CRYOPRESERVATION AND TRANSPLANTATION OF OVARIAN TISSUE IN JAPANESE QUAIL (*Coturnix japonica*)

by

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A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Animal Science)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

December 2009

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Abstract

Cryopreservation of avian genetic material is limited in terms of practicality, efficiency, and success. The complex structure of female gametes in birds makes the application of cryopreservation extremely difficult, and the fertilization obtained from frozen/thawed poultry semen is low and unpredictable. Embryonic germ cells can be frozen and used to generate germline chimeras, but the low efficiency and complex procedures limit these techniques for genetic conservation.

The techniques of transplantation of ovaries and testes in newly hatched chicks have recently been developed and donor-derived offspring could be efficiently produced from cryopreserved testicular transplants and from fresh ovarian tissue transplants in chicken. This success in chicken gonadal transplantation provides an effective method for recuperating live birds from cryopreserved gonadal tissue. Research efforts are needed to develop cryopreservation technique for ovarian tissues and further apply cryopreservation and transplantation to the other avian species.

The Japanese quail is a much smaller bird than the chicken and has a slightly different reproductive biology. Preliminary research has demonstrated that live offspring could be obtained from fresh ovarian grafts in one-week old quail. Using the Japanese quail as a model, the objective of this thesis is to develop a feasible and reliable cryopreservation technique for ovarian tissue and to recover it by subsequent transplantation, with the hope that this protocol of cryopreservation and transplantation can attribute to female germplasm conservation for other avian species in the future.
Both slow-freezing and vitrification were used as cryopreservation approaches in this study. The efficiency was evaluated at three levels: cell viability, tissue histology and the recovery of the donor fertilities after surgical transplantation. Donor-derived offspring were obtained from the ovarian tissues that had been cryopreserved by either slow-freezing or vitrification. The vitrification protocol used in this study showed better outcomes at each level of evaluation. Thus the current study verified that the function of ovarian tissue in an avian species can be successfully preserved at subzero temperatures and recovered by transplantation. The vitrification protocol is recommended for future use concerning its low cost, high efficiency and overall simplicity in optimization to benefit more avian species.
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Acknowledgements

I would like to thank my research supervisor Dr. Fred Silversides, whom I regard as a very good mentor and friend. It is his constant encouragement and guidance that motivate me to explore my “wild idea” in my studies with enthusiasm and to face and cope with difficulties in the experiments with calmness and wisdom.

I would like to gratefully acknowledge Dr. Yonghong Song, with his profound knowledge and rich experience, he guided me to the field of germplasm conservation.

I am deeply indebted to my academic supervisor, Dr. Kim Cheng for his generous support and invaluable advice. It is Kim who welcomed me to this country and helped me to step to a new stage of my life, which means a lot to me.

Special gratitude is owed to Dr. Gregory Lee, Dr. Rajadurai Rajamahendran, Dr. Anthony Cheung and Dr. Marina von Keyserlingk for reviewing my thesis and providing constructive criticism.

I owe deep thanks to our lovely barn crew in Agriculture and Agri-Food Canada (AAFC), Harold Hanson, Karli Ryde, Kathy Ingram, Lee Struthers, Martin Fraser and Wendy Clark for taking care of the birds and all the barn work. Special thanks to our technician Mairi Robertson for record keeping and lab maintenance. I really enjoyed the time when we were working together. Her extensive experience and knowledge and naturally sweet personality are very impressive to me and I learned so much from her.

I would also convey my heartfelt gratitude to the faculty members and staff of the Faculty of Land and Food Systems (LFS) in the University of British Columbia (UBC),
especially Sylvia for helping us ordering experimental materials; Lia, Allison, Kirsten for assisting us with administration issues with great patience. Similarly, I am thankful to the folks working for AAFC, Dr. Tom Forge, Dr. Moussa Diarra, Dietmar, Helen, Linda, Lynne, Marion, Mary Ann, Sarah, Stephen, and Todd, who welcomed me to Agassiz and helped me in various aspects.

I would also like to give thanks to the funding and scholarship providers, especially research funds from the British Columbia Ministry of Agriculture and Land (funds administered by the UBC Specialty Birds Research Committee) and also the support from AAFC via Agassiz Research Centre.

Thanks to our Research Associate Dr. Darin Bennett who takes care of us like a big brother. Thanks to our fellow graduate students in Avian Research Centre in UBC who showed me love and care after I came to UBC.

Thanks to all my friends I know through Inter Varsity, especially the Williams, the Epps, the Eftodas for helping me survive in Vancouver and in Fraser Valley.

I spent terrific time in the students’ house of AAFC in Agassiz. I cherished the friendship with our “French Connexion” and other oversea students. Special thank goes to my lovely roommate Emilie Bullo for being with me on those long working nights.

I will give my biggest gratitude to my parents and my brother, though they are far from me, they are always the source of my confidence and courage on my journey of perusing my dreams. I feel so blessed to have such a wonderful family.

“Thanks be to God for His indescribable gift!”- 2 Corinthians 9:15
Dedication

To all experimental birds

They are my little heroes
Co-authorship statements

All the experiments and analysis were completed by me, Jianan Liu. The following authors are listed on the manuscripts: Drs. Yonghong Song, Kimberly M. Cheng and Fred G. Silversides. The co-authors were involved in the preparation of manuscripts included in this thesis.
Chapter 1 Literature review

1.1 Principles of cryobiology

1.1.1 Water and aqueous solution in biological systems under low temperature

Water is of significant importance to living systems. It is widely involved in biophysical and biochemical reactions, contributes to the maintenance of biological structures and serves as a medium at the macroscopic level [1, 2]. Thus it is not surprising that the phase change associated with water and aqueous solutions under low temperature becomes a major concern in cryobiology.

1.1.1.1 Properties and molecular structure of water

The reason that water is favorable to biological systems may relate to some of its unique properties, such as high heat capacity and viscosity compared with its analogous hydride in the periodic table, which are determined by its unique molecular structure. Each water molecule is made up of an oxygen atom attached to two hydrogen atoms through covalent bonds. The two hydrogen atoms together with the two pair of “lone-pair” electrons of the oxygen atom form the four vertices of a tetrahedron. Thereby, two vertices are negatively charged by the electrons of the oxygen atom while the other two are positively charged as a result of the hydrogen protons [2]. By forming hydrogen bonds, each water molecule can be attached to four neighboring water molecules. This repeated framework gives the structure of Ice Ih [3]. As for the structure of liquid water, different hypotheses have been proposed and a divergence of opinions still exists. However, what can be safely borne in mind is that, whether as ice or liquid water, each
water molecule has a tendency to be tetrahedrally bound by four other water molecules [4].

1.1.1.2 Ice nucleation

After liquid water is cooled below the temperature of 0 °C, it will sustain a liquid state before the formation of ice crystals takes place, a stage denoted as supercooling or undercooling [5], which is described by the temperature difference between 0 °C and the temperature at which ice formation begins. Ice formation has to be initiated by ice nucleation. In pure water, clusters of hydrogen-bond associated water molecules serve as the original nuclei, which trigger the process termed “homogeneous nucleation”. The critical size of the clusters is affected by temperature and sample volume [6]. The surface curvature of the clusters is important as well [7]. The crystallization will expand quickly after nuclei interaction and arrangement [8]. In reality, homogeneous nucleation is rare; instead, ice nucleation is usually catalyzed by factors other than water itself, such as structures associated with the container wall or impurities in the water [9], which are common in aqueous solution in biological systems [10]. Though the exact knowledge of the chemical nature and origin of this phenomenon is still fragmentary [9], it is considered to be the basis of an important procedure, seeding, in cryopreservation protocols under slow-freezing strategies.

1.1.1.3 Freezing and vitrification of aqueous solution in biological systems

In comparison with that of pure water, thermodynamics of aqueous solutions in biological systems turn out to be much more complex. This is partly because the solutes (for example, chemicals used for protection, see below for details) have a dramatic
impact on the thermodynamic features of the water that they are dissolved in. As well, the behaviors of aqueous solutions under subzero temperatures are affected by the properties of the biological structures that they are present in, mostly, the membrane structure. Investigations are usually started with simple models such as a suspension of a certain type of cells and add assumptions fitting the classic thermodynamic theories.

When a cell suspension is supercooled, the cytoplasm will not go through ice nucleation immediately, even if ice nucleation is induced in the extracellular medium by either spontaneous formation of large enough nuclei or mechanical agitation. This phenomenon is affected by the freezing point depression and the membrane barrier. The freezing point depression [11] is expressed as:

$$
\Delta T = \frac{1000K_f W_{SO}/W_T}{(1-W_{SO}/W_T)M_S}
$$

in which $\Delta T$ is the difference in the freezing point between pure water and the solution, $K_f$ is the freezing point constant, $W_{SO}$ and $W_T$ are the weight of dissolved solids and the total weight of solution, respectively, and $M_S$ is the effective molecular weight of dissolved solids. The dissolved solids could refer to the protective chemicals or the solid part of the cytoplasm itself, which can be regarded as a complex solution [12].

