THE STUDY AND MANIPULATION OF PIGLET GONOCYTES

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By

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ABSTRACT

The studies in this thesis examined piglet gonocyte identification, isolation, purification, preservation and potential for initiation of spermatogenesis after transplantation into irradiated recipient testes. As a first step, we characterized a previously non-described auto-fluorescence in the piglet testis tissue. This auto-fluorescence mainly originated from granules among the testis interstitial cells, and we found that its interference with immuno-fluorescence can be overcome using Sudan black staining. We also showed that porcine gonocytes can be specifically labelled with the lectin Dolichos biflorus agglutinin (DBA). To optimize gonocyte isolation, we found that ~9-fold more live cells could be harvested by enzymatic digestion of testis tissues than with mechanical methods. However, the proportion of gonocytes (~7%) did not differ between the mechanical and enzymatic methods of testis cell isolation. We then developed a novel three-step strategy for isolation of gonocytes by combining enzymatic digestion and vortexing, resulting in a gonocyte proportion of ~40% (~5-fold more than that from conventional methods). For short-term preservation of testis cells, we found that the survival of testis cells under hypothermic conditions was dependent on the cell type, and affected by storage duration, temperature and medium used. More than 80% of live testis cells survived the 6-day hypothermic preservation period in 20% FBS-L15, without visible changes to the cell culture potential or gonocyte proportion. In another experiment where testis tissues were maintained under hypothermic conditions, we found that ~25% of testis cells could survive for 6 days if preserved in HypoThermosol-FRS solution (HTS-FRS), without morphological changes. To purify gonocytes, we showed that centrifugation of testis cells using 17% Nycodenz can lead to precipitation of gonocytes in pellets (with a purity of > 80%). We also found that pre-coating tissue

culture plates with both fibronectin and poly-D-lysine can result in the negative selection of gonocytes (with a purity of up to 85%). We subsequently showed that further purification of gonocytes (to > 90%) could be achieved by combining the two latter approaches. To prepare recipients for germ cell transplantation, we used local irradiation of piglet testes which reduced testis growth, decreased seminiferous tubule diameters and completely eliminated spermatogenesis at 4 months post-irradiation. Compared with the absence of endogenous spermatogenesis in the control testes, spermatogenesis up to elongating spermatids was observed in the irradiated testes after gonocyte transplantation. In summary, we investigated several critical elements in the study and manipulation of gonocytes in a large animal model.

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DEDICATION

This dissertation is dedicated to my parents Changjiang Yang and Junfang Zhou, and my wife Fang Shi.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
AAV	adeno-associated virus
AMH	anti-müllerian hormone
AP-2γ / TFAP2C	transcription factor activator protein 2
ATP	adenosine triphosphate
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CSF1	colony stimulating factor 1
DAPI	4,6-diamino-2-phenyl indole
DBA	Dolichos biflorus agglutinin
DMEM	Dulbecco's modified Eagle's medium
DPBS	Dulbecco's phosphate buffered saline
dpc	days post coitum
EBs	embryoid bodies
ECMs	extracellular matrices
EG	ethylene glycerol
EGCs	embryonic germ cells
EGF	epidermal growth factor
ESCs	embryonic stem cells
ESGRO	murine leukemia inhibitory factor
FACS	fluorescent activated cell sorting
FBS	fetal bovine serum
FCS	fetal calf serum
GalNAc	N-acetylgalactosamine residues
GCT	germ cell transplantation
GDNF	glial cell line derived neurotrophic factor
GM-CSF	granulocyte macrophage-colony stimulating factor
HTS-FRS	HypoThermosol-FRS solution
IGF-1	insulin-like growth factor1
L15	Leibovitz L15
LIF	leukemia inhibitory factor
MACS	magnetic activated cell separation
MEF	mouse embryonic fibroblasts
MIS	müllerian-inhibiting substance

NSERC	natural sciences and engineering research council
OSM	Oncostatin M
PDGF	platelet-derived growth factor
PFCs	Perfluorocarbons
PGCs	primordial germ cells
PGP9.5/UCHL-1	ubiquitin-C-terminal hydrolase 1
RA	retinoic acid
rAAV	recombinant adeno-associated virus
RG	refrigeration temperature
RT	room temperature
SCF	stem cell factor
SSCs	spermatogonial stem cells
SSEA-1	stage-specific embryonic antigen-1
STO	sim mouse embryo-derived thioguanineand ouabain resistant
TGFß	tumor necrosis factor-beta
TGFα	tumor necrosis factor-alpha

CHAPTER 1 GENERAL INTRODUCTION

Germ cells in the form of gametes (oocytes and spermatozoa) pass the genetic information from one generation into another. Faithful formation of these gametes not only enables the continuation of the species, but also impacts the quality of the individual's life. In mature testes, spermatogenesis constitutes the basis of male fertility and provides virtually unlimited numbers of spermatozoa during the entire adulthood.

Development and establishment of the male germline is a prolonged multi-phase process that spans almost the entire foetal development and the animal's neonatal life until puberty. Primordial germ cells (PGCs) are considered the most primitive germ cells initiating the male germline development (Chiquoine 1954; Ginsburg *et al.* 1990). Following a rapid increment in number by mitosis, PGCs are arrested in G1/G0 phase of the cell cycle in the seminiferous cords and thereafter are referred as gonocytes. Active mitotic divisions of gonocytes usually start before birth and continue in the neonatal seminiferous cords (Coucouvanis *et al.* 1993; de Rooij 1998; Jiang and Short 1998b). Later, gonocytes develop into spermatogonial stem cells (SSCs) which maintain spermatogenesis in mature testes (de Rooij 1998; Jiang and Short 1998b).

Although the existence of stem spermatogonia had been postulated for many decades (Clermont and Leblond 1953; de Rooij 1969; de Rooij and Kramer 1968), solid evidence was only presented in 1994 when germ cells from a fertile individual generated donor-derived full spermatogenesis (demonstration of all stage of spermatogenesis) after transplantation into the seminiferous tubules of an infertile recipient (Brinster and Avarbock 1994; Brinster and Zimmermann 1994). This technique is now commonly referred to as germ cell transplantation (GCT). Ever since the introduction of GCT, this

transplantation system has been applied successfully as a unique bioassay in the investigation of a number of fundamental aspects of spermatogenesis and male fertility. Additionally, GCT has offered new alternative approaches in preservation of male fertility, propagation of genetic potential and production of transgenic animals.

A variety of studies have focused on improving the efficiency of GCT, including work on donor cell preparation, transplantation techniques, and treatment of recipient testes to deplete endogenous spermatogenesis. However, a number of factors remain to be investigated or improved before GCT can be broadly applied, especially in farm animals. These factors include unequivocal identification of SSCs among isolated testis cells, accumulation of sufficient amounts of SSCs with high purity, and enhanced colonization efficiency of transplanted SSCs in recipient testes.

After transplantation, SSCs must migrate from the lumen of the seminiferous tubule, where they are deposited, into the stem cell niches (specialized microenvironments in close proximity to the basement membrane of the tubule) before spermatogenesis can occur. The unavoidable interaction between the donor SSCs and recipient's Sertoli cells is crucial during such "acclimation" process, and while spermatogenesis resulting from the transplanted germ cells is supported by recipient's Sertoli cells, it follows the donor germ cells' characteristics (Clouthier *et al.* 1996; Fran ça *et al.* 1998; Russell and Brinster 1996).

As the direct progenitor of SSCs and the only germ cell type found in neonatal testes, gonocytes can be recognized by their characteristic morphological attributes and topography within the seminiferous cords (McGuinness and Orth 1992b; Orwig *et al.* 2002b). These unique properties may facilitate the search for specific bio-markers for the

identification and characterization of gonocytes. The extended lifespan of gonocytes in large animal neonatal testes provides a prolonged window of opportunity for the study and manipulation of these germline stem cells. Before or soon after birth, gonocytes resume their proliferation and active amoeboid movement (migration) directed to the basement membrane of the seminiferous cords/tubules (McGuinness and Orth 1992b; Nagano *et al.* 2000b; Pelliniemi 1975; Van Vorstenbosch *et al.* 1987). This renewed migration capability has been suggested to promote relocation of gonocytes into the basement membrane (Orth *et al.* 1998; Orwig *et al.* 2002b). Gonocytes can also be cultured where they mimic the *in vivo* behaviour by proliferating, developing colonies, and forming specialized cytoplasmic processes which adhere to Sertoli cells.

Although mouse SSCs can initiate full spermatogenesis after GCT, conflicting reports exist on the ability of rodent gonocytes in initiating spermatogenesis after GCT (McLean *et al.* 2003; Ohbo *et al.* 2003; Shinohara *et al.* 2001). It is unclear whether transplanted gonocytes are indeed competent in producing full spermatogenesis in recipient testes, especially in farm animals.

The overall objective of the present work was to study and manipulate gonocytes from piglets as a farm animal model. We set out to characterize, isolate, purify and preserve porcine gonocytes and test their potency in generating full spermatogenesis after transplantation into recipient testes.

CHAPTER 2 LITERATURE REVIEW AND OBJECTIVES

2.1 Testis Structure and Spermatogenesis

As the primary reproductive organ in the male, the mature testis produces spermatozoa and androgens. Within the scrotum, the testis is covered with multiple layers including the visceral vaginal tunic and the tunica albuginea. Connective tissue projections extend from the tunica albuginea into the testis parenchyma, dividing it into several lobules. Each lobule consists of seminiferous tubules and interstitial tissue. The Leydig cells within the interstitial tissue produce androgens, while spermatozoa develop within the seminiferous tubules. The seminiferous tubules open at both ends into the rete testis, through which the spermatozoa are transported out of the testis and into the epididymis, where they are stored and matured before ejaculation (Almeida *et al.* 2006; Hafez and Hafez 2000; King 1993).

