CRYOPRESERVATION AND XENOGRAFTING OF TESTIS TISSUE

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ABSTRACT

The objective of this thesis was to investigate and expand the use of testis tissue xenografting as means of maintaining the developmental potential of donor testis tissue.

The objective of the first study was to investigate the effect of donor age on spermatogenesis in canine testis tissue after xenografting into immunodeficient recipient mice. Fragments of testis tissue from 12 dogs of different ages were xenografted under the back skin of mice. Donors were categorized based on testis developmental status at the time of grafting into: less than four months (immature), four to six months (young), and greater than six months of age (adult). The grafts were evaluated at four, six or eight months post-grafting. At four months post-grafting, immature and young groups had higher graft recovery rates (92 ± 5.8 and $88 \pm 4.4\%$ versus $69 \pm 3.5\%$; $P = 0.001$ and $P = 0.001$), graft weights (34 ± 8.1 and 32 ± 11.0 mg versus 7 ± 2.6 mg; $P = 0.001$ and $P = 0.02$), vesicular gland indices (1.1 ± 0.20 and $0.6 \pm 0.18\%$ versus $0.1 \pm 0.03\%$; $P < 0.0001$ and $P = 0.02$), seminiferous tubule numbers (517 ± 114.8 and 364 ± 161.0 versus 10 ± 5.1; $P < 0.0001$ and $P = 0.03$), and larger seminiferous tubular diameters (140 ± 17.8 and 130 ± 3.4 µm versus 55 ± 21.9 µm; $P = 0.003$ and $P = 0.001$), compared to adult donor xenografts. Xenografts from immature donors maintained the growth and development for eight months, as exhibited by greater graft weights (17 ± 4.6 mg, $P = 0.002$), seminiferous tubule numbers (547 ± 210.3, $P < 0.01$) and tubular diameters (93 ± 15.9 µm, $P < 0.0001$), and induced greater vesicular gland indices ($1.5 \pm 0.46\%$, $P = 0.0005$), compared to adult donor xenografts. The growth and development of testis tissue xenografts from immature and young donors were not different after eight months ($P > 0.05$). Young donor xenografts had greater seminiferous tubule number and diameter compared to adult donor

xenografts (P = 0.009 and P = 0.004, respectively) at eight months post-grafting. Elongated spermatids were the most advanced germ cell type present at four and eight months post-grafting in the testis grafts of immature and young age groups.

The objective of the second study was to evaluate three different strategies to preserve/cryopreserve immature porcine testis tissue. Immature porcine testes were cooled at 4 °C for 24, 48 or 72 hours, and testis tissue fragments were cryopreserved using programmed slow freezing with dimethyl sulfoxide (DMSO), glycerol, or ethylene glycol, or vitrified using DMSO or glycerol at 5, 15 or 30 min exposure time. *In vitro* cell viability was determined by trypan blue exclusion, and *in vivo* developmental potential was evaluated by xenografting into immunodeficient mice. Compared to fresh tissue, short-term cooling of porcine testis tissue resulted in similar *in vitro* cell survival rates (93 ± 2.2% for fresh versus 95 ± 0.3, 93 ± 1.7 and 87 ± 4.3% after 24, 48 and 72 hours at 4 °C, respectively; P = 0.74) and *in vivo* development, with generation of elongated spermatids and sperm after four months of grafting. Cryopreservation of testis tissue with programmed slow freezing using glycerol and vitrification with DMSO (5 min equilibration) or glycerol (5 or 15 min equilibration) did not compromise the developmental competence of xenografts when compared to fresh tissue (control), characterized by the formation of elongated spermatids and sperm.

These findings suggest that canine testis tissue from immature donors and cooling of immature porcine testis tissue to refrigerator temperature for up to 72 hours or cryopreservation with slow controlled freezing or vitrification could be suitable methods to restore male fertility following xenografting.

ACKNOWLEDGMENTS

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LIST OF ABBREVIATIONS

µm	Micrometer
A_{al}	$A_{aligned}$
ANOVA	Analysis of variance
A_{pr}	A_{paired}
ART	Assisted reproductive technologies
A_s	A_{single}
bFGF	Basic fibroblast growth factor
CPA	Cryoprotectant agent
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNase	Deoxyribonuclease
DPBS	Dulbecco's phosphate buffered saline
eCG	Equine chorionic gonadotrophin
EDTA	Ethylene diamine tetraacetic acid
EGF	epidermal growth factor
ERM	Ets-related molecule
ES	Equilibration solution
FBS	Fetal bovine serum
FSH	Follicle stimulating hormone
GDNF	Glial cell line-derived neurotrophic factor
GFP	Green fluorescent protein
hCG	Human chorionic gonadotrophin
Hr	Hour

ICSI   Intracytoplasmic sperm injection

IGF   Insulin-like growth factor

IVF   *In vitro* fertilization

LH   Leuteonizing hormone

LIF   Leukemia inhibitory factor

LN$_2$ Liquid nitrogen

Mg   Milligram

S.C.   Subcutaneous

SCID   Severely combined immunodeficient

SEM   Standard error of mean

SSC   Spermatogonial stem cell

VEGF   Vascular endothelial growth factor

VS   Vitrification solution

CHAPTER 1: GENERAL INTRODUCTION

Production of sperm is a very organized process that generates virtually unlimited numbers of gametes during adulthood. This continuous proliferation and differentiation of germ cells requires a delicate balance of function among all testis compartments [1]. Many complex aspects of testis function remain elusive due to the lack of suitable *in vitro* or *in vivo* models.

Cross-species transplantation of heterogeneous populations of testis cells, including spermatogonial stem cells, recovered from donor rats or hamsters into recipient mice testis, has resulted in the establishment of rat or hamster spermatogenesis in the mouse testis [2, 3]. This has not been the case with the transplantation of spermatogonial germ cells from phylogenetically more distant species into mouse testes, and did not result in spermatogenesis beyond spermatogonial proliferation. The failure of spermatogenesis is likely a result of the incompatibility of microenvironments [4, 5]. This has led to the recent development of an alternative approach, namely testis tissue xenografting. Xenografting involves the grafting of testis tissue from a wide range of immature donors under the back skin of immunodeficient recipient mice. This has successfully resulted in the establishment of spermatogenesis [6-13]. Sperm collected from such grafts are fertilization-competent, support the development of embryos [6, 8, 12], and lead to the birth of offspring using the tissues of neonatal donor animals [14].

Preservation of genetic material is crucial for conservation of rare animal breeds or lines. The loss of genetic diversity associated with infertility or premature death of valuable

species is also a major ongoing problem in wild life conservation. Current attempts to address this problem through assisted reproduction involve the collection of sperm or ova from the valuable animals, before or after death. These attempts have several limitations. The most important concern relates to the collection of sperm from males that die before reaching sexual maturity. The use of fresh or cryopreserved immature donor testis tissue in xenografting could circumvent such limitations. This novel approach can also be used to study spermatogenesis. The accessibility of grafted tissue located along the back in the recipient mouse makes it possible to manipulate spermatogenesis and steroidogenesis in a controlled manner. This may not be easy or feasible in the donor animal. This, in turn, would allow the analysis of the effects of new hormone regimens, drugs, or toxicants on testis function of a target species via xenografting in a laboratory mouse.

There has been a wide range of species that have been used as donors for testis tissue xenografting [6-11], but there are no reports of studies using dogs as donor animals. Development of canine testis tissue xenografting methodology could improve the investigation and manipulation of testis function in this species. It could also offer the possibility of preserving genetic potential of canids undergoing castration before maturation. Testis tissue xenografting may prove to be a valuable tool in the conservation of the threatened or endangered canine species that die prematurely. This could further aid in the conservation and maintenance of the existing biodiversity in wild life. Many studies indicate the benefits of xenografting in the establishment of spermatogenesis of the neonatal and prepubertal testes in immunodeficient recipient mice [6-12].

The first objective of this study was to investigate the effect of donor age on spermatogenesis in canine testis tissue after xenografting into immunodeficient recipient mice. As part of our attempt to expand the application of testis tissue xenografting, the second objective was to determine the enhanced preservation conditions of immature porcine testis tissue, as a model, to maintain the testis developmental potential. Short-term preservation would mimic shipment on ice, whereas long-term preservation would reflect the storage of the testis tissue for months or years. Difficulties finding donors of the same age-range or breed for replicate experiments made it unfeasible to consider dog testis tissue for cryopreservation. Therefore, it was decided to use immature porcine testis tissue to study the effects of cryoprotectants, equilibration times and freezing rates on the survival of testis tissue.

CHAPTER 2: LITERATURE REVIEW

I. TESTIS TISSUE XENOGRAFTING

Recently, considerable research has been dedicated to finding practical techniques for male germ line conservation, manipulation and propagation. As the scope and significance of biodiversity loss have become more apparent, positive actions such as xenografting must be examined in order to deal with the crisis, especially in rare and endangered species. This novel method may also, in the future, provide an important contribution to the treatment of human infertility caused by genetic defects or cytotoxic therapies.

Xenografting of testis tissue under the back skin of recipient immunodeficient mice has provided a tool for the study and manipulation of the male germ line [6]. Completion of spermatogenesis in neonatal or immature testis tissue xenografts, from diverse species, establishes the appropriateness of this *in vivo* model. The immunocompromised nude mouse serves as an *in vivo* incubator which provides a suitable environment for the growth and development of the donor-derived testis tissue grafts.

This procedure has allowed the development of spermatogenesis in testis tissue xenografts obtained from several species including immature: mice, hamsters, rabbits, cattle, horses, pigs, goats, cats, monkeys, and sheep in immunodeficient recipient mice [6-15]. Complete spermatogenesis including sperm production from immature donor mice, hamsters, cats, rabbits, pigs, goats, cattle or rhesus monkeys was successfully produced using the xenografting technique [6-10, 16]. Fertility competence of sperm derived from testis tissue xenografts has been verified by the generation of blastocysts in the pig and monkey and

also offspring in the mouse and rabbit using intracytoplasmic sperm injection (ICSI) [8, 12, 16]. Xenografting of testis tissue, therefore, may pave the way for the restoration of the male germ line and fertility preservation. It has been suggested that testis tissue xenografting and using the resultant xenogeneic sperm could be an alternative option for recovered cancer patients to circumvent the risk of re-introduction of neoplastic cells by methods such as auto-transplantation of isolated germ cells [17].

In large animals and humans, the study and manipulation of spermatogenesis and steroidogenesis is challenging and costly. Consequently, xenografting into a laboratory rodent model would facilitate this challenge [18, 19]. Spermatogenesis requires optimal conditions for successful sperm production, where the slightest changes in testis environment such as temperature variation might disrupt the process. Therefore, using a proper *in vivo* model is much more desirable than the corresponding *in vitro* model in controlled studies to produce optimal results. This approach, although in the experimental stage, could provide new avenues for clinical intervention in assisted reproductive technologies, where alternative techniques are not available or applicable.

Several issues need to be addressed in order to effectively improve testis tissue xenografting in different species. This manuscript attempts to provide a general review of past and current efforts for xenografting of testis tissue, prospective implications, and future clinical applications. The critical *"what's next"* question in xenografting of the testis tissue impinges on controversial issues related to preservation of human fertility. There are both technical and ethical issues concerning the acquisition of human testis biopsy, re-establishment, preservation and restoration of testis tissue.

2.1 Key concepts of testis tissue xenografting

2.1.1 Immunodeficient mouse: a suitable 'in vivo' lab model

The availability of proper *in vitro* and *in vivo* models is critical to provide a well-balanced environment for spermatogonial cell differentiation and self-renewal. The study of testis tissue development using an *in vivo* model, without working directly on the target species, could be cost-effective and may help address some of the ethical issues limiting the maintenance and treatment of large animals or non-human primate species [8]. The NCr nude (nu/nu) and severely combined immunodeficient (SCID) mice have been used as mouse models for grafting and transplantation studies [6, 17, 20]. The immune response is altered in these mice to circumvent foreign tissue rejection. The autosomal recessive nude gene in homozygous (nu/nu) mice prevents the growth of hair, and development of normal thymus [21]. The compromised T-lymphocyte function allows athymic mice to accept and grow grafted tissues. SCID mice are homozygous for the severe combined immunodeficiency mutation which leads to a blockage in both B- and T-lymphocyte development [22]. Shortage of natural killer cells, macrophages and complement activity has also been suggested as contributing factors in the prevention of foreign tissue rejection in immunodeficient mice [23]. No significant difference has been reported between nude and SCID mice concerning the re-establishment, growth, and development of spermatogenic cells in xenografts [10, 17].

Recipient mice are usually castrated at the time of grafting in order to remove the source of testosterone, leading to a dramatic rise in gonadotrophins, and thereby providing a stimulus for the newly grafted tissues. In other words, the pathway of the hypothalamus-

pituitary-gonadal feedback loop in the recipient mice switches to the hypothalamus-pituitary of the recipient mouse and Leydig cells of the xenograft [6]. Lowered levels of serum LH in the grafted recipient mice demonstrate that Leydig cells in the grafted tissues are able to produce sufficient testosterone to provide the negative feedback on gonadotrophin production [6]. In castrated mice, in the absence of androgens, vesicular glands regress to about 10% of their normal size in intact mice. Therefore, measuring the weight of the vesicular glands can be used as an indicator of the levels of biologically-active testosterone in castrated recipient mice [6].

2.1.2 Ideal grafting site

Spermatogenesis in mammals usually takes place at a temperature that is a few degrees below that of the normal body core temperature. Therefore, the site of grafting into the recipient mouse is critical for growth and development of the ectopically grafted testis tissue. In general, the ideal site should provide an environment for the grafts that is comparable to the *in situ* condition, in terms of both blood supply and temperature. Possible sites for transplantation of the testis tissue that have been examined include the intraocular region [24], the skin of the thoracic area [24], the scrotum [18, 25], beneath the kidney capsule [19], under the skin of the outer ear [18, 26], and the axillary area [25]. Complete spermatogenesis was reported in the scrotum and under the skin of outer ear of rats [18]; whereas the anterior chamber of the eye and thoracic skin allowed only for the recovery of the grafted tissues [24]. Exposure to different temperatures is an obvious, and perhaps the most important difference among these regions, which might influence the late stages of spermatogenesis [26]. Grafting of immature testis tissue under the back skin,

between the shoulders and rump of recipient mice, has achieved spermatogenesis from different mammalian donor species [6-8, 10, 14]. The area under the dorsal skin appears to be a superior environment for the ectopically grafted testis tissues. The lack of hair in the recipient mice has been suggested as an additional factor contributing to the lowered temperature under the back skin which enhances spermatogenesis [27]. In most mammals, normal spermatogenesis occurs in an environment 4-6 °C cooler than body temperature [28]. Spermatogenesis arrest in cryptorchid testes affirms the impact of temperature, which halts the formation of round and elongating spermatids [29]. In one study, however, using a limited number of nude and SCID mice with complete fur, no difference in the degree of spermatogenesis was observed [10]. The lower core body temperature in the mouse (about 36-37 °C), compared to usually higher temperatures for most other species, may explain the suitability of the mouse model.

Vascularization is likely another key factor for the successful development of grafted tissues. Most grafted tissues show minor initial damages which might be related to an episode of hypoxic ischemia shortly after implantation [11]. Intraocular [24], intramuscular [24], and sub-capsular kidney grafting sites [19] have been proven to be suitable vascular beds for testis tissue transplants. However, these sites might not provide adequate physical features, nor an appropriate temperature for tissue growth. In addition, it has been suggested that immature grafts more readily undergo angiogenesis and resist post-grafting ischemia better than the mature testis tissue [7]. Tissue damages related to hypoxia during grafting appears to be more detrimental for differentiated germ cells than for the earlier stages [11]. This may explain the lower success rates with tissue from mature donors as compared to tissues from immature donors [30].

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2.1.3 Spermatogenesis follow-up

Spermatogenesis is a regular, cyclic proliferation and differentiation of germ cells resulting in production of virtually unlimited numbers of sperm during the life of a male. Germ cells of different types must have closely coordinated interplay with Sertoli cells to be able to undergo mitosis, meiosis, and differentiation [31]. Spermatogonial stem cell (SSC) renewal is also believed to be highly dependent on Sertoli cell support to guarantee the continual production of sperm during adulthood [32]. Leydig and Sertoli cells produce testosterone and estrogen, which are necessary to maintain the hormonal milieu of testis tissue through the hypothalamus-pituitary-testis feedback mechanism [31].

Spermatogonial stem cells are enclosed in seminiferous cords and tubules and form the foundation of spermatogenesis. They periodically undergo self-renewal and give rise to differentiating daughter cells. These stem cells are mostly characterized as "A_{single}" (A_s), which account for the generation of proliferative spermatogonia ($A_{aligned}$ or A_{al}, A_{paired} or A_{pr}). A_{al} are also thought to be a part of the self-renewal pool of spermatogonia [31]. A_{single} cells may be transplanted into the seminiferous tubules of recipient testes and are capable of colonization [33]. This could offer novel applications in the production of transgenic animals via transduction of desired genetic materials into spermatogonia before transplantation. Different visual reporter genes such as GFP and LacZ have been added to monitor spermatogenic activities [5]. These approaches could further address the study and manipulation of spermatogenesis using this model.

During the seminiferous epithelium differentiation, A_s cells give rise to a controlled cyclic generation of A_1 to A_2, A_3, A_4, intermediate, and B spermatogonia. B spermatogonia enter

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in meiosis to generate preleptotene and leptotene cells. Morphological changes of nuclear chromatin provide a basis of spermatogenic cell classification. Zygotene and pachytene cells, in which chromosome pairing occurs, then advance through the second meiosis with the generation of spermatids [31].

The cellular kinetics of pre-meiotic, meiotic, and post-meiotic phases in the testis tissue are extremely organized. Testis tissue xenografting can provide conditions that are sufficiently identical to those of the donor testes to allow the continuation or the re-establishment of spermatogenesis [4, 34]. Analysis of ectopically grafted testis tissues usually includes histological evaluation of the recovered graft, spermatogonial proliferation, and progression through spermatogenesis [8]. Formation of a tubular lumen within seminiferous cords indicates maturation of Sertoli cells with complete regulatory and nursing capacity to support the progression of spermatogenesis [6, 35]. It appears that only SSCs have the potential to induce or restore spermatogenesis after transplantation into recipient mouse testes [36]. Low numbers of SSCs in the adult mouse testis and identical morphology to their immediate daughter progeny cells make it quite challenging to distinguish and to isolate SSCs [36, 37]. Xenografting of testis tissue might provide a powerful tool for *in vivo* confirmation of the ability of SSCs in a specific tissue sample to undergo spermatogenesis.

2.1.4 Endocrine and paracrine support of grafted tissues

In castrated animals, the absence of testosterone and its suppressive effects on gonadotrophins cause the levels of gonadotrophins to increase to higher than normal values. After xenografting of testis tissue into castrated immunodeficient recipient mice, Leydig cells in the newly grafted testis tissue begin to produce testosterone in response to LH and eventually re-establish the cyclic negative feedback on LH [6]. Gonadotrophins affect testis growth, steroidogenesis and gametogenesis [38]. Despite the generally perceived species-specificity of gonadotrophins, these observations collectively indicate that the ectopically grafted testis tissues from a wide range of donor species are steroidogenically active in the recipient mouse and responsive, to a large degree, to mouse gonadotrophin stimulation. Nevertheless, low efficiency of testis tissue xenografting for certain donor species such as the bovine and equine species, may be attributed to the lack of compatibility between the donor-derived testosterone and recipient mouse gonadotrophins [11].

Internal gene expression and surrounding environment of SSCs are responsible for stability of the cellular interactions in the testis tissue [39]. Although the function of SSCs is critical for spermatogenesis, little is known about the essential factors controlling their activity. Glial cell line-derived neurotrophic factor (GDNF) is a secretory protein of Sertoli cells acting as one of the critical factors facilitating SSC-Sertoli cell communication [39]. The first report describing the important role of GDNF on SSC function *in vivo* was based on observations characterizing reduced spermatogonial proliferation in adult heterozygous mice testes [40]. The explanation for the reduced

spermatogonial proliferation in adults was over-expression of GDNF in testis [40]. On the other hand, excessive GDNF has been shown to induce SSC proliferation and self-renewal during the perinatal period [1].

Sertoli cell transcription factor ERM (Ets-related molecule) is reported to be essential for SSC self-renewal after puberty [1]. It has been demonstrated that other factors such as basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF), leukemia inhibitory factor (LIF), and epidermal growth factor (EGF) may support the proliferation of spermatogonia *in vitro* and *in vivo* [39, 41, 42]. Finding critical species-specific factors and cellular pathways may assist the establishment of spermatogenesis in species which have not exhibited successful testis tissue xenografting, such as marmosets, horses, and bulls.

2.1.5 General procedures for ectopic grafting of testis tissue

Following collection of the donor tissue through castration of neonatal or immature donors and removal of the tunica albuginea, small fragments of dissected donor testes are arbitrarily assigned to be used for ectopic testis grafting in different recipient mice. Some are fixed for use as a reference for histology. Usually eight fragments of donor testis tissues are grafted under the back skin of castrated immunodeficient mice (SCID or NCr nude). Different donor species vary in the time to testis maturation. Therefore, the time of graft retrieval for analysis varies depending on the progression of spermatogenic cells in the intact testis tissue *in situ*. The histological examination of xenografts includes the assessment of general morphological changes in the tissue and the diameter of seminiferous tubules. Differentiation status and characterization of the most advanced

germ cells are recorded to estimate developmental progress. In addition to the evaluation of xenograft development, serum levels of testosterone and the gonadotrophins can be determined in blood. The relative weight of the vesicular glands is highly androgen-dependent and reflects the bioactivity of testosterone produced by the xenografts [6, 8, 11, 15, 43].