It is postulated that at least above certain temperatures, the cell membrane may play a role as a barrier [13]. However, the growth of extracellular ice crystals leads to a vapor pressure gradient between intracellular water ($p_i$) and extracellular water ($p_e$), which
can be given as follows according to Raoult’s law and the Clausius-Clapeyron equation [14]:

\[
\frac{d \ln \frac{p_e}{p_t}}{dT} = \frac{L_f}{RT^2} - \frac{nV}{(V + nv)V} \frac{dV}{dT}
\]  \hspace{1cm} (2)

in which \( T \) is temperature, \( L_f \) is the heat of fusion of ice, \( R \) is the gas constant, \( n \) is osmoles of solute in the cell, \( v \) is the molar volume of water, and \( V \) is the volume of water in the cell. In response, to maintain the osmotic equilibrium, the cell will lose its intracellular water at a rate described as:

\[
\frac{dV}{dt} = \frac{kART}{v} \ln\left(\frac{p_e}{p_t}\right)
\]  \hspace{1cm} (3)

where \( t \) is the time, \( k \) is the permeability constant which is temperature-dependent, \( A \) is the area of cell surface. Ideally, the supercooled cell water will constantly flow out of the cell to form ice until equilibrium is reached. Otherwise, intracellular freezing will begin.

Theoretically, if the concentration of solution and the cooling rate are high enough, before the equilibrium is established, the temperature may drop below \( T_g \), the glass transition temperature [15], which is shown in Fig 1.1 (darkened region) [16]. Under this circumstance, the viscosity of water becomes extremely high which impedes the rearrangements of the water molecules. In consequence, ice nucleation will be
affected to a large extent because ice nucleation requires the formation of hydrogen bonds among a sufficiently high number of molecules [8]. Water in this phase is denoted glassy water or amorphous ice, and the process is called vitrification [17]. The arrangement of molecules in glassy water is random, similar to what is exhibited by liquid water but in glassy water the molecules are almost immobilized as a result of the high viscosity. Although the glass transition in pure water is hard to obtain in practice [18], some aqueous solutions turn out to be very efficient glass formers.

**1.1.1.4 Recrystallization and devitrification**

In reality, a perfect intracellular ice-free state is hard to achieve, especially when the cooling rate is high. The fact is that the ice crystals formed during the freezing process are so tiny that they are innocuous to cell survival; however they do bear higher surface energies [19, 20]. Thus when frozen samples are thawed, these small crystals tend to grow into large crystals, known as recrystallization. For vitrified samples, the intracellular glass may convert back into supercooled state by devitrification, which can be followed by recrystallization. Both are considered to be harmful to cells and are associated with slow warming [21].

**1.1.2 Principles of cryoinjury**

**1.1.2.1 Intracellular ice formation**

The formation of intracellular ice crystals is associated with cell damage and/or cell death [5, 19, 22, 23], which could happen during both cooling and warming processes, and a primary concern of all cryopreservation technology is to prevent or reduce intracellular ice formation (IIF). As mentioned in the previous section, IIF will
take place when the cooling rate is not high enough to vitrify intracellular water but is too high for the cell to allow supercooled intracellular water to flow out. However, the mechanisms associated with the propagation of IIF are currently not clear.

One possibility is that at a critical degree of supercooling, the heterogeneous nuclei with a critical radius would spontaneously initiate the IIF [24]. It is known that the critical radius of ice nuclei, \( r_c \), was estimated to be approximately inversely proportional to the extent of supercooling, which is equal to \( T_m - T_f \), where \( T_m \) and \( T_f \) are melting and freezing temperature, respectively [7]. It can be proposed that in theory under a certain cooling rate, when \( r_c \) and \( T_f \) fit the curve shown in Fig 1.2, ice nucleation is induced spontaneously.

Given the evidence that few effective ice nucleators exist in the cytoplasm [25, 26], it is speculated that extracellular ice as well as the plasma membrane may be involved as catalytic factors in IIF. However, controversy exists in whether the membrane is intact during IIF. Mazur [13] suggested that the cell membrane acts as an effective barrier to extracellular ice above certain temperatures, but the supercooled intracellular water could still be seeded by extracellular ice which grows through aqueous pores in the membrane. According to the Kelvin equation, the freezing point depression (\( \Delta T \)) of the water in a pore is correlated inversely with the radius (\( a \)) of this pore:

\[
\Delta T = \frac{2v_f T_f \sigma_{sl} \cos \Theta}{a L_f}
\]  

(4)
in which $v_1$ is the molar volume of water, $T_f$ is the freezing point of planar water, $\sigma_{SL}$ is the interfacial energies between ice and water, $\Theta$ is the contact angle, and $L_f$ is the molar heat of fusion. Therefore a given pore radius corresponds to a certain temperature below which the ice dendrite should be able to grow through the pore. This has been supported by evidence observed at the intercellular level, that is, the cell-cell contact structure such as gap junctions would facilitate both intracellular and intercellular ice nucleation [27, 28].

Another theory with the premise of an intact membrane is named “surface-catalyzed nucleation” [29]. That is, the properties of the membrane are changed as a result of the complex reactions with the extracellular ice. These changes make the membrane more efficient in catalyzing ice nucleation. The nature of the reactions between extracellular ice and membrane can be, as listed by the authors, chemical, electrical, mechanical, ionic and/or thermal. Little experimental evidence is available to support this hypothesis.

Given the fact that disruption of the membrane sometimes occurs with IIF [30], it is assumed that seeding may occur when extracellular ice contacts the cytoplasm through the damaged part of the membrane. The disruption of the membrane may be due to the electrical transients [31] or osmotic pressure gradients [5, 32] that are common with the freezing process.
1.1.2.2 Solution effects

Early studies revealed that the plots of cell viability against cooling rate were always in the shape of an “inverted U” [33], which means that cells can easily be killed by slow or rapid cooling. Evidences have shown that rapid cooling may lead to IIF [34, 35], which is detrimental, shown as the right hand of the “inverted U”. According to the classic “two-factor theory” [36], high mortality of cells with respect to slow freezing is always referred to as “solution effect”. As has been mentioned above, during slow freezing, water molecules in the extracellular medium were dragged to form the growing extracellular ice crystals, cells in turn respond by dehydration so as to maintain osmotic homeostasis. Two direct consequences are the increase of the concentration of the intracellular as well as extracellular solution and the decrease of cell volume, which may contribute to the “solution effect”.

The hypothesis of “electrolyte stress” was first investigated by Lovelock [37]. Generally, the increase in the concentration of the electrolytes in the extracellular solution occurs during freezing because water molecules are used to form ice crystals, which causes an increase in the concentration the electrolytes of the intracellular solution. When the solute concentration is higher than a critical value under a critical region of temperature, red blood cells become sensitive to and thus can easily be damaged by thermal and mechanical shock. When the concentration becomes even higher, the cellular structures are dispersed by the “lyotropic effect” from the electrolytes in and outside of cells.

An alternative hypothesis is that the damage is caused by the response of cells to the osmotic stress. Under osmotic stress caused by the high concentration of the
extracellular solution, cells will shrink to some extent. Meryman and colleagues [38-40] believed that there is a minimum critical volume related to injury during the cooling process. Beyond this critical value, cells are unable to shrink further to maintain the osmotic equilibrium, and are instead mechanically constrained, causing membrane lesions.

Note that the pattern of the shrinkage of cells exposed to hyperosmotic solutions during slow freezing and cells subjected to hyperosmotic solutions without the freezing process is different [41, 42], suggesting that stress may not be the only factor that destroys cell structure during slow-freezing. Mazur and Rigopoulos [43] have observed that during slow freezing, cells are confined in channels of the unfrozen fraction of the extracellular solution formed between the ice crystals, which become narrower as the freezing process progresses. Cells may simply be damaged by the physical forces induced when the width of the channels becomes smaller than that of the cell diameter.

1.1.2.3 Cryoinjury in tissue

It has long been recognized that ice formation in tissue is harmful to the structural integrity of the frozen tissue [44-46]. Ice crystals formed in the interstitial matrix are in the shape of convex lens as has been observed in soil (Fig 1.3) [47]. The growth of ice lenses is facilitated by water channels such as cell junctions or capillary vessels. Consequently, the surrounding matrix may be damaged by the direct mechanical crush and/or osmotic stress.
1.1.3 Cryoprotectants and their activities

Cryoprotectants or cryoprotective agents (CPAs) are the chemicals that can help to diminish or eliminate the adverse effects of the cooling-warming procedure. Organisms adapted to extreme environments such as polar regions or harsh winters are usually observed to produce certain compounds, known as natural CPAs. In the practice of cryopreservation, CPAs are added with the aim of promoting survivability of cells, tissues or organs.

1.1.3.1 Cryoprotectants in nature

One common strategy for various organisms is to produce antifreeze proteins (AFPs) [48-50]. Two major mechanisms are involved in their protective activities, namely thermal hysteresis and ice recrystallization inhibition. As has been introduced, once seeded, ice crystals will expand quickly. However, the AFPs tend to adsorb to the surface of ice crystals, which modifies the pattern of ice growth and lowers the freezing point, thus producing a gap between the freezing point and the melting point. This phenomenon is known as hysteresis and the gap is called the hysteresis gap [51, 52]. Similarly, by modifying ice morphology, recrystallization is inhibited during the warming stage [53]. The identification of the ice-binding surface of AFPs with the help of molecular techniques confirmed this protein-ice crystal interaction [54, 55].

The formation of ice crystals is usually deleterious to biological systems. However in some cases it is impossible to avoid. Rather than inhibit nucleation, some organisms actively control the propagation of ice nucleation by using ice nucleating agents. By this means, the threats from the formation of ice will be reduced in that ice crystals are confined to large extra organ spaces such as body cavities, subdermal lymph
sacs and haemolymph systems [56, 57]. Most biological ice nucleators contain proteinaceous motifs [58-62], while some particles with non-proteinaceous chemical natures, such as fat body cells and calcium phosphate spherules in gall fly [63], known as crystalloid deposits can also serve as ice nucleator in biological systems. In most situations, biological ice nucleating agents are produced by the freeze-tolerant organisms themselves whereas sometimes they are derived from bacteria residing in plants [64] and animals [65].