Every second, a boar can generate approximately 100,000 spermatozoa, as a result of spermatogenesis within the seminiferous tubules in a mature testis (Almeida *et al.* 2006; Kemp *et al.* 1988; King 1993). The highly efficient and continuous production of spermatozoa is maintained by spermatogonial stem cells (SSCs). SSCs undergo mitotic divisions to maintain the stem cell pool and to differentiate to advance spermatogenesis. Mammalian spermatogenesis is classified into spermatocytogenesis and spermiogenesis which happen in sequence. Throughout spermatogenesis, Sertoli cells remain in close contact with various types of germ cells. Sertoli cells by forming tight junctions also separate the basal and adluminal compartments, mainly to protect haploid germ cells from the immune system (Almeida *et al.* 2006; Hafez and Hafez 2000; King 1993). Spermatocytogenesis occurs in both basal and adluminal compartments, spanning from

SSCs to spermatids, through different stages of spermatogonia (e.g., types A_1-A_4, intermediate and B), and two rounds of meiosis (primary and secondary spermatocytes). Spermiogenesis takes place entirely in the adluminal compartment where spermatids transform into spermatozoa. Subsequently, the spermatozoa are released from Sertoli cells into the lumen of seminiferous tubules, and transported into the rete testis (Almeida *et al.* 2006; Hafez and Hafez 2000; King 1993).

In pigs, the primary spermatocytes first appear at approximately 10 weeks and spermatozoa at 20 weeks of age in the seminiferous tubules, and in the ejaculate at 22 weeks of age. Generation of spermatozoa from spermatogonia takes 35 days in pigs, and 10.2 days for transportation of spermatozoa through the epididymis. A boar is usually considered sexually mature at approximately 30 weeks of age (Hafez and Hafez 2000).

2.2 Origin and Development of the Male Germline Progenitor Cells

2.2.1 Origin and Development of Primordial Germ Cells

Primordial germ cells (PGCs) are the first traceable germline-directed progenitors for both male and female germ cells. In mice, PGCs become identifiable initially at 7-7.5 days post-coitum (dpc) as a cluster of 50-100 alkaline phosphatase positive cells at the base of the allantois (Chiquoine 1954; Ginsburg *et al.* 1990). Migrating through the allantois (at 8 dpc) and the hindgut, PGCs reach and aggregate in the genital ridge at 9.5-11.5 dpc (Anderson *et al.* 2000). In pigs, PGCs could be initially identified after staining with stage-specific embryonic antigen-1 (SSEA-1), and observed as elongated cells or with distinct pseudopods in the 18 dpc embryos. After proliferation for a few days, PGCs begin to differentiate according to sex of embryos from 26 dpc (Black and Erickson 1968; Pelliniemi 1974; Pelliniemi 1976). In humans, PGCs could be originally

distinguished in the allantois endoderm and mesenchyme of the stalk at the 22nd day of gestation (Falin 1969), and colonize the genital ridge by 4.5 weeks (Francavilla *et al.* 1990; Rabinovici and Jaffe 1990).

On arrival in the genital ridge, PGCs go through several rounds of mitotic divisions in both male and female primordial gonads. However, from 12.5 dpc in mice, PGCs start to behave differently depending on the gender. In the male, PGCs are arranged in shape of rows and enter mitotic arrest until after birth (Hilscher *et al.* 1974; Jost *et al.* 1973; McLaren and Southee 1997), whereas in the female they are arranged in random arrays and enter meiosis by 13.5 dpc (Jost *et al.* 1973; McLaren 2000). Interestingly, the fate of PGCs is not determined by their chromosomal sex, but by the somatic surroundings (Adams and McLaren 2002; McLaren *et al.* 1995). Organ (tissue) culture studies indicate that PGCs will enter meiosis if not grown with embryonic testis tissue, or if the testis architecture is disrupted (Dolci and De Felici 1990; McLaren and Southee 1997; Yao *et al.* 2003). Sertoli cells likely play a critical role in PGCs' differentiation depending on sex (Bowles *et al.* 2006).

2.2.2 Origin and Development of Gonocytes

Gonocytes are a temporary population of germline stem cells after the mitotic arrest of male PGCs and before differentiation into spermatogoinal stem cells (SSCs) (de Rooij 1998; Jiang and Short 1998b). Morphologically, gonocytes can be identified as distinctively large round cells in the center of the seminiferous cords with one or two nucleoli in a prominent nucleus (McGuinness and Orth 1992b). Their existence starts when the foetal seminiferous cords are formed and lasts until days or even years after birth, depending on the species (Hughes and Varley 1980; McLean *et al.* 2003; Olaso and

Habert 2000; Russell *et al.* 1990; Shinohara *et al.* 2001). Before or soon after birth, gonocytes resume proliferation and many migrate to the basement membrane, while others degenerate as a result of apoptosis (Coucouvanis *et al.* 1993). This degeneration process has been observed in virtually all mammalian species, except sheep that lack mitotic arrest (Olaso and Habert 2000). For instance in the mouse, two phases of germ cell apoptosis occur, one at 13 dpc and the other at 10-13 post-partum (dpp) (Wang *et al.* 1998); while in rats this apoptosis happens at 15.5-18.5 dpc and from 2 dpp which peak at 7 dpp, similar to the apoptotic patterns observed in cultured testicular tissue (Boulogne *et al.* 1999). In pigs, gonocyte degeneration was observed even though no precise timing was reported (Black 1971; Gondos 1980; Pelliniemi 1975; Van Straaten and Wensing 1977; Van Vorstenbosch *et al.* 1984). A decrease in germ cell number per testicular transverse section was observed approximately 2 weeks after birth, although total germ cell number increased (Van Straaten and Wensing 1977). Morphological studies showed that gonocytes resume active amoeboid movement (migration) directed to the basement membrane *in vivo* (McGuinness and Orth 1992b; Nagano *et al.* 2000b; Pelliniemi 1975; Van Vorstenbosch *et al.* 1987). The migration of gonocytes to the basal compartment is critical to their survival, cells that continue residing in the cords center will eventually degenerate (Edward C. Roosen-Runge 1968). In rats, gonocytes with pseudopods were able to migrate into the basement compartment and derive full donor spermatogenesis after transplantation, while most round gonocytes underwent apoptosis (Orwig *et al.* 2002b).

2.2.3 Origin and Development of Spermatogonial Stem Cells

While most gonocytes develop into SSCs after arrival in the basement compartment, it has been suggested that a few gonocytes directly transform into differentiating spermatogonia and initiate the first wave of spermatogenesis (Yoshida *et al.* 2006). The transition from gonocytes to SSCs happens shortly after birth with unclear timelines, although it has been estimated to start from ~3 dpp in mice (McLean *et al.* 2003) and 1-2 months in pigs (Goel *et al.* 2007; Hughes and Varley 1980). Continuation of the efficient and highly orchestrated process of spermatogenesis depends on SSCs. It was proposed that two populations of SSCs exist in the mouse testis, the "working" and "potential" SSCs. Under certain conditions such as when the working SSCs are damaged, a non-self-renewing sub-population of SSCs (probably Nanos3-positive) may become active and recover the SSC loss by self-renewal (Nakagawa *et al.* 2007; Suzuki *et al.* 2009). Working SSCs undergo proliferation to renew the stem cell pool, and differentiation to form differentiating spermatogonia which eventually lead to the formation of spermatozoa (de Rooij and Russell 2000). SSCs are small in number (~0.02% to 0.2% of cells in the mouse and rat adult testes (Huckins 1971; Kanatsu-Shinohara *et al.* 2005c; Tegelenbosch and de Rooij 1993)), but have great potential as the only stem cells in an adult body that can contribute genes to the next generation. Thereby, if genetically modified, they can potentially pass the genetic modifications onto progeny after natural breeding (Brinster and Avarbock 1994; Honaramooz *et al.* 2003b; Honaramooz *et al.* 2008) or microinsemination (Goossens *et al.* 2003; Honaramooz *et al.* 2002b; Shinohara *et al.* 2006). Therefore, SSCs could be a unique target for producing transgenic farm animals. SSCs can be genetically modified (Nagano *et al.* 2001a; Nagano *et al.* 2000a), cultured and cryopreserved, and still maintain their ability to initiate

spermatogenesis in recipient testis after transplantation (Avarbock *et al.* 1996; Nagano *et al.* 1998). This provides a new option for the preservation of highly valued or endangered species (individuals) and for propagation of desired male genetics (Fig. 2.1). However, largely due to the rareness of SSCs and the lack of unequivocal identifying bio-markers, practical utilization of SSC potential has been limited (Oatley and Brinster 2008).