In situations where the sperm can be retrieved by mechanical dispersion of the testis grafts, fertility competence of the sperm can be determined by intracytoplasmic sperm injection (ICSI) into the cytoplasm of metaphase II oocytes [8]. Verification of embryo parentage has been used to further confirm that the blastocysts formed are indeed due to fertilization and not due to parthenogenesis [8].

2.2 Grafting of testis tissue from different donor species

Spermatogenesis has been extensively studied only in some species, but xenografting can provide an *ex situ* assessment of testis development and spermatogenesis from a variety of donor species in an *in vivo* rodent model. Since testis tissue from different donor species behaves differently following ectopic grafting into mice, it is necessary to briefly review the development from each donor species to determine the role of the donor on testis tissue growth and developmental progression.

2.2.1 Testis tissue xenografting in laboratory and companion animals

2.2.1.1 Allografting of murine testis tissue

Allografting (grafts sourced from a genetically non-identical member of the same species) of newborn mouse testis tissue into nude mice lead to completely functional donor-derived spermatogenesis four weeks after grafting. Regardless of the mixed recovery rate of the seminiferous tubules in the grafted tissue, the kinetics of spermatogenesis were similar to those of the intact testis [6, 7, 14]. Grafting of newborn mouse testis tissue is a valuable technique to evaluate the potential of spermatogonia for spermatogonial stem cell proliferation, and seminiferous tubular development. Testosterone levels of the castrated recipients of testis allografts were comparable to those of intact mice indicating that the androgen concentration is normalized after allografting immature mouse testis tissue [7]. It should be noted that the overall mass of the grafted testis tissue in the recipient mice was greater than total mass of two testes in an intact mouse [14]. Accumulation of excessive fluid causing expansion of the luminal part of seminiferous tubules may cause pressure necrosis of the seminiferous epithelium after two months. This major drawback was ironically seen only when mouse testis tissue was allografted in mice [6, 14]. Seminiferous tubule dilation was also observed following surgical obstruction of efferent ducts, and in the estrogen receptor knockout mice which may endure defects or lack of efferent ducts [44, 45]. Therefore, tubular dilation of the allografts appears to be the result of a non-functional duct system to drain the tubular fluid. This was confirmed by allografting an intact neonatal mouse testis along with its epididymis which resulted in the development of morphologically normal tubules in the grafted tissue [14]. The testis allografts re-establish a strong androgenic negative feedback on gonadotrophin release from the recipient mice. Furthermore, compartmental damage in testis allografts did not inhibit the capability of interstitial leydig cells to produce testosterone or to interrupt the feedback mechanisms [14]. Since this damage does not affect the function of Leydig cells and the

androgen levels, testis tissue grafting in mice can provide an excellent strategy to assess androgen production or its biological mechanisms in the testis tissue [14]. The ICSI of the graft-derived sperm into oocytes, and consequent embryo transfer to a surrogate mother gave rise to the birth of fertile male and female mouse pups [14]. Therefore, allografting of the testis tissue can be used to investigate testis developmental potential and spermatogenic function of testes from neonatally lethal mutants [46].

Spermatogenesis in adult testis allografts was not comparable to that of immature allografts. Allografts of an adult donor mouse into recipient mice produce qualitatively inferior degrees of spermatogenesis, severe atrophy, and tissue degeneration. This may be associated with the fact that very few stem cells re-establish spermatogenesis in allografts of adult donors [7]. The resultant donor-derived sperm in the allografts of adult donors might have originated from germ cells which completed differentiation after grafting [7]. In all probability, there is a greater chance of survival of immature testis tissue after a period of ischemia following grafting procedures compared to that of adult testis tissue. It is also thought that there is more effective vascularization in immature testis tissue allografts compared to than that of adult donor testis tissues [7].

2.2.1.2 Xenografting of rat and hamster testis tissue

The initiation, development, and maintenance of spermatogenesis in testis tissue xenografts from newborn rats followed a pattern similar to that of immature mouse grafts, from both quantity- and quality perspectives. A high ratio of meiotic to post-meiotic germ cells followed by completion of spermatogenesis was reported from newborn rat testis tissue xenografts. The classic cellular morphology and hormonal function of Leydig cells

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were seen in the xenografts. In contrast, when the photoperiod-regressed adult hamster testes, which were used to mimic a prepubertal state, were grafted into mice, the grafts showed extreme degeneration and inefficiently recovered Leydig cells. This was probably associated with the inability of the tissue to stimulate angiogenesis, and subsequent ischemia [7, 47].

Gassei et al. (2006) developed a new model of xenografting using *in vitro*-generated cell aggregates derived from dissociated immature rat testis tissue [48]. They suggested that the composition of immature testis cell has the potential to initiate Sertoli cell aggregation *in vitro* and proceed further to *in vivo* development and differentiation of germinal cells after grafting [48].

2.2.1.3 Xenografting of feline testis tissue

Feline testis tissue xenografts have shown gradual progression in growth and development. Kitten testis tissues were capable of initiating spermatogenesis after xenografting into immunocompromised mice; however, quantitatively few sperm could be retrieved from these xenografts. The time frame of testis maturation was reported to be similar to that of intact cats, indicating that mouse-derived gonadotrophins and xenograft-gonadotrophin interplay were effective. Increased weight of the vesicular glands indicated the release of bioactive androgens from the xenografts [10]. However, these authors could not carry out a complete investigation, due to the premature death of a number of recipient mice infected with an unrelated viral infection [10]. In another study, sperm were successfully obtained from xenografts of immature donor cats aged 8 to 16 weeks [49].

Xenografts from mature donors more than 8 months of age showed complete degeneration [49].

The efficiency of sperm production in feline xenografts and the possibility of testis tissue xenografting from endangered feline species are yet to be determined. Nevertheless, the initial outcome of feline testis tissue xenografting is encouraging, especially for future research on germ line preservation of endangered feline species. Additionally, feline testis tissue xenografting might provide an accessible *in vivo* model which would facilitate the study and manipulation of spermatogenesis and the prevention of diversity loss in feral feline species.

2.2.2 Testis tissue xenografting in farm animals

2.2.2.1 Xenografting of equine testis tissue

Xenografting of equine testis tissue into recipient mice resulted in spermatogenesis up to the meiosis stages. Presence of pachytene spermatocytes, as the most advanced germ cells in xenografts at four months period, confirmed the meiotic arrest [11]. The underlying reasons for grafting inefficiency to develop post-meiotic stages are not completely clear. It has been suggested that the development of xenografts in horses is highly age-dependent. Testis xenografts from very immature donor horses (about 2 weeks old) were not able to begin differentiation and showed a very low survival rate. Xenografts from peripubertal equine testes (i.e. 10 and 12 months old horses) had a higher survival rate and displayed limited developmental progress beyond the pachytene stage over the first four months of grafting. The limited efficiency of xenografts from peripubertal testes might be due to

degeneration of existing differentiated germ cells and impaired differentiation of remaining SSCs. Sexually mature donor testis tissue xenografts (4 year old horses) showed severe degeneration of seminiferous tubules which was likely associated with the low tolerance to ischemia shortly after grafting [11, 30].

Levels of serum testosterone in recipient mice were low, suggesting the insufficient endogenous gonadotrophin release or inadequate bioactivity of murine gonadotrophins on equine Leydig cells [11]. Administration of exogenous gonadotrophins into recipient mice enhanced germ cell differentiation by formation of elongated spermatids in some xenografts, compared to untreated recipient mice, but had no effect on the percentage of seminiferous tubules with differentiated germ cells or the weight of the vesicular glands [11]. Elimination of these possible deficiencies might improve the performance of equine xenografts. Using donors of the same breed, specific ranges of donor age, and time adjustment of exogenous gonadotrophin therapies might reduce the variability of the responses and elucidate the observed inefficiencies in the reported data by Rathi et al (2006) [11]. Equine testis tissue xenografting could provide an option for fertility preservation of valuable and rare horse breeds, where other techniques of fertility preservation and restoration are not feasible.

2.2.2.2 Xenografting of bovine testis tissue

Testis tissue xenografts from prepubertal donor bull calves yielded good recovery rates with complete cellular differentiation. Regardless of the age of the pre-pubertal bull donors, spermatogenesis proceeded to produce elongated spermatids. In older donors, the progression of spermatogenesis declined over time [15, 43, 50]. Therefore, the age of the

donor and the time period allowed for the grafts to mature in recipient mice, are critical factors for the development ofspermtogenesis development in bovine testis tissue xenografting [43, 51]. The timing of post-grafting spermatogenesis has proved controversial. While spermatogenesis in testis tissue xenografts was reported to be equivalent to that of the testis *in situ* condition [9], accelerated spermatogenesis was demonstrated in another study by the same group [43]. In general, substantial acceleration of lumen formation, accelerated pre-meiotic phases of spermatogenesis, and generation of pachytene spermatocytes, as well as, low efficiency of post-meiotic differentiation have been reported as the major characteristics of bovine testis tissue xenografting by Rathi et al. [15]. In addition, as suggested by Huang et al. (2008), early onset of spermatogenesis was observed in xenografts that mainly contained undifferentiated germ cells at the time of grafting; whereas, the presence of more advanced germ cells in the donor tissue resulted in poor graft development [50].

Evidently, the excessively low efficiency of spermatogenesis ($\leq 10\%$) and germ cell loss in xenografts are challenging aspects of bull calf testis tissue xenografting [9, 15, 43]. Moreover, low initial numbers of germ cells in xenografts might be due to an ineffective re-population or primary pre-meiotic arrest of these germ cells in xenografts. Seminiferous tubule dilation related to fluid accumulation also occurs in bovine xenografts [15], although with a much lower incidence than reported for mouse testis tissue xenografts [6]. In contrast to primate testis tissue xenografts [8], Huang et al. (2008) observed no differences in the development in bovine testis tissue xenografts between castrated and non-castrated recipient mice [50].

Improvements in the methods of xenografting, such as the stimulation of vascularization and endocrine / paracrine support of early differentiating spermatogonial cells, may contribute to the spermatogenesis efficiency [50]. Schmidt et al. (2006), for the first time, indicated that exposure of bovine testis tissue, prior to grafting, to vascular endothelial growth factor (VEGF) assists spermatogonial stem cell growth, proliferation and differentiation [52]. It has been suggested that discovery of the gene expression patterns during different phases of angiogenesis and growth of xenografts might provide a new approach to manipulate sperm production [53]. Strategies for the generation of transgenic livestock with genetic manipulation of germ cells via transduction, prior to grafting, may also prove promising [9].

2.2.2.3 Xenografting of porcine, ovine, and caprine testis tissue

Porcine, ovine, and caprine neonatal testis tissue xenografting into mice was reported to produce complete spermatogenesis, comparable to the cellular developmental trend of the intact testes [6, 13]; however, irregular formation of some seminiferous tubules in porcine xenografts was reported by Honaramooz et al. (2002) [6]. Moderate acceleration by a few weeks in testis maturation and sperm production were remarkable characteristics of both porcine and ovine testis tissue xenografting [6, 54]. Moreover, porcine and caprine xenografts developed efficient spermatogenesis along with quantitatively comparable sperm production per gram of tissue to that of testes *in situ* [6]. Xenogeneic pig and goat sperm obtained from testis grafts were able to fertilize oocytes [6, 12]. It is believed that the duration of cellular events in spermatogenesis is inherited [55]. Zeng et al. (2006) proposed that the acceleration of development in porcine and ovine testis tissue xenografts

could be due to gonadotrophin stimuli leading to a reduction in the time required for testis maturation rather than the duration of cellular events in spermatogenesis [54].

Porcine testis xenografts were suggested to be a preferred model for investigation of gene expressions in the testis because the profile of gene expression in xenografts was similar to that of porcine testis *in situ* [56]. This model could provide a useful approach for the manipulation of spermatogenesis and the study of gene expression during different stages of growth, proliferation and differentiation of porcine germinal cells.

2.3 Xenografting of non-human primates and human testis tissue

2.3.1 Xenografting of non-human primate testis tissue

Xenografts of premature rhesus monkey (*Macaca mulatta*) testis tissue in recipient mice displayed a remarkably high recovery rate and significant growth. Histological evaluation at different time points after xenografting exhibited normal spermatid morphology along with a typical increase in the diameter of the seminiferous tubules. Surprisingly, accelerated lumen formation with significant shortening of the time required for testis maturation and complete spermatogenesis was noted in rhesus monkey testis tissue xenografts [8]. In the same report, rhesus monkey testis tissue xenografts from a one year old donor developed unusually rapid maturation in only seven months after grafting; giving them the same appearance as fully mature four year old control testes. Honaramooz et al. (2004) also found that supplementation with exogenous recombinant rhesus gonadotrophins was not necessary and rhesus testis tissue was responsive to mouse gonadotrophins [8]. Assessment of fertility competence of the recovered sperm from

prepubertal primate xenografts was performed via ICSI of rhesus monkey oocytes followed by the evaluation of the pronucleus and blastocyst formation. The xenogeneic sperm were found to be completely functional and capable of supporting embryo development as verified by parentage analysis [8].

Unlike the rhesus monkey, marmoset monkey (*Callithrix jacchus*) testis xenografting did not proceed beyond germ cell meiosis [47]. Testis development was arrested at pre-meiotic phase and no androgen was detected in the serum of recipient mice [7], even after co-grafting of marmoset and hamster testis tissues [47]. However, Honaramooz et al. (2003) suggested that poor graft development in marmoset monkeys is likely attributed to a mutation on exon 10 of LH receptor gene. This may lead to the lack of responsiveness to recipient mouse LH in marmoset testis tissue xenografts [57]. Induction and maintenance of germ cell differentiation after autologous testis grafting in marmosets verified the specificity of these unique LH receptors in this species [58].

Successful testis xenografting from prepubertal primate testes was considered promising for future fertility preservation and restoration in prepubertal cancer patients.

2.3.2 Xenografting of human testis tissue

Xenografting of immature testis tissue from several species into recipient mice has encouraged many studies with human testis xenografting, with the intention to conserve and restore fertility. Exposure of the testes to chemotherapic agents or total body radiotherapy for the purpose of harming propagating malignant cells, could inevitably affect healthy cells involved in proliferation. Thus, spermatogenic deficits and infertility

are consequential challenges for cancer patients [17]. Semen cryopreservation before fertility-compromising treatments, while not applicable in prepubertal boys, is an option for adult patients [17, 59]. Testis tissue xenografting is, therefore, a convincing theoretical approach with the potential for the restoration of fertility in prepubertal cancer survivors. Additionally, unlike germ cell transplantation, testis tissue xenografting with recovery of sperm from graft recipients would circumvent the possibility of re-introduction of neoplastic cells into the patient [17, 59]. At present, ethical concerns serve to constrain the source and availability of human testis tissue for xenografting studies.

Schlatt et al. (2006) used a readily available source of human testis tissue from adult individuals who had undergone testis biopsies for infertility treatments. This study was also able to use whole testes of transgender individuals undergoing castration. However, as might have been expected, ectopic grafting of adult human testis tissue into recipient immunodeficient mice resulted in insufficient graft recovery, lack of meiotic activity in germ cells, initiation of hyalinization in seminiferous tubules, and subsequent gradual sclerosis [60]. Steroidogenesis deficiency was also evident in adult human testis tissue xenografts [17, 60]. However, Schlatt et al. (2006) demonstrated superior survival of spermatogonia over advanced germ cells in adult human xenografts, even after germinal cell regression caused by cytotoxic treatment prior to xenografting [60]. Nonetheless, the chance of survival and developmental potential of xenografts from prepubertal boys remains unknown.

Although testis tissue xenografting has improved during recent years, this alternative approach is not a viable method for fertility protection and restoration of prepubertal boys due to ethical concerns. Although merely experimental, there have been innovative breakthroughs in the storage of spermatogenic cells by cryopreservation. Cryopreservation of prepubertal testis biopsies and xenografting at a later date, or xenografting of fresh testis tissue followed by cryopreservation of resultant spermatids or spermatogonia, offer a theoretical alternative for fertility preservation of prepubertal boys to circumvent the risk of cancer relapse [17]. The first study on the survival and developmental capacity of testis tissue xenografts from cryptorchid young boys was conducted by Wyns et al. (2007) [25]. They demonstrated a reduction in spermatogonial cells in recovered grafts, but similar cell proliferative activity of spermatogonial and Sertoli cells of both cryopreserved and fresh tissue xenografts was retained [25]. Nonetheless, the hazard of viral infections and / or unknown zoonotic risks which may give rise to an epidemic, as well as ethical issues are serious concerns that need to be considered prior to clinical application of this technique [25, 60].

2.4 Xenografting of cryopreserved testis tissue

The use of cryopreserved neonatal and prepubertal testis tissues from pig and hamster and recently prepubertal boys for xenografting has been reported [6, 7, 25]. Although preliminary in scope, the outcomes were promising. However, a qualitatively optimal technique specifically developed for testis tissue cryostorage is not yet available. In order to improve practical cryopreservation of testis tissue, efficacious cryoprotectants, less

damaging methods of freezing and thawing, and safe techniques of tissue handling are required. Cryostorage is a breakthrough strategy in testis tissue banking for cancer patients; even though associated risks of neoplastic recurrence, safety and ethical challenges of xenografting, and many unknown interrelated issues are areas of high concern.

2.5 Relevance of testis tissue xenografting

Testis xenografting could facilitate the experiments on large animals or primates by performing ectopic grafting of donor testis tissue into immunodefficient mice, which offers a major step forward, both ethically and logistically. The *in vivo* study of spermatogenesis using testis tissue xenografts is more cost-effective, could minimize the generation of inconsistent data due to the individual variation in target species [11]. The study and manipulation of spermatogenesis and steroidogenesis by changing the hormonal milieu in recipient mice is a far more versatile and practical approach than the *in situ* approach in larger animal models and humans [6, 8]. It is, for example, possible to easily castrate a recipient mouse and if necessary, also perform hypophysectomy to remove the influence of major reproductive hormone by the recipient mouse, and to supply any combination of exogenous hormones to study their effects on testis tissue xenografts. This technique also has the potential to save and manage present genetic diversity, especially in rare and endangered species. Assisting the movement of genetic material between animals of the same species by means of testis xenografting might provide a solution for the preservation of endangered species and to prevent a biodiversity crisis [10].

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The successful application of xenografting could offer a robust alternative to the cloning of genetically valuable species. Animal cloning via nuclear transfer has been successful in several species, although technical challenges of this technique might limit routine expansion into other species. Other problems of cloning such as a low success rate and high mortality of cloned animals, can be avoided by grafting [61]. In fact, cloning is not a prudent option for increasing the number of individuals for an endangered species as it does not provide genetic diversity. Xenografting is a technique that can provide millions of genetically unique sperm produced in a graft, ensuring diversity in endangered species.

Xenografting of testis tissue from experimental animals can also offer the possibility of germ cell development and gametogenesis from resourceless animals such as cloned, neonatally lethal, mutant, transgenic or knock-out animals [6, 61]. Moreover, innovative techniques of gene incorporation, using for instance, viral vectors or electroporation into spermatogonial stem cells can lead to expression of the transgene(s) of interest in sperm. Genetic manipulation of male germ cells *in vitro* followed by xenografting of testis tissue may offer a potential alternative for production of transgenic sperm for scale up production of transgenic livestock [9].

In summary, xenografting of testis tissue could introduce new strategies in the conservation and propagation of genetic material by allowing complete spermatogenesis to occur in grafts from newborn or premature animals of different species into immunodeficient mice. Obtaining donor-derived fertility-competent sperm in recipient mice might provide practical methods for protection of rare or endangered species.

Restoration of germ lines of otherwise resourceless animals that die prematurely has provided proof of principle for future practical use of testis tissue xenografting. Reduction of repeated sampling from different individuals and consistency of outcome, are some of the possible applications of testis tissue xenografting from large animal or primate donors. This technique is potentially applicable to fertility preservation of cancer patients undergoing whole-body radiotherapy or cytotoxic chemotherapy. However, the ethical issues and safety concerns would have yet to be resolved.

II. TESTIS TISSUE CRYOPRESERVATION

2.6 Introduction to cryopreservation

The survival secret for overwintering of certain insects and some arctic fish, or other native inhabitats of frozen parts of the world, is their ability for cryoprotection [62, 63]. The study of their freezing tolerance or cryo-injury avoidance phenomena could help us understand the underlying protection mechanism and possibly devise methodologies for cryopreservation. Cryostorage has been a topic of interest especially in fertility preservation for which banking of gonadal cells and tissues is critical for future experiments or diagnoses. To date, many different cryopreservation strategies have been proposed or developed to serve as a mechanism to restore all functional properties of the cryopreserved cells and tissues.

The first successful cell cryopreservation was carried out by accidental freezing of fowl sperm in diluents containing glycerol [64]. Later on, cryopreservation of bull sperm using the same cryoprotectant, revolutionized the bovine artificial insemination industry [65, 66]. At about the same time in the early 1950's, cryopreservation of unfertilized oocytes was also studied following exposure to glycerol and low temperatures [67]. After initial success with *in vitro* embryo manipulation in the 1950's [68], research involving embryo freezing intensified. Many methods have been developed for embryo cryopreservation. Some have become routine procedures since the 1980's [69-71]. Cryopreservation of mature oocytes has also been achieved in recent years [72-74], with high survival rates and development of normal pregnancies after IVF (*in vitro* fertilization); however, the technique has been neither simple nor convenient.

