach to target organs or donor grafts to attack them, resulting in T cell exhaustion; and this would mean that donor grafts could be protected from being rejected (Thorp et al., 2015). In another study, a technique was provided to prevent transplant rejection without drugs for kidney transplants (Mitka, 2002); the method was conducted by injecting blood stem cells of donors into recipients. In the process, these recipients were provided dose of radiation with the target of immune system to decrease the vulnerable cells to immune responses. Then, those stem cells were absorbed into the bone marrow of recipients and incorporated with its blood circulation to facilitate accept the donor grafts as their own (Mitka, 2002).

In summary, vitrification will be considered as the right procedure that may be applied to many biological materials of diverse species, especially on bird gonadal tissues, if its conditions are satisfied correspondently. Moreover, vitrification has shown more benefits regarding the simplicity and the low cost (Comizzoli et al., 2012). The broad application of the strategies, including vitrification and subsequent transplantation, will help the recovery of species and protect the population from extinction. In this thesis, vitrification-warming procedure and immune response of recipients to donor grafts have emerged as main factors, which contributed to the success of gonadal tissue transplantation. Besides, several factors should be considered, such as the compatibility of breeds and optimal surgical technique.

CHAPTER 7. FUTURE DIRECTIONS

This thesis concentrated on the vitrification-warming procedure and subsequent transplantation to cryopreserve chick gonadal tissues for long term regeneration of the genetic resources of the poultry breeds, including heritage chicken breeds. Thus, an optimal vitrification-warming procedure is necessary to secure the strategy. During our studies, several limitations emerged for tissues post-procedure regarding cell structural integrity or apoptosis execution; thus, a novel method was establish based on the related conditions. In further works, improved mathematical models that can account for mechanical tissue disruption (as opposed to just apoptosis and necrosis) and from them an improved vitrification-warming procedure will need to be tested with the chick gonadal tissues, which can be compared with the previous processes. The re-evaluation will help to provide an appropriate and applicable procedure. Also, the transplantation techniques need to be improved to improve their effectiveness. The improvements could be regarding the prevention of bleeding with concise technique, ablation of gonadal tissue with an electrocautery device, for instance.

Moreover, the results showed issues related to the post-transplantation incursion of immune cells, which appeared in the histological morphology of ovarian tissues at maturity, suggesting some levels of rejection. Thus, the immunosuppressive treatment will need to be optimized depending on recipients to achieve functional and active ovarian grafts. To increase the efficiency of the immunosuppressants, birds can be tested at different times for the presence or absence of the drug and their immune response at those time points. This experiment should be designed with a larger number of chicks to achieve enough statistical power to facilitate evaluation through aging stages. Finally, different chicken breeds should be selected to find appropriate recipients for transplantation to prevent rejection.

CHAPTER 8. GENERAL CONCLUSIONS

The thesis confirmed the potential of the vitrification method and subsequent transplantation of the chick gonadal tissues into recipients, which help maintain the genetic resources of heritage chicken breeds. In addition, donor-derived offspring can be produced through ovarian tissue transplantation. Thus, producers or industries can cryopreserve gonads from one to three days old chick using the vitrification technique and regenerate those tissues when needed. To minimize tissue damages and increase the efficiency in preserving and regeneration of gonadal tissues, it will require an optimization of the procedure and the immunosuppressive treatment to allow the ovarian graft to develop at maturity with folliculogenesis activity.

Also, the vitrification-warming procedure was evaluated by the morphological damages and the execution of apoptotic cells on gonadal tissues, including testes and ovary. The studies showed some negative effects of the procedure, which were based on the alterations of the cellular structure. From these assessments, the current mathematical model developed to predict tissue response to CPA equilibration could not be used and requires further research. However, to facilitate this analysis and future work, a biophysical study of the diffusion of cryoprotective agents in gonadal tissues was also undertaken, establishing the relative rates of permeation of CPAs in chick gonadal tissues. However, it is necessary to evaluate damage mechanisms and relevant factors for further research to improve the vitrification protocol for chick gonadal tissue.

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