2.3 Isolation and Purification of the Male Germline Progenitor Cells

A major step toward improving the study and manipulation of the male germline is to purify or at least enrich these cells. Gonocytes account for approximately 1.4% of cells in the neonatal rat testis or 7% among the piglet seminiferous tubule cells (Honaramooz *et al.* 2005; Orwig *et al.* 2002b). The proportion of SSCs is even lower than gonocytes, comprising an estimated 0.02% to 0.2% of cells in mouse and rat adult testes (Huckins 1971; Kanatsu-Shinohara *et al.* 2005c; Tegelenbosch and de Rooij 1993). It is generally agreed that, after depositing the mixed population of donor testis cells in the lumen of the recipient seminiferous tubules, Sertoli cells recognize and allow SSCs access to the stem cell niche at the basement compartment of the tubule (Chuma *et al.* 2005; Hasthorpe *et al.* 1999; Jiang and Short 1995; Jiang and Short 1998a; Nagano *et al.* 1999; Ohta *et al.* 2000; Shinohara *et al.* 2001; Yuji Takagi 1997). However, the extent of colonization in recipients is directly proportional to the number and availability of donor SSCs (Dobrinski *et al.* 1999b; Jiang 2001; Nagano *et al.* 1999; Shinohara *et al.* 1999; Shinohara *et al.* 2000), emphasizing the importance of target cell selection.

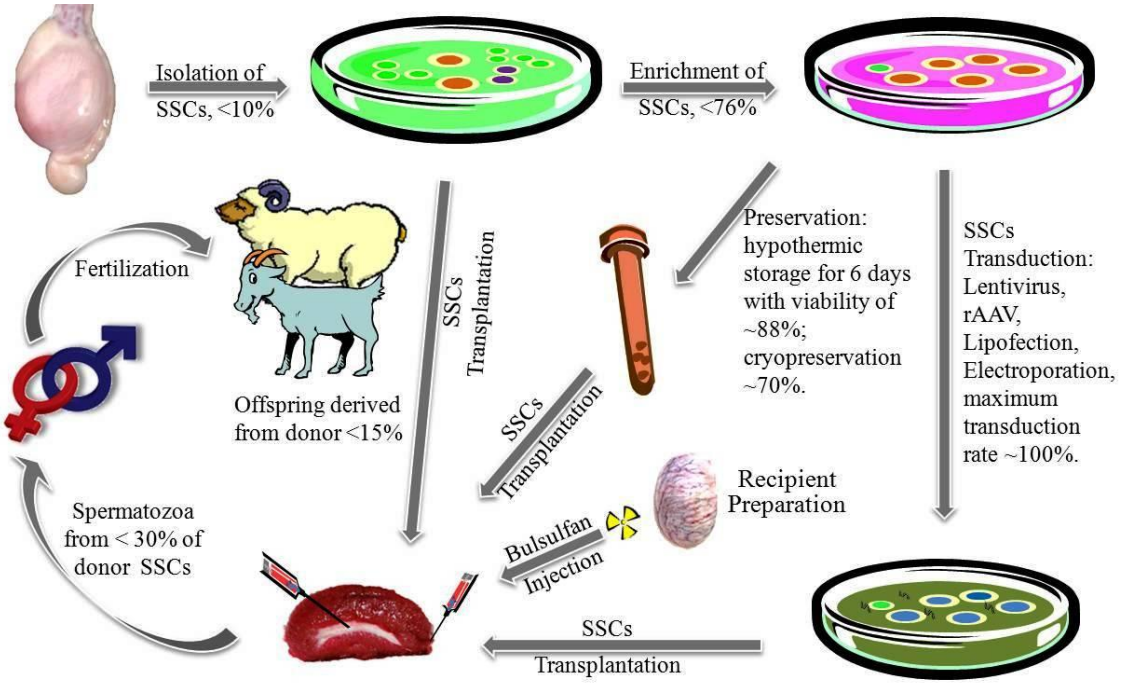


Fig. 2.1. A schematic representation of the procedures involved in germ cell transplantation in farm animals. A single-cell suspension of germ cells is prepared after enzymatic digestion of the donor testis for transplantation into recipient testes. The number of spermatogonial stem cells (SSCs) can be enriched in the donor cell population and the resultant cells can be used fresh or preserved (for short term through hypothermic preservation or long-term by cryopreservation) and/or transfected with genes of interest before transplantation. The recipient animal can be treated with busulfan or undergo local irradiation of the testes to reduce the number of endogenous SSCs, in preparation for germ cell transplantation. Transplanted SSCs can form colonies of donor-derived spermatogenesis and produce spermatozoa to allow the recipient to sire progeny carrying the donor haplotype.

Germline stem cells could potentially arise from PGCs, gonocytes or spermatogonia (Jiang 2001; Jiang and Short 1998b). Isolation of the male germline progenitor cells is the first step in subsequent enrichment, modification or transplantation of these cells (de Rooij and Mizrak 2008; Dobrinski and Travis 2007; Khaira *et al.* 2005; Oatley and Brinster 2008). Therefore, it is important to maximize the proportion and recovery rate of germ cells in the freshly isolated cells, because low efficiency of cell isolation may hamper the subsequent studies and applications.

2.3.1 Isolation and Purification of PGCs

Embryos at different developmental stages may be dissected for the collection of migratory and post-migratory PGCs. The gonadal sex could not be morphologically distinguished before 12 days in mice, 18 days in rabbits, 21 days in pigs, 40 days in cattle, and 7 weeks of gestation in humans (Francavilla *et al.* 1990; Leichthammer *et al.* 1990; Pelliniemi 1974; Swain and Lovell-Badge 1999). Therefore, it might be desired to select male gonads at later stages for specific isolation of male PGCs. To do so, the urogenital complex needs to be dissected from the sexed embryo and removed of its attached mesonephros using fine needles, and the genital ridge disassociated by repeated pipetting, with or without enzymatic digestion. The resultant freshly isolated cells are heterogeneous and include various genital ridge somatic cells and PGCs, depending on the technique used and species. PGCs have been successfully harvested with proportions ranging from 0.5% to 68% among the freshly disassociated genital ridge cells from mouse (Mayanagi *et al.* 2003; Pesce and De Felici 1995), pig (Shim and Anderson 1998), goat and human foetuses (Kühholzer *et al.* 2000; Shamblott *et al.* 1998). The isolated

PGCs were suggested to contain sub-populations with different stem cell potential in mice (Matsui and Tokitake 2009; Morita-Fujimura *et al.* 2009); however, validation is necessary in other species.

2.3.2 Isolation and Purification of Gonocytes

The parenchyma of the neonatal testis is made of seminiferous cords, with gonocytes as the only type of germ cells present, and interstitial tissue (Fran ça *et al.* 2000; Frankenhuis *et al.*1981; Goel *et al.* 2007; Hughes and Varley 1980; Ryu *et al.* 2004). Topologically, gonocytes reside in the center of the seminiferous cords before they are incorporated into the basement of seminiferous cords turning into SSCs. Sertoli cells inter-connect along with the surrounding peritubular myoid cells to support germ cell development and to separate the seminiferous cords/tubules from the interstitial tissue. The interstitial tissue is mainly made of Leydig cells and also contains vasculature and mesenchymal cells. Since the stage and timing of transition from gonocytes to SSCs is not clear, if the goal is to collect gonocytes, it is safer to use testes from animals shortly after birth. Procedures for gonocyte/SSC isolation vary among laboratories depending on the target cell types and the species. However, two-step enzymatic digestion strategies are widely applied to isolate both cell types in many species. The rationale for using a two-step enzymatic strategy is that the first step will largely remove testis interstitial cells using enzymes specific for extracellular matrices (ECMs, e.g., collagenase and hyaluronidase) and the second step is to break down the seminiferous cords/tubules using trypsin with or without other enzymes, including DNase to prevent cellular aggregation (Bellve *et al.* 1977). These approaches usually result in a maximum of 10%

gonocytes/SSCs in the freshly isolated testis cells (de Rooij and Van Pelt 2003; Herrid *et al.* 2009a; Li *et al.* 1997; Lo *et al.* 2005; Luo *et al.* 2006; Orth *et al.* 1997; Orwig *et al.* 2002b; Van Dissel-Emiliani *et al.* 1989). However, the use of donor animals which are cryptorchid, vitamin-A deficient or have certain mutations (e.g., Steel) may provide higher SSC proportions in freshly isolated testis cells, because germ cells from these animal models were reported to be comprised largely of non-differentiating spermatogonia (Ogawa *et al.* 2000; Shinohara and Brinster 2000; van Pelt *et al.* 1996; Van Pelt *et al.* 1995).

2.3.3 Isolation and Purification of SSCs

SSCs could be highly enriched (but rarely purified) with diverse strategies in different species (Khaira *et al.* 2005). These strategies can be divided into those with or without using fluorophore labelled antibodies, and include methods such as fluorescent activated cell sorting (FACS) (Herrid *et al.* 2009a; Izadyar *et al.* 2002b; Kubota *et al.* 2004a; Lo *et al.* 2005; Moudgal *et al.* 1997; Shinohara *et al.* 2000), magnetic activated cell separation (MACS) (Gassei *et al.* 2009; Giuili *et al.* 2002; Herrid *et al.* 2009a; Kubota *et al.* 2004a; Schönfeldt *et al.* 1999), forward/side scatter measurements in FACS (Kubota *et al.* 2003; Lo *et al.* 2005; Shinohara *et al.* 2000), density gradient centrifugation (Dirami *et al.* 1999; Herrid *et al.* 2009a; Izadyar *et al.* 2002b; Luo *et al.* 2006; Marret and Durand 2000; Rodriguez-Sosa *et al.* 2006) and differential plating (Dirami *et al.* 1999; Herrid *et al.* 2009a; Izadyar *et al.* 2002b; Luo *et al.* 2006; Rodriguez-Sosa *et al.* 2006). Using such approaches, SSCs have been enriched to purity levels of as high as 75% in testis cells from farm/large animals (Herrid *et al.* 2009a; Izadyar *et al.* 2002b; Luo *et al.* 2006; Rodriguez-Sosa *et al.* 2006). However, there is concern that the

binding of cellular bio-markers (e.g., antigens/receptors on/in cells) by antibodies may potentially influence the cell behaviour or its fate in response to manipulations (Bashamboo *et al.* 2006; Bendel-Stenzel *et al.* 2000; Gilner *et al.* 2007; Yan *et al.* 2000). While it is possible that PGCs and gonocytes could be collected by manually selecting individual cells based on morphology (Goto *et al.* 1999; Leichthammer *et al.* 1990; Orwig *et al.* 2002b), it is not possible for SSCs because they cannot be morphologically distinguished from other spermatogonia.