Though ice nucleation can be inhibited or controlled by the forgoing mechanisms, freeze-tolerant organisms still need to counter the stresses with respect to solution-effects. In response, most of them accumulate certain protective solutes such as sugars, polyols, amino acids, which usually play an important role in two types of protection, that is, colligative protection and membrane protection. Colligative protection is usually provided from sugars or sugar alcohols, which have low molecular weight, soluble in water in high concentrations, and can move across cell membranes. They are accumulated in high concentrations and counteract the osmotic stresses (cell-volume reduction) by reducing the concentration of the electrolyte in the unfrozen portion of the solution because under a given pressure, the total mole fraction of solute in a partly frozen solution is solely dependant on the temperature [66, 67]. Some sugars and amino acids can also stabilize the phospholipid bilayer of the membrane when the cells are dehydrated under cold stress, targeting especially certain compounds that are important to maintain membrane structures and functions. For example, trehalose may bind to the hydration shell in the phospholipid by forming hydrogen bonds [68] while proline modifies biopolymers with hydrophobic side chains, converting the latter into hydrophilic
groups [69-71]. They also interact with lipid, contributing to inhibition of the fusion of the dry vesicles of membranes and decreasing the melting point, hence even when dehydrated, lipid is still in the active liquid crystalline phase [72-74].

1.1.3.2 Cryoprotectants in cryopreservation

Chemicals used in cryopreservation are traditionally categorized as penetrating and non-penetrating CPAs. Examples of penetrating CPAs include sugars [75, 76], diols [77, 78] and amides [79]. They are small molecules with high solubility in water and can penetrate membrane structures as indicated by their name. Based on these important features, it is easy to predict that they protect cells by virtue of colligative activations. In his study using sodium chloride solution containing cryoprotective neutral solutes as a model, Lovelock quantified the colligative protection of different CPAs as follows [67]:

\[
y = \frac{\Delta T - 2Knx}{Kn}
\]  

where \( y \) is the concentration of a particular CPA needed to prevent damage, \( \Delta T \) is freezing point depression, \( K \) is a constant indicating the observed freezing point depression of the aqueous solution by unit solute concentration, and \( nx \) means when damage occurs, the concentration of sodium chloride is \( n \) times the initial concentration \( x \). Using the criteria deduced from this equation, Lovelock tested the protective action of different chemicals and found that glycerol is most suitable for cryopreservation of human red blood cells.
Non-penetrating CPAs are usually polymers characterized by large molecular mass and poor penetrating ability through membrane structures. The exact mechanism of their protective action is not very clear. They are most likely involved in inhibiting ice formation, showing similar activities to those of natural antifreeze proteins [80]. “Salt-buffering” action has also been reported [81], which means that they protect cells by lowering the extracellular salt concentration thus reducing the “solution effect”. Some of the polymers are preferable for cryopreservation by vitrification since they enhance the glass forming tendency of penetrating CPA-based solutions [82] by way of increasing the viscosity of the solution at subzero temperatures [83] or reducing the critical cooling rate above which the vitreous state is obtained [84]. In addition, the presence of polymers was shown to lower the critical heating rates, above which devitrification is suppressed [85].

1.1.3.3 Toxicity of cryoprotectants

Toxic effects associated with cryoprotectants have been recognized in various manners [86]. First of all, before a freezing procedure is started, the highly concentrated CPAs themselves will lead to a sudden water efflux across the cell membranes, as a result, cells may be killed by volume collapse [41]. In addition CPAs may have a direct effect on cellular structures. For example, Hammerstedt and Graham explained that glycerol has a direct effect on the membrane bilayer structure and also affects the polymerization of microtubules and the association of microtubules and microtubule-associated proteins [87]. Further evidence for toxicity is the “chaotropic effect” shown by butane diol [88], which is responsible for membrane blebbing. Finally, chemical reactions between CPAs and macromolecules may lead to irreversible effects. A case in point is denaturation of proteins surface hydrogen bonding [89]. Another example is the Maillard
reaction between sugars (usually used as cryoprotectants) and amino-containing compounds in biological systems [90].

1.1.4 Cryopreservation of biological materials

1.1.4.1 Slow freezing

In early 1950s, the discovery of the protective property of glycerol by Polge and his colleagues [91, 92] kindled biologists’ interests in long-term storage of biological materials by way of cryopreservation. In 1963, Mazur first demonstrated the kinetics of water in and around cells during freezing through mathematical methods [14]. The classic “two-factor hypothesis” was then proposed to construed the freezing injury to cells, based on investigations in different cell types [33, 36, 93]. Briefly, each cell type has an optimal cooling rate, at which the maximal viability is obtained after the cooling-warming procedure. If the cells are cooled at rates less than the optimal one, they may be killed by injuries related to the exposure to concentrated solutions, known as “solution effects”. On the other hand, if the cooling rate is higher than the optimal rate, cells may be threatened by the formation of ice intracellularly because the supercooled intracellular water does not have adequate time to flow out of cells. This is regarded as the fundamental principle for slow-freezing or controlled-rate freezing that has been used in preserving cells and embryos.

In the slow-freezing or equilibrium freezing strategy, the rule of thumb is to cool the samples at the fastest rate at which an equilibrium is established between the rate of cell dehydration and extracellular ice formation, thus protecting samples from both intracellular ice formation and solution effect mentioned in the two-factor theory. The
ideal outcome is that by the end of the cooling procedure, the small amount of residual intracellular water will be vitrified.

Several points need to be taken into account when this strategy is applied. First, the optimal cooling rate varies dramatically among different cell types [33]. The theoretical optimal rate could be calculated according to classic thermodynamic theories. Nevertheless caution must be taken since the quantitative approach is always simplified by ideal assumptions as well as simple models, and dissatisfaction can be formidable when extrapolation is used. What has been used in most studies today is largely based on empirical data. Accurate control of the cooling rate is usually with the assistance of special devices such as programmable freezers, which is prevalent in today’s studies; passive cooling devices [94], open freezing systems [95], multi-step cooling in an alcohol bath [96] or commercial freezing containers in freezers [97, 98]. Second, seeding is usually conducted either manually or automatically at temperatures around -6 to -8 °C to favor the efflux of supercooled water. Third, to neutralize the solution-effect, cryoprotectants are added at relatively low concentrations considering the injurious effects of long exposure to CPAs during slow-freezing. Loading and unloading CPAs per se may be harmful to cells of which the osmotic tolerance is limited. In this regard, some useful approaches were summarized by Meryman in a recent review [99]. Last but not least, it is widely accepted that rapid thawing benefits cell survival on account of reduced ice recrystallization [96, 98, 100]. Stepwise removal of cryoprotectants is employed by some researchers to alleviate the solution effect associated with thawing [101].
1.1.4.2 Vitrification

In addition to the conventional slow-freezing approach, vitrification, also known as ultra-rapid cooling approach is attracting more and more attention. As early as the first half of last century, Luyet and colleagues have endeavored to put vitrification theory into practice of cryopreservation [35, 102-104]. More attempts were made by Boutron and colleagues, revealing the glass-forming tendency of a series of cryoprotectants [105-107]. In their neat work with mouse embryos, Rall and Fahy successfully applied the fundamental principles of vitrification in cryopreservation and obtained comparable results to slow-freezing methods [108], and they introduced the concept of a “vitrification solution”. Since then, more breakthroughs have been made in the area of embryonic vitrification [109-111], and more and more studies have shed light on vitrification of gametes [112, 113], tissue [114, 115] and intact organs [116].

Cryopreservation by vitrification is characterized by both an ultra-high cooling rate and high concentrations of CPAs which are designed to facilitate the phase transition of supercooled water within and outside of cells from the liquid phase to the amorphous glassy phase, rather than undergoing ice crystallization [108, 117]. Obviously, efforts are needed to put to reduce the toxicity resulting from the overwhelming high concentrations of CPAs [118, 119].

The first and most straightforward approach is to neutralize the toxic CPAs by “toxicity neutralizers”. In his review [16], Fahy suggested that non-penetrating CPAs, usually macromolecules or polymers such as polyvinyl pyrrolidone (PVP) could be used as neutralizers. This has been supported by various studies [120,121]. In addition, acetamide and dextrose were also considered as potential candidates as neutralizers [16].
A second strategy is to make use of stepwise procedures to load and remove the CPAs [122, 123], which might help to circumvent the osmotic stresses [124]. Toxicity can also be reduced indirectly by diminishing the concentration while enhancing the cooling rate, in view of the reciprocal relationship between the two [85]. To maximize the cooling rate, a practical approach is to minimize the insulation of the containers by certain manual modifications (e.g. open pulled straw) or moreover, allowing the liquid nitrogen to contact pretreated samples directly, which can be achieved with the help of a number of special devices or methods (Table 1.1). Like in slow-freezing, rapid thawing and stepwise removal of CPAs are usually preferable for vitrification so as to avert devitrification and osmotic stress.

1.2 Ovarian tissue cryopreservation and transplantation

1.2.1 As a way of germline conservation

Ovarian cryopreservation and transplantation has been used in different areas as a effective means of restoring and recovering female reproductive potential.