The first gonadal tissue cryopreserved successfully was ovarian tissue, resulting in preservation of cell viability and normal function [75]. In some cases, cryopreservation of structurally intact tissues could be more beneficial than cryopreservation of cells, especially when it is equally important to retain all of the tissue's potential. However, providing a robust freezing protocol to maintain all the compartments of the tissue could be more complicated. Rodent ovarian tissue was cryopreserved successfully after exposure to glycerol and autografted back into the animals [75-77]. Subsequent reports of live rat offspring, sheep ovarian cyclic function, and pregnancy after freezing the ovary prior to grafting, represented the first important steps demonstrating the feasibility of this approach [78, 79]. Cryopreservation of testis tissue prior to its grafting has also yielded complete

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spermatogenesis [6, 7]. Restoration of spermatogenesis was also obtained after cryopreserved testis cells were transplanted into recipient testes [3, 20, 80]. Major advances have been made in the cryopreservation of reproductive tissues. The following is a review of the primary contributing factors for optimal cryopreservation and the possible applications of cryopreservation in future experimental and clinical settings in reproduction medicine.

2.7 Biophysics of cryopreservation

A clear understanding of cellular biophysical behavior at the time of cooling and exposure to different cryoprotectants is critical to improve the cell structural and functional potential after freezing. At slow cooling rates, extracellular ice crystal formation begins with the presence of a nucleation site in the extracellular medium. Since ice is pure crystalline water, the extracellular space becomes hypertonic due to the removal of water, as ice crystals develop. Intracellular water, therefore, moves outward across the cell membrane due to the differential osmotic gradient, and cells dehydrate and shrink. This is the opportunity when certain cryoprotective compounds could come into play and permeate the cells to protect them against high solute concentration or ice crystal damage. Since various cryoprotectant agents (CPA) permeate different types of cells at varying rates, it is of great benefit to understand the biophysics of cryopreservation to minimize damages [81, 82].

Vitrification is a method of cryopreservation in which cells or tissues are exposed to high concentrations of CPA and high freezing rates. This technique prevents ice crystal formation, which is one of the major damaging consequences of freezing. The optimal CPA concentration and exposure time to prevent toxicity in different cell types also needs to be addressed specifically for each cell and tissue type. It is, therefore, critical to consider the biophysical theories behind different freezing protocols to minimize intracellular ice formation, risk of toxicity of CPA, solution effects and osmotic shock [81, 82].

2.8 Freezing injuries

2.8.1. Ice crystal damage

When live cells are subjected to freezing, many of them will suffer from damages caused by the cryopreservation process. Ice formation is one of the biophysical changes which causes irreversible breakdown of cells and tissues [81]. Due to the destructive effects of intracellular ice formation, conventional approaches to cryopreservation as yet, have not proven suitable for multicellular tissues [83]. Unlike cell suspensions, the highly organized structure of the tissue may vary in its response to cryopreservation; therefore, distribution of ice formation could be extremely hazardous to the complex tissue structure [84, 85]. Ice formation, initiated in the extracellular space, leading to an osmotic gradient across the cell membrane, causing intracellular water to move toward the concentrated extracellular space surrounding the cells [86, 87]. Previous studies have revealed that optimal cooling

rates for various cell types are directly associated with the degree of water permeability of cell membranes at different temperatures during freezing [88, 89]. Freezing injury is also attributed to intracellular ice crystal formation and osmotic dehydration (solution effect) [86, 87].

2.8.2. Osmotic changes

Extracellular ice formation causes elevated solvent concentrations and cell dehydration. As ice grows, the excluded solutes cause hyperosmotic stress to cells. The so-called 'freeze-dehydration' is another biophysical consequence of cryopreservation [81, 82, 86, 87, 90]. Prolonged exposure to hypertonic conditions might permanently damage cell membranes and destabilize proteins [87]. However, a short exposure of cells to optimized concentrations of hypertonic media before freezing might protect them from supercooled water retention within cells and subsequent intracellular crystallization during freezing [87]. Where cooling is faster than optimal, intracellular ice formation could occur due to inadequate time for water to follow osmotic gradient across the cell membrane [81, 82, 87]. The osmotic tolerance of cells is another critical factor to be considered during addition and removal of different cryoprotectants. Physical destruction, subsequent organelle disruption, and functional damage are some of the known consequences of intracellular ice formation [91].

2.9 Cryoprotectants: protection and toxicity

The idea of cell and tissue preservation by adding cryoprotectants before freezing has been investigated for decades. Sufficient concentration of cryoprotectants could minimize ice crystallization and/or promote amorphous solidification (vitrification). Glycerol was introduced as a cryoprotective agent in 1949 by Polge et al. [64]. Later, cryoprotective properties of dimethyl sulfoxide (DMSO) were reported by Lovelock and Bishop (1959) [92]. These two cryoprotectants have mainly been used since then, as classic cryoprotective additives, although many other CPA products have also been introduced. Permeating CPAs such as DMSO, glycerol, methanol, propanediol, ethylene glycol, and dimethyl acetaldehyde, and non-permeating CPAs including sucrose, dextran, albumin, polyvinyl pyrollidone, and hydroxyethyl starch, have been shown to afford effective cryoprotection [86, 87]. As described below, cryoprotective agents are known to act through different pathways to protect cells against freezing injuries.

2.9.1. Modulation of hydrogen bonding and interaction with water molecules

Hydrogen bonding gives CPAs high solubility and high permeability across cell membranes [87].

2.9.2 Salt-buffering effect

During freezing, cells experience osmotic dehydration and shrinkage; therefore, the addition of CPAs into the cells, maintains salt dilution. Basically, CPA replaces water in cells, which dilutes the intracellular salts and prevents intracellular crystal formation. The amount of CPA and water that permeates into the cells depends on the concentration of permeable solutes and final cell volume. The properties of CPAs and those of cell membranes will influence the degree of cryoprotection for different cell types [81, 87].

2.9.3 Stabilizing biomembrane critical macromolecules

Under normal conditions, water stabilizes the membrane bilayers. Loss of water during cryopreservation may disrupt normal membrane permeability and damage the membrane itself. The CPAs stabilize proteins as well as phospholipid bilayers of cell membranes and help to protect the membrane against freezing and dehydration stresses [93]. Studies have collectively demonstrated that CPAs such as DMSO and disaccharide sugars such as sucrose and trehalose may electrostatically interact with membrane phospholipids to provide stabilization [94, 95].

2.9.4 Scavenging oxygen free radicals

CPAs may prevent oxygen free radicals and oxidative stress to the cells [87]. CPAs block the action of unstable intermediate products, such as oxygen free radicals, by binding their hydrogen atoms to them [96, 97].

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2.9.5 Inhibition of nucleation

Ice formation occurs in the media through nucleation. During cooling, initial heterogeneous nucleation sites, such as small particles, change in shape and increase in size within media reaching a stage that forms ice crystals. Alternatively, induced nucleation could be beneficial to provide consistent extracellular crystallization. This is the basis for 'seeding', which induces nucleation onto supercooled media enabling embryo cryopreservation [87]. Seeding can be achieved by clamping the side of vials or straws with a forceps cooled in liquid nitrogen to stimulate the local ice growth into the solutions. Intracellular nucleation can also be lethal or damaging for cells and tissues. Some CPAs such as DMSO or glycerol inhibit nucleation by increasing the high viscosity of intracellular water [87]. Non-permeating CPAs, on the other hand, increase and promote cellular dehydration by increasing the extracellular solute concentration, thereby reducing intracellular crystallization [86].

Despite the protective potential of CPAs, a side effect of the addition of CPAs is *cytotoxicit*y. Tissue tolerance to CPAs is limited and overexposure to CPAs may cause damage [90]. However, toxicity is difficult to assess precisely [87]. Cytotoxicity is exacerbated by increasing CPA concentration during ice formation [90]. It is believed that optimizing the freezing rate and rates of CPA addition and removal could reduce the toxicity of CPAs [90].

2.10 Cryopreservation techniques

Finding the optimal cryopreservation protocol for certain cells and tissues depends on the application of a proper cryoprotectant, and cooling rate. Critical factors for effective cryopreservation such as cell permeability to water or CPA, and subsequent osmotic changes, are directly affected by the rate of cooling [89]. Prolonged overexposure to hypertonic conditions leads to osmotic stress, cell shrinkage, and irreversible membrane and protein impairment [87].

The alternative route which avoids ice crystal formation and solute damage is transformation of aqueous milieu to the amorphous character of a glassy state, known as vitrification. Vitrificaiton involves exposure of tissue to extreme viscosity CPAs and ultrafast freezing [82, 87, 90]. However, this approach is compromised by the cytotoxic effects of CPAs during lengthy periods of exposure, and osmotic effects on cells [81, 87]. To deal with this problem, some researchers have suggested using a combination of CPAs to improve vitrification. Proper media may include disaccharides such as sucrose or trehalose, and proteins or polymers [98, 99].

2.11 Thawing methods

Optimal thawing and CPA removal are critical factors for survival after freezing [86]. Earlier studies pointed out that consistent cooling and thawing rates (slow-freezing / slow-thawing and fast-freezing / fast-thawing) might improve cell and tissue survival after

cryopreservation [71]. Moreover, osmotic changes during CPA removal might damage the cells by extensive cell shrinkage or swelling associated with the rapid movement of water into the cell as compared to slower movement of CPA out of the cell [82]. Limited amount of water replacement, however, is needed to restore osmotic equilibrium and physiologic cell volume [82].

2.12 Applications of cryopreservation in reproduction medicine

Development of cryoconservation methods for long-term storage of cells and tissues is critical for *ex situ* preservation of biological diversity and to maintain genetic stability in different species. There has been a substantially increased interest in biomedical research on infertility. Assisted reproductive technologies (ART) are some of the prominent features of clinical therapies [86], although many unsolved problems have remained. Preservation is one of the major challenges of routine ART, since short-term or long-term storage is required where immediate use of fresh cells or tissues is not desired or possible. To deal with fertility concerns in cancer patients, preservation of sperm, zygotes, embryos, or ovarian tissues is a common practice [100-103]; however, cryopreservation techniques for unfertilized oocytes, ovarian, and testis tissue, need to be improved for routine use in prepubertal individuals.

Ovarian and testis toxicity are the inevitable long-term consequences of certain therapeutic oncological regimens, leading to premature fertility failure or sterility in cancer patients

[59, 104, 105]. Gonadal cells or tissue cryopreservation before high-dose gonadotoxic chemo- and radiotherapy could be beneficial [86, 105].

Cryopreservation of testis tissue or cell suspensions could be an alternative fertility option for prepubertal boys with cancer or azoospermic men, since spermatogenesis is not completed in these patients [34, 106]. Reversible suspension of testis tissue growth and development via cryopreservation is currently under extensive investigation. Salvaging genetic potential through banking of gonadal tissues is also an issue of clinical significance in animal reproduction. Optimal cryoconservation methods could also be combined with transplantation or xenografting techniques to overcome some of the complications in the biodiversity crisis of rare or endangered species [10]. In fact, experimental methods for the generation of fertility-competent gametes from cryopreserved ovarian or testis tissue have paved the way for future clinical use. Searching for optimal techniques for reproductive cell and tissue cryopreservation is an exciting endeavour in current reproductive science and technology. Although, successful gamete and gonadal tissue restoration could have huge impact on the enhancement of fertility preservation, serious ethical implications associated with collection and preservation of human gametes and gonadal tissues have yet to be resolved.

theIn summary, experimental gonadal tissue and cell conservation by cryopreservation can serve a platform for further evaluation of the potential for long-term storage. After discovering the cryoprotective effects of glycerol and DMSO, several other cryoprotective agents have also been proposed and widely used for cryoconservation. Many challenges

are associated with the optimal maintenance of tissue structure and the subsequent functional restoration of cryopreserved samples. Optimal osmotic conditions, cooling and thawing rates and cryoprotectant concentrations are crucial elements that need investigation.

Based on this information, we propose to investigate testis tissue xenografting and to determine the enhanced preservation conditions of immature testis tissue. This may provide a novel insight into the study, manipulation, and restoration of male fertility in different species.

HYPOTHESES AND OBJECTIVES

▸ ***Hypothesis 1:*** Xenografting of testis tissue from donor dogs into recipient mice will result in the establishment and maintenance of spermatogenesis in testis tissue xenografts.

▸ ***Objective 1*:** To investigate testis tissue xenografting from donor dogs into recipient mice.

▸ ***Hypothesis 2:*** Grafted testis tissue from immature and young donors will have superior recovery, growth and development to that of adult donors.

▸ ***Objective 2*:** To examine the effect of donor age on progression of spermatogenesis in dog testis tissue after xenografting into recipient mice.

▸ ***Hypothesis 3:*** Short-term refrigeration or long-term cryopreservation of donor piglet testis tissue prior to xenografting will maintain the developmental potential of testis tissue xenografts.

▸ ***Objective 3:*** To test the effects of short-term refrigeration or long-term cryopreservation of immature porcine testis tissue on its survival and developmental potential.

CHAPTER 3: THE EFFECT OF DONOR AGE ON PROGRESSION OF SPERMATOGENESIS IN CANINE TESTIS TISSUE AFTER XENOGRAFTING INTO IMMUNODEFFICIENT MICE

3.1 Abstract

The purpose of this study was to examine the effect of donor age on progression of spermatogenesis in dog testis tissue after xenografting. Testes of dogs 2.5 to 22 months of age were obtained by surgical castration. Donors were categorized based on developmental pattern of spermatogenesis at the time of grafting. Immature, young, and adult categories corresponding to less than four months, four to six months, and greater than six months of age, respectively were established. Fragments of testis tissue were implanted under the back skin of immunodeficient nude mice. The xenografts were retrieved and analysed at intervals of four, six and eight months after grafting. At four months post-grafting, immature and young groups had higher graft recovery rates ($92 \pm$ 5.8 and 88 ± 4.4% versus 69 ± 3.5%; $P = 0.001$ and $P = 0.001$), graft weights (34 ± 8.1 and 32 ± 11.0 mg versus 7 ± 2.6 mg; $P = 0.001$ and $P = 0.022$), vesicular gland indices ($1.1 \pm$ 0.20 and 0.6 ± 0.18% versus 0.1 ± 0.03%; $P < 0.0001$ and $P = 0.015$), seminiferous tubule numbers (517 ± 114.8 and 364 ± 161.0 versus 10 ± 5.1 ; $P < 0.0001$ and $P = 0.028$), and larger seminiferous tubular diameters (140 ± 17.8 and 130 ± 3.4 µm versus 55 ± 21.9 µm; $P = 0.003$ and $P = 0.001$) compared to adult donor xenografts. Immature donor xenografts maintained the growth, and development for eight months as exhibited by greater graft weights (17 ± 4.6 mg, $P = 0.002$), vesicular gland indices (1.5 ± 0.46%, $P = 0.0005$), seminiferous tubule numbers (547 ± 210.3, $P < 0.01$) and tubular diameters (93 ± 15.9 µm,

P < 0.0001) compared to adult donor xenografts. The growth and development of testis tissue xenografts from immature and young donors were not different after eight months (P > 0.05). Young donor xenografts had greater seminiferous tubule number and diameter compared to adult donor xenografts (P = 0.009 and P = 0.004, respectively) at eight months post-grafting. Elongated spermatids were the most advanced germ cell type present at four and eight months post-grafting in immature and young age groups. This study showed that immature and young donors (less than six months of age) were the most promising donors for testis xenografting, offering a potential alternative for male germ line preservation for canids that die prematurely or must be castrated before maturation.

3.2 Introduction

Reversible suspension of testis cells and tissue growth and development has been tested using different approaches [102]. The purpose of these studies was to propagate and to manipulate gonadal tissue for fertility restoration. Ectopic grafting of testis tissue into recipient mice has allowed for the maturation and the establishment of spermatogenesis in grafts of a variety of species including mouse, hamster, rabbit, bull, horse, pig, goat, cat, monkey, and sheep [6-13]. The results from testis tissue xenografting in different studies also varied depending on the donor species and age, but generally have led to the induction of spermatogenesis with dynamics similar to those of the donor species. Recovered sperm from the grafts have been shown to be fertilization-competent [6, 8, 12].

Testis tissue xenografting could be of great benefit, especially from immature donors where other techniques such as cryopreservation of ejaculated sperm to preserve an individual's fertility are not applicable [59]. Many characteristics of spermatogenesis and steroidogenesis can also be examined using this *in vivo* model, since all the compartments of testis tissue remain intact.

Despite the range of species that have been used as donors in testis tissue xenografting, there are no reports from canine donors. Dogs kept as pets, are usually castrated at about six months of age; however, some dog owners may later regret the decision, wishing they could produce offspring from their favorite dog. For this reason, commercial cloning of pets has become a popular viable option, although it is costly and technically demanding.

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Preservation of the testes after castration, however, may offer an alternative since xenografting of preserved tissue could generate viable sperm even from neonatal donors. Furthermore, survival of threatened or endangered species is dependent upon conservation of the existing biodiversity within wild life isolated populations [107]. The movement of genetic materials from wild animals maintained in captivity or protected areas is especially challenging [107]. Testis tissue xenografts from neonatal donor males could offer a previously unavailable option to produce gametes from captive or free animals facing premature death. Xenografting has already offered an alternative method for preservation and re-establishment of prepubertal testis development in felids [10, 49]. For cats, the most practical donor age was found to be prior to the onset of puberty [49]. The same may be true for many other domestic species [30]. However, testis maturation and normal spermatogenesis have not been comprehensively studied using a range of donor ages and particularly not for the canine species.

Among canids, the Ethiopian wolf (*Canis Simensis*) is believed to be the most endangered canine species in Africa with only about 400 surviving [108]. Therefore, investigation of canine testis tissue xenografting could provide a new insight into fertility restoration of domestic and wild life canids.

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3.3 Materials and methods

3.3.1 Study design

Dog testes from donors of different ages were obtained following surgical castration. Fragments of testis tissue were grafted under the back skin of castrated immunodeficient mice. Half of the recipient mice received exogenous gonadotrophins. The mice were sacrificed at four, six or eight months following grafting. Xenografts were retrieved to assess their recovery rate, weight, and histological characteristics. Vesicular gland weights of recipient mice were obtained to indirectly evaluate the testosterone output of xenografts.

3.3.2 Donor animals and testis tissue preparation

Testes of 12 dogs were obtained after routine surgical castration at the Small Animal Surgery Service of the University of Saskatchewan or at a private clinic in Saskatoon, SK. The dogs were of mixed breeds (*Table 3.1*) and were 2.5 (n = 3 littermates), 3.5 (n = 2 littermates), 4.5, 5.5, 7, 8, 10, 13, and 22 months of age (n = 1 donor each) at the time of castration. The testes were immediately transferred into Dulbecco's phosphate buffered saline without calcium or magnesium (DPBS, Cat# 20-031-CV; Mediatech, Herndon, VA). The DPBS was maintained on ice and transferred to the laboratory within 1 hour. After rinsing three times with DPBS supplemented with 1% antibiotic/antimycotic solution (10000 IU penicillin, 10,000 µg/ml streptomycin, 25 µg/ml amphotericin B; Cat# 30-004-CI, Mediatech), the tunica albuginea and overt connective tissues were removed.

The donor testes were cut into small fragments approximately five mg in weight or two mm^3 in volume. Testis fragments were kept in Dulbecco's modified Eagle's medium (DMEM; Cat# 50-003-PB; Mediatech) on ice until grafting. The xenografting procedure took place within two hours after castration. Comparable pieces of testis tissues were fixed in Bouin's solution overnight. They were washed with and stored in 70% ethanol solution. These fragments served as a reference for subsequent histological analysis of development in testis xenografts. The cross sections of seminiferous cord/tubules in each graft section were counted and measured (μm) to characterize the testis tissue xenografts at the time of grafting.

3.3.3 Recipient animals and xenografting procedures

Male immunodeficient nude mice (NCr, nu/nu, Taconic, Germantown, NY), four to six weeks of age at arrival, were used as recipient animals. Mice were housed in groups of four and maintained aseptically under controlled photoperiod conditions (lights-on 6 a.m. through 6 p.m.) with sterile water and mouse chow provided ad libitum. After surgical preparation and anaesthesia with intra-peritoneal injection of ketamine hydrochloride (100 mg/kg; Ketalene, Bimeda-MTC, Cambridge, ON, Canada) and xylazine hydrochloride (10 mg/kg; Vet-A-Mix, Shenandoah, IA), castration was performed. During the surgery, eight transverse linear incisions (about 0.5–1 cm in length) were made on the back skin of each mouse, four on each side. One testis tissue fragment was inserted under the subcutaneous fascia through each incision and the incisions were closed with stainless steel wound clips (Michel Clips 7.5 mm, Miltex, York, PA). Four to 15 mice were grafted per donor age. In

the case of donor littermates that were 2.5 and 3.5 months old, four to six recipient mice were grafted with tissue from each donor animal. Based on previous reports of successful testis tissue xenografting from premature donors, we decided to use a higher number of recipients for immature donors. In total, 53 mice received canine testis tissue xenografts (*Table 3.1*). All animal experimental procedures and subsequent treatments were carried out according to the guidelines of the University of Saskatchewan's Institutional Animal Care and Use Committee.

3.3.4 Gonadotrophin treatment

Half of the recipient mice per donor age were randomly assigned to receive gonadotrophin treatments as a pilot project to determine if exogenous luteinizing hormone (LH) and follicle stimulating hormone (FSH) would affect the growth and development of canine testis tissue xenografts. These mice were administered with 10 IU equine chorionic gonadotrophin (eCG, Folligon, Intervet, Whitby, ON, Canada) and 10 IU human chorionic gonadotrophin subcutaneously (hCG, Chorulon, Intervet). The remaining untreated mice served as control animals. The treatment started immediately after grafting and continued twice a week until the time of sacrifice at four, six or eight months after xenografting (*Table 3.1*).