2.4 Short-term Preservation of the Male Germline

Advances in the biological studies of the male germline cells necessitate development of proper preservation strategies. Preservation of semen/spermatozoa is now commonly used in many farms and conservation/research facilities (Bagchi *et al.* 2008; Barbas and Mascarenhas 2009). Compared with spermatozoa, conservation of germline progenitor cells has additional benefits such as: retaining of the male germline at much earlier age (from newborn or even foetuses) when spermatozoa do not exist; deriving pluripotent stem germ cells from these progenitor cells; allowing extensive studies on the entire male germline development process; and more efficiency because a single progenitor cell is capable of producing thousands of spermatozoa (Brinster 2002). Depending on the application scenarios, germline cells and gonadal tissues could be stored long-term using cryopreservation or short-term using hypothermic temperatures.

Cryopreservation is to maintain living cells and tissues in media supplemented with cryoprotectants at very low temperatures (e.g., at -196 ℃ in liquid nitrogen) for extended periods of time. This causes cessation of all biological activities while being preserved and resumption of such activities once returned to the body temperatures. Slow

freezing (controlled and non-controlled) and vitrification are two commonly used techniques for cryopreservation of germ cells and tissues (Wyns *et al.* 2010; Brook *et al.* 2001; Geens *et al.* 2008; Wyns *et al.* 2007; Wyns *et al.* 2008). Although cropreservation is a great tool in preservation of germ cells and gonadal tissues, it was not pursued in this thesis.

2.4.1 Hypothermic Preservation of Testis Cells and Tissue

There are situations where short-term storage of testis cells or tissues would be more suitable and necessary for future applications. Such applications include short-term maintenance of cells and tissues during transportation of samples, tissue maintenance prior to transplantation/grafting/cryopreservation (Honaramooz *et al.* 2004; Honaramooz *et al.* 2002b; Yang and Honaramooz 2010; Yang *et al.* 2010a). Hypothermic conditions could be divided into levels of mild (35 to 32 ℃), moderate (32 to 27 ℃), profound (27 to 10 ℃) and ultra-profound (10 to 0 ℃) (Taylor 2000). Hypothermic temperatures cause a decrease in cellular metabolism and oxygen/intracellular energy consumption, thereby prolong cell viability during storage (Belzer and Southard 1988; de Perrot *et al.* 2003; Taylor 2000).

While several investigations attempted hypothermic preservation of testis tissues, no reports of studies on short-term preservation of testis cells is available for any species. Storing testis tissues at 4 ℃ for one or two days in DPBS, DMEM/F12 with HEPE or L15 was reported not to affect primate spermatogenesis after subcutaneous grafting of the tissue fragments into mice (Jahnukainen *et al.* 2007a), or adversely affect the viability of isolated testis cells in the preserved pig testis tissue (Fujihara *et al.* 2008; Zeng *et al.* 2009).

2.5 Culture of the Male Germline Progenitor Cells

The collection of target cells with higher purity usually coincides with considerable cell loss in the process and relatively smaller target cell numbers in the remaining cell population. As a result, up to 90% of germline progenitor (stem) cells could be discarded during tissue/cell handling process such as in cell enrichment/purification operations. Although the desired number of target cells with a satisfactory purity level could be achieved by increasing the initial pool of cells prior to purification, this may not always be possible especially when the donor tissue is in limited supply. On the other hand, manipulation of germline progenitor cells such as transduction and transplantation of SSCs requires large cell numbers. Therefore, it may be necessary to propagate purified germline progenitor cells in culture. Culture of gonadal cells *in vitro* could also provide a controlled system to facilitate the study of germline development.

2.5.1 Maintenance and Propagation of SSCs in Culture

Over the last decade, attempts were made aiming at long-term *in vitro* maintenance and propagation of SSCs in culture from different species, such as mice (Hamra *et al.* 2004; Jeong *et al.* 2003; Kanatsu-Shinohara *et al.* 2011; Kanatsu-Shinohara *et al.* 2005b; Kanatsu-Shinohara *et al.* 2003a; Kanatsu-Shinohara *et al.* 2005c; Kubota *et al.* 2004a; Kubota *et al.* 2004b; Nagano *et al.* 1998; Nagano *et al.* 2003; Ogawa *et al.* 2004; Van Der Wee *et al.* 2001), rats (Hamra *et al.* 2005; Ryu *et al.* 2005; Van Pelt *et al.* 2002), hamster (Kanatsu-Shinohara *et al.* 2008), cattle (Aponte *et al.* 2008; Aponte *et al.* 2006; Izadyar *et al.* 2003a), pigs (Dirami *et al.* 1999; Kuijk *et al.* 2009) and humans (Lim *et al.* 2010; Sadri-Ardekani *et al.* 2009). Establishment of an efficient culture system

involves integrating the optimized culture conditions, mainly basal media, medium supplements, feeder cells, plate coatings, type and concentration of serum or its replacement, combination of growth factors/cytokines, and incubation settings such as temperature.

Once it was believed that germ cells could only survive *in vitro* for several weeks (Kierszenbaum 1994); however, after the availability of GCT as a functional assay for SSCs, it was shown that culture of mixed populations of mouse testis cells could maintain SSCs for at least 4 months (Nagano *et al.* 1998). This conclusion was based on observing full spermatogenesis after allotransplantation of the cultured cells into recipient mice (Nagano *et al.* 1998). The culture conditions in the latter study included a DMEM based medium supplemented with 10% FBS on STO (SIM mouse embryo-derived thioguanine and ouabain resistant) feeder cells at 32^{o}C with 5% CO_2. Highly enriched type-A mouse spermatogonia also survived 25 days of co-culture with Sertoli cells in DMEM with 5% Nu serum (Van Der Wee *et al.* 2001).

Beyond survival, expansion of mouse SSC numbers was then achieved during 3 months of culture in DMEM with 10% FBS on STO feeder cells, and a combination of growth factors which mainly included the platelet-derived growth factor (PDGF) and leukemia inhibitory factor (LIF), along with basic fibroblast growth factor (bFGF), stem cell factor (SCF), murine oncostatin M (OSM) and insulin-like growth factor-1 (IGF-1) (Jeong *et al.* 2003). A systematic study of culture conditions found that the incubating temperature (32 *vs.* 37 ^{o}C) did not affect the maintenance of mouse SSCs, but the composition of feeder cells, culture media and growth factor (especially the glial cell

line-derived neurotrophic factor, GDNF), could influence the SSC self-renewal during the week-long culture (Nagano *et al.* 2003).

Long term propagation of mouse SSCs for 5 months was accomplished in culture of mixed mouse testis cells on mouse embryonic fibroblasts (MEF), with introduction of an expanded basal media components, namely Stempro-34 SFM (formerly used in culture of human hematopoietic stem cells) with various reagents and foetal calf serum (FCS), and a combination of growth factors such as LIF, bFGF, a murine leukemia inhibitory factor (ESGRO), epidermal growth factor (EGF) and GDNF (Kanatsu-Shinohara *et al.* 2003a). Additionally, progeny were produced using spermatids from the cultured SSCs after allotransplantation (Kanatsu-Shinohara *et al.* 2003a). Similar culture conditions applied for more than 2 years could substantially expand SSC populations while retaining the potential to produce progeny using spermatids derived from the cultured SSCs after transplantation (Kanatsu-Shinohara *et al.* 2005c). It was suggested that neonatal SSCs have higher dividing activity than adult SSCs (Nagano In press; Nagano *et al.* 2000a; Nagano *et al.* 2002b).

Despite the proven importance of STO and Sertoli cells as feeder cells for SSC survival in mice and rats (Hamra *et al.* 2004; Jeong *et al.* 2003; Nagano *et al.* 1998; Nagano *et al.* 2003; Ryu *et al.* 2005; Van Der Wee *et al.* 2001), mouse SSCs were expanded on laminin coated plates in Stempro-34 SFM basal media without feeder cell or serum for more than 6 months while maintaining the competence to produce progeny after transplantation (Kanatsu-Shinohara *et al.* 2005b). Enriched mouse SSCs were also propagated for several months with a doubling time of approximately 6 days in a serum-free MEM-alpha medium on STO with addition of only GDNF, or along with GDNF

family receptor alpha 1 (GFRα1) and bFGF depending on mouse strains (Kubota *et al.* 2004b). Since SSCs did propagate in culture in the absence of both feeder cells and serum (Kanatsu-Shinohara *et al.* In press), they may not be indispensable for survival and proliferation of mouse SSCs (Kanatsu-Shinohara *et al.* In press; Kanatsu-Shinohara *et al.* 2005b; Kubota *et al.* 2004b). However, GDNF was recognized as a single most critical growth factor for mouse SSC self-renewal in culture (Kanatsu-Shinohara *et al.* 2005b; Kanatsu-Shinohara *et al.*2003a; Kubota *et al.* 2004a; Oatley and Brinster 2008; Ogawa *et al.* 2004). Recently, it was shown that supplementation of colony stimulating factor 1 (CSF1), fetuin and lipid substances can also promote proliferation of SSCs in culture (Kanatsu-Shinohara *et al.* In press; Oatley *et al.* 2009).