A number of medical treatments are now available for cancer patients; however a notorious side effect is that the treatments always lead to sterility. Sexually mature female patients may benefit from the cryopreservation of oocytes and/or embryos, which has long been investigated and standard protocols are available in many labs. For young patients who have not reached puberty, the only solution is to preserve their ovarian tissues or ovaries in advance of the administration of the cancer therapies. Their fertility can be regained by way of autotransplantation (transplant to the same person) of the preserved ovarian tissues or whole ovaries as long as conditions permit.
The corresponding research in animals is so far largely restricted to mammals, including livestock and lab animals. Animal models contribute considerably to the refinement of the cryopreservation protocols and surgical techniques as a reference for human clinical concern. Large farm animals and nonhuman primates provide good models for humans but the experiments may be limited by time and space. While lab rodents are easier to manipulate and maintain, giving us more freedom to conduct intensive study, caution must be taken when fitting the experimental results to practical operation.

Another important application of ovarian cryopreservation and transplantation is in germplasm banking of lab animals bearing valuable genes, which has already been put into practice in mice [144]. Using the same strategy, both commercial lines and local stocks of interests in animal industries can be maintained economically and securely.

Practical benefits can also be foreseen in the conservation of the diversity of wildlife. In comparison with oocyte and embryo cryopreservation, the advantages of ovarian cryopreservation and transplantation are clear. In the first instance, special prerequisite of the donor or pre-treatments are not required. Collection of ovarian tissue or ovaries can even be carried out regardless of donor’s age, even shortly after the donor’s demise (though donor and recipient’s age may affect the efficiency of the subsequent transplantation, which will be discussed later). Whereas in the case of oocyte and embryo cryopreservation, ovarian stimulation of the donor when she is fertile is necessary and it takes considerable of time before oocytes can be collected. Even so, the number of mature oocytes collected or the derived embryos is very limited. A small strip of ovarian cortex, on the contrary, can contain large numbers of oocytes at different
stages including those in primordial follicles, which are believed to bear full reproductive potential and are more resistant to freezing protocols [145]. Furthermore, the manipulation of bulk tissues is easier than that of delicate cells.

1.2.2 Cryopreservation of ovarian tissue

1.2.2.1 Slow freezing

Dating back to 1950s, scientists have tried to demonstrate that transplanted frozen-thawed ovarian tissues could regain their functions, using lab rodents as models. These studies, however, were mostly concentrated on the endocrine function of the grafts (summarized in [146]), until 1960, when Parrott [146] first reported a live birth from frozen-thawed mouse ovarian grafts. In this study, glycerol was used as cryoprotectant and the slow-freezing strategy was applied. Samples were stored at -79°C, which is unpopular today and the exact cooling rate used was not very clear. In 1994, Gosden et al. [147] reported the first live birth from another popular animal model, the ewe. The cryoprotectant used in this study was dimethyl sulphoxide (DMSO) and the slow-freezing procedure was carried out using a programmable freezer. After that, the samples were plunged into liquid nitrogen (-196°C) for storage. With modification, this protocol was widely adopted thereafter in cryopreservation of ovarian tissues. Apart from programmable freezers, other devices, for instance open freezing system, were also used [95].

As for cryoprotectant, penetrating CPAs such as dimethyl sulphoxide (DMSO), ethylene glycol (EG), propanediol (PROH) and glycerol (GLY), are used in various studies in different species. The concentration is usually around 1.5M. Some studies also
combine sugars such as sucrose or fructose with the cryoprotective agents. Although investigations have been made to compare the efficiency of at least two of the above CPAs, simple conclusions should never be drawn considering that the efficiency of CPAs is dramatically impacted by the specific experimental design, for example, animal species, cooling rate, and seeding temperature.

Fast thawing is conducted by either brief air warming followed by immersion in a water bath or in a water bath only. The CPAs need to be removed by putting the samples in a series of media containing progressively lowered concentrations of CPAs. As an alternative, samples can be shaken and rinsed repeatedly in medium without CPAs.

**1.2.2.2 Vitrification**

Similar to what has been applied in oocytes and embryos, a combination of high concentrations of cryoprotectants and a high cooling rate is essential for ovarian vitrification. Cryoprotectants are similar to those used in slow-freezing (e.g. DMSO, EG) except for the concentration (usually 20%-40% for vitrification). Sugars and sometimes polymers are included to reduce the proportion of the toxic CPAs. The first step is to expose the samples to cryoprotectants. Small sized samples such as those from small animals or from large animals but in pieces can be simply immersed in protective solutions. Special perfusion techniques may be required for large samples such as whole ovaries from the ewe [148]. Stepwise exposure is employed by most studies these days. After exposure to cryoprotectants, the samples, contained in straws or vials, are plunged into liquid nitrogen.
As has been mentioned, to ensure an ultrahigh cooling rate (around 2500°C/min), some special devices and methods have been developed (Table 1.1) and used for ovarian tissue. For example, samples held by copper grids can be plunged directly into liquid nitrogen [127]. In two recent studies, a similar strategy was used except that the samples were carried by vials and needles, and the corresponding methods were named direct cover vitrification (DCV [116]) and needle immersed vitrification (NIV [143]). Using solid surface vitrification (SSV) [115], tissues were vitrified by being placed on a metallic surface which is partly immersed in liquid nitrogen. Further, commercial vitrification kits that are similar to what has been designed and used for embryo vitrification are now available. Such examples are Cryotop [140] and CVM-cube [142], which have been used for mouse ovarian tissues.

1.2.3 Transplantation of ovarian tissues

Transplantation is the in vivo approach of recovering the preserved gametes. Both autotransplantation and xenotransplantation have been adopted in practice. Autotransplantation, with the meaning of transplanting the tissues back to the body where they are derived from, is usually used to help cancer patients regain their fertility after the sterility-causing treatments. Experiments in mice [146] and sheep [147] have demonstrated its feasibility. Using a similar strategy, Donnez and colleagues [149] reported the first successful pregnancy and live birth from autotransplanted cryopreserved ovarian tissues in humans.

Transplantation can also be conducted from one species to another, namely xenotransplantation. The consequent immune rejection is reduced by using
immunodeficient mice or nude rats as recipients or administrating immunosuppressive medications to recipients. In research on cryopreservation of human ovarian tissues, xenotransplantation provides an easy access to monitoring the recovery of the grafts [150, 151], which helps to refine the cryopreservation protocols as well as surgical regimens. Moreover, mature oocytes from xenografts can be used to produce offspring with the help of in vitro fertilization and embryo transfer, in the case that the conditions of the donor fail to meet the requirements of autotransplantation. Xenotransplantation also benefits studies in animal reproduction, especially wildlife, since samples can be obtained shortly after the death of the animals and can be stored by cryopreservation. Besides, samples from one individual can be xenotransplanted to different recipients, providing more research opportunities without killing more animals. Using this strategy, research has been conducted in wild species such as marsupials [152] and elephants [153]. In addition, xenotransplantation allows oocytes from one species to go through in vivo maturation in another species, which can be further used to produce offspring, particularly for species in which assisted reproductive technologies, such as gamete banking, in vitro fertilization and embryo transfer, are not well-established. A breakthrough has been made in lab rodents as live offspring have been gained from fresh mouse ovarian tissues xenografted into the rat [154].

1.2.4 Application in avian genetic conservation

Avian genetic diversity has declined enormously in the past few decades. According to the International Union for Conservation of Nature and Resources, nearly 500 avian species are “vulnerable”, “endangered” or “critically endangered”, which is 5% of the known 9,672 existing avian species [155]. Meanwhile, the poultry breeding
industry has encountered shrinkage of the available variation, represented by both commercial as well as local stocks [156]. Therefore, an economical and reliable means of genetic conservation of avian species is needed.

There are two major approaches available for avian genetic material conservation: preserving live birds or building cryobanks. Using the first strategy, wild species are secured in their natural or artificial habitats, while in poultry breeding industry, duplicated stocks consisting of additional birds of the pedigreed pure lines are placed in different geographical locations for the sake of biological and economic security [157]. To date, this is the only method that can be put into practice effectively. However, the associated high cost in maintenance and vulnerability to potential hazards such as outbreak of diseases argues for the alternative, cryopreservation, mainly referred to as cryobanking of germplasm, which can be in the form of gametes (primordial germ cells, semen, oocytes), different stages of embryos, and gonadal tissues (testicular and ovarian tissues). Semen cryopreservation and subsequent artificial insemination are expected to assist poultry breeding and the conservation of endangered wild birds [155]. However, fertility rates using cryopreserved semen tend to be very low and lack consistency, even within a line [156]. Even if the semen could be cryopreserved with satisfactory fertility, cryopreservation of the female gametes-oocytes is still beyond the reach of current cryopreservation techniques. Similarly, late-stage embryos can not be cryopreserved either. Cells such as primordial germ cells, embryonic stem cells and blastodermal cells can be cryopreserved and have been used to produce transgenic lines. Nonetheless, the actual procedures are very complex and the outcome is poor and inconsistent, making the techniques less practical for genetic conservation [158].
Considering the special structure of the female reproductive system and the associated oogenesis in birds, cryopreservation and orthotopic transplantation of ovarian tissues might be a feasible way of storing and recovering the female reproductive potential. There are no data available on ovarian tissue cryopreservation in avian species. However, surgical orthotopic transplantation techniques have recently been developed for the chicken and the Japanese quail (*Coturnix Japonica*) [159, 160], which should allow cryopreservation and transplantation to contribute to the preservation of avian germplasm.

The Japanese quail is a useful model for avian reproductive research. Some of the favorable characteristics are ease of handling, high egg laying potential and short breeding cycle [161]. Japanese quail and chickens have the same number of chromosomes (2n=78) [162]. Taxonomically they belong to the same family known as Phasianidae, but not to the same genus [163]. The structure and physiology of their reproductive systems are quite similar. Like in most other avian species [164], only the left ovary and oviduct are functional. Quail and chickens have a similar mechanism of sex determination, which largely depends on estrogen synthesis and its effects on the hormonal milieu of the developing embryo [165-167]. Similarity between quail and chickens is also observed in their ovulation-oviposition cycle (the time from ovulation of an ovum to the oviposition of the egg) [164].