3.3.5 Analysis of recipient mice

Four months after grafting, two to three mice per donor were randomly selected and sacrificed to evaluate graft recovery rate, weight, and spermatogenesis status. Half of these mice were recipients of gonadotrophin treatment and half were control mice. This procedure was repeated at six months post-grafting for the mice carrying the 2.5 months old donor grafts. The remainder of donor testis tissue xenografts were retrieved at eight months post-grafting to further examine their growth and development (*Table 3.1*). At the time of sacrifice, mice were anaesthetized and sacrificed by cardiac bleeding. Vesicular glands were dissected out and weighed to calculate the vesicular gland index (vesicular gland weight / body weight), as a measure of biological testosterone activity. Grafts were recovered, weighed and fixed overnight in Bouin's solution followed by three washes with 70% ethanol before histological processing. Fixed testis tissues were paraffin embedded, sectioned with a thickness of 7 µm at the largest cross-section and stained with hematoxylin and eosin. For histological analysis of the grafts, all seminiferous tubules in the largest cross-section of each testis graft were counted and measured. Histological assessment of testis tissue xenografts was performed using a microscope equipped for digital photomicrography (Northern Eclipse Image Analysis software, version 7.0, Empix Imaging, Mississauga, ON, Canada).

Each slide was scored at 200X and 400X magnifications according to a previously described screening system [7], to characterize the incidence of developmental events present in each tubule cross section. The slides were anonymously labelled, and the operator blindly analyzed and scored all the tubular cross-sections of each xenograft. The

developmental patterns were scored as: (i) complete tubular degeneration and, or fibrosis and, or Sertoli cell only tubules; (ii) gonocytes and spermatogonia as the only germ cells; (iii) spermatocytes as the most advanced germ cells; (iv) round spermatids as the most advanced germ cells; (v) elongated spermatids as the most advanced germ cells; or (vi) mature sperm present in the lumen of the tubule. Data from different grafts within each mouse were averaged for the mouse and data are presented as the Mean ± SEM values for the group.

3.3.6 Statistical analyses

Donors were categorized by age and based on histological characteristics at the time of grafting. Categories were based on developmental pattern of spermatogenesis at the time of grafting. Immature, young, and adult categories corresponding to less than four months, four to six months and greater than six months of age respectively, were established. The outcomes of interest in this analysis included the mean graft weight, graft recovery rate (%), vesicular gland index (%), number of seminiferous tubules, and seminiferous tubule diameter (µm). The differences in graft outcome were compared across age categories using generalized estimating equations (GEE) to adjust for use of grafts from the same donor on more than one recipient animal (PROC GENMOD, SAS for Windows version 9.1, SAS Institute, Cary, NC). The data were modeled assuming a normal distribution and the model included a repeated statement with subject listed as the donor identification. The residuals were examined for outliers and to assess the model assumptions of normal distribution and equal variance. All differences between age categories were considered

statistically significant where P < 0.05 and the data were expressed as mean ± SEM. Each recipient mouse was considered as an experimental unit. Mean seminiferous tubule diameter prior to xenografting was compared among different donor age groups by one-way analysis of variance. No difference was observed in the growth, development or maturation between the gonadotrophin treated and control mice; therefore, the data from all recipient mice of the same donors were pooled. ***Table 3.1*** summarizes our experimental data at xenografting.

Table 3.1 Experimental data and histological analysis of donor testis tissue at the time of grafting.

Donor breed	Donor age (months)	No. of recipient mice per donor	No. of testis xenografts per recipient mouse	No. of mice in gonadotrophin treatments	Graft period (months)	Seminiferous tubule number per 5 mg tissue before grafting	Seminiferous tubule diameter before grafting (µm)
Boxer X	2.5	4	8	2	4 6	272±23.3	65±0.5[a]
Boxer X	2.5	5	8	2	4 6	269±25.0	61±0.8[a]
Boxer X	2.5	6	8	3	4 6	270±23.3	71±0.8[a]
Miniature Poodle X	3.5	4	8	2	4 8	132±16.0	63±0.8[a]
Miniature Poodle X	3.5	6	8	3	4 8	136±10.0	63±0.7[a]
Chihuahua	4.5	4	8	2	4 8	138±15.5	98±1.3[b]
Rottweiler X	5.5	4	8	2	4 8	150±19.5	140±2.4[c]
Lab. Retriever X	7	4	8	2	4 8	140±21.0	138±1.7[c]
Chihuahua	8	4	8	2	4 8	75±4.0	169±2.5[d]
Shih Tzu X	10	4	8	2	4 8	111±3.0	172±2.6[d]
German shepherd X	13	4	8	2	4 8	114±8.5	186±14.2[d]
Shih Tzu X	22	4	8	2	4 8	168±26.5	156±2.9[e]

Donors, recipients, and testis tissue xenograft properties prior to xenografting. ANOVA was used to compare the seminiferous tubule diameter of testis tissue xenografts. Means with different superscript letters within columns are significantly different ($P < 0.05$). Data are presented as Mean ± SEM.

3.4 Results

3.4.1 Graft recovery and growth

From a total of 53 recipients, grafts were retrieved from 42 mice, while no grafts were detectable in eight mice. Three of the mice died prior to the scheduled time-points and were excluded from the analysis. *Tables 3.2 and 3.3* summarize the data for testis grafts collected at four, six or eight months post-grafting. To simplify analyses and general trends, donors were categorized based on histological characteristics and developmental pattern at the time of grafting into: Immature (less than 4 months old) with spermatogonia as the only type of germ cells present; young (4-6 months old) with round spermatids as the most advanced germ cell type present; adult (more than 6 months old) donors with elongated spermatids as the most advanced germ cell type present.

Graft recovery rate is defined as the relative percentage of detectable grafts collected at the time of sacrifice. At four months post-grafting, immature and young testis xenografts showed a higher recovery rate (detectable grafts) than adult donor grafts (P = 0.001), while no significant difference was detected between immature and young groups graft recovery rate (P = 0.57). Xenografts of 3.5 months age group had the highest numerical recovery rate *(Table 3.2)*. Xenografts of greater than 6 months old donors had a varied graft recovery rate. Graft recovery rates at eight months post-grafting were not different among donor groups (P > 0.05) and generally had more variation *(Table 3.3)*.

Testis tissues xenografts were about five mg at the time of grafting. At four months post-grafting, immature and young donor groups did not differ in graft weight (P = 0.92), though revealed greater graft weight than adult donor xenografts (P = 0.001 and 0.02, respectively; *Table 3.2)*. At eight months post-grafting, xenografts from immature donors had higher weight than those from adult groups (P = 0.002). Young groups did not significantly differ in graft weight from immature and adult donors (P = 0.59 and 0.14, respectively). In general, testis xenografts from adult donors had poor overall growth at both four and eight months post-grafting periods *(Table 3.3)*. The exception to this was the eight months old donor that at four months post-grafting showed both low and high graft weight variations (6 to 27 mg). Testis tissue xenografts of 2.5 months old donor groups which were collected at six months post-grafting were 7 to 62 mg in weight (*Table 3.3)*.

At four months post-grafting, vesicular gland indices (percentages of the vesicular gland weight divided by the body weight) were generally higher in the mice carrying immature and young donor grafts than mice with adult donor xenografts (P < 0.0001 and P = 0.02, respectively). At this time point, however, no significant difference was evident between vesicular gland indices of mice carrying immature testis xenografts and mice with young donor grafts (P = 0.07; *Table 3.2)*. At eight months post-grafting, mice carrying immature donor grafts had considerably higher vesicular gland indices than those in adult age groups (P = 0.0005). Mice with young age donor xenografts displayed no significant difference in the vesicular gland indices with those of immature or adult groups (P = 0.39 and 0.08, respectively; *Table 3.3)*. Exogenous gonadotrophin treatment of the recipient mice did not

significantly alter the recovery and development of xenografts within age groups; therefore, the data were pooled for each age group. Gonadotrophin treatment also did not alter vesicular gland indices of the treatment group, compared to control mice carrying xenografts (P > 0.05).

Table 3.2 Characterization of dog testis tissue xenografts at four months after grafting.

	Donor age (months)	Graft recovery (%)	Graft weight (mg)	Seminiferous tubule # per graft cross section	Graft seminiferous tubule diameter (μm)	Vesicular gland index (%)
Immature	2.5	90 ± 8.2	41 ± 8.9	597 ± 114.9	186 ± 4.6	1.18 ± 0.26
	3.5	97 ± 3.1	36 ± 10.3	546 ± 215.2	124 ± 1.7	0.97 ± 0.29
Young	4.5	94 ± 6.3	18 ± 3.9	591 ± 339.5	125 ± 2.7	0.83 ± 0.45
	5.5	81 ± 6.3	82 ± 22.0	136 ± 64.0	138 ± 3.3	0.3 ± 0.29
Adult	7	63 ± 12.5	N/A	N/A	N/A	0.08 ± 0.05
	8	63 ± 25.0	39 ± 32.9	22 ± 5.5	125 ± 6.0	0.1 ± 0.03
	10	69± 6.3	7 ± 0.2	52 ± 0.0	156 ± 6.9	0.23 ± 0.13
	13	75 ± 0.0	N/A	N/A	N/A	0.04 ± 0.0
	22	81 ± 6.3	6 ± 0.7	28 ± 24.0	111 ± 3.7	0.07 ± 0.04

Donors were categorized based on developmental pattern of spermatogenesis at the time of grafting. Immature, young, and adult categories corresponding to less than 4 months, 4-6 months and greater than 6 months of age respectively were established for statistical analysis. At four months post-grafting, these groups were compared in graft recovery (defined as the number of detectable grafts collected at the time of sacrifice), graft weight, seminiferous tubule number and diameter in the largest cross-sections of xenografts, and vesicular gland indices of the mice carrying the xenografts. Immature and young donors exhibited markedly greater values in all aspects than adult donors ($P < 0.05$). Data are presented as mean ± SEM. N/A = Not applicable, where grafts were detectable, but were either completely degenerated or had negligible graft weight changes, compared to the time of implantation.

Table 3.3 Characterization of dog testis tissue xenografts at six or eight months post-grafting.

	Donor age (months)	Graft recovery (%)	Graft weight (mg)	Seminiferous tubule # per graft cross section	Seminiferous tubule diameter (µm)	Vesicular gland index (%)
Immature	2.5[*]	97 ± 3.8[*]	52 ± 9.0 *	669 ± 242.5*	--	0.96 ± 0.29[*]
Immature	3.5	88 ± 7.2	26 ± 2.4	670 ± 233.6	124 ± 2.2	1.70 ± 0.61
Young	4.5	19 ± 18.8	19 ± 0.0	67 ± 66.5	119 ± 3.7	0.32 ± 0.27
Young	5.5	94 ± 6.3	52 ± 8.1	217 ±1.5	134 ± 3.7	1.45 ± 0.02
Adult	7	56 ± 6.3	12 ± 1.9	100 ± 35.0	117 ± 2.8	0.25 ± 0.05
Adult	8	56 ± 18.8	N/A	N/A	N/A	0.48 ± 0.02
Adult	10	50 ± 12.5	5 ± 1.5	N/A	N/A	0.03 ± 0.01
Adult	13	N/A	3 ± 0.0	N/A	N/A	0.14 ± 0.05
Adult	22	75 ± 12.5	1.5 ± 0.0	20 ± 19.0	144 ± 6.7	0.10 ± 0.06

Donors were categorized based on developmental pattern of spermatogenesis at the time of grafting. Immature, young, and adult categories corresponding to less than 4 months, 4-6 months and greater than 6 months of age respectively were established for statistical analysis. At eight months post-grafting, these groups were compared in graft recovery (defined as the number of detectable grafts collected at the time of sacrifice), graft weight, seminiferous tubule number and diameter in the largest cross-sections of xenografts, and vesicular gland indices of the mice carrying the xenografts. Immature and young donors exhibited markedly greater values in all aspects than adult donors (P < 0.05). Data are presented as mean ± SEM. N/A = Not applicable, where grafts were detectable, but were either completely degenerated or had negligible graft weight changes, compared to the time of implantation. Asterisks indicate mice that were sacrificed at six months after grafting, while other groups were sacrificed at eight months after grafting.

3.4.2 Histological analysis

3.4.2.1 Tubular morphology

At the time of grafting, immature donor testis tissues contained seminiferous cords with Sertoli cells and gonocytes / spermatogonia as the only germ cell types present. Interstitial tissue contained immature Leydig cells *(Fig. 3.1* and *3.4A)*. The tissue from older donors displayed varying degrees of germ cell differentiation. Spermatocytes were mainly the most advanced germ cell types in young donors. Elongated spermatids or sperm were the most advanced germ cell type present in adult donor xenografts *(Fig. 3.1* and *3.4G)*.

At four months post-grafting, seminiferous tubules of immature donors exhibited initiation of spermatogenesis with spermatocytes being the most abundant *(Fig. 3.2)*. Round spermatids were the most advanced germ cells in the 2.5 months old donor groups, while elongated spermatids were the most advanced germ cell type in the 3.5 months old donor group *(Fig. 3.2* and *3.4B)*. Among young donor groups, 4.5 months old donor xenografts revealed re-initiation of development and spermatogenesis up to formation of elongated spermatids. In contrast, 5.5 months old donor xenografts showed more than 60% degenerative tubules and spermatocytes as the most prevalent types of germ cells *(Fig. 3.4E* and *3.2)*. In adult donor xenografts, the majority of seminiferous tubules displayed degenerative changes as a common feature. Degenerative changes with fibrosis and vacuolization of seminiferous tubules were observed in 54 to 100% of the seminiferous

tubules. Round spermatids were identified as the most advanced germ cell types in 3% of seminiferous tubules in 10 months old donor xenografts *(Fig. 3.2* and *3.4H)*.

At six or eight months post-grafting, from 2.5 and 3.5 months old donors, the established spermatogenesis had continued to develop round and elongated spermatids as the most advanced germ cell types *(Fig. 3.3* and *3.4C)*. Tubules from one of the three 2.5 months old donors showed complete degeneration. The grafts from the 4.5 months old donor contained no differentiated germ cells after eight months of grafting (*Fig 3.4F*). On the other hand, grafts from donors older than 5 months contained variable degrees of development and tubular degeneration. The 5.5 and seven months old donor grafts displayed more than 50% spermatocytes, and 2-3% round spermatids as the most advanced germ cells. Moreover, at eight months post-grafting, grafts from adult donors showed complete tubular degeneration with partial recovery of a few seminiferous tubules only in the 22 month old donor group *(Fig. 3.3* and *3.4I)*. *Figure 3.4* shows representative histological appearances of testis tissue xenografts before and after grafting.

3.4.2.2 Tubular number

The average number of cross sections of seminiferous cords or tubules in the widest cross-section of grafts was determined for each age group, pre- and post-grafting. At four months post-grafting, no difference was indicated in number of seminiferous tubule sections between testis grafts of immature and young donor groups (P = 0.44), although these groups contained a greater number of tubule sections than adult donor groups (P < 0.0001 and P = 0.03, respectively).

At eight months post-grafting, testis grafts obtained from immature and young donor dogs had markedly increased numbers of seminiferous tubule cross sections than those of adult donors (P = 0.01). Grafts of 2.5 months old donors were examined at six months post-grafting to verify the continuance of testis xenograft growth and proliferation status between the four and eight months periods. After six months of grafting, the number of seminiferous tubules of 2.5 months old donors were comparable to that of 3.5 months old donors at eight months post-grafting (P = 0.45).

3.4.2.3 Tubular diameter

Seminiferous tubule diameter was also measured to examine the impact of donor age on histological characteristics of the grafts. At the time of grafting, immature donor testes exhibited smaller seminiferous tubule diameters as compared with those of older donors (P < 0.001, *Table 3.1*). There was increased seminiferous tubule diameter as donor age increased (P < 0.001). Testes from adult donors contained the largest seminiferous tubule diameters at the time of grafting (P < 0.001, *Table 3.1*).

At four months post-grafting, immature and young donor grafts exhibited greater seminiferous tubule growth compared to adult donor groups (P = 0.003 and 0.0008, respectively). At four months post-grafting, testis grafts from immature and young groups exhibited seminiferous tubule diameters comparable to those in the intact mature testis (*Table 3.2*). At eight months post-grafting, the recovered immature and young donor xenografts had markedly higher tubular diameters than those of adult donor grafts (P <

58

0.0001 and P = 0.004, respectively). Immature and young donor grafts did not exhibit any