Compared to mouse SSCs, hamster SSCs required bFGF as an essential growth factor, along with GDNF, for vigorous proliferation during the year-long culture on laminin coated plates *in a* TX-WES basal medium. But although round spermatids could be generated after transplantation of the cultured SSCs, no progeny was produced (Kanatsu-Shinohara *et al.* 2008). Rat SSCs were also successfully propagated *in vitro* in serum-free basal media of either Stempro-34 with GDNF-GFRα1 on MEF, or MEM-alpha with GDNF-GFRα1-bFGF on STO. It was estimated that as many as a million SSCs could be generated from a single SSCs after 7 months of culture (Hamra *et al.* 2005; Ryu *et al.* 2005).

In farm animals, bovine type-A spermatogonia were maintained in co-culture with Sertoli cells in a MEM basal medium supplemented with FCS for more than 3 months when spermatid-like cells were produced (Izadyar *et al.* 2003a), and addition of GDNF improved SSC survival and self-renewal (Aponte *et al.* 2006). More than 10,000-fold

propagation of SSCs was achieved during a one-month culture in Stempro-34 SFM basal media with a combination of GDNF, LIF, EGF and bFGF, (Aponte *et al.* 2008).

Similarly, populations of human SSCs were also substantially expanded (by approximately 18000-fold) when cultured on laminin-coated plates with StemPro-34 SFM basal media in presence of GDNF, EGF, LIF and bFGF (Sadri-Ardekani *et al.* 2009).

In contrast, approximately 60% of porcine type-A spermatogonia were reported to survive after 2 days of culture in the serum-free medium KSOM with SCF and/or granulocyte macrophage-colony stimulating factor (GM-CSF) (Dirami *et al.* 1999). Colonies of cells were observed during culture in StemPro-34 SFM basal medium with 1% FCS and growth factors, but not in MEM basal medium with 10% FBS and LIF (Aponte *et al.* 2008). Similar to results of culturing bovine testis cells, morphologically different colonies arose from the early culture of week-old piglet testis cells; however, number and magnitude of SSC-like colonies were only enhanced by EGF and FGF rather than GDNF or LIF. SSCs could not be propagated beyond 9 passages (i.e., 1 or 2 months) (Kuijk *et al.* 2009).

2.5.1.1 Derivation of Pluripotent Embryonic Stem Cell-like Cells from SSCs in Culture

After long-term maintenance and proliferation of SSCs *in vitro*, colonies with different morphologies start to appear during the first couple of weeks. Multipotent/pluripotent stem cells could be derived from these colonies in neonatal and adult mice (Guan *et al.* 2006; Huang *et al.* 2009; Kanatsu-Shinohara *et al.* 2004; Ko *et al.*

2009; Seandel *et al.* 2007) and adult human (Conrad *et al.* 2008; Golestaneh *et al.* 2009; Kossack *et al.* 2009; Mizrak *et al.* 2010).

Colonies with appearances similar to colonies of embryonic stem cells (ESCs) have been observed after several passages of testis cells in culture (with some or all of the following growth factors: bFGF, GDNF, LIF and TGFβ1) on uncoated or gelatin-coated plates with no feeder cells (Conrad *et al.* 2008; Golestaneh *et al.* 2009; Guan *et al.* 2006; Kanatsu-Shinohara *et al.* 2004; Kossack *et al.* 2009; Mizrak *et al.* 2010), or on testis stromal cells (Seandel *et al.* 2007). These colonies could be manually selected and further maintained and expanded in a DMEM basal medium with bFGF and/or LIF and FCS on MEFs (Guan *et al.* 2006; Kanatsu-Shinohara *et al.*2004; Kossack *et al.* 2009; Seandel *et al.* 2007) or feeder-cell-free gelatin (Conrad *et al.* 2008; Golestaneh *et al.* 2009; Guan *et al.* 2006) or Matrigel coated plates (Mizrak *et al.* 2010).

Cells derived from mouse or human multipotent/pluripotent stem cells during culture showed normal karyotype with various ESC genotypes and phenotypes. These cells also have other characteristics in common with ESCs including: differentiation into somatic cells of all three germ layers (ectodermal, mesodermal and endodermal lineages); sharing typical phenotypes or function *in vivo* and *in vitro* (Conrad *et al.* 2008; Golestaneh *et al.* 2009; Guan *et al.* 2006; Kanatsu-Shinohara *et al.* 2004a; Kossack *et al.* 2009; Mizrak *et al.* 2010; Seandel *et al.* 2007); formation of teratoma after transplantation into seminiferous tubules and grafting subcutaneously or under the kidney capsule in recipient mice (Conrad *et al.* 2008; Golestaneh *et al.* 2009; Guan *et al.* 2006; Kanatsu-Shinohara *et al.* 2004; Kossack *et al.* 2009; Seandel *et al.* 2007); the potential to generate spermatogenesis after transplantation (Guan *et al.* 2006; Kanatsu-Shinohara *et*

al. 2004; Seandel *et al.* 2007); and development of chimera after early blastocyst injection in mice (Guan *et al.* 2006; Kanatsu-Shinohara *et al.* 2004; Seandel *et al.* 2007).

Interestingly, mouse SSCs/gonocytes aggregated with foetal/neonatal mesenchymal and epithelial cells on agar, survived and grew after grafting under the renal capsule. More importantly, they directly gave rise to somatic cells of all three germ layers, suggesting that SSCs/gonocytes may retain the capability to trans-differentiate into other cell types in suitable microenvironments (Simon *et al.* 2009). Recently, trans-differentiation of mouse SSCs into hematopoietic cells was reported after transplantation into bone marrow (Ning *et al.* 2010).

2.5.2 Maintenance and Propagation of Gonocytes in Culture

Although SSCs derived from cultured gonocytes could be potentially expanded for more than 2 years in mice (Kanatsu-Shinohara *et al.* 2005b; Kanatsu-Shinohara *et al.* 2005c), to date the long-term propagation of gonocytes has not been reported in any species. In most studies, gonocytes were maintained in a simple culture system for several days or weeks, typically in Eagle's MEM, IMDM or DMEM/F12 basal media with or without FBS (Fujihara *et al.* 2008; Goel *et al.* 2007; Hasthorpe *et al.* 2000; Hasthorpe *et al.* 1999; Tu *et al.* 2007; Yu *et al.* 2009; Yu *et al.* 2005). Organ culture was also developed in evaluating effects of certain additives, such as placing gonad tissue fragments on a thin perforated film that floats on the culture media (Lehraiki *et al.* 2009; Li and Kim 2004; Petre-Lazar *et al.* 2007; Zhou *et al.* 2008).

In culture, gonocytes have been shown to develop specialized cytoplasmic processes and form colonies mimicking *in vivo* behaviour, probably through a SCF/c-kit pathway (Hasthorpe *et al.* 1999; McGuinness and Orth 1992b; Orth *et al.* 1997; Orth and

Boehm 1990; Orth and Jester 1995; Orth *et al.* 2000; Orth and McGuinness 1991; Orth *et al.* 1998). Co-culture with Sertoli cells and ECM coatings have promoted gonocytes proliferation and migration in rats (Orth and McGuinness 1991; Van Dissel-Emiliani *et al.* 1993). On the other hand, Sertoli cells have also been reported to have no enhancing effects or even have inhibitory influence on gonocytes colony formation (Hasthorpe 2003; Hasthorpe *et al.* 2000; Orth and Boehm 1990; Piedrahita *et al.* 1997).

In rodents, bFGF, LIF, ciliary neurotrophic factor and activin have been reported to enhance survival of gonocytes and colony formation in culture (Hasthorpe 2003; Kanatsu-Shinohara *et al.* 2007; Meehan *et al.* 2000; Piedrahita *et al.* 1997; Van Dissel-Emiliani *et al.* 1996), while SCF, TGFß, RA, inhibin and androgens exerted negative effects on gonocytes proliferation (Boulogne *et al.* 2003; Hasthorpe 2003; Kanatsu-Shinohara *et al.* 2004; Li and Kim 2004; Merlet *et al.* 2007; Spangrude 2003; Tu *et al.* 2007). It was reported that PDGF was the single growth factor to potentially promote gonocyte proliferation in culture (Basciani *et al.* 2008; Li *et al.* 1997; Wang and Culty 2007).

Certain gonocyte sub-populations showed multiple pluripotency markers, and teratoma was observed after subcutaneous transplantation of cultured piglet gonocytes (Goel *et al.* 2009; Hoei-Hansen *et al.* 2005; Niu and Liang 2008; Tu *et al.* 2007). Gonocytes might also directly differentiate into other somatic cells of the three germ layers (Simon *et al.* 2009). However, to date, no pluripotent stem cells have been derived from the cultured gonocytes in any species.