However, quail are much smaller birds than chickens (about 1/4 to 1/3 the size of chicken at the same stage) and lay relatively larger eggs per gram body weight [168]. The time of oviposition is usually in the afternoon which is opposite to that of chicken (before noon) [169]. It has been shown that the onset of folliculogenesis is earlier in Japanese
quail chicks than in chicken chicks [170]. As observed in a previous study [160], the mortality of quail chicks is very high in the first week, especially after surgical manipulation, but after they achieve a certain age (5 weeks), they tend to recover faster from hemorrhage than chickens [171]. When applying ovarian tissue cryopreservation and transplantation to Japanese quail and other small birds, these factors might be considered in the modification and evaluation of the protocols.

1.3 **Objective of the current study**

In the current study, using the Japanese quail as a model, we tested the hypothesis that the female reproductive potential of ovarian tissue of avian species could be cryopreserved, which means that the tissue could survive the freezing/thawing or vitrification/warming procedure and regain its function after subsequent transplantation. The production of donor derived offspring would demonstrate successful cryopreservation and transplantation.

Furthermore, we would like to develop a simple and practical cryopreservation protocol that could be used for other avian species with minor modification in the future. In this regard, two major cryopreservation strategies namely slow-freezing and vitrification were employed. To evaluate their efficiency, cell viability, tissue morphology and recovery of fertility were examined.
### TABLE 1.1. Special devices/methods used in vitrification facilitating rapid cooling.

<table>
<thead>
<tr>
<th>Devices/methods</th>
<th>Application</th>
<th>References</th>
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<td>Grid</td>
<td>Oocytes</td>
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<td></td>
<td>Embryos</td>
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<td>Ovarian tissues</td>
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<tr>
<td>Open pulled straw (OPS)</td>
<td>Oocytes</td>
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<td>Sperm</td>
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<td>Solid surface vitrification (SSV)</td>
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<td>Embryos</td>
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<td>Ovarian tissues</td>
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<td>Cryoloop</td>
<td>Oocytes</td>
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<td>Embryos</td>
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<td>Cryotip</td>
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<td>Cryotop</td>
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<td>Cryologic vitrification method (CVM)</td>
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<td>Ovarian tissues</td>
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<td>Direct cover vitrification (DCV)</td>
<td>Ovarian tissues</td>
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<td>Needle immersed vitrification (NIV)</td>
<td>Ovarian tissues</td>
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FIG. 1.1. Supplemented phase diagram of a hypothetical cryoprotectant. In which
Tm, Th, Tg, Td are the equilibrium freezing (melting point) curve, homogeneous
nucleation temperature, glass transition temperature, and devitrification curve.
Modified from Ref. [16].
FIG. 1.2. The correlation between the critical radius of ice nuclei $r_c$ and the freezing temperature $T_f$ for ultra-clean micro-DI water droplets. Data from Ref. [7].
FIG. 1.3. The schematic of “ice lens” formed in the interstitial matrix in tissue, modified from Ref. [47].
1.4 References


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Chapter 2 Production of donor-derived offspring from cryopreserved ovarian tissue in Japanese quail (*Coturnix japonica*)

2.1 Introduction

Cryopreservation of ovarian tissue has been used in mammals including humans to preserve female reproductive potential. Live offspring from cryopreserved ovarian tissue or whole ovaries have been reported in various mammalian models [1-3]. In human clinical practice, it is used as a treatment to restore female patients’ fertility threatened by cancer therapies [4]. There are currently two main approaches to cryopreserving ovarian tissues. One is slow-freezing (also known as slow-rate freezing or equilibrium freezing), by which tissues are usually frozen in a programmable freezer at controlled slow cooling rates and cryoprotective agents (CPAs) are required. This method has been used to preserve embryos, oocytes, ovarian tissues and whole ovaries in previous studies [3, 5-7].

As an alternative, vitrification preserves tissues by achieving a vitreous state of the whole tissue, which can be obtained by a high cooling rate and a high concentration of CPA. Vitrification does not require special devices and is usually very fast. It may also be preferable for cryopreservation of multi-cellular structures such as tissues or entire organs [8, 9]. Exploration and applications of vitrification in ovary cryopreservation have been documented [10-12].

In combination with transplantation, ovarian cryopreservation can be used as a means of germplasm banking [13-15], especially for those species in which the cryopreservation of germplasm is challenging such as avian species. Avian oocytes can

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1 A version of this chapter will be submitted for publication. Liu J, Song Y, Cheng KM, Silversides FG. Production of donor-derived offspring from cryopreserved ovarian tissue in Japanese quail (*Coturnix japonica*).
not be cryopreserved because of the presence of the yolk, which diminishes the protective effectiveness of existing cryopreservation methods, leaving oocytes vulnerable to the challenges induced by freezing protocols in the microenvironment [16, 17]. Though germline chimeras can be generated by freezing early embryonic germ cells [18, 19], low efficiency and inconsistency in the outcomes make them impractical when applied to avian genetic resource conservation [16, 20].

Recent studies demonstrated techniques of orthotopic ovarian transplantation in newly hatched chickens [21] and in week-old Japanese quail [22]. Donor-derived offspring from fresh transplants were obtained in both species, suggesting the possibility of combining cryopreservation and transplantation as a universal and practical approach to female fertility preservation in avian species. The purpose of the current study is two-fold: first, using the Japanese quail as a model, we evaluated slow freezing and vitrification cryopreservation protocols for use in birds. In addition, we evaluated whether the reproductive function of cryopreserved ovarian tissue in quail could be recovered by transplantation.

2.2 Materials and methods

2.2.1 Birds and tissue preparation

Week-old female chicks of (recessive) White-breasted (WB) [23] and Wild-type (QO) [24] Japanese quail maintained at the Agassiz Research Centre were used as donors and recipients, respectively. All the methods involved were approved by the Animal Care Committee of the Agassiz Research Centre and followed principles described by the Canadian Council on Animal Care (2009).
Ovaries were removed immediately after euthanasia by cervical dislocation and immersed in Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma) with 10% fetal bovine serum (FBS) (Sigma) on ice. The surrounding connective tissue and fat were then gently trimmed off and each ovary was cut into two approximately equal pieces under a dissecting microscope. All the tissue pieces were kept on ice before further treatment within 4 hours.

### 2.2.2 Ovarian tissue cryopreservation

#### 2.2.2.1 Slow freezing

Two pieces of ovarian tissue were loaded in each 0.5 ml CBSTM High Security Straw (Cryo Bio System, 75008 Paris, France) filled with DMEM (Sigma) containing 10% (v/v) dimethylsulphoxide (DMSO) (Sigma) and 10% (v/v) FBS (Sigma). Straws were then put on ice to allow the tissue pieces to equilibrate for at least 20 min. The freezing procedure was carried out using a programmable freezer (Kryo 360-1.7, Planer Plc., Middlesex, UK) following the protocol described by Silversides et al. [25]. Briefly, straws were placed in the freezing chamber which was cooled to 10°C. The temperature was then lowered to -7°C at the rate of 0.5°C/min. After manual seeding at this temperature, the straws were held at -7°C for 10min and then further cooled to -55°C at 0.5°C/min. Finally the straws were plunged into liquid nitrogen and stored for at least 72 hours.

Thawing was similar to that used for cryopreserved chicken testicular tissue [26] with slight modifications. The straws were removed from liquid nitrogen and quickly plunged into ice-water until the ice in the straws melted. The contents of each straw were then emptied into Petri dishes containing DMEM supplemented with 10% FBS. The
cryoprotective agent was removed by gently shaking the dishes plus repeated rinsing for at least 3 times with media. The tissue pieces were then suspended in media on ice within 4 hours before further use.

2.2.2.2 Vitrification

The procedure used in this study was based on two methods, direct cover vitrification (DCV) [11] and needle immersed vitrification (NIV) [27], which have been applied in mouse ovarian tissue vitrification. Four tissue pieces were put on each acupuncture needle (Cloud & Dragon Medical Device Co. Ltd, China) which had been manually modified to fit the cryovials (Fisher). The tissue pieces held by the needles were first immersed into an equilibration solution containing 7.5% (v/v) ethylene glycol (EG) (Sigma) and 7.5% DMSO in Dulbecco’s phosphate-buffered solution (DPBS) (Sigma) supplemented with 20% FBS (Sigma). After 10 min equilibration at room temperature, the needles were moved to a vitrification solution containing 15% EG (Sigma), 15% DMSO (Sigma) and 0.5 M sucrose (Sigma) for 2 min at room temperature. Tissue pieces were touched by a piece of gauze to remove the surrounding vitrification solution and then plunged directly into liquid nitrogen in an insulated box. The final step was to transfer the needles into cryovials filled with liquid nitrogen which were then covered with pre-cooled caps and stored in liquid nitrogen for at least 72 hours.