difference in tubular diameter after eight months of grafting (P = 0.91, *Table 3.2 and 3.3)*.
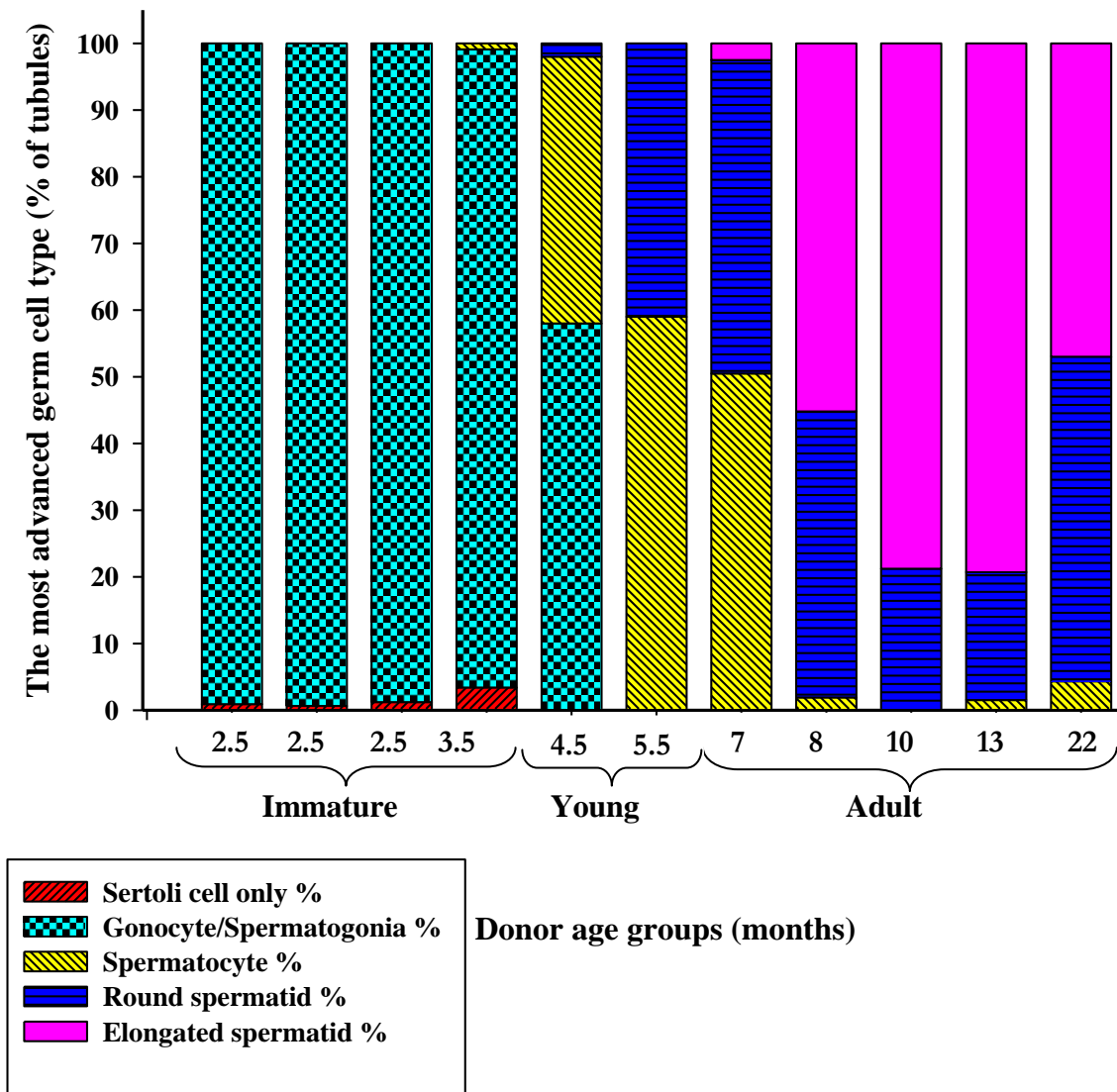
Figure 3.1 The most advanced germ cell types prior to grafting.
Donors were categorized based on developmental pattern of spermatogenesis at the time of grafting. Donor categories correspond to immature, young and adult (< 4, 4-6, and > 6 months old, respectively). Histological appearance of testis tissue xenografts was scored based on the percentages of seminiferous tubule cross sections with Sertoli cell only or with spermatogenesis containing the cells specified as the most advanced germ cell types present.
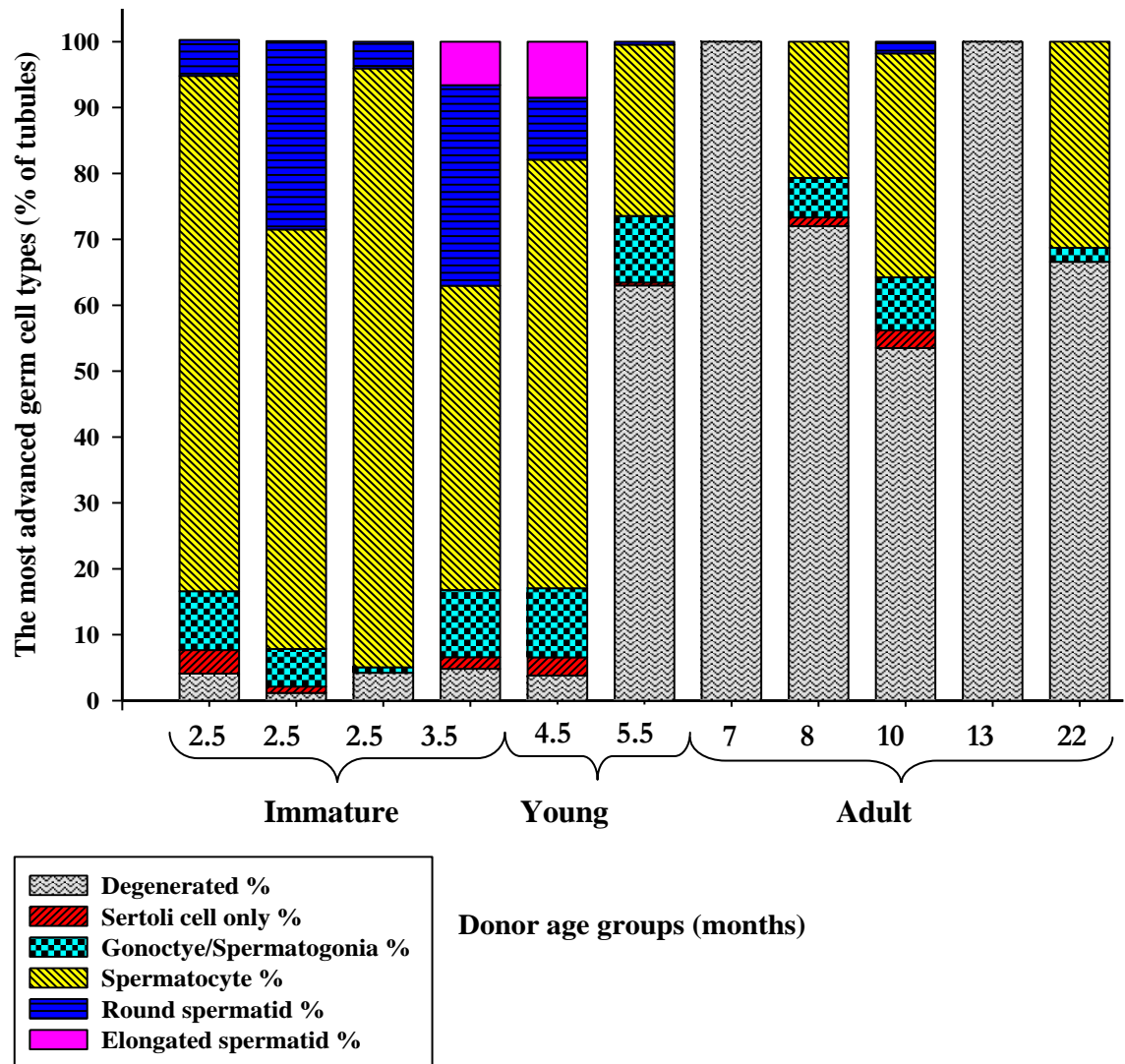
Figure 3.2 The most advanced germ cell types at four months post-grafting.
Donors were categorized based on developmental pattern of spermatogenesis at the time of grafting. Donor categories correspond to immature, young and adult (< 4, 4-6, and > 6 months old, respectively). Histological appearance of testis tissue xenografts was scored based on the percentages of seminiferous tubule cross sections with degeneration, Histological appearance of testis tissue xenografts was scored based on the percentages of seminiferous tubule cross sections with Sertoli cell only or with spermatogenesis containing the cells specified as the most advanced germ cell types present.
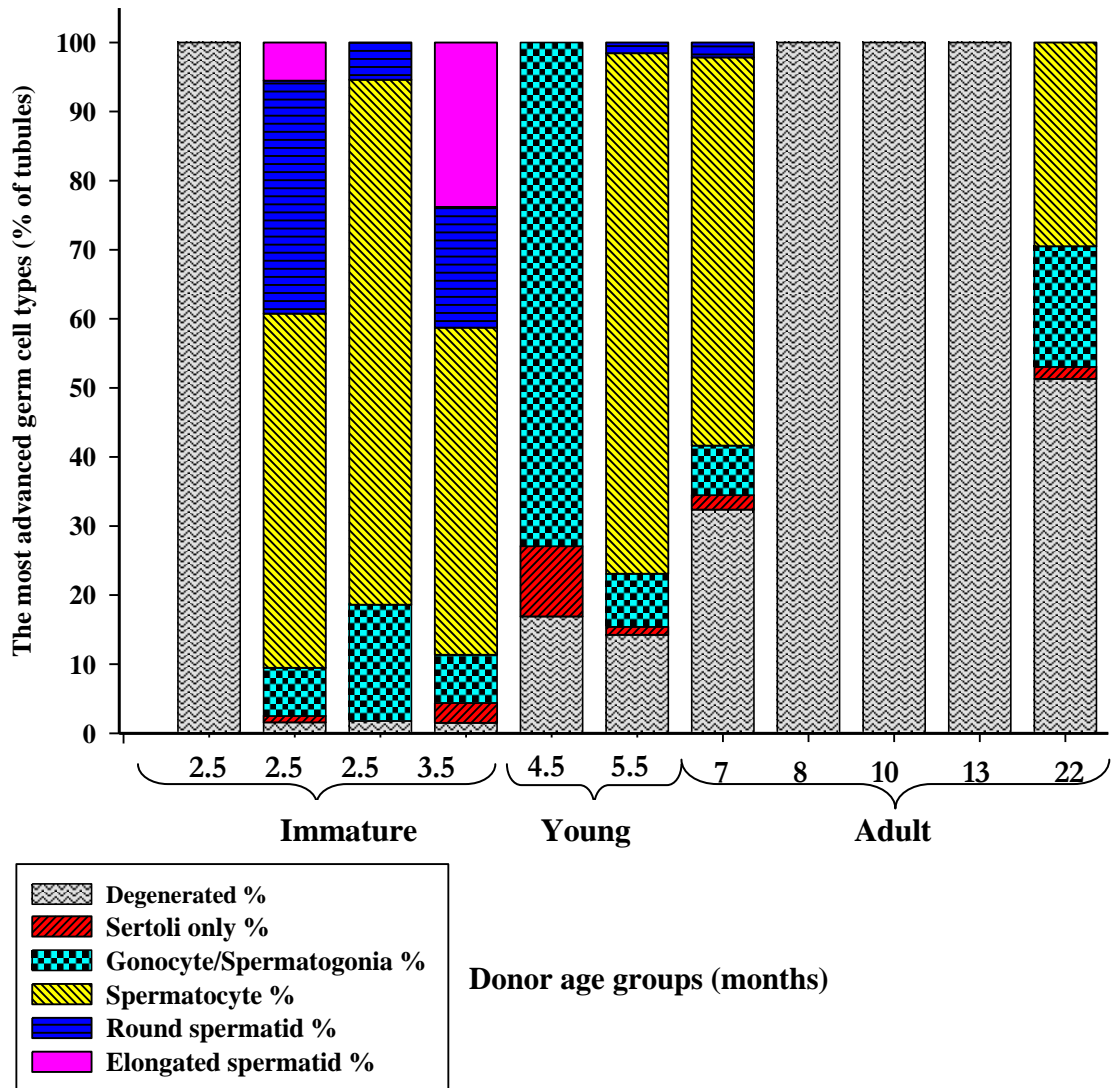
Figure 3.3 The most advanced germ cell types at six or eight months post-grafting.

Donors were categorized based on developmental pattern of spermatogenesis at the time of grafting. Donor categories correspond to immature, young and adult (< 4, 4-6, and > 6 months old, respectively). Histological appearance of testis tissue xenografts was scored based on the percentages of seminiferous tubule cross sections with Sertoli cell only or with spermatogenesis containing the cells specified as the most advanced germ cell types present.
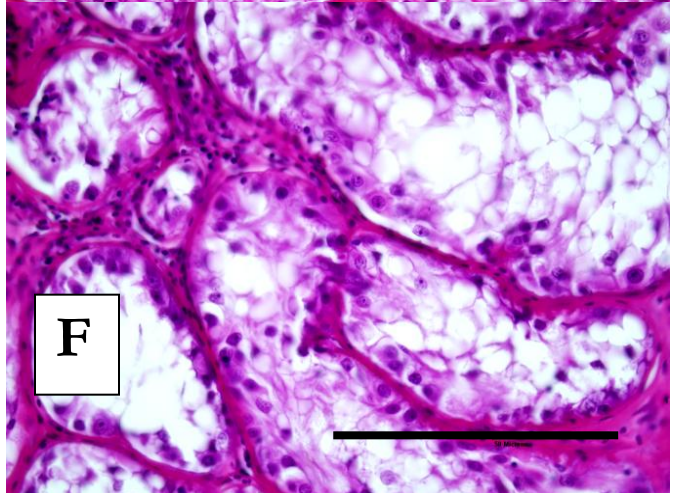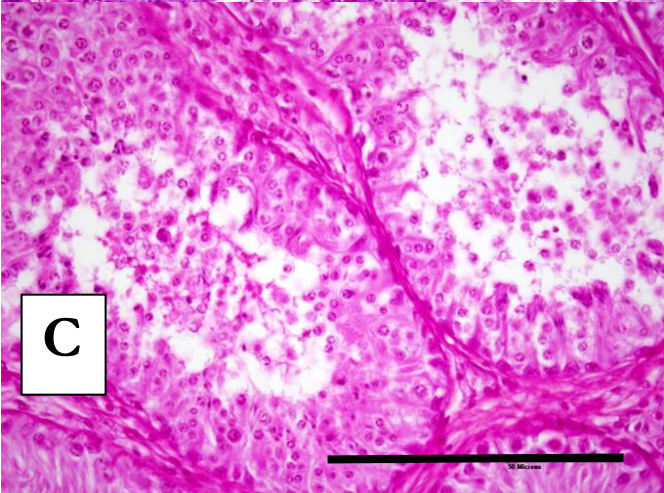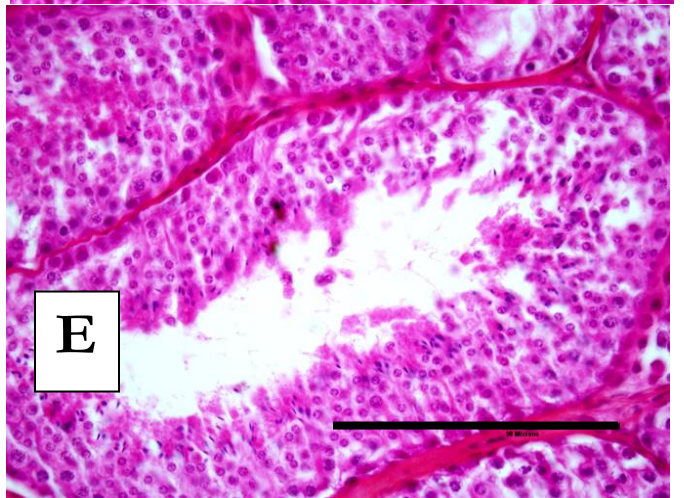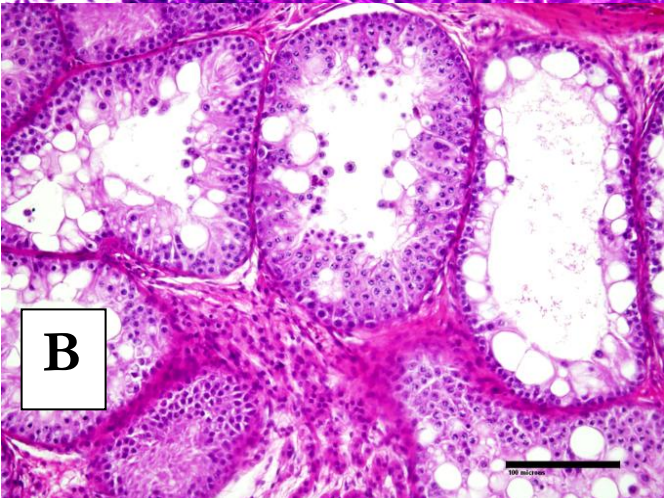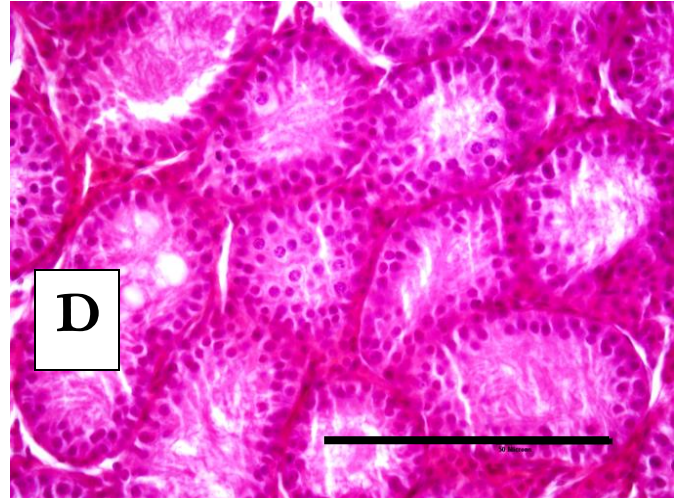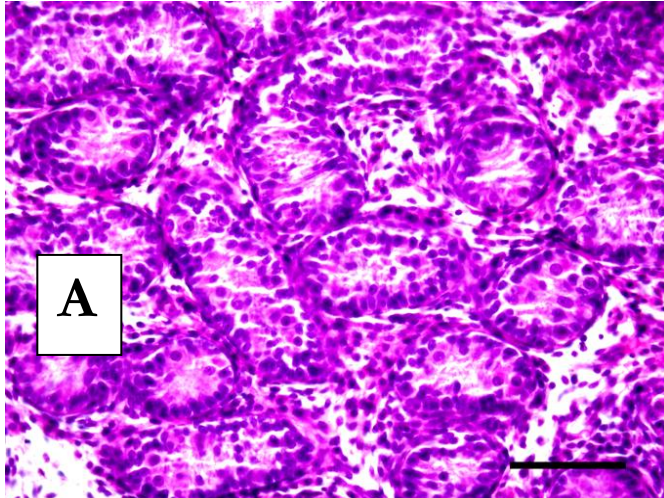
Figure 3.4 Histological appearances of testis tissue xenografts at the time of grafting, and at four, six or eight months post-grafting.

A, B, C: 2.5 months old donor at the time of grafting and at four and six months post-grafting, respectively.

D, E, F: 4.5 months old donor at the time of grafting and at four and eight months post-grafting, respectively.

G, H, I: 8 months old donor at the time of grafting and at four and eight months post-grafting, respectively.

Scale bar = 100 μm at 200X and 400X magnifications.

3.5 Discussion

Testis tissue xenografting has allowed complete spermatogenesis up to the generation of viable sperm from diverse donor species in recipient mice [6, 8, 11-14, 16, 47, 51]. The efficiency of spermatogenesis of testis xenografts has, however, been inconsistent in different donor species. Spermatogenesis in xenografts has been shown to be comparable to intact testis tissue of donor species from pigs and goats [6], while less efficient spermatogenesis was observed in testis tissue xenografts from cats, marmoset monkeys, and bulls [8-10, 15, 51]. The underlying mechanisms of these species-specific differences have not been clearly identified. Successful outcome of testis tissue xenografting and its widespread applicability may also pertain to domestic and wild canids.

The present study provided the first experimental evidence that canine testis tissue can undergo complete maturation after xenografting into immunodeficient recipient mice *(Fig 3.2, 3.3, 3.4B, C)*. Immature canine testis xenografts initiated and maintained complete spermatogenesis after grafting. Most notably, immature dog testis tissues exhibited considerably greater developmental potential than tissues from older donors over a long period of time *(Fig 3.4B, C)*. However, successful testis xenografts exhibited decreased sperm production, compared to the same age intact canine testis tissue *(Fig 3.1, 3.3)*.

Donors were categorized based on developmental pattern of spermatogenesis at the time of grafting. Immature, young, and adult categories corresponding to less than four months, four to six months and greater than six months of age, respectively, were established for statistical analysis. Immature and young dogs were identified as the most promising donor

age groups to achieve high graft recovery rate, growth and development. At four months post-grafting, immature and young donor xenografts demonstrated markedly higher graft recovery rate, graft weight, vesicular gland index, seminiferous tubule number, and seminiferous tubule diameter than those of the adult donors. Immature donor xenografts also exhibited greater graft weight, vesicular gland index, seminiferous tubule number and tubular diameter than those of adult donor xenografts at eight months post-grafting. However, the young donors showed variable graft recovery rate (88 ± 4.4 and 56 ± 26.5 at four and eight months post-grafting, respectively), growth and development, along with less efficient spermatogenesis quantitatively and qualitatively. Lower efficiency of spermatogenesis has been reported likely as a result of initial germ cell loss and subsequent developmental dysfunction in testis tissues after xenografting [15]. Grafts from adult donors, on the other hand, revealed degeneration with only partial maintenance or recovery of spermatogenesis in a few instances. Nevertheless, the partial recoveries of spermatogenesis in some mature testis tissue xenografts and the development of spermatocytes and round spermatids at four and eight months are noteworthy. These findings, which are in agreement with previous studies on testis tissue xenografting of adult hamsters, horses, cats, and humans, suggest that adult dog testis tissue might not be suitable for xenografting [7, 11, 30, 49, 60]. The degenerative tissue response is the prominent feature of adult testis tissue xenografting, which may be associated with lower tolerance of the tissue to periods of ischemia after xenografting compared to younger donors [7].

Immature dog testis tissue grafts showed no differentiation along the seminiferous cords at the time of grafting. This group developed varying degrees of normal spermatogenesis up to the generation of round and elongated spermatids and also maintained the newly established spermatogenesis up to eight months after grafting. Testis tissue xenografts from one of the 2.5 months old donors, however, unexpectedly regressed and no detectable grafts were found six months post-grafting. The higher efficiency of testis tissue xenografts from immature vs. mature dogs is demonstrated by a relatively higher number of seminiferous tubule cross sections per harvested graft with more advanced germ cell types. The immature littermate donors were consistent in recovery, growth and development of round and elongated spermatids at different time-points after grafting. Evidence indicates that the resulting spermatogenesis in immature and young testis xenografts was clearly not equivalent to intact testis of dogs of a similar age. In the immature donors, we expected to observe a higher proportion of seminiferous tubules containing elongated spermatids or sperm after six or eight months. In one case, the prevalence of elongated spermatids in a 2.5 months old donor at four months post-grafting was comparable to intact testis from a seven months old dog, but no further development was observed at six months post-grafting. Unfortunately, the prevalence of elongated spermatids in all other donor grafts after four or eight months was reduced and not comparable to the intact testis tissue of dogs of the same age.

Young dog donor groups were classified by initiation of spermatogenic development and partial differentiation with round spermatids as the most advanced germ cell type at the time of grafting. These groups showed no elongated spermatids at the time of grafting.

Young testis xenografts had remarkable growth, variable graft recovery rate (88 ± 4.4 and 56 ± 26.5 at four and eight months post-grafting, respectively) and less efficient spermatogenic development up to formation of round and elongated spermatids at the four and eight months time frames. A greater graft recovery rate and partial spermatogenic development have also been shown in testis tissue xenografts of young horses, donkeys, and monkeys, when compared to adult testis tissue xenografts with poor recovery and no spermatogenic development [30]. In our study of canine testis tissue xenografting, the variation in testis maturation among young groups may be attributed to different breeds or donor effect. This finding has been supported by previous reports of testis tissue xenografting with other species [11, 15, 109]. Due to these aforementioned studies, variations in donor breed may limit our ability to conclusively characterize spermatogenic development in immature and young donor grafts.

Adult donor grafts exhibited partial recovery (0 - 45%) at four months post-grafting. Most failed to maintain spermatogenesis and underwent degeneration. Our findings are consistent with previous observations that indicate spermatogenesis cannot generally be maintained in testis tissue xenografts from adult donors [7, 30]. Nevertheless, we observed partial recovery and maintenance of spermatogenesis in a few seminiferous tubules in a graft from a 22 months old donor, although spermatogenesis in these tubules only advanced as far as the primary spermatocyte stage. This partial recovery of some seminiferous tubules along with excessive degeneration and spermatogenic regression has also been observed in adult mice and human testis grafts [7, 60]. The chance of partial recovery of seminiferous tubules or maintenance of spermatogenesis in both young and

adult donor xenografts could be related to the re-establishment of the existing spermatogenesis occurring in the intact testes at the time of grafting.

In this study, we often observed variation between grafts in the same recipient, as well as, within-individual grafts. Often there were more differentiated tubules toward the edges of the graft than the center of the grafts. Vascularisation deficiencies, as well as, different rates of Sertoli cell maturation in various seminiferous tubules, and possible germ cell loss after grafting are suggested reasons that may contribute to these variations [11, 15, 109].

Histological characteristics of the donor testis tissue at the time of grafting appear to be a reliable indicator of grafting success. Regardless of the donor species, the more advanced spermatogenesis is at the time of grafting, the greater is the chance of degeneration in the graft. Poor recovery of adult testis grafts has been suggested to be due to more intense spermatogenic activity in adult xenografts at the time of grafting. Possibly, this tissue is more sensitive to hypoxic ischemia before vascularisation [7, 30]. It is not known if this observation is consistent for all species. The degree of efficient vascularisation which is controversial [7, 30, 109] needs to be further defined in grafts of immature and adult testis. The inability of Sertoli cells of sexually mature testes to divide may be offered as another likely reason for developmental breakdown in adult grafted testis tissues [110]. Our current knowledge of testis tissue xenografting does not clearly explain why adult testis grafts develop or degenerate, nor does it explain what determines the partial recovery or development of these grafts over extended periods of time.

Contrary to an earlier study which indicated a positive effect of exogenous gonadotrophin treatments on graft growth and maturation [109], our study did not support those observations. This illustrates that results of testis tissue xenografting from one species can not necessarily be extrapolated to another species. Vesicular gland indices, as indicators for production of bioactive testosterone by Leydig cells of the grafts, were evaluated in all recipients. As anticipated, mice carrying grafts of immature and young donor tissues had higher vesicular gland indices than mice carrying adult donor testis xenografts. This indicates that immature and young donor testis xenografts are more responsive to mouse gonadotrophins.