2.5.3 Maintenance and Propagation of PGCs in Culture

PGCs proliferated during *in vitro* culture before undergoing mitotic arrest, a pattern similar to their *in vivo* behaviour, suggesting an age-dependent cell autonomous mechanism (Donovan *et al.* 1986; Matsui *et al.* 1991; Ohkubo *et al.* 1996). A similar mechanism was also proposed to regulate proliferation and differentiation of SSCs in culture (Wu *et al.* 2009b).

Tumor necrosis factor-alpha (TGFα) has been shown to enhance the proliferation of PGCs prior to reaching the genital ridge, but not those in growth arrest (Kawase *et al.* 1994). In addition, LIF (De Felici and Dolci 1991; Farini *et al.* 2005; Matsui *et al.* 1991; Pesce *et al.* 1993), SCF (Dolci *et al.* 1991; Godin *et al.* 1991; Pesce *et al.* 1993), bFGF (Resnick *et al.* 1992), interleukin-4 (Cooke *et al.* 1996), retinoic acid (Koshimizu *et al.* 1995) and cAMP (De Felici *et al.* 1993) were demonstrated to promote survival and proliferation of PGCs *in vitro*.

Long-term propagation of PGCs beyond the growth arrest in culture was achieved on feeder cells after the addition of growth factors bFGF, LIF and SCF in rodents (Labosky *et al.* 1994; Matsui *et al.* 1992; Resnick *et al.* 1992).

Although PGCs are believed to normally give rise to male and female germ cells *in vivo*, it has been shown that LIF can derive and maintain the ESC-like cells from cultured PGCs (Pease and Williams 1990; Smith *et al.* 1988; Williams *et al.* 1988). Similarly, the combination of growth factors bFGF and SCF (Labosky *et al.* 1994; Matsui *et al.* 1992; Resnick *et al.* 1992; Shim *et al.* 2008; Stewart *et al.* 1994) or the addition of bFGF in culture were shown to stimulate the transformation of PGCs to embryonic germ cells (EGCs) (Kawase *et al.* 1994; Matsui *et al.* 1992; Resnick *et al.* 1992), which share many morphological, phenotypical and pluripotent characteristics with ESCs (De Felici

et al. 2009; Laible and Alonso-González 2009; Shamblott *et al.* 1998; Solter and Knowles 1978; Stewart *et al.* 1994). Interestingly, PGCs were also derived from EGCs in culture (Eguizabal *et al.* 2009).

Aside from mouse cells, rabbit EGC-like cells, with certain pluripotent phenotypes, were also generated from cultured PGCs in the presence of LIF, bFGF and forskolin on MEF; however, they could only propagate for less than a month, no teratomas were formed after grafting under the recipient's renal capsule (Kakegawa *et al.* 2008).

When human PGCs were cultured in the presence of LIF, bFGF and forskolin on STO feeder cells, ESC/EGC-like colonies appeared. Some of the colonies formed embryoid bodies (EBs), and the EB-derived cells could propagate for long-term and differentiate into somatic cells showing numerous markers of the three germ layers *in vitro*; although no teratomas were formed after transplantation into the host mice (Shamblott *et al.* 2001; Shamblott *et al.* 1998). Liver, myogenic and neural lineage cells were also produced during the *in vitro* culture, and after transplantation of the EB-derived cells from cultured PGCs into the liver, muscle or brain cavity (Chen *et al.* 2007; Shao *et al.* 2009; Teng *et al.* 2009).

It was reported that many PGCs could not survive the primary culture due to apoptosis, supplementation of protease inhibitors (especially α_2-macroglobulin) and antioxidants may enhance maintenance of pig PGCs (Lee *et al.* 2000). LIF and SCF were also suggested to have inhibitory effects on apoptosis of mouse PGCs (Pesce *et al.* 1993). Interestingly, PGCs could survive and proliferate in culture with DMEM and FBS on STO feeder cells without supplementation with growth factors. EGCs were also derived

and propagated for more than 29 passages (6 months) in culture, differentiated into multiple somatic cell types *in vitro*, and piglet chimeras produced after blastocyst injection (Mueller *et al.* 1999; Shim *et al.* 1997). Growth factors SCF, LIF and bFGF were also added in culture media DMEM/F10 for maintenance of pig PGC-derived EGCs on STO, and it was reported that PGC-derived colonies developed into EBs in a few days even in the absence of growth factors (Piedrahita *et al.* 1998).

Week-long primary culture of pig PGCs on STO, SCF and LIF but not bFGF promoted survival and proliferation of PGCs (Redwan 2009). Synergistic effects of LIF, bFGF and SCF were suggested in promoting survival and maintenance of porcine PGC-derived EGCs in culture, and feeder cells especially those releasing SCF were necessary for the development of EGCs (Lee and Piedrahita 2000).

Bovine PGCs have been maintained in culture on SNL or cattle embryonic fibroblast feeder cells for 7 weeks, with EBs formed and ESC-like cells derived in culture. Incorporation of PGC-derived cells into inner cell mass was also observed *in vitro* after blastocyst injection (Cherny *et al.* 1994).

One study reported maintenance of goat PGCs for 3 months on STO in DMEM with FCS and LIF; however, colonies were formed only in the first month, spontaneous differentiation of PGCs into multiple somatic cell types was also observed in culture (Kühholzer *et al.* 2000).

2.6 Transduction of the Male Germline Progenitor Cells

To date, a biopharmaceutical protein (ATryn) produced by transgenic animals has entered the market (http://www.gtc-bio.com/products/atryn.html), and it was estimated that the production cost will be about 7% (using transgenic dairy goat) or 0.03% (using

transgenic hens) of that using conventional methods (Redwan 2009). Improvement in economically-important phenotypic characteristics of livestock could be expedited considerably using transgenic technologies as compared with the traditional select breeding (Niemann and Kues 2007). Through modification (deletion/replacement) of impaired genes, genetic diseases could be potentially cured and prevented from passing into the next generations (Toelen and Deprest 2010; Wagner *et al.* 2009).

Several technologies have been developed for production of transgenic animals, including pronuclear microinjection (Brinster *et al.* 1981; Gordon *et al.* 1980; Hammer *et al.* 1985), somatic cell nuclear transfer (Brunetti *et al.* 2008; Nottle *et al.* 2007; Schnieke *et al.* 1997), oocyte-mediated (Cabot *et al.* 2001; Chan *et al.* 2001; Chan *et al.* 1998; Hofmann *et al.* 2004; Sato *et al.* 2003; Tsukui *et al.* 1996; Yang *et al.* 2007a) and spermatozoa-mediated methodologies (Arezzo 1989; Brackett *et al.* 1971; Gandolfi 2000; Lavitrano *et al.* 1989; Webster *et al.* 2005; Wu *et al.* 2008). Nevertheless, these approaches have low efficiency, for example, no more than 1% of the microinjected zygotes led to the birth of transgenic animals (Pinkert and Murray 1999), and low survival rate of the cloned embryos (1-5%) (Yang *et al.* 2007b).

Transgenesis via viral vectors is indeed highly efficient, and could potentially produce up to 100% transgenic animals born (Hofmann *et al.* 2004; Nagano *et al.* 2002b; Punzon *et al.* 2004; Whitelaw *et al.* 2008; Whitelaw *et al.* 2004). However, the use of viral vectors has limitations including the limited capacity for carrying large foreign genes, possibility of causing mutation or tumors, and posing higher biohazard risks impeding their clinical applications (Goff 2001; Hofmann *et al.* 2006; Park 2007; Themis *et al.* 2005; Whitelaw *et al.* 2008).

ESCs have been commonly used to produce various transgenic mouse models (Capecchi 1989; Gertsenstein *et al.* 2002; Gossler *et al.* 1986; Robertson *et al.* 1986). Transgenesis of ESCs followed by their injection into blastocysts can lead to incorporation into embryonic layers with a possibility of germline transmission (Robertson *et al.* 1986). However, ESC technology could not be readily adapted in farm animals, because to date ESCs are still not available in these animals (Muñoz *et al.* 2009). In contrast, transgenesis via spermatozoa has been successfully applied in a wide range of species (Marialuisa Lavitrano *et al.* 2005; Smith and Spadafora 2005); however, spermatozoa are terminal male gametes, each transgenic spermatozoon normally produces a single transgenic individual after fertilization, and huge variations in success rate are expected between labs and among different species (Brinster *et al.* 1989; Marialuisa Lavitrano *et al.* 2005; Smith and Spadafora 2005).

Male progenitor germ cells (SSCs, gonocytes and PGCs) could also be transducted *in vitro*, and because spermatogenesis is a very efficient process that continues throughout the adult life in male mammals, a single transgenic germline progenitor cell could potentially generate millions of transgenic spermatozoa. Furthermore, these progenitor cells can be transplanted into recipient testes where they can produce transgenic gametes which upon mating can result in germline transmission.

2.6.1 *In vivo* Transduction of SSCs

Attempts were initially made to transduct rodent testis cells *in vivo*. While direct injection of adenoviral vectors into the interstitial tissue or seminiferous tubules mainly transducted Leydig and Sertoli cells, germ cells were not infected (Blanchard and Boekelheide 1997). Interestingly, injection of plasmid DNA followed by *in vivo*

electroporation of the testis transducted spermatogenic cells, with reported transient expression of the transgene for up to ~2 months (Coward *et al.* 2006; Hibbitt *et al.* 2006; Kubota *et al.* 2005; Muramatsu *et al.* 1997; Umemoto *et al.* 2005; Yamazaki *et al.* 1998), and transgenic mouse progeny were produced by ICSI of the collected transgenic spermatozoa (Huang *et al.* 2000). In addition, when recipient mice and rats were mated only a few days after testis injection of DNA plasmid/constructs mixed with a lipofection reagent, eventually transgenic progeny were produced, showing germline transmission (Celebi *et al.* 2002; Chang *et al.* 1999b; Sato *et al.* 1999a; Sato *et al.* 1999b; Yonezawa *et al.* 2001). This suggested that the foreign DNA constructs were rapidly transported through rete testis into the efferent ducts and epididymis as early as in one day, and mostly acquired by spermatozoa 3-4 days after injection (Chang *et al.* 1999a; Sato *et al.* 2002).