For warming, the vials were moved out of the liquid nitrogen and the needles holding the ovarian tissues were quickly immersed into 1 M sucrose solution at room temperature for 5 min. They were subsequently moved into 0.5 M, 0.25 M sucrose solution and DPBS containing 20% FBS for 5 min each. The tissue pieces were then suspended in media on ice within 4 hours before further use.
2.2.3 Cell viability estimation

Cell viability after freezing-thawing or vitrification-warming procedures was estimated by trypan blue assay [28, 29]. Fresh tissue was used as control. Tissue pieces were finely chopped using two scalpels in 0.25% trypsin-EDTA (Sigma) solution and 1.5 mg/ml collagenase (Sigma). The fine fragments were then incubated at 37°C and were gently pipetted every 10 min to promote the digestive reaction. After 30 min, 50% FBS was added to inhibit the enzyme activity and the digestive products were centrifuged at 500 g for 10 min. The precipitate of each sample (2 tissue pieces) was re-suspended thoroughly in 20 µl DMEM. Samples were stained by 0.4% trypan blue (Sigma) at room temperature. The cell viability was examined under a light microscope using a hemacytometer (Hausser Scientific, Horsham, PA, USA); dead cells were stained blue and living cells were not stained.

2.2.4 Histological examination

Fresh and frozen-thawed or vitrified-warmed tissue samples were fixed in Bouin’s solution for 24 hours, dehydrated in alcohol and subsequently embedded in paraffin. The embedded samples were cut into 7-micrometer serial sections, mounted on slides and stained with hematoxylin and eosin. Images were captured and examined using a digital camera (1300R, Qimaging Corp., Burnaby, British Columbia, Canada) mounted on a microscope (Olympus BX51, Olympus Corp., Tokyo, Japan).

In this study, follicles with the following characteristics as shown in Fig. 2.1 were defined as morphologically normal: a) intact follicular epithelium; b) intact ooplasm with
a visible germinal vesicle; c) widest diameter larger than 20 µm. The number of morphologically normal follicles was counted from five different sections of each sample.

### 2.2.5 Ovarian transplantation

The frozen-thawed or vitrified-warmed ovarian tissue from WB birds was surgically transplanted into week-old QO birds, using the techniques that had recently been developed in chickens [21] and quail [22]. Each recipient was anesthetized by subcutaneous injection of 0.1 mg of ketamine (Ketaset®, Ayers Laboratories, Guelph, ON, Canada) and 0.05 mg of xylazine (Rompun®, Bayer Inc., Toronto, ON, Canada). The anesthetized bird was then placed on a warm operating surface. An incision was made on the left side of the abdominal cavity distal to the last rib. The gizzard was carefully detached from the peritoneal membrane and displaced, along with the intestine to expose the ovary. After gently breaking the greater abdominal air sac, the host ovary was removed using a pair of fine forceps. Two pieces of donor ovarian tissue were put on the original site of the recipient’s ovary and were covered by the remains of the greater abdominal air sac. Four or five interrupted sutures were used to close the skin. Fresh tissue pieces were transplanted using the same technique to be used as a control treatment. An immunosuppressant (CellCept, Hoffmann-LaRoche Ltd., Mississauga, ON, Canada) was given orally to all the operated birds at a dose of 100 mg/kg per bird per day for 2 weeks after the operation.

### 2.2.6 Progeny test

To assess the function of the grafts, a progeny test was conducted using plumage color as a genetic marker [23]. The white breast trait carried by the WB quail is
determined by a homozygous recessive genotype of a single autosomal gene \( wb (wb/wb) \), and the QO line has wild type (+/+ ) coloration which is a dark plumage color [23, 24]. Thus heterozygous offspring (+/\( wb \)) from the cross of the WB and QO lines would be phenotypically wild type. In our study, after the QO recipients obtained their sexual maturity at the age of 6 weeks, each was paired with a fertile WB male bird. Eggs were collected and incubated, and most were opened after 12-14 days’ incubation. Some chicks were allowed to hatch. The feather color of the embryos and chicks indicated their maternal origin, that is, the white-breasted embryos (\( wb/wb \) ) were derived from the WB donor ovarian tissue whereas the dark colored embryos (+/\( wb \) ) originated from regenerated ovarian tissue of the host QO recipients. The test was continued until the recipients were 20-week old.

2.2.7 Statistical analysis

All statistical analyses were conducted with SASBatch 4.1.1. Cell viability, egg production, fertility, and age at first egg were analyzed using the GLM procedure. The ANOVA procedure was used to test the difference in the means of follicle counts among treatments. Chi-square analyses in the FREQ procedure were used to compare the difference in the number of surviving recipients, recipients producing fertile eggs, recipients producing only host-derived offspring, recipients producing only donor-derived offspring and recipients producing both host- and donor-derived offspring. Statistical significance was set as \( P \leq 0.05 \).
2.3 Results

2.3.1 Cell viability estimation

Cell viability of samples (Table 2.1) that had been treated with either the slow-freezing or the vitrification protocol was significantly lower than that of the control group (81.9 %). It was significantly higher in vitrification group (77.1 %) than in slow-freezing group (70.0 %).

2.3.2 Histological examination

Table 2.2 summarizes the results of histological examination. No significant difference were seen in the mean number of morphologically normal follicles between the control group (26.8 ± 4.5) and the vitrification group (24.6 ± 1.4); but both of these groups had significantly more morphologically normal follicles than in the slow-freezing group (14.3 ± 2.9).

2.3.3 Surgical transplantation and progeny test

The results of the surgical transplantation and progeny test are presented in Tables 2.3 and 2.4. Egg production, fertility, and age at first egg of recipients that produced at least one egg were not influenced by the treatment (Table 2.3). The recipients that survived surgery and lived until the end of the test at 20 weeks were considered to be “surviving recipients” (Table 2.4). There was no significant difference between groups in surviving recipients out of recipients receiving surgery. Likewise, the number of recipients producing fertile eggs of surviving recipients was comparable among groups. Of recipients that produced at least one offspring, 1 out of 6 in the control group and 2 out of 7 in the vitrification group produced only host-derived offspring, while this ratio was significantly greater in slow-freezing group (7 out of 9). No significant difference
was seen in the number of recipients that produced only donor-derived offspring for control, slow-freezing and vitrification groups. Four out of 6 recipients in the control group and 5 out of 7 recipients in the vitrification group produced both donor-and host-derived offspring, while no recipients in the slow-freezing group produced both.

2.4 Discussion

Ovarian tissue cryopreservation and transplantation enable us to preserve female germplasm. Current applications are exclusively in lab mammals, farm mammals and human clinical cases. It is urgent to develop a practical and reliable means of female fertility preservation in birds. The present study demonstrated that the fecundity of ovarian tissue in an avian species could be cryopreserved in a simple and inexpensive manner and reconstituted satisfactorily by surgical transplantation. Donor-derived offspring were obtained from transplanted ovarian tissue that had been cryopreserved by either slow-freezing or vitrification. Vitrification had greater efficiency as measured by cell viability, tissue morphology and overall reproductive performance of the cryopreserved transplants.

Ovarian tissues cryopreserved by slow-freezing in the present study had lower cell viability, fewer morphologically normal follicles, and a lower efficiency in producing donor-derived offspring when compared with their vitrified counterparts. Our protocol originated from protocols used for mammalian ovarian tissue cryopreservation [30], big differences may exist in how tissues from different species respond to the freezing protocol. Therefore more refinements may be needed, such as the rate of freezing/thawing, the type and concentration of the cryoprotectants, the length of equilibration, and the seeding temperature. A more important reason is that to date slow-freezing
protocols were mostly based on those for cell or embryo cryopreservation. To protect cells from lethal intracellular ice formation, the efflux of the supercooled intracellular water is facilitated by inducing extracellular ice nucleation, i.e. seeding. This might be detrimental to the extracellular matrix of ovarian tissue which is of great importance in ovarian follicle development [31] and thus to the reestablishment of fertility. In the future, instead of using a single penetrating cryoprotectant such as DMSO, a combination of both penetrating and non-penetrating cryoprotectants such as polymers may help to reduce or eliminate the adverse effects of extracellular ice crystallization [32].

In contrast to conventional slow-freezing, a vitrification protocol could theoretically preserve tissues both intracellularly and extracellularly through ice-free solidification. This could be achieved by a high cooling/warming rate plus a high concentration of cryoprotective agents (CPAs). The concentration of CPAs could be lowered to a small extent to reduce the toxicity as long as the cooling/warming rate is enhanced correspondingly, making use of the reciprocal relationship of the two [33].

A practical way to gain a higher cooling rate is to let the sample contact liquid nitrogen directly, which has been facilitated by special devices such as cryoloops [34] and cryotops [35] that have been exploited in the study of oocyte or embryo vitrification. Unfortunately, these devices are not ready for tissue vitrification. Chen and colleagues [11] have developed a new method named “direct cover vitrification”, in which liquid nitrogen is applied directly onto the mouse ovaries contained in cryovials. In this way, ovaries could be effectively vitrified by using cryoprotectants with relatively lower concentration, hence lower toxicity. However, this method may not be appropriate for a large number of samples because it requires too much manipulation and the resultant
prolonged time of exposure to CPAs may lead to damage. Wang and colleagues [27] improved the method by introducing acupuncture needles as carriers to hold multiple samples and immersing the samples into liquid nitrogen, in the process they called needle immersed vitrification (NIV). The method ensured the homogeneity and was faster for a large number of samples but the investigators did not suggest a practical way to store the samples. In the present study, we used acupuncture needles as carriers and stored samples in cryovials to meet the requirement of routine use.

Furthermore, it is easier to extrapolate vitrification protocols to preserve the ovarian tissue of local stocks and commercial lines in the poultry industry and endangered wild avian species because there are fewer variables. These variables include the type and concentration of the cryoprotective agents and the length of the time that samples are exposed to equilibration and vitrification solution.