This study was intended to provide information on the developmental potential of dog testis tissue from a variety of ages and breeds. However, further research using more donors per age group, preferably of the same breed, and a higher number of recipients could be beneficial to reduce variability. The development of improved research techniques such as testis cell-specific gene expression or germ cell-specific markers could improve the ability to track spermatogenic maturation and would be beneficial to attain more consistent and reliable outcomes.

In conclusion, this study demonstrated that xenografting of immature canine testis tissue could offer an excellent practical technique for complete *ex situ* spermatogenesis in the recipient mouse. This is the first report of canine testis tissue xenografting with successful initiation and maintenance of testis tissue growth and development which produced round and elongated spermatids as the most advanced germ cells. Developmental progress in testis tissue xenografts from immature donors proved to be successful, although it was less

efficient than that of the intact testis tissue of the same age dogs. Testis tissue from young dog donors produced variable graft recovery rates, growth, and development of the xenografts over time. Although we did not test the functional competence of dog sperm from xenografts, xenogeneic sperm have been shown to be fertile in studies of other species [6, 8, 12]. Our findings open new possibilities for the preservation of wild life male germ-lines, especially of canine species. Most importantly, dog testis tissue xenografting provides a feasible *in vivo* model for exploration of testis function across all canids.

CHAPTER 4: CRYOPRESERVATION OF IMMATURE PORCINE TESTIS TISSUE TO MAINTAIN ITS DEVELOPMENTAL POTENTIAL AS XENOGRAFTS

4.1 Abstract

The purpose of this study was to develop effective strategies for cooling and cryopreservation of immature porcine testis tissue that maintain its developmental potential. Testes from one week old piglets were subject to one of twelve cooling/cryopreservation protocols: as intact testes, cooling at 4 °C for 24, 48, or 72 hr (*Experiment 1*); or as fragments, programmed slow-freezing with dimethyl sulfoxide (DMSO), glycerol, or ethylene glycol (*Experiment 2*); or solid-surface vitrification using DMSO, glycerol, or ethylene glycol, each using 5, 15, or 30 min cryoprotectant exposure times (*Experiment 3*). For testis tissue xenografting, four immunodeficient recipient mice were assigned to each protocol, and each mouse received eight grafts. The recipient mice were sacrificed four months post-grafting to assess the status of graft development. Morphology and *in vitro* assessment of cell viability showed cooling of testis tissue at 4 °C for up to 72 hr maintained the structural integrity, cell viability, *in vivo* growth, and developmental potential with spermatogenesis up to formation of elongated spermatids and sperm, comparable to fresh tissue (control). In frozen-thawed testis tissues, higher numbers of viable cells were present using a programmed slow freezing protocol with glycerol as compared to DMSO or ethylene glycol (P < 0.001). Among the vitrified groups, the use of DMSO with a 5-min exposure time yielded numerically higher viable cell numbers than other groups. Frozen-thawed tissue fragments recovered after xenografting showed normal spermatogenesis; germ cells advanced to round and

elongated spermatids after programmed slow-freezing using glycerol as well as after vitrification using glycerol with 5 or 15 min exposure times or using DMSO with a 5-min exposure time. In conclusion, these findings suggest that cooling / cryopreservation and xenografting of immature porcine testis tissue offer suitable methods for short-term and long-term preservation of testis tissue, which could be used to develop these new experimental approaches for restoration of male fertility.

4.2 Introduction

Transplantation of dissociated testis cells [20, 111] and grafting of small fragments of testis tissue [6] from a donor into recipient mice are two novel experimental tools with potential therapeutic applications for restoring fertility in males. These systems have allowed the study and manipulation of different aspects of the dynamic process of spermatogenesis, an otherwise seemingly impossible task. However, immediate transplantation or grafting of fresh testis cells or tissues is not always possible or desirable. Therefore, the ability to preserve donor testis tissue for future use is critical. Testis tissue harbours spermatogonial stem cells that exhibit tremendous potential for self-renewal and proliferation. Effective preservation of testis tissue may maintain this potential until the tissue is allowed to resume its development.

Although cryopreservation of isolated testis cells has been successfully achieved for animals and humans [80, 106, 112, 113], so far very little attention has been paid to cryopreservation techniques aimed at maintaining the developmental potential of *structurally intact testis tissue*. Cryopreservation of testis tissue theoretically offers a practical method when other techniques such as cryopreservation of ejaculated sperm [59] are not available or applicable. Preservation of testis tissue has many applications, including salvaging the genetic potential of immature endangered and valuable animals or conservation of fertility for pre-pubertal boys undergoing gonadotoxic cancer therapies.

To our knowledge, no cryopreservation protocol specifically designed for immature testis tissue can offer preservation of functional competence and cellular viability of testis

needed for such applications. Several studies have examined cryopreservation of testis cell suspensions or tissue fragments using glycerol, ethylene glycol, DMSO, or propanediol [114-116], but lack any functional assessment of spermatogenic cells. However, even if many cells of a multicellular system survive freezing and thawing, preservation of all functional compartments of the tissue is not guaranteed [82]. Furthermore, no report has characterized or provided a vitrification technique for immature testis tissue capable of maintaining the potential of the tissue for completion of spermatogenesis. Merely maintaining the physical characteristics of the cryopreserved testis tissue is not adequate; an efficient approach to overcome the deficiencies in developmental (re)establishment of spermatogenesis is also required.

This study was designed to develop and compare a number of strategies for cooling or cryopreservation of porcine testis tissue that both minimize damage to the tissue and maintain its developmental potential. Damage to the tissue was determined by an *in vitro* assessment of cell viability. Development potential was evaluated through a histological examination of testis tissue grafted under the back skin of immunodeficient recipient mice.

4.3 Materials and methods

4.3.1 Study design

Immature porcine testes were subject to one of twelve cooling/cryopreservation protocols: cooled at 4 ºC for 24, 48, or 72 hr as intact testes (*Experiment 1*); or cut into 5 mg testis tissue fragments and cryopreserved using programmed slow-freezing with DMSO,

glycerol, or ethylene glycol (*Experiment 2*); or subjected to solid-surface vitrification with sucrose, ethylene glycol, and either DMSO or glycerol (*Experiment 3*). We also examined the effects of using larger diameters of tissue fragments (15, 20, or 30-mg) on cryopreservation outcome. Cooled and frozen-thawed tissues were assessed for *in vitro* cell viability and developmental potential after xenografting into recipient mice. Half of the recipient mice were treated with exogenous gonadotrophins to examine whether such treatments enhanced the xenografting outcome. Recipient mice were sacrificed at four months post-grafting to assess the efficiency of xenografting and the status of spermatogenesis in the xenografts.

4.3.2 Preparation of donor testis tissue

Donor testes were obtained from a university-affiliated swine facility after routine castration of Yorkshire-cross piglets (< 1 week old). Testes were shipped to the lab within 1 hr of collection in Dulbecco's phosphate buffered saline without calcium and magnesium (DPBS, Cat# 20-031- CV; Mediatech, Herndon, VA) on ice. The testes were then rinsed three times in DPBS and underwent cooling or were decapsulated, with the parenchyma cut into 5-mg fragments and maintained in Dulbecco's modified Eagle's medium (DMEM, Cat# 50-003-PB; Mediatech) on ice for 1-2 hr until use in slow freezing or vitrification procedures. A subset of testis tissues were also cut into larger fragments (15, 20, and 30 mg) in the shape of strips. Comparable tissue fragments were fixed before and after equilibration in cryoprotectants, as well as after freezing- thawing, as reference for histological examination of tissue integrity and development.

4.3.3 Preservation of testis tissue

Experiment 1- Refrigeration: Intact testes (enclosed within the tunica albuginea) were stored in DPBS at 4 ℃ for 24, 48, or 72 hr.

Experiment 2- Programmed slow-freezing: Fragments of testis tissue (5, 15, 20, or 30 mg each) were equilibrated in different freezing media with varying concentrations of cryoprotectants comprising: **1.** DMEM + 5% fetal bovine serum (FBS, Cat# 26400-036, Gibco, Carlsbad, CA) + 10% dimethyl sulfoxide (DMSO, Cat# 210002, BioVeris, Gaithersburg, PA); **2.** DMEM + 5% FBS + 7% glycerol (Cat# G2025, Sigma, St. Louis, MO); **3.** DMEM + 5% FBS + 7% ethylene glycol (Cat# 102466, Sigma) + 0.1 M/L sucrose (Cat# S1888, Sigma). Testis tissue fragments along with 0.45 mL of the freezing medium were packaged into 0.5-mL plastic mini-straws at room temperature (one testis tissue fragment per straw). Straws were sealed and loaded into a programmable freezer (IceCube 14S, Cat# 16821/2000, Minitube, Ingersoll, ON, Canada) and underwent a defined freezing program. Sample and chamber temperatures were monitored during the entire freezing process by inserting a thermocouple in one of the straws and one placed in the chamber. The freezing program was developed and modified based on a previous report on human testis tissue cryopreservation [115]. The process was initiated by maintaining the straws at 22 ℃ for 10 min, cooling to 4 ℃ at -1 ℃/min, holding at 4 ℃ for 5 min, cooling at 0.3 ℃/min from 4 ℃ to -8 ℃, holding at -8 ℃ for 10 min, cooling at 0.5 ℃/min from -8 ℃ to -50 ℃, then 10 ℃/min from -50 ℃ to -90 ℃, and holding for 10 min at -90 ℃. At this point, straws were plunged directly into liquid nitrogen (LN$_2$) and

stored until analysis. The freezing program was similar for the three freezing media as described above, and no seeding was applied in these protocols.

Experiment 3 - Solid-surface vitrification: Groups of 5-mg testis tissue fragments were equilibrated in either DMSO-vitrification solution 1 (DMSO-VS1; DMEM + 7.5% DMSO + 7.5% ethylene glycol; v/v) or glycerol-vitrification solution 1 (glycerol-VS1; DMEM + 3.5% glycerol + 7.5% ethylene glycol; v/v) at 22 ºC for 10 min. To evaluate the effects of exposure time to the vitrification solutions, tissue fragments were then exposed for 5, 15, or 30 min to either DMSO vitrification solution 2 (DMSO–VS2; DMEM + 20% v/v FBS + 15% v/v DMSO + 15% v/v ethylene glycol + 0.5 M sucrose) or glycerol-vitrification solution 2 (glycerol–VS2, DMEM + 20% v/v FBS + 7% v/v glycerol + 15% v/v ethylene glycol + 0.5 M sucrose), respectively *(Fig. 4.1A)*. The tissues were then immediately transferred onto aluminum boats floating on LN_2 using very fine forceps *(Fig. 4.1B)* to minimize the carryover of medium drops around the tissues [117]. After vitrification, three pieces of tissue were transferred into each cooled cryovial (Cat# 5012-0020, Nalgene Nunc, Northbrook, IL) floating on LN_2 *(Fig. 4.1C)*. Cryovials were immediately plunged and stored in LN_2 until evaluation or xenografting.

4.3.4 Thawing process

Cryopreserved testis tissue fragments were thawed using one of the following procedures:

1) Straws containing cryopreserved tissues using programmed slow-freezing were transferred from the LN_2 tanks and immersed into a water bath (37 ºC) until the ice melted

(~11 sec). The sealed end of the straws were cut and the tissues drained into 2 mL of the first thawing solution (DMEM + 20% v/v FBS + 0.5 M sucrose) at 37 ℃ and incubated for 1 min. The tissues were then washed in the second solution (DMEM + 20% v/v FBS) at 37 ℃ for 1-2 min and kept in this medium on ice until immediate examination or xenografting. After thawing, the larger tissue fragments (15, 20, or 30-mg) were cut into 5-mg fragments prior to *in vitro* and *in vivo* analyses.

2) Cryovials containing vitrified tissues were removed from the LN2 tank and kept at room temperature for about 30 sec. The cryovials were then filled with the thawing solution (DMEM + 20% FBS v/v + 0.5 M sucrose) and the tissues transferred into the same thawing solution at 37℃ for 1-2 min. The tissues were then washed in DMEM + 20% v/v FBS at 37 ℃ for 1-2 min and kept in the same solution on ice until further assessment or immediate xenografting.

4.3.5 In vitro assessment of cell viability

Fresh (control) cooled and frozen-thawed testis tissues underwent a sequential enzymatic digestion [6, 118, 119] to investigate the extent to which the preservation techniques affected cell viability. Briefly, we used 0.2% w/v collagenase type IV (Cat# C5138, Sigma) in DMEM at 37 ℃ for 8 to 10 min with occasional agitation, followed by the addition of 0.1% w/v hyaluronidase (Cat# H3884, Sigma) in DMEM for 15-20 min, and 0.01% w/v DNase type I (Cat# DN-25-IG, Sigma) in DMEM for an additional 5-10 min. After centrifugation at 500 *g* and removal of supernatant, the resulting cell pellet was resuspended in 0.25% w/v trypsin with 2.21 mM EDTA (Cat# MT-25-053-CI,

Mediatech), and the trypsin reaction stopped by the addition of an equal volume of FBS (equal to the total volume). To calculate the resultant cell concentration and viability, 50 μL of trypan blue (0.4% solution Cat# T8154, Sigma) was added to 50 μL of the cell suspension, a sample of which was placed on a haemocytometer and observed under a bright-field microscope (at 400X magnifications). Tissue digestions and *in vitro* cell viability assessments were performed in triplicate.

4.3.6 Xenografting of testis tissue fragments into immunodeficient mice

The developmental competence of the fresh (control) and preserved testis tissue fragments was assessed by grafting under the back skin of recipient mice as previously described [6]. All animal experiments were approved by and performed under the guidance of the University of Saskatchewan Animal Care and Use Committee. Male immunodeficient mice (nu/nu NCr nude, Taconic, NY) about 8 week old were anaesthetised using intra-peritoneal injection of a mixture of ketamine hydrochloride (100 mg/kg; Ketalene, Bimeda-MTC, Cambridge, ON, Canada) and xylazine hydrochloride (10 mg/kg; Vet-A-Mix, Shenandoah, IA). Throughout the study, the mice were housed in microisolator cages in groups of four, maintained under controlled photoperiod (lights-on 6 a.m. through 6 p.m.) and handled aseptically with sterile water and mouse chow provided *ad libitum*. After surgical preparations, the mice were castrated and grafted with eight testis tissue fragments (about 5 mg each) subcutaneously on their back skin. The incision sites were closed using wound clips (Michel Clips 7.5 mm, Miltex, York, PA). Groups of mice received fresh (control) and cooled/cryopreserved testis tissue fragments preserved by different protocols (2 grafts / protocol / mouse, 4 mice/group, n = 28) as follows: Group 1:

Fresh and cooled tissues for 24, 48, or 72 hr; Group 2: Fresh and frozen tissues with programmed slow-freezing protocols using DMSO, glycerol, and ethylene glycol, with fragments that were 5 mg each throughout the preparation; Groups 3, 4, 5: Same as the previous group but using fragments that were originally 15, 20, or 30 mg each, respectively; Group 6: Fresh and vitrified tissues using DMSO after 5, 15, or 30 min of cryoprotectant exposure time; Group 7: Fresh and vitrified tissues using glycerol after 5, 15, or 30 min of exposure time.

The effects of exogenous gonadotrophins on development of xenografts were investigated by treating half of the mice in each group with equine chorionic gonadotrophin (eCG, Folligon, Intervet, Whitby, ON, Canada) and human chorionic gonadotrophin (hCG, Chorulon, Intervet) twice a week (s.c., 10 IU each hormone), starting immediately after xenografting and continuing until sacrifice at 4 months post-grafting.

4.3.7 Analysis of xenografts

At 4 months post-grafting, mice were anesthetised and sacrificed after cervical dislocation. The mice were weighed, the back skin dissected open and photographed, and the number of detectable grafts recorded. After dissection from the skin, grafts were weighed and fixed in Bouin's solution for 24 hr followed by rinsing with and storage in 70% ethanol until processing for histological analysis.

Histological slices (7 μm) were prepared from the largest diameter of the grafts and stained with haematoxylin and eosin. Histological analyses were performed using a

microscope equipped for digital photomicrography (Northern Eclipse Image Analysis software, version 7.0, Empix, Mississauga, ON, Canada). Analyses included assessing tissue integrity, counting the number and measuring the diameter of all seminiferous tubules in the slide, and scoring each tubular cross-section according to a previously used scale [7, 14] by characterizing the incidence of the most advanced germ cell types present in each tubule cross section. The operator was blinded to the source of the grafts to be analyzed. Each seminiferous tubule was scored as: (i) complete tubular degeneration and / or fibrosis and / or Sertoli cell only tubules; (ii) gonocytes or spermatogonia as the only type of germ cells; (iii) primary or secondary spermatocytes as the most advanced germ cells; (iv) round spermatids as the most advanced germ cells; (v) elongated spermatids as the most advanced germ cells; or (vi) mature sperm present in the lumen of the tubule.

4.3.8 Statistical analyses

The effects of refrigeration time (Experiment 1), different cryoprotectants in slow-freezing (Experiment 2), as well as different vitrification media and exposure times to these media (Experiment 3) were analyzed using one-way or two-way ANOVA with the Holm-Sidak test as post-ANOVA analysis. Data without a normal distribution were assessed using the non-parametric Kruskal-Wallis ANOVA on ranks with Dunn's test. Spearman's correlation coefficient was determined between the weight of the recovered grafts and seminiferous tubule numbers or diameters. Statistical analyses were performed using SigmaStat (version 3.5, Systat, Chicago, IL) and $P < 0.05$ was used as the level of statistical significance. Average values were expressed as mean $\pm$ SEM. One mouse died prior to the time of analysis and was excluded from the data.

4.4 Results

4.4.1 In vitro assessment of cell viability

The effects of cooling and cryopreservation on cell viability, growth, and developmental potential are summarized in Tables 4.1 and 4.2.

Experiment 1: Intact (uncut) testes cooled at 4 ºC for 24, 48, or 72 hr retained very high cell viability after storage, comparable to fresh control tissue, with no significant reduction in the number of viable cells during the length of cooling (P = 0.74; *Table 4.1*).

Experiment 2: Cell viability (%) was not affected by the original size of the testis tissue fragment (5, 15, 20, and 30 mg) undergoing the same cryopreservation treatment (P = 0.32), and therefore the data were pooled for all further analyses. Cell viability of testis tissues cryopreserved via programmed slow-freezing was higher (P < 0.001) with glycerol-based freezing medium (88 ± 3.3%) compared to DMSO-based (75 ± 7.8%) and ethylene glycol-based (55 ± 11.5%) freezing media. In addition, significantly more cells (by about 5-fold) were isolated from the programmed slow frozen tissues using glycerol-based medium (P < 0.001) than from DMSO- or ethylene glycol- based media (*Table 4.2*).

Experiment 3: The vitrified tissue showed no significant effect of cryoprotectant exposure times (5, 15, or 30 min) for either of the cryoprotectants (P = 0.22). The interaction of equilibration time and cryoprotectant did not affect the number or percentage of viable cells in testis tissues (P = 0.27). However, a numerically higher cell viability (%) and

number was evident in vitrified tissues equilibrated in DMSO for 5 min as compared to the other treatments.

4.4.2 Assessment of testis tissue xenografting

Histological analysis was performed at four time points: on fresh tissue before any manipulations; after incubation in different cryoprotective agents; and after freezing-thawing procedures but prior to grafting or viability assessment; and at four months after xenografting into mice. The structural integrity of all cooled and cryopreserved tissues appeared comparable to fresh testis tissue, and no histological damage was evident under light microscopy after cryoprotective exposure or after freezing-thawing. Exogenous gonadotrophin treatment of the recipient mice did not affect the recovery and development of xenografts (P = 0.90), and therefore the data from treated and non-treated mice were pooled.

Experiment 1: Pre-grafting storage of intact testes at 4 ºC for 24, 48, or 72 hr did not significantly alter the recovered graft weight (P = 0.18) or the number of seminiferous tubules (P = 0.49), as compared to fresh (control) tissues. However, the diameter of seminiferous tubules significantly differed between the fresh and cooled tissue xenografts (P < 0.001; *Table 4.1*). No significant relationship was evident between the number of seminiferous tubules and the weight of the corresponding grafts (P > 0.05).

Experiment 2: Pre-grafting cryopreservation of testis tissue fragments using programmed slow freezing with DMSO or ethylene glycol resulted in a low graft weight (after 4

months, the grafts were still comparable in weight to the testis tissue fragments at the time of grafting) and a low recovery rate, and consequently grafts contained very few seminiferous tubules (*Table 4.2*). These groups were consequently excluded from further statistical analyses to avoid imbalanced comparisons. Growth of testis tissue grafts was not affected by the original size of the testis tissue fragments (5, 15, 20, and 30 mg; P = 0.32), and therefore these data were pooled.

Programmed slow-freezing using glycerol caused no significant changes in the graft weight (P = 0.19) or seminiferous tubule number (P = 0.76) compared to fresh controls, but seminiferous tubule diameters were smaller than in fresh (control) grafts (P < 0.001; *Table 4.2*).

Experiment 3: Tissues vitrified in glycerol-VS2 using 5- and 15-min exposure time and fresh testis tissue xenografts were not significantly different with respect to graft weight (P = 0.35) or number of seminiferous tubules in the recovered grafts (P = 0.13). However, the 5-min exposure time in glycerol-VS2 provided better protection to testis tissues against freezing damage than 15- or 30-min exposure times, as evidenced by significantly larger seminiferous tubule diameters in recovered grafts (P < 0.001). The seminiferous tubule diameters from the DMSO-VS2 5-min exposure time group were comparable to grafts of fresh tissues (121 ± 1.8 *vs.* 130 ± 5.9 μm, respectively; P = 0.05) and significantly higher than tissues exposed to DMSO-VS2 for 15 min (P = 0.009). No grafts were recovered from tissues exposed to DMSO-VS2 for 30 min (*Table 4.2*). Due to the small number of recovered grafts, the impact of different cryoprotectants on vitrified graft weight and seminiferous tubule number was not considered in further statistical analyses.