At least in mature mice, it seems that viral vectors could not successfully transduct SSCs *in situ* after injection into seminiferous tubules, even though differentiated spermatogenic cells might be observed to express the transgene (Blanchard and Boekelheide 1997; Honaramooz *et al.* 2008; Ikawa *et al.* 2002; Kanatsu-Shinohara *et al.* 2004b; Nagano *et al.* 2000a; Takehashi *et al.* 2007). However, one study reported that SSCs in neonatal rather than mature mouse testes were transducted *in vivo* after injection of a retroviral vector (the Moloney murine leukemia virus) into the seminiferous tubules. About half of the injected testes showed the transgenic SSCs, of the 86% fertile mice injected with the vector, transgenic mice were produced from 26%, after natural mating (~22% of all mice injected), showing germline transmission of the transgene in ~2.8% of the sired offspring (Kanatsu-Shinohara *et al.* 2004b). The observed difference between

the neonatal and mature testes could be due to the blood-testis barrier and multiple layers of germ cells in mature seminiferous tubules which may have blocked the access of the transgene to the SSCs *in situ* (Chang *et al.* 1999a; Kanatsu-Shinohara *et al.* 2004b; Nagano *et al.* 2000a; Sato *et al.* 2002).

2.6.2 *In vitro* Transduction of SSCs/Gonocytes

Since SSCs can generate full donor derived spermatogenesis after injection into recipient seminiferous tubules, it is reasonable to transduct SSCs *in vitro* to increase their exposure to the transgene prior to transplantation.

While no SSCs were transducted after injection of retroviral vectors into mouse seminiferous tubules, transgenic SSCs were observed in recipient mouse testes (for ~6 months) after transplantation of *in vitro*-transducted SSCs, or after co-transplantation of freshly collected testis cells with retroviral vectors. Up to 20% of neonatal SSCs expressed the transgene in recipient testes after *in vitro* transduction followed by transplantation, and transgene was carried in ~4.5% offspring after mating of the transplanted recipients. Neonatal SSCs showed higher transduction efficiency *in vitro* compared with those of mature testes (Nagano *et al.* 2001a; Nagano *et al.* 2000a).

Similarly, SSCs were successfully transducted *in vitro* with adenoviral or adeno-associated viral vectors resulting in 49-76% transduction efficiency and production of transgenic progeny (Honaramooz *et al.* 2008; Takehashi *et al.* 2007). The use of lentiviral vectors resulted in transduction of 29-100% of SSCs in recipient mice, with SSCs from neonatal testes resulting in ~10-fold higher transduction efficiency than those from mature testes (Nagano *et al.* 2002b).

In rats, although retroviral vectors resulted in low transduction efficiency (~0.5%) (Orwig *et al.* 2002a), ~45% of SSCs were transducted *in vitro* with lentiviral vectors without significant toxicity, and after mating of the transplanted recipients, 6% of the offspring carried the transgene and passed it on to at least three generations (Hamra *et al.* 2002; Ryu *et al.* 2007).

Compared with viral-mediated gene delivery methods, non-viral methods are simple and easier to operate, and carry virtually no biosafety risks to the operator or the public; however, they are also very inefficient. Although calcium phosphate- and DEAE-dextran-mediated *in vitro* transfection of neonatal mouse SSCs with a plasmid vector resulted in hardly any transfected SSCs (0.6%), electroporation led to relatively high transfection efficiency (~20%) but low survival rate (~9%), while lipofection resulted in low transfection efficiency (~4%) with very high survival rate (~96%). After mating of recipients that were transplanted with transfected SSCs by a lipofection reagent, ~49% of progeny carried the transgene and showed Mendelian pattern of germline transmission (Kanatsu-Shinohara *et al.* 2005d).

Despite the failure of *in vivo* electroporation of mouse SSCs (Coward *et al.* 2006; Hibbitt *et al.* 2006; Huang *et al.* 2000; Kubota *et al.* 2005; Muramatsu *et al.* 1997; Umemoto *et al.* 2005; Yamazaki *et al.* 1998), *in vitro* electroporation of bovine testis tissue resulted in transfection of SSCs, as assessed by xenografting into the back skin of immunodeficient mice (Oatley *et al.* 2004a). Transfection of pig germ cells was reported after injection of a plasmid and lipofection reagent mixture into busulfan-treated testes; however, from the report it was not clear whether SSCs were indeed transfected (Kim *et al.* 1997).

As a proof-of-principle study, testis cells from transgenic goats were injected into the seminiferous tubules of recipient goat testes, and transgenic progeny were produced after mating of the recipients (Honaramooz *et al.* 2003b). To date, *in vitro* transduction of isolated SSCs from large animals has been studied only in one report using goats, where SSCs were transducted using adeno-associate viral (AAV) vectors and transplanted into recipient goats, resulting in the presence of transgenic spermatozoa in ~35% of ejaculates, giving rise to ~10% transgenesis of embryos after IVF (Honaramooz *et al.* 2008).

Only one study could be found on gonocyte transduction (pig), where more than 90% cell survival was reported after using lentiviral vectors *in vitro*, with a transduction efficiency of ~11%. After xenotransplantation into the seminiferous tubules of immunodeficient recipient mice, the transgenic gonocytes survived and colonized the recipient testes (Kim *et al.* 2010).

2.7 Transplantation of Male Germline Progenitor Cells

Germ cell transplantation (GCT), also referred to as spermatogonial stem cell transplantation, is a process in which testis cells harvested from a fertile donor male are microinjected into the seminiferous tubules of infertile recipients (Brinster and Avarbock 1994; Brinster and Zimmermann 1994). Transplanted spermatogonial stem cells could migrate to the basolateral compartment of the seminiferous tubules and proliferate to initiate donor-derived spermatogenesis, with the support from the recipient testis somatic cells (Chuma *et al.* 2005; Clouthier *et al.* 1996; Franç*a et al.* 1998; Jiang and Short 1998a;

Nagano *et al.* 1999; Ohta *et al.* 2000; Orwig *et al.* 2002b; Parreira *et al.* 1998; Parreira *et al.* 1999; Russell and Brinster 1996).

2.7.1 Transplantation of SSCs / Spermatogonia in Different Species

Full donor-derived spermatogenesis was first reported after direct microinjection of mouse testis cells into the testes of recipient mice, resulting in progeny production (Brinster and Avarbock 1994; Brinster and Zimmermann 1994). Complete spermatogenesis was also observed in mouse testes after heterologous transplantation of testis cells from donor rats (Clouthier *et al.* 1996; Russell and Brinster 1996) and hamster (Ogawa *et al.* 1999a); however, only colonization/proliferation of SSCs but not full spermatogenesis was found in recipient mouse testes after transplantation of donor germ cells from rabbits (Dobrinski *et al.* 1999a), dogs (Dobrinski *et al.* 1999a), cats (Kim *et al.* 2006), bulls (Dobrinski *et al.* 2000), boars (Dobrinski *et al.* 2000), horses (Dobrinski *et al.* 2000), baboons (Nagano *et al.* 2001b) and humans (Nagano *et al.* 2002a). Interestingly, recipient mice supported full/complete spermatogenesis (demonstrated all stages of spermatogenesis) of subcutaneously grafted testis tissue from a wide range of donor species including pigs, goats, cats, bull calves, sheep, horses and monkeys (reviewed in Rodriguez-Sosa and Dobrinski 2009). Nevertheless, autologous/homologous transplantation of testis cells also resulted in spermatogenesis in rats (Jiang and Short 1995; Ogawa *et al.* 1999b), dogs (Kim *et al.* 2008), goats (Honaramooz *et al.* 2003a; Honaramooz *et al.* 2003b), pigs (Honaramooz *et al.* 2002a; Mikkola *et al.* 2006), sheep (Herrid *et al.* 2009b; Rodriguez-Sosa *et al.* 2009) and cattle (Herrid *et al.* 2006a; Izadyar *et al.* 2003b; Oatley *et al.* 2005a; Rathi *et al.* 2005; Stockwell *et al.* 2009) (Table 2.1).