Apart from cryopreservation strategy, failure to retain reproductive potential of grafts may also be associated with the transplantation procedure itself, for instance, ischemia-reperfusion injury and failure of the reestablishment of hormonal cyclist [36, 37]. In the progeny test in this study, only 2 out of 9 recipients in the slow-freezing group produced donor-derived offspring, and the offspring from both of them were only donor-derived, indicating complete removal of the hosts’ ovaries but a low rate of recovery of the grafts. In the vitrification group, 5 out of 7 recipients produced donor-derived offspring, and all of them produced host-derived offspring as well, indicating incomplete ovariectomy and regeneration of host ovarian tissue but better recovery of the grafts. It is possible that the host residual ovarian tissue may facilitate the recovery of the grafts.
As in rodents, the fragments of quail ovarian tissue for grafting are small and angiogenesis can not be obtained by surgically reanastomosis as in the transplantation of larger organs but merely by the process of revascularization [38]. The wound healing of the host ovary may induce an endogenous process that benefits rebuilding the vasculature of the grafts thereby reducing the loss of the follicle pool due to ischemia-reperfusion injury. On the other hand, it has long been known that the ovary is an active endocrine organ and its activities including follicle development and ovulation are dependent on both intra and extra ovarian hormones or other signal molecules. The grafts may benefit from the host endocrine products, for example, growth factors and signal receptors [39, 40] enabling them to recover faster and more effectively. These postulations require further investigation.

In future practice, instead of removing the entire host ovary, incomplete ovariectomy could be used to promote the recovery of the graft and avoid excessive bleeding which is the primary cause of death during surgery in very small birds [21]. Although this allows the production of host-derived offspring, it may be compensated for by a higher recovery rate and better performance of the grafts. The extent to which the host’s ovary should be removed or destroyed needs to be determined by future studies.

In conclusion, our production of live donor-derived offspring from cryopreserved ovarian tissue demonstrated that cryopreservation and transplantation of ovarian tissue can be used as a practical and reliable way to preserve female germplasm in birds and represents a major step towards the genetic resource conservation of endangered commercial lines of poultry and endangered wild avian species.
2.5 Acknowledgements

We would like to thank Harold Hanson, Karli Ryde, Kathy Ingram, Lee Struthers, Mairi Robertson and Wendy Clark for taking care of the experimental birds. Dr. Tom Forge is also acknowledged for providing devices for microphotography.
### TABLE 2.1. Cell viability (mean ± SEM) of cryopreserved ovarian tissues.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sample Size</th>
<th>Cell viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9</td>
<td>81.9 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Slow-freezing</td>
<td>15</td>
<td>70.0 ± 0.9&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vitrification</td>
<td>9</td>
<td>77.1 ± 1.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a-c</sup> Values with different superscripts are significantly different (P<0.05).
TABLE 2.2. Number of morphologically normal follicles (mean ± SEM).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sample size</th>
<th>Normal follicles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>26.8 ± 4.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Slow-freezing</td>
<td>8</td>
<td>14.3 ± 2.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vitrification</td>
<td>8</td>
<td>24.6 ± 1.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a, b</sup> Values with different superscripts are significantly different (P<0.05).
TABLE 2.3. Egg production to 20 weeks, fertility and age at first egg (mean ± SEM) of quail receiving ovarian transplants.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Recipients producing eggs</th>
<th>Egg production*</th>
<th>Fertility (%)*</th>
<th>Age at the first egg (d)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>38.5 ± 10.2</td>
<td>58.8 ± 10.4</td>
<td>76.5 ± 6.4</td>
</tr>
<tr>
<td>Slow-freezing</td>
<td>11</td>
<td>42.0 ± 7.0</td>
<td>64.5 ± 9.2</td>
<td>61.9 ± 5.1</td>
</tr>
<tr>
<td>Vitrification</td>
<td>7</td>
<td>56.9 ± 5.3</td>
<td>63.6 ± 6.6</td>
<td>66.9 ± 3.1</td>
</tr>
</tbody>
</table>

*No significant difference was seen between any two treatments.
### TABLE 2.4. Reproductive performance of quail receiving ovarian transplants.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Recipients</th>
<th>Surviving recipients</th>
<th>Recipients producing fertilized eggs</th>
<th>Recipients producing only host-derived offspring</th>
<th>Recipients producing only donor-derived offspring</th>
<th>Recipients producing both host- and donor-derived offspring</th>
<th>Host-derived offspring</th>
<th>Donor-derived offspring</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14</td>
<td>7</td>
<td>6</td>
<td>6</td>
<td>1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1</td>
<td>4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43</td>
</tr>
<tr>
<td>Slow-freezing</td>
<td>29</td>
<td>14</td>
<td>10</td>
<td>9</td>
<td>7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>223</td>
</tr>
<tr>
<td>Vitrification</td>
<td>10</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
<td>5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>177</td>
</tr>
</tbody>
</table>

1. Surviving recipients of recipients.
2. Recipients producing fertilized eggs of surviving recipients.
3. Recipients producing only host-derived offspring of recipients producing offspring.
4. Recipients producing only donor-derived offspring of recipients producing offspring.
5. Recipients producing both host- and donor-derived offspring of recipients producing offspring.

<sup>a,b</sup> Values in a column with different superscripts are significantly different (P<0.05).
2.6 References


Chapter 3 General discussion

3.1 Cryopreservation strategy

This study is the first to report that live offspring can be obtained from cryopreserved ovarian tissues in avian species, which is of great importance to avian germplasm conservation because avian oocyte cryopreservation has not been possible. In contrast, intensive studies on germplasm cryopreservation have been conducted in a number of mammalian species and ovarian tissues can be cryopreserved by either slow-freezing or vitrification.

Both methods are aimed at preventing intracellular ice formation, which is believed to have irreversible consequences leading to cell death. By slow-freezing, ideally, a delicate equilibrium is established between the rate of water loss from the cell and the rate at which this water forms extracellular ice crystals, preventing the lethal intracellular ice formation from supercooled intracellular water. The extracellular ice nucleation is usually induced by seeding at a subzero temperature. Slow-freezing has been well studied in the cryopreservation of oocytes and embryos in mammals and humans and is widely accepted. Most of the classic principles of cryobiology are on the basis of slow-freezing studies. However, it is time-consuming (averages several hours) and requires special devices such as a programmable freezer. When it is applied to ovarian tissue cryopreservation, additional problems need to be taken into account. For one thing, the induction of extracellular ice formation is required, which is harmful to the extracellular matrix of ovarian tissue and further affects the function after thawing. For another, the optimization is extremely complex because ovarian tissue contains different
types of cells and they may have different requirements for optimization. This has been exemplified by a study showing that stromal cells are more susceptible to cryoinjury than primordial follicles [1]. In addition, many other factors, including the nature and concentration of cryoprotectants, the equilibration time, the seeding temperature and the warming rate need to be established. Special experimental designs such as the fractional experimental design described by a recent study [2] may help to simplify the optimization.

In contrast, with vitrification, both intracellular and extracellular media solidify without ice crystallization within a very short time and turn into a glassy-like state, with viscosity so high that rearrangement of water molecules is largely impeded, thus preventing ice crystallization. This is facilitated by an ultra-rapid cooling rate as well as a high concentration of cryoprotectants (around 45% w/v [3]). Vitrification is particularly preferable to slow-freezing for tissues because both intracellular and extracellular components are protected from ice formation. Furthermore, vitrification is very rapid and no expensive freezing devices are required. However, the available data in ovarian tissue cryopreservation using vitrification are still scarce and there are conflicting results on its efficacy. The apparent drawbacks are that the high concentration of cryoprotectant is likely to cause tissue injuries and there is still a risk of ice recrystallization after devitrification at the warming step [4].

In the current study, live offspring were obtained from cryopreserved ovarian tissues using both slow-freezing and vitrification strategies, indicating that quail ovarian tissues can survive both protocols and that their function can be reinstated by orthotopic
transplantation. In accordance with recent studies in mice and humans [5, 6], vitrification seemed to be superior to slow-freezing in preserving quail ovarian tissue, demonstrated by high cell viability, normal tissue morphology and a greater number of recipients producing donor-derived offspring. It should be pointed out that we can not conclude simply that vitrification is better than slow-freezing in general, but the particular slow-freezing protocol involved may not be optimal for quail ovarian tissue cryopreservation, given the fact that donor-derived offspring were still obtained from a small number of samples. With appropriate refinements, slow-freezing could still be considered as a back-up strategy in avian ovarian tissue cryopreservation. Refinements could be considered in several aspects.

Non-penetrating polymers can be included as secondary cryoprotectants. As a well-studied and extensively-used cryoprotectant, DMSO was used in the present study to protect ovarian tissues in slow-freezing. However, the toxicity of penetrating cryoprotectants such as DMSO can sometimes become very significant and neutralization may be taken into account. One practical strategy is to mix penetrating cryoprotectants with non-penetrating cryoprotectants such as sugars (e.g. sucrose or trehalose) or polymeric chemicals (e.g. polyvinylpyrrolidone, PVP). In a study with mouse 2-cell embryos [7], the addition of PVP-Percoll was shown to increase oocyte survival and reduce zona damage without affecting the developmental potential of the embryos, which could be explained by the low toxicity of PVP and its role in reducing physical cryoinjury by modifying ice crystallization. Addition of polymers could also help to diminish the
osmotic stress caused by penetrating cryoprotectants simply by replacing a certain amount of penetrating cryoprotectants [8] in cryoprotective solutions.