4.4.3 Assessment of spermatogenic development after xenografting

Prior to grafting, the donor testis tissue contained seminiferous cords with gonocytes / spermatogonia as the only germ cell type present (**Fig. 4.2A**).

Experiment 1: Regardless of the pre-grafting storage duration at 4 ºC, complete spermatogenesis with elongated spermatids was the most advanced germ cell development observed in testis tissue grafts in all groups at four months post-grafting (**Fig. 4.3**).

Experiment 2: At four months post-grafting, no differences in developmental status of the tissue were observed between testis tissue grafts of originally different sizes (5, 15, 20, and 30 mg, $P > 0.05$), and therefore the data were pooled. Complete differentiation of spermatogenic cells was observed in grafts from the group of testis tissues frozen using programmed slow-freezing with glycerol (**Fig. 4.2F** and **4.4A**). However, few grafts and seminiferous tubules survived the programmed slow-freezing with DMSO or ethylene glycol. Only three out of 32 grafts survived in the groups of testis tissues cryopreserved with DMSO and no graft survived in the ethylene glycol group. These groups were therefore excluded from statistical analyses.

Experiment 3: Histological assessment indicated that vitrification in glycerol VS2 with 5- and 15-min exposure times or DMSO-VS2 with a 5-min exposure time more effectively preserve the developmental potential of testis tissues. Recovered grafts had elongated spermatids as the most advanced germ cell types present, which was comparable to grafts from fresh (control) tissue (**Fig. 4.2C, D, E** and **4.4B**).

4.5 Discussion

Despite its potential importance, little attention has been paid to developing and evaluating workable protocols for slow-freezing and vitrification of immature testis tissue with evidence of functional restoration. We used a number of strategies to preserve immature testis tissues and to evaluate preservation effects on the tissues, including xenografting preserved testis tissue into recipient mice. Testis tissue xenografting into immunodeficient recipient mice has been used as a unique approach to achieve testis tissue maturation and complete spermatogenesis from fresh immature testis tissue from a donor species [6-13]. Grafting of frozen-thawed testis tissues has also been demonstrated [6, 16, 25, 120], albeit with low efficiency and without a critical evaluation of different preservation strategies.

This study provided strong evidence that 24, 48, and even 72 hr storage of intact testes at 4 ℃ does not impair the structural integrity, survival, or proliferative potential of immature porcine testis tissue. This has practical implications as shipment of testes to other labs for cryopreservation or xenografting may take more than a day. Our results generally concur with an earlier study in which non-human primate testis tissues were cooled for 24 hr prior to grafting, then analyzed at 12 weeks post-grafting [120]. However, our results show a far higher graft survival rate and 10-30 fold greater graft weights at 4 months post-grafting. Moreover, we demonstrated that cell viability, graft survival, and developmental competence of tissues cooled for 24, 48, or 72 hr were comparable to fresh (control) testis tissue. Interestingly, we found a higher percentage of seminiferous tubules with more advanced germ cell types in tissues cooled for 48 or 72 hr than in fresh or 24 hr cooled testes. We speculate these results may collectively indicate that the developmental

potential of testis tissue is positively impacted by low but stable metabolism prior to grafting; proper cooling may help the graft acclimate to the hypoxic conditions that probably exist immediately after xenografting and therefore improve subsequent development.

Nine different cryopreservation strategies were compared to evaluate the impact of freezing rate (slow *vs.* ultrafast), cryoprotectants, and length of exposure to cryopreservative agent (CPA) on testis tissue. Slow controlled freezing with glycerol has been in common use for embryo preservation since the 1980's [121]; however, applying the same cryopreservation conditions for tissues is more challenging because the cryopreservation protocol must be modified for all different cell types present in the tissue.

With respect to slow-freezing, cryopreservation of testis tissue with glycerol showed higher (~88%) post-thaw cell survival than with DMSO or ethylene glycol. Cell viability was comparable to cooled or even fresh tissues. This finding is in contrast to a recent report by Milazzo et al. (2008) showing high cell viability (~90%) with programmed slow-freezing of immature mouse testis tissue using DMSO as a cryoprotectant [122]. Keros et al. (2005) have also shown harmful effects of glycerol on testis tissue of human adult patients [115]. They suggest that DMSO was a suitable cryoprotectant for testis tissue, based on morphological assessment, ultra-structural data, and *in vitro* testosterone production after cryopreservation. DMSO has also been found to be a more suitable cryoprotective agent than ethylene glycol for immature mouse, rat, and non-human primate testis tissue [123-125]. DMSO has been effectively employed for cryopreservation of immature mouse testis tissue [7, 16, 126, 127], with no morphological changes to the

testis tissue and with maintenance of functional Leydig cells [115]. Shinohara et al. (2002) reported the birth of mouse offspring from sperm retrieved from cryopreserved pre-pubertal testis tissue with DMSO after transplantation under tunica albuginea of the recipient testes [16]. Species differences could account for differences between our results and these earlier observations as cryopreservation protocols are cell, tissue and organ species-specific. Glycerol has a strong affinity for the cellular phospholipid head groups during freezing [34], buffers salts at low temperatures, binds with metallic ions, dehydrates cells, and reduces ice expansion during water solidification [128, 129]. High cryoprotection of glycerol observed in our study, may have been associated with the slow-freezing protocol we employed. This technique could therefore offer a reliable cryopreservation technique for maintenance of the potential of immature porcine testis tissue for complete maturation and spermatogenesis. We also observed higher graft survival, weight, and seminiferous tubule number with complete spermatogenesis in testis tissue grafts preserved using programmed slow-freezing with glycerol compared to DMSO or ethylene glycol. Damage, if any, was possibly minimized due to the well-defined osmolality control (solution effect) and a limited concentration of glycerol to avoid the toxic effect of glycerol-based solutions [81, 82]. Our programmed slow-freezing protocol using DMSO or ethylene glycol did not offer promising results, which could be attributed to the long exposure time. Due to their lower molecular weights, DMSO and ethylene glycol have higher penetration rates than glycerol, and thus prolonged exposure can cause toxicity leading to the inferior graft recovery rate and growth.

We also developed a vitrification protocol for cryopreservation of immature testis tissue. In vitrification, ice crystal formation is by-passed by using higher concentration of cryoprotectants (5M) and an ultrafast cooling rate. As the cryoprotectants have toxic effects, vitrification of cells or tissues should be conducted with minimum exposure time. In the present study, we focused on the type of cryoprotectant and the most effective exposure times to vitrification solutions. Our graft survival and recovered graft weight data strongly suggest that protocols of 5 or 15 min in glycerol-VS2 or 5 min in DMSO-VS2 are preferred for immature testis tissue. The poor graft survival observed when longer CPA exposure times of 15 and 30 min in DMSO-VS2 were used was likely due to toxic effects of high concentrations of this permeable cryoprotectant on the testis tissue [117]. Moreover, histological examination of the recovered grafts from the groups of tissues vitrified by 5 or 15 min exposure times to glycerol-VS2 showed a high proportion of round spermatids; however, the number of elongated spermatids was 20-fold higher in the 5-min exposure time group. The absence of surviving grafts from the tissues exposed to glycerol-VS2 for 30 min may also be due to the longer exposure time and subsequent glycerol toxicity [117].

Vitrification requires careful handling of the tissue and an appropriate choice of final cryoprotectant exposure [130]. However, this process could provide preferential conditions for freezing with superior results in restoration of immature testis tissue. In our protocols, ultrafast freezing of the tissue aqueous milieu into a non-crystalline glassy phase was achieved by exposing testis tissues to highly viscous solutions of cryoprotectants, and ultrafast cooling via placement on a super-cooled aluminum surface

(-194.9 °C). This strategy maintains all tissue compartments in a vitreous state [117, 131, 132] under desirable aseptic conditions; direct plunging of the tissues into liquid nitrogen, a commonly used procedure, poses a greater risk of contamination. The solid-surface vitrification of testis tissue, as applied in our study, is an easy, safe, and applicable cryopreservation technique for the preservation of tissue structural integrity and developmental potential.

To our knowledge, this is the first report of immature testis tissue vitrification showing maintenance of cell viability and developmental potential to actively (re)establish complete spermatogenesis after xenografting into immunodeficient mice. Interestingly, similar post-thaw cell survival rates were observed for the two suitable cryopreservation protocols: *programmed slow freezing with glycerol* and *vitrification following 5-min exposure time to DMSO-VS2*. Additionally, our results confirm *in vitro* cell viability assessment of cryopreserved tissue could be used as a relatively reliable assay to predict testis tissue potential for development.

Reversible suspension of testis tissue growth, development, and spermatogenesis is a topic of interest for fertility preservation of immature individuals especially when immediate transplantation or xenografting of testis cells or tissues are not applicable. Although the effects of cryoprotectant concentration and cooling rate are not similar in all tissues [133] or species, our findings offer a number of strategies that can be used to effectively cryopreserve immature porcine testis tissue as a model for other species. Restoring the developmental potential of immature porcine testis tissue after programmed slow cryopreservation and solid-surface vitrification may prove useful in clinical and

experimental applications of tissue/gonadal banking. Cryopreservation of testis tissue obtained by biopsy is a possible choice for human adults whose only source of sperm is the testis parenchyma, especially when combined with sperm detection in frozen-thawed cell suspensions of testis tissues for ICSI [134]. But more importantly, cryopreservation of immature testis biopsies can offer a unique alternative for prepubertal boys undergoing gonadotoxic cancer treatments whose only potential source of spermatogenesis, such as spermatogonial stem cells, is at risk.
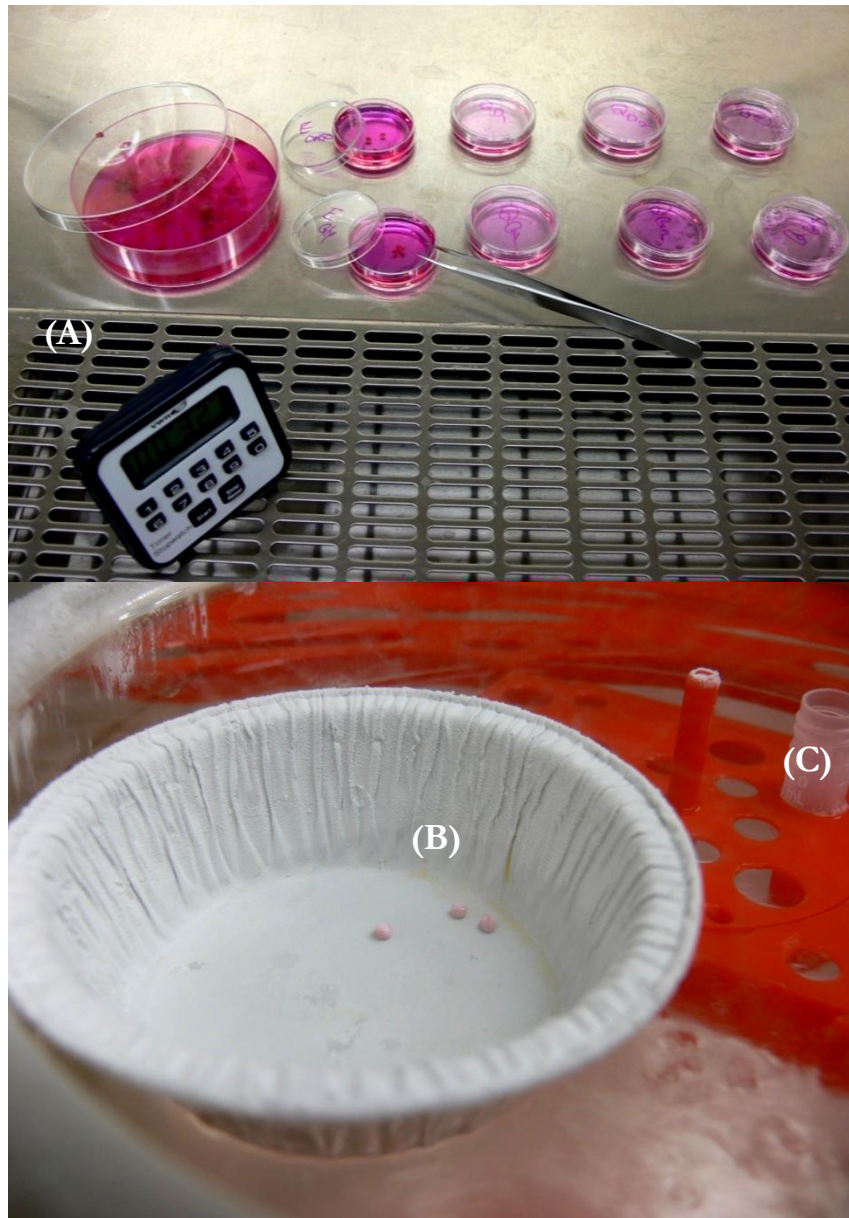
Figure 4.1 Testis tissue vitrification procedures. After exposure of testis tissue fragments to vitrification solutions for different lengths of time (5, 15 or 30 min) (**A**) testis tissue fragments were placed on aluminum boats (**B**) floating on liquid nitrogen, then transferred into cooled cryovials (**C**) followed by plunging into liquid nitrogen.

Table 4.1 *In vitro* viability of testis tissue cooled at 4 °C for 24, 48 or 72 hr before grafting and *in vivo* developmental competence at four months post-grafting.

	Fresh (Control)	24 hr at 4 °C	48 hr at 4 °C	72 hr at 4 °C
No. of viable cells $\times 10^3$ per 5 mg tissue	3868 ± 790^a	3935 ± 236^a	3878 ± 435^a	3077 ± 823^a
Cell viability (%)	92.7 ± 2.2^a	95.4 ± 0.3^a	92.8 ± 1.7^a	86.9 ± 4.3^a
Recovered grafts (%)	100	87.5	87.5	100
Graft weight (mg)	129 ± 58.5^a	109.2 ± 40^a	321 ± 116.7^b	294.5 ± 81.7^b
No. of seminiferous tubule cross sections per graft	377 ± 159^a	582 ± 107^a	951 ± 332^a	890.5 ± 175^a
Seminiferous tubule diameters (µm)	108.7 ± 0.84^a	101.7 ± 0.86^b	104.8 ± 1.01^c	116.3 ± 0.84^d

Number and percentage of viable cells were determined per 5 mg of tissue fragments. Histological attributes were also determined in cooled testis tissue xenografts at 4 months post-grafting by the number of seminiferous tubule cross sections per graft and seminiferous tubule diameter. Graft recovery rate was defined as the percentage of detectable grafts collected at the time of sacrifice. Means with different superscript letters within rows are significantly different ($P < 0.05$). Data are presented as Mean ± SEM.

Table 4.2 *In vitro* viability of cryopreserved testis tissue before grafting and *in vivo* developmental competence at four months post-grafting.

Programmed slow freezing				Vitrification					
	DMSO	Glycerol	Ethylene glycol	DMSO			Glycerol		
				5 min	15 min	30 min	5 min	15 min	30 min
No. viable cell (in 5 mg) $\times 10^3$	453 ± 120^b	$2{,}539\pm363^a$	395 ± 197^b	$1{,}645\pm494^a$	830 ± 149^b	988 ± 212^b	955 ± 256^b	785 ± 112^b	$1{,}044\pm171^b$
Viability(%)	74.8 ± 7.8	88.4 ± 3.3	55.3 ± 11.5	80 ± 9.9	66.2 ± 5.1	60.4 ± 14	77.7 ± 6.3	61.4 ± 7.9	72.2 ± 8.9
Recovered grafts(%)	6.25	40.6	N/A	50	12.5	N/A	66.6	50	16.6
Graft weight (mg)	4.5 ± 1.5	91.1 ± 34.6	N/A	19.5 ± 9.5	30 ± 0	N/A	55.5 ± 30.5	179.5 ± 116.5	40 ± 0
No. of seminiferous tubule cross sections per graft	17 ± 0	363 ± 104.3	3 ± 0	109.5 ± 30.5	43 ± 0	N/A	128 ± 58	495 ± 301	183 ± 0
Seminiferous tubule (µm)[1,2] diameter	70.3 ± 2.8	94.5 ± 1.0	149.5 ± 24.4	120.5 ± 1.8	94.9 ± 4.5	N/A	136.5 ± 3.7	73.3 ± 1.9	86.4 ± 2.6

Number and percentage of viable cells were determined per 5 mg of tissue fragments. Graft recovery was defined by the number and weight of detectable grafts collected at the time of sacrifice. Histological attributes were also defined by number of seminiferous tubule cross sections per largest mid section of recovered graft and tubule diameter at 4 months post-grafting.

Means with different superscript letters within rows are significantly different ($P < 0.05$). Data are presented as Mean ± SEM.
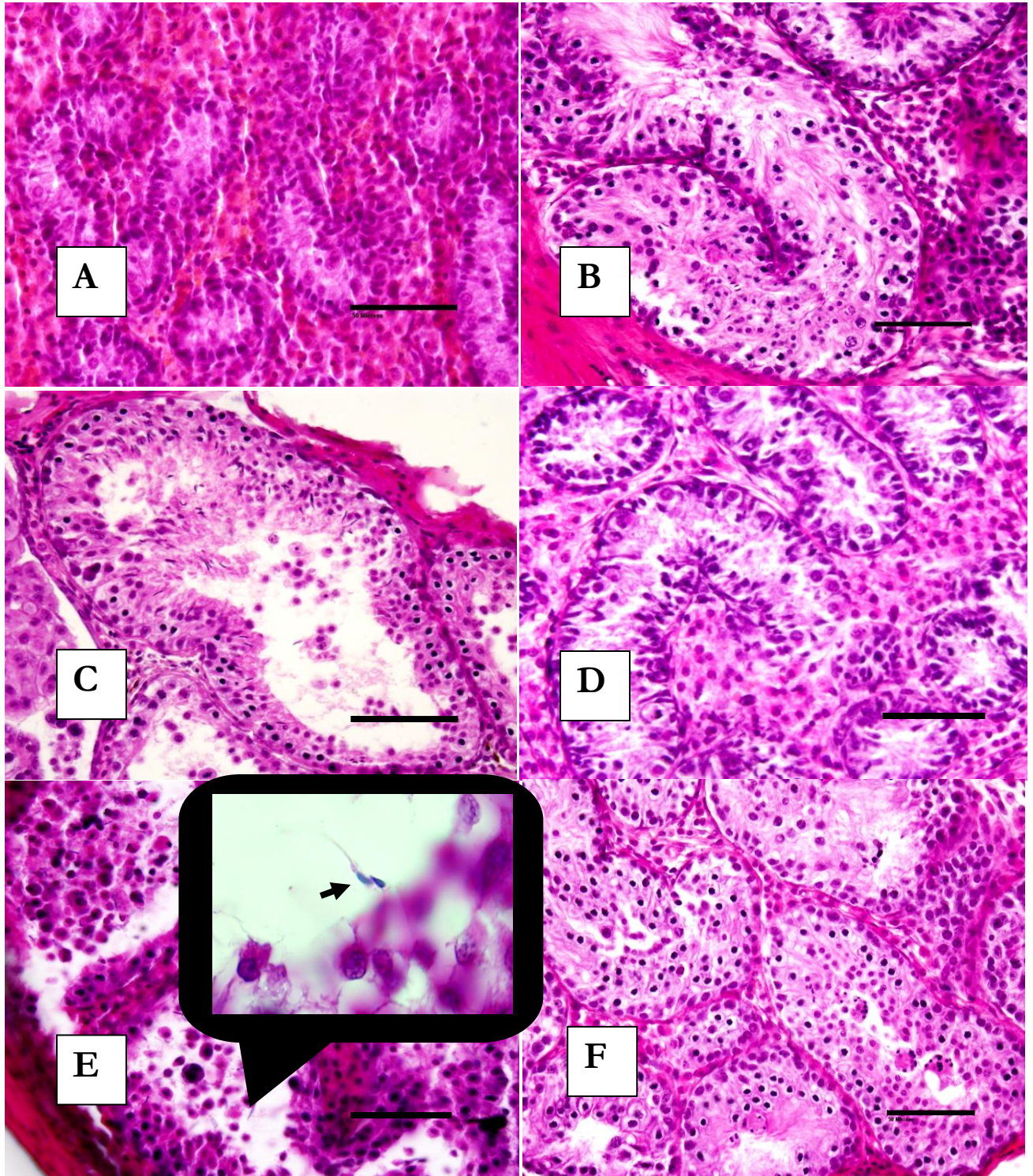
Figure 4.2 Histological appearances of fresh and cryopreserved immature porcine testis tissues before and four month post-grafting into recipient mice. Fresh immature porcine testis tissue at the time of grafting (**A**); and testis tissue xenografts recovered at four months post-grafting from fresh (control) (**B**); vitrified by exposure to glycerol-vitrification solution 2 (VS2) for 5 min (**C**); vitrified by exposure to glycerol-VS2 for 15 min (**D**); vitrified after exposure to DMSO-VS2 for 5 min. The inset shows presence of sperm in lumen of seminiferous tubule (at 1000X magnification) (**E**); or cryopreserved using programmed slow freezing in glycerol (**F**). Scale bar = 50 μm at 400X.
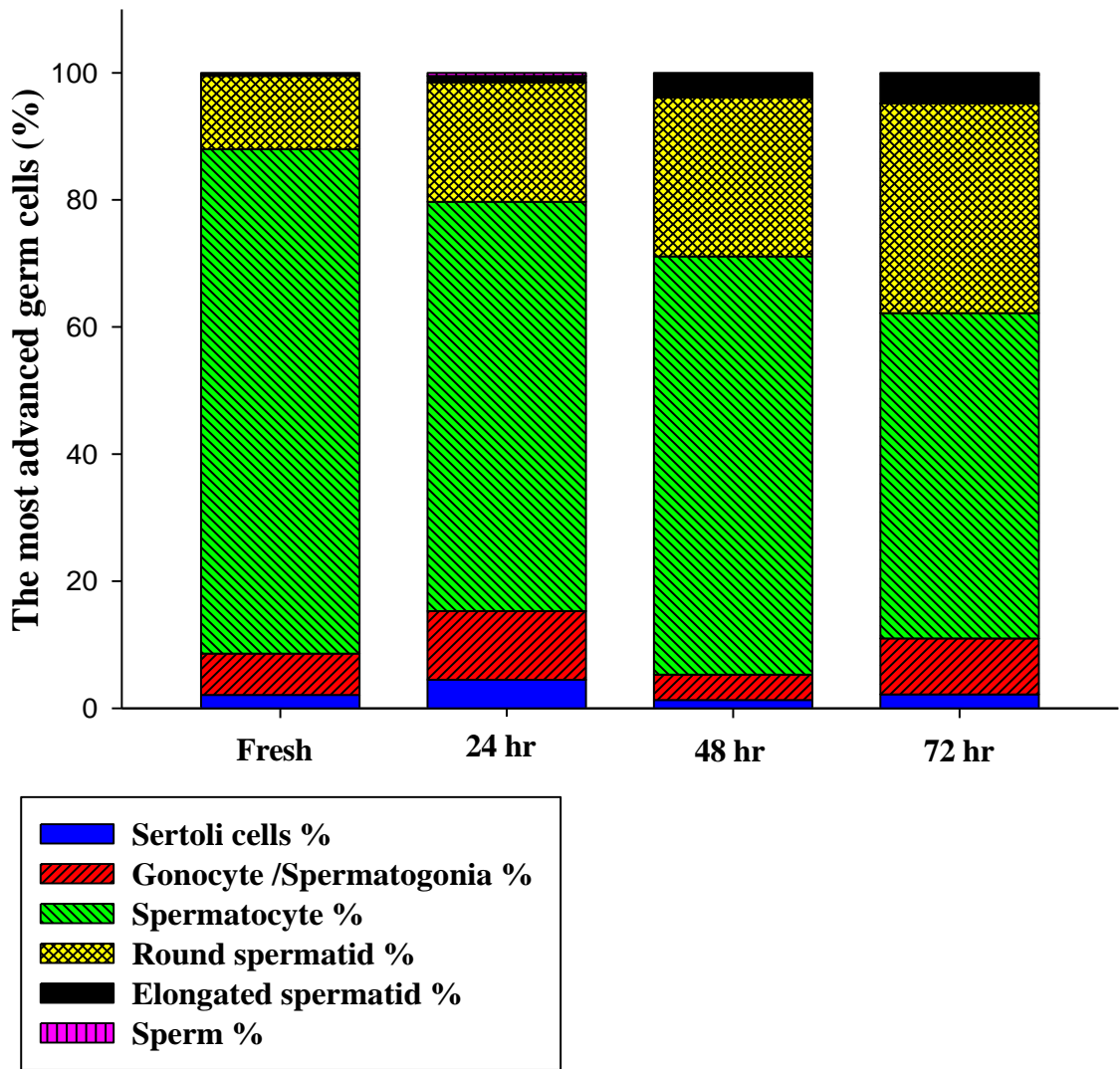
Figure 4.3 The most advanced germ cell types present in the seminiferous tubules of fresh or cooled testis tissue. Relative abundance (%) of the most advanced germ cell types present in the seminiferous tubules of recovered testis tissue grafts at four months post-grafting from fresh (control) and cooled testis tissue grafts (at 4 ℃ for 24, 48 or 72 hr).

(A)

(B)
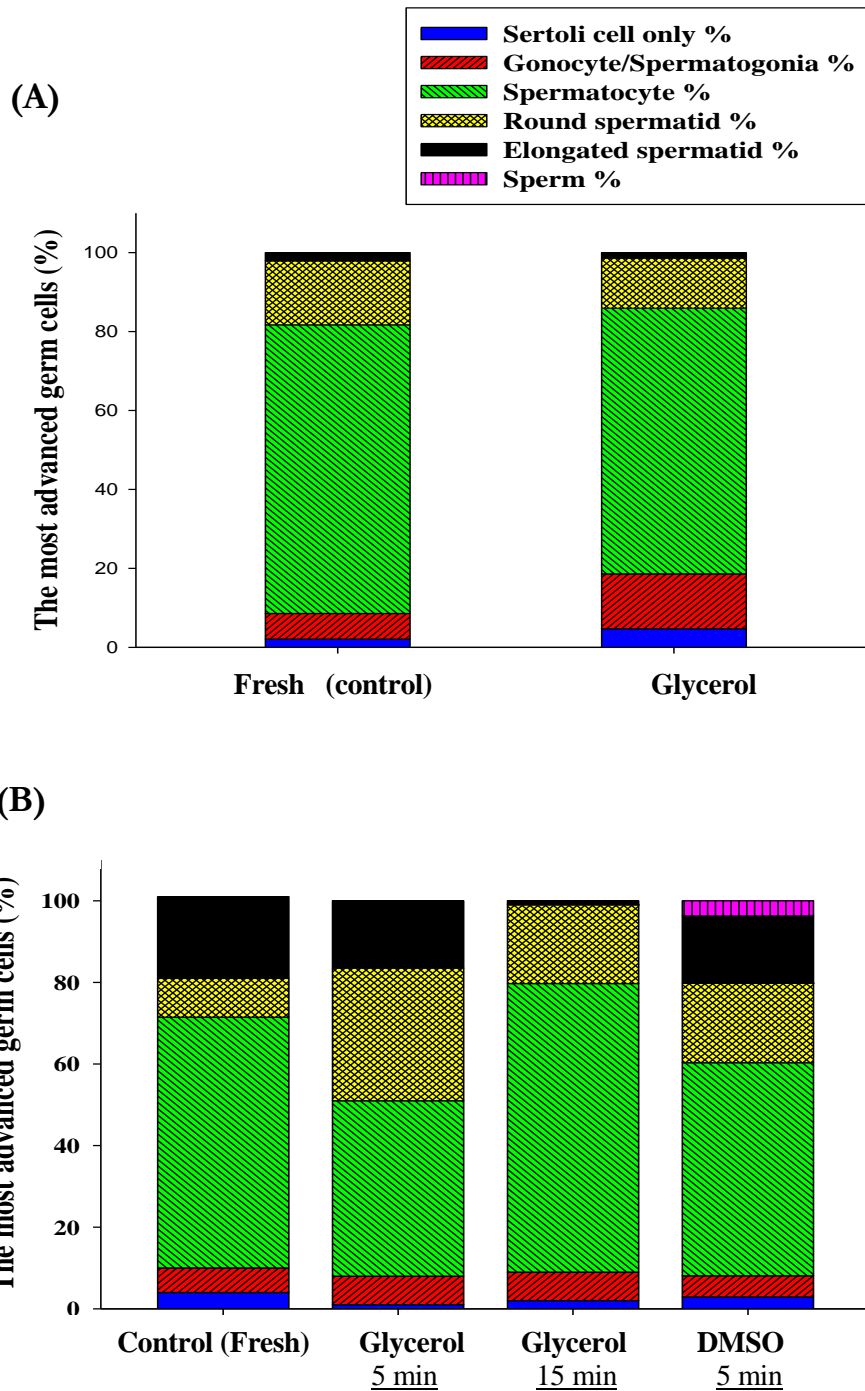
Figure 4.4 The most advanced germ cell types present in the seminiferous tubules of fresh or cryopreserved testis tissue. Relative abundance (%) of the most advanced germ cell types present in the seminiferous tubules of recovered grafts from fresh (control) or cryopreserved testis tissue using programmed slow freezing with glycerol (A), or vitrification in DMSO (5 min exposure time) or glycerol (5 or 15 min exposure time) (B).

CHAPTER 5: GENERAL DISCUSSION AND CONCLUSIONS

The experiments presented in this thesis are pilot studies to advance research on preservation of gonadal tissue. We examined the (re)establishment of spermatogenic activity of donor testis tissue in different donor age groups of a canine model after testis tissue xenografting, and preservation of immature testis tissue using short-term cooling, programmed slow freezing or vitrification procedures in the porcine model.

Despite the wide range of species that have been used as donors for testis tissue xenografting, there were no reports of studies using dogs as the donor animal. Canine species are excellent models for many scientific and practical applications. Development of canine testis tissue xenografting will provide a new insight into the study and manipulation of canid testis function in an accessible mouse model. This technique may be used to investigate the *in vivo* potential of testis tissue in (re)establishment of spermatogenesis in xenografts. It could also be an alternative to selective breeding of dogs to promote genetic diversity in canine population. The selective breeding might produce modern breeds with specific physical characteristics, which has put some purebred breeds in danger by increasing the risk of genetic, physical or behavioural problems. More importantly, testis tissue xenografting may also provide a previously unavailable tool to produce gametes from prepubertal males as an alternative to the more challenging approach of cloning. This may also improve the possibility of preserving the genetic potential of canids that undergo castration before the age of maturity. Preservation of

genetic material is crucial for conservation of rare animal breeds or lines. The loss of genetic diversity associated with infertility or premature death of valuable species is also a major ongoing problem in wild life conservation. Current attempts to address this problem through assisted reproduction involve the collection of sperm or ova from the valuable animals, before or after death. These attempts have several limitations. The most important concern relates to the collection of sperm from males that die before reaching sexual maturity. Testis tissue xenografting is a process to salvage genetic potential from neonate who may never reach maturity. Testis tissue xenografting will also offer a suitable approach to cope with the biodiversity crisis by allowing the movement of genetic material, particularly in wild life canine species which are limited by environmental or geographical barriers. Xenografting is a leading edge technique that provides millions of genetically unique sperm produced in a graft, enabling sperm banks to provide widespread diversity in endangered species. Furthermore, there are several genetic diseases which involve similar genes in both canine and human species. Therefore, successful xenografting of dog testis tissue might open new opportunities to study and address human genetic fertility issues.

We also examined the effect of donor age on progression of spermatogenesis in dog testis tissue by xenografting into recipient mice. In this study, complete testis tissue development with the generation of elongated spermatids was achieved in immature and some young dog testis tissue xenografts. The newly established spermatogenesis was

maintained for four, six and eight months after grafting. Our findings confirm that the developmental stage of testis tissue at the time of grafting could be a critical factor affecting the progressive growth and development of xenografts. Although the reason is not well understood, similar studies in other species also indicate that neonatal and immature testis tissue xenografts have the ability to achieve efficient maturation and complete spermatogenesis in recipient mice. Testis tissue xenografting has also shown successful development of spermatogenesis in several species including immature: mice, hamsters, rabbits, cattle, horses, pigs, goats, cats, monkeys, and sheep in immunodeficient recipient mice [6-15]. Complete spermatogenesis with generation of sperm has been reported in immature donor mice, hamsters, cats, rabbits, pigs, goats, cattle or rhesus monkeys [6-10, 16]. Fertility competence of sperm derived from testis tissue xenografts has been verified by the generation of blastocysts in the pig and monkey and also offspring in the mouse and rabbit using intracytoplasmic sperm injection (ICSI) [8, 12, 16]. It has been speculated that higher survival rates of immature testis tissue stems from its superior ability to tolerate hypoxia and the more rapid establishment of vascularization [12, 28, 103]. Hence, as discussed in Chapter 3, this study shows the successful use of immature testis tissue in xenografting in a new donor species. Immature and young dogs were identified as the most promising donor age groups to achieve high graft survival, growth and development. Four months post-grafting, immature and young donor xenografts had markedly higher graft recovery rate, graft weight, vesicular gland index, seminiferous tubule number, and seminiferous tubule diameter than adult donors.

Immature donors showed higher graft weight, vesicular gland index, seminiferous tubule number and tubular diameter than adult donors eight months after grafting, as well. However, young donors showed variable graft recovery rates, growth and development, along with quantitatively and qualitatively less efficient spermatogenesis *(Fig. 3.2, 3.3, 3.4E, F)*. Lower efficiency of spermatogenesis might be due to initial germ cells loss and subsequent developmental dysfunction in testis tissues after xenografting [15]. Grafts from adult donors revealed degeneration with partial maintenance or recovery of spermatogenesis. Although partial recovery of very few seminiferous tubules is significant, other reports have also demonstrated degenerative changes as the major outcome in xenografting of adult hamsters, horses, cats, and humans [7, 11, 49, 60]. Therefore, mature testis donors might not be well suited for testis tissue xenografting. Indeed, building on this successful study could be helpful to demonstrate fertility competence of graft-originated sperm by generating embryos from these immature testis donors, a task which was beyond the scope of this MSc project. Fertility competence of xenogeneic sperm and production of new progeny are the final goals of this approach in various species. Assessment of fertility competence of dog xenogeneic sperm is the next step in canine testis tissue xenografting. Broad applicability of testis tissue xenografting could offer new experimental and practical methods which could be applied in domestic and nondomestic canids.

It is not always possible, however, to xenograft the testis tissue immediately; hence, we needed to preserve the testis tissue in a protected status to conserve the fertility potential. Salvaging genetic potential through cryopreservation of testis tissue has great clinical significance in preservation and restoration of fertility in animals and humans. This could be highly beneficial where testis tissue is the only source of potential gametes for some immature rare or threatened species. Other approaches such as semen freezing are not applicable to immature testis tissue where sperm production is not yet manifested. Cryopreservation will greatly expand the feasibility of testis tissue xenografting and add to its versatility as a powerful tool in experimental and clinical restoration of fertility.

The porcine model was chosen since it provided consistent and continuous supplies of testis tissue, available through the local university affiliated swine facility. We investigated two cryopreservation conditions for immature porcine testis tissue. Cryopreservation using precisely controlled conditions via automated programmed slow freezing, and by ultrafast-freezing using vitrification methods were studied. Programmed slow freezing with glycerol-based solutions led to high *in vitro* cell survival and restoration of *in vivo* developmental potential. With respect to slow-freezing, cryopreservation of testis tissue with glycerol showed higher (~88%) post-thaw cell survival than with DMSO or ethylene glycol. This cell viability was comparable to cooled or even fresh tissues. This finding is in contrast to a recent report by Milazzo et al. (2008) showing higher cell viability (~90%) with programmed slow-freezing of immature mouse

testis tissue using DMSO as a cryoprotectant [122]. Keros et al. (2005) have also shown detrimental effects of glycerol on testis tissue of human adult patients [115]. However, our finding demonstrated spermatogenesis with generation of elongated spermatids after testis tissue xenografting into immunodeficient recipient mice. Furthermore, testis tissue vitrification was investigated and verified to be an extremely efficient cryopreservation method. We accomplished this task with testis tissue vitrification using DMSO (5-min exposure time) and glycerol (5 and 15 min exposure times). Successful *in vitro* cell survival along with *in vivo* developmental potential up to the production of elongated spermatids illustrated the improved conditions of ultrafast testis tissue cryopreservation. The results of vitrification studies are particularly important as this method is feasible in less elaborate laboratories or even under field conditions. To the best of our knowledge, this is one of the first reports of testis tissue vitrification and xenografting into recipient mice. Vitrified tissues successfully exhibited complete spermatogenesis after xenografting.

It is intuitively known that optimal cryopreservation requires refinement of the freezing and thawing rates, osmotic conditions, choice and concentration of cryoprotectants, and equilibration times in cryoprotective solutions [81, 82, 86, 133]. Indeed, improvement of all aspects of freezing techniques will ensure survival rates of tissue structure and subsequent functional restoration of cryopreserved cells within those tissues. Testis tissue cryopreservation could offer a unique approach in preservation of gonadal germ cells or tissues for valuable experimental animals, as well as, rare or endangered species that die

prematurely. Although ethical and technical issues have yet to be addressed, gonadal cell or tissue cryopreservation might also serve as a novel alternative for preservation of fertility in prepubertal boys undergoing gonadotoxic cancer treatments. Moreover, functional restoration of gonadal cells and tissues via cryopreservation could offer an experimental platform for further evaluation of reproductive cell and tissue potential after long-term storage.

Our studies also revealed that short-term storage of intact testis tissue at 4 °C up to 72 hr is a practical option which would enable the shipment of testis tissue. Short-term storage of tissue at 4 °C maintained the cell viability, *in vivo* growth, and tissue developmental potential with complete spermatogenesis after grafting. These results are comparable to those of fresh tissue xenografts. This finding is in agreement with the previous report on successful xenografting of 48 hr refrigerated testis tissue [6]. Our results generally concur with an earlier study in which non-human primate testis tissues were cooled for 24 hrs prior to grafting, then analyzed at 4 months post-grafting [120]. However, our results show a far higher graft survival rate and a 10-30 fold greater graft weight at four months post-grafting.

Based on these studies, conclusions can be summarized as follows: After xenografting, development of immature donor dog testis tissue and establishment of spermatogenesis can be achieved. Cryopreservation is a feasible method which enhances the relevance of testis tissue xenografting when immediate grafting is not applicable or desired. Automated

slow freezing with glycerol and vitrification of testis tissue are both suitable approaches for successful testis tissue cryopreservation enabling tissue survival and developmental potential. These findings advance the knowledge for the preservation of male fertility using either an automated programmable freezer or a simple vitrification procedure. Moreover, *in vitro* viability assessment of testis tissues after enzymatic digestion could serve as a fast and highly predictive approach for evaluating different cooling/cryopreservation protocols.

Overall, these results are pertinent to wide-ranging applications of tissue *in vitro* preservation and *in vivo* restoration. These techniques could be considered as important steps to develop feasible approaches for gonadal tissue preservation which could be applicable in human and animal clinical settings. Current findings certainly suggest that age-related differences exist and impact on the success of dog testis tissue xenografting. There is also a need for additional studies to determine the fertility competence of xenogeneic sperm or elongated spermatids. Our findings in short-term and long-term preservation of testis tissue are applicable for clinical and experimental purposes.

CHAPTER 6: RELEVANCE AND FUTURE APPLICATIONS

6.1 Relevance of this research

These results could have profound impacts on further studies aimed at both *in vitro* and *in vivo* gonadal tissue preservation in animals. This research also provides applicable protocols for *in vitro* preservation and / or cryopreservation of testis tissue for future applications.

6.1.1   Applications in animal conservation

Many wild life species are protected by laws to save them from extinction. Wild life law enforcement, sustainable economic procedures, environmental education and prevention of illegal wild life trade have all been partially successful in dealing with the crisis in habitat protection and wild life conservation. Research is increasingly recognized as one means to improve the understanding of risk factors and to seek the best solutions for wild life preservation. Gonadal cell and tissue preservation is an important addition to other assisted reproductive technologies to sustain endangered species. Short-term testis tissue maintenance and cryopreservation offer a possible alternative in rescuing genetic resources from, exceedingly rare or endangered male species that die prematurely.

In our studies, we developed the conditions for cryopreservation of testis tissue in order to be able to maintain the structural integrity and developmental potential (Chapter 4).

Future studies could seek to expand these methods of testis tissue cryopreservation to a variety of different species.

6.1.2 Future applications in human infertility issues

Germ cells undergoing rapid self-renewal and differentiation are especially at risk of cytotoxic damage after exposure to oncological therapies [124]. Infertility concerns for certain patients, particularly prepubertal boys undergoing cancer therapies or suffering from genetic defects, are a further reason to continue the study of cryopreservation and xenografting. However, the advances in scientific technology and experimental approaches can not overlook the ethical issues surrounding the risk of disease transmissions and inherent nature of a specific technique and its context.

Testis tissue has the potential to restore fertility with a reduced risk of re-introduction and transmission of oncogenic cells [6, 60]. Grafting of fresh or cryopreserved testis tissue provides a method to study and manipulate primate spermatogenesis and steroidogenesis in a laboratory model, as it is not feasible in humans *in situ* [6].

6.1.3 Experimental applications

Successful establishment of spermatogenesis with generation of elongated spermatids in dog donor testis tissue xenografts in recipient mice further confirms that testis tissue xenografting could serve as a model to study dog testis function in a laboratory model.

Xenografting might also facilitate gamete production from transgenic lines for biomedical research. Additionally, these methods could offer new approaches to preserve the neonatal lethal transgenic, mutant or cloned animals for experimental purposes [6].

6.2 Areas of future research on extra-corporeal preservation of testis tissue

This study proved the feasibility of growth and development of fresh and cryopreserved testis tissue after xenografting into recipient mice. Although there are serious technical and ethical problems involved in the application of these methods, there are other research applications for xenografting.

- The effect of different cytotoxic drugs on human testis tissue must be examined to determine their selective influence on specific spermatogenic cells [120]. This could be achieved using testis tissue xenografts as a model. Particularly, it would be an important tool in exploring approaches for avoiding the irreversible damages of these drugs in cancer patients undergoing gonadotoxic oncological treatments.

- Finding exclusive markers for particular stages of spermatogenic cells could be beneficial in cases where functional evaluation of spermatogenesis is required for future clinical practice.

- Problems such as low success rate and high mortality of cloned offsprings after nuclear transfer can be avoided by grafting [61]. In fact, cloning does not provide genetic

diversity in a way that is provided by millions of genetically unique sperm produced in a graft. Therefore, practical improvements in xenografting of testis tissue from experimental animals can offer the possibility of germ cell development and gametogenesis from resourceless animals such as cloned, neonatally lethal, mutant, transgenic or knock-out animals.

- Study and manipulation of spermatogenesis and steroidogenesis could be facilitated using improved cryopreservation conditions and xenografting techniques offered in our study. Since spermatogenesis is an extremely organized process, even slight changes could be intrusive for the system. However, applying such manipulations in large animals, wild life, and humans is considerably easier in a laboratory environment.

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