In the current study, the introduction of cryoprotectants was undertaken on ice to reduce the temperature-dependent toxic effect [9]. However, low temperatures may lead to poor penetration of cryoprotectant, thus weakening the protective effect. In the future, cryoprotectants could be introduced at room temperature or higher, and the concurrent increase in toxicity could be mitigated by shortening the exposure time or by using multi-step introduction, as is used in vitrification. More studies are needed to investigate this further.

Finally, thawing is important to ensure the protection of tissues. Early studies in embryos used a slow warming strategy [10-12]. However, slow warming is less beneficial than fast warming which inhibits the process of recrystallization [4, 13]. In this sense, the thawing strategy in the current study could be improved by directly plunging the samples into a water bath with a temperature of 37°C. During thawing, stress can also be induced by the removal of the cryoprotectants. In vitrification, this is relieved by stepwise dilution with sugars, which could be used in slow-freezing as well.

Even if slow-freezing protocols could be refined, vitrification is promising in avian ovarian tissue cryopreservation, especially when large numbers of samples are to be preserved in the field or the protocols need to be extrapolated to different species. For optimization, it is essential to reduce the toxicity of cryoprotectants by enhancing cooling rate and/or reducing the length of dehydration. In practice, an ultra-rapid cooling rate was
achieved by allowing tissue samples to contact liquid nitrogen directly or to contact a liquid nitrogen-chilled surface, which is facilitated by a number of modified carriers or containers. For example, Isachenko and associates [14] used a copper grid as a carrier to plunge ovarian tissue pieces directly into liquid nitrogen. Another example of a special carrier is the polyester sheet in the kit named “Cryotop”, which can be soaked and capped in liquid nitrogen [15]. Using solid surface vitrification, instead of direct contact with liquid nitrogen, samples were placed on a cold metallic surface that was partially immersed in liquid nitrogen [16]. A commercial kit based on this strategy has been used but the results were not as good as those of slow-freezing for protecting the developmental potential of primordial follicles in tissues [17].

The vitrification protocol in this study was derived from two studies that described successful cryopreservation of ovarian tissues in mice and humans [5, 6]. Chen and co-workers [5] vitrified mice ovaries contained in cryovials by pouring liquid nitrogen directly into the vials, subsequent to two steps of brief exposure of the equilibration solution (10 min) and the vitrification solution (2 min). This novel method was named direct cover vitrification (DCV). The ovarian tissues cryopreserved by DCV in this study showed higher viability of primordial follicles (92.3%) compared to slow-freezing (77.8%) and conventional vitrification using standard straws (59.6%). Similarly, the pregnancy rate (87%) of the recipients in the DCV group was the highest of the three cryotreatment groups and was comparable to that of the control group. However, this method may be less useful if large numbers of samples are to be vitrified at the same time, and too much manipulation may jeopardize the tissue integrity and functional
recovery. Recently, Wang and co-workers [6] developed a new container-less method, which they called needle immersed vitrification (NIV), using fine acupuncture needles to hold the tissue. Tissues held by needles were immersed into liquid nitrogen after the two-step dehydration described by Chen et al. [5]. In comparison to slow-freezing and another container-less vitrification method, direct dropping vitrification [18], the results from NIV were superior in all assessments including histological analysis, ultrastructural evaluation and follicle counts after transplantation. However, as the authors indicated, appropriate methods for storing are needed. In the present study, fine acupuncture needles were adapted to hold tissue fragments and cryovials were used as storage containers. The results indicated that quail ovarian tissue could be preserved in a practical manner. Including the current study, the original protocol has been successfully used for three very different species (mouse, human, and quail), which may suggest that it could be extrapolated to more species with optimization being simple or not required.

Promoting penetration is another important way to reduce the toxic effects of cryoprotectants. Because the concentration of cryoprotectants used in vitrification is very high, the exposure time is usually short to avoid toxicity. At the same time, the tissues need to be well saturated with cryoprotectants to avoid cooling injury. In most studies to date, including the current study, ovaries were cut into pieces to facilitate penetration before they were submerged in solutions containing cryoprotectants prior to cooling. However, whole ovaries in rats [19] and mice [20] can also be vitrified with outcomes comparable to those of fragmented tissues. For larger ovaries such as those from sheep which have blood vessels that are relatively easy to access, in addition to the conventional
way of preserving ovarian cortical strips, perfusion of cryoprotectants into whole ovaries could be carried out through specialized pump systems [21]. Cryopreservation of whole ovaries in large avian species might benefit from this in the future. Visual methods such as high-performance liquid chromatography (HPLC) [22] and magnetic resonance imaging (MRI) [23] are available to monitor the penetration of cryoprotectants in tissues. These could be introduced in future studies to help in refining cryopreservation protocols.

### 3.2 Ovarian tissue transplantation

It has been shown that early-stage follicles rather than those at late stages are more resistant to cryopreservation protocols [24, 25], possibly due to their small size and slow metabolism. Transplantation is an effective way for these follicles to achieve maturation in vivo meaning that the reproductive potential can be recovered.

Autotransplantation of cryopreserved ovarian tissues is used in human clinical practice to help cancer patients regain their fertility after remission. Alternatively, together with other assisted reproductive technologies such as in vitro fertilization and embryo transfer, xenotransplantation can be used for fertility reconstitution, preventing the risk of reintroduction of cancer to the donor [26]. Xenotransplantation can also be used in wildlife conservation [27, 28] or in monitoring the recovery of ovarian tissues after cryopreservation and short-term transplantation [29]. Immunodeficient lab rodents can be used as recipients in xenotransplantation for mammals, however, there is currently no such avian model available for birds. Efforts should be made to overcome barriers in interspecies transplantation so that cryopreservation and transplantation of ovarian tissue could be used for wild avian species conservation in the future.
Ovarian tissues can be transplanted to the anatomical normal site, which is known as orthotopic transplantation. Transplantation to a different site is called heterotopic transplantation. For example, the kidney capsule is a competent heterotopic site for transplantation in lab rodents while in humans the uterus, the rectus abdominal muscle, or the subperitoneal tissue beneath the abdominal fascia between the umbilicus and the pubic bone could be used [30-32].

In the current study, we used orthotopic transplantation within one avian species (quail) between different lines. Similar surgical techniques were first used in adult chickens by Guthrie [33], but a later study [34] demonstrated that the offspring in Guthrie’s study were derived from the regenerated hosts’ ovarian tissue. Further attempts in chickens between 24 days and 30 days were still not successful [35]. Breakthrough was made recently in one-day old chicks [36], suggesting that in birds, it is critical to conduct the transplantation in younger recipients to reduce the rejection and promote the recovery of the grafts. Similar conclusions were also drawn from the studies in mammals [37]. However, one-day old quail chicks used as recipients showed high post-surgery mortality (77.8%) compared to the week-old recipients (14.3%) in the study using fresh transplants [38]. In the current study we used one-week old quail chicks as donors and recipients and the post-surgery mortality was around 50%.

One possible reason for mortality is the excessive hemorrhage during ovariectomy which is very common in surgeries such as ovariectomies, hysterectomies and castrations on small avian species such as fiche, quail and canaries [39]. Instead of the current
method, a bipolar electrosurgical unit may be used to help bluntly dissecting the ovary in future studies [39].

For the recipients surviving the surgery, there was no significant difference in overall fertility among groups receiving different treatments, described as recipients producing fertile eggs of surviving recipients. However, a significant difference was seen in those producing only host-derived offspring and those producing both donor- and host-derived offspring. Interestingly, 9 out of 14 surviving recipients in the slow-freezing group produced offspring, of which 7 produced only host-derived offspring and 2 produced only donor-derived offspring. In the vitrification group, all 7 surviving recipients produced offspring, and 5 of these produced donor-derived offspring, all of which produced host-derived offspring as well. There seemed to be a correlation between high regeneration rate of host ovarian tissue and high recovery rate of the grafts. This may be interpreted by the studies of ischemia-perfusion injury in mammals.

Ischemia-perfusion injury is considered to be a major factor leading to the loss of the growing follicular pool [40], particularly for small ovaries or slices of ovarian tissues because surgical reanastomosis is not available and angiogenesis can only rely on the process of revascularization [41]. Administration of exogenous factors including antioxidants, growth factors and hormones were attempted in various studies to relieve ischemic stress. However, the results were controversial and these factors may have toxic effects [37, 42]. Alternatively, a recent study in lab rodents showed that the reestablishment of vasculature and the recovery of transplants may benefit from the wound-healing process in the transplantation site [41]. The authors postulated that when
implanting ovarian grafts into a wounded site, the triggered endogenous process related to revascularization could shorten the ischemic period and improve the recovery of the endocrine system and follicular development of the grafts. The results in the current study suggest that similar mechanism may work for birds, considering that the anatomic site of avian ovaries is highly vascularized. Another benefit of partial removal of the host ovary is that the viability of recipients during and after surgery may be improved because the risk of massive hemorrhage can be reduced. However, uncertain issues such as the extent the host tissues should be removed to prevent negative effects seen in mammals need to be confirmed by future investigations.

3.3 Summary

- Using the Japanese quail as a model, we demonstrated that live offspring can be obtained from cryopreserved ovarian tissue in avian species, which means that the female reproductive potential can be preserved and recovered by cryopreservation and subsequent transplantation.

- Cryopreservation and transplantation of ovarian tissue is a practical method of female genetic material conservation in avian species. It can now be used to maintain commercial quail lines and preserve valuable local quail stocks that may be of interest to the poultry industry in the future.

- Vitrification can be used as a practical strategy of ovarian tissue cryopreservation in avian species. Slow-freezing can also be used but thorough optimizations are required.
• Partial removal of host ovarian tissue before transplantation may benefit the recovery of the ovarian transplants through a wound-healing process.
3.4 References