Table 2.1. Progress of germ cell transplantation

Donor	Recipient	Colonization	Spermatocyte	Spermatozoa	Progeny	Transgenic Offspring
Mice	Mice	+	+	+ (Brinster and Zimmermann 1994)		+ (Brinster and Avarbock 1994; Honaramooz *et al.* 2008; Kanatsu-Shinohara *et al.* 2005d; Nagano *et al.* 2001a)
Rat	Rat	+	+	+	+	+ (Hamra *et al.* 2002; Ryu *et al.* 2007)
Rat	Mice	+	+	+ (Clouthier *et al.* 1996; Franca *et al.* 1998; Russell and Brinster 1996)		
Hamster	Mice	+	+	+ (Ogawa *et al.* 1999a)		
Rabbit	Mice	+ (Dobrinski *et al.* 1999a)				
Dog	Mice	+ (Dobrinski *et al.* 1999a)				
Pig	Mice	+ (Dobrinski *et al.* 2000)				
Bovine	Mice	+ (Dobrinski *et al.* 2000)				
Horse	Mice	+ (Dobrinski *et al.* 2000)				
Baboon	Mice	+ (Nagano *et al.* 2001b)				
Mice	Pig	- (Honaramooz *et al.* 2002a)				
Goat	Goat	+	+	+	+ (Honaramooz *et al.* 2003a; Honaramooz *et al.* 2003b)	+ (Honaramooz *et al.* 2003b)
Cattle	Cattle	+ (Herrid *et al.* 2006a)		+ (Stockwell *et al.* 2009)		
Cat	Mice	+ (Kim *et al.* 2006)				

Pig	Pig	+ (Honaramooz *et al.* 2002a)		+ (Mikkola *et al.* 2006)	
Chicken	Chicken	+	+	+	+ (Lee *et al.* 2006)
Dog	Dog	+	+	+ (Kim *et al.* 2008)	
Sheep	Sheep	+	+	+	+ (Herrid *et al.* 2009b)

+: Positive results obtained

2.7.2 Germ Cell Transplantation Techniques

In the first report of GCT in recipient mice, testis cells were directly microinjected by a fine glass needle into the lumen of superficial seminiferous tubules visible through the tunica albuginea under a microscope. This required about 0.2-0.3 ml of cell suspension and took 5-30 min per testis, resulting in filling of 50-100% of surface tubules with donor cells (Brinster and Avarbock 1994; Brinster and Zimmermann 1994; Clouthier *et al.* 1996). In mice and rats, donor testis cells could also be transplanted through microinjection into efferent ducts (Dobrinski *et al.* 1999a; Franç a *et al.* 1998; Nagano *et al.* 1999; Ogawa *et al.* 1997; Ogawa *et al.* 1999a) or rete testis (Hamra *et al.* 2002; Jiang and Short 1995; Nagano *et al.* 2001b; Ogawa *et al.* 1997; Ogawa *et al.* 1999b). While all three routes could fill more than 50% of seminiferous tubules on the testis surface, the rete testis route is technically more difficult than the efferent duct or tubule lumen routes. Efferent duct injection is relatively quicker to perform and less cell suspension is required (100-150 µl with 70%-100% surface tubule coverage within 15-30 min for both testes), but direct injection into the tubule lumen requires less preparation and tissue dissection (Ogawa *et al.* 1997). Currently the latter two methods are frequently utilized in transplantation of donor testis cells into rodent testis.

However, these techniques could not be readily adapted to use in large animals mainly because of major anatomical structure differences including thick tunica albuginea and very long and convoluted tubules in the testis of large animals (Honaramooz *et al.* 2003a; Honaramooz *et al.* 2002a; Schlatt *et al.* 1999). Therefore, an ultrasound-guided intra-testicular rete testis infusion system was developed which could result in filling of about half of the recipient seminiferous tubules with ~5 mL of cell suspension within 30 min (Honaramooz *et al.* 2002a; Schlatt *et al.* 1999). To date, this

approach has been applied successfully in species such as boars (Honaramooz *et al.* 2002a), bulls (Joerg *et al.* 2003; Schlatt *et al.* 1999), goats (Honaramooz *et al.* 2003a; Honaramooz *et al.* 2003b) and primates (Schlatt *et al.* 2002; Schlatt *et al.* 1999). An extra-testicular rete testis method with ultrasound guidance or epididymal dissection was also reported capable of introducing donor cells into sheep seminiferous tubules (Rodriguez-Sosa *et al.* 2006; Rodriguez-Sosa *et al.* 2009).

2.7.3 Approaches to Improve Transplantation Efficiency

Widespread application of GCT is currently limited due to challenges in improving the transplantation efficiency (reviewed by Honaramooz and Yang, 2011; Khaira *et al.* 2005; Oatley and Brinster 2008; Dobrinski and Travis 2007; Geens *et al.* 2008). Studies on GCT demonstrated that donor-derived spermatogenesis in the recipient testes could be improved significantly when recipient endogenous germ cells were absent due to genetic mutations (Boettger-Tong *et al.* 2000; Brinster *et al.* 2003; Geissler *et al.* 1988; Ogawa *et al.* 2000; Ohta *et al.* 2001; Shinohara *et al.* 2001; Silvers 1979) or were depleted after ablative strategies such as busulfan injection (Brinster 2002; Brinster and Avarbock 1994; Brinster and Zimmermann 1994; Ogawa *et al.* 1999b; Okabe *et al.* 1997) or testis local irradiations (Creemers *et al.* 2002; Giuili *et al.* 2002; Herrid *et al.* 2009b; Honaramooz *et al.* 2005; Izadyar *et al.* 2003b; Kim *et al.* 2008; Oatley *et al.* 2005a; Schlatt *et al.* 2002; Zhang *et al.* 2006).

Busulfan is a DNA-alkylating agent that destroys proliferating cells and is frequently used to deplete recipient germ cells before GCT (Brinster and Avarbock, 1994; Honaramooz *et al*, 2005; Moisan *et al*, 2003). As a side effect, high doses of busulfan

have been reported to cause severe hematopoietic suppression and even death in rats, mice and pigs (Honaramooz *et al.* 2005; Mikkola *et al.* 2006; Ogawa *et al.* 1999a; Udagawa *et al.* 2001). Therefore, busulfan treatment of pregnant females with lowered doses was also utilized to reduce the potential lethal toxicity, especially to the progeny (Brinster *et al.* 2003; Honaramooz *et al.* 2005). Currently rodents with genetic mutations or after busulfan treatment are commonly used as recipients for GCT. Due to the lack of proper farm animal models with genetically-depleted germ cells, and the high toxicity of busulfan treatment, local irradiation of testes has also been used to prepare recipient goats for transplantation (Honaramooz *et al.* 2005).

Aside from depletion of recipient endogenous germ cells, the efficiency of GCT is highly related to the number of SSCs transplanted (Dobrinski *et al.* 1999b). In mice, most transplanted donor cell populations contained ~100-200 SSCs, resulting in ~7-20% successful colonization in the recipient testes (Nagano *et al.* 1999; Shinohara *et al.* 1999). It was also suggested that only two donor-derived spermatogenic colonies could be produced after transplantation of every one million testis cells (i.e., ~1% of transferred SSCs) (Jiang 2001). Therefore, enrichment of donor germ cells for SSCs could increase the potential number of spermatogenic colonies in recipients (Shinohara *et al.* 1999; Shinohara *et al.* 2000). Several strategies have also been applied/suggested to improve the colonization of donor germ cells in recipient testis. Younger animals were demonstrated to be more suitable in providing a microenvironment for colonization of donor germ cells (Brinster *et al.* 2003; Ogawa *et al.* 1999b; Ryu *et al.* 2003; Shinohara *et al.* 2001). Recipient treatment with GnRH agonists was also shown to improve the

efficiency of colonization (Dobrinski *et al.* 2001; Ogawa *et al.* 1998; Ogawa *et al.* 1999b).

2.7.4 Transplantation of Gonocytes and PGCs

The ability of SSCs in colonization of recipient testes and differentiation into spermatozoa after GCT are well-established and have been shown in both homologous and heterologous transplantations between mice and rats (Clouthier *et al.* 1996; França *et al.* 1998; Ogawa *et al.* 1999b; Russell and Brinster 1996; Zhang *et al.* 2003). However, only colonization and proliferation of mouse gonocytes (and not full spermatogenesis) were observed in recipient testis following homologous transplantation (McLean *et al.* 2003; Ohbo *et al.* 2003; Ohmura *et al.* 2004; Ohta *et al.* 2004; Shinohara *et al.* 2002a). On the other hand, full spermatogenesis was generated after homologous transplantation of rat gonocytes (Jiang and Short 1995; Jiang and Short 1998a; Orwig *et al.* 2002b; Ryu *et al.* 2003). To date, homologous transplantation of gonocytes has not been tested in any large animals, although piglet gonocytes were reported to colonize but not differentiate in immunodeficient recipient mouse testes (Goel *et al.* 2009; Kim *et al.* 2010). In some aspects, gonocytes are believed to be different from SSCs (Forand *et al.* 2009a; Hasthorpe 2003; McLean *et al.* 2003; Meehan *et al.* 2000; Ohbo *et al.* 2003; Shinohara *et al.* 2001; Van Den Ham *et al.* 2002), and further investigation are needed to determine if gonocytes possess the potential to produce donor-derived spermatogenesis following GCT. This is particularly important in farm animals, where gonocytes could be easily collected at a much earlier age compared with SSCs.

As the progenitor of gonocytes, rat and mouse PGCs have produced donor-derived spermatozoa in recipient testes after homologous transplantation (Chuma *et al.* 2005; Jiang and Short 1995; Jiang and Short 1998a; Ohta *et al.* 2004). PGCs are the first identifiable germ cells in early embryos with limited number but capable of giving rise to both male and female germ cells (Adams and McLaren 2002; Chuma *et al.* 2005; Jiang and Short 1998a; Ohta *et al.* 2004; Wilhelm *et al.* 2007). Although at the beginning, preparing sufficient amount of PGCs for transplantation might be a challenge, it could be solved by propagation in culture. For genetic preservation and dissemination of foetus died at earlier ages, PGCs transplantation could be an appealing strategy. Transplantation of PGCs has not been reported in any large animals, with chicken as the most studied non-rodent species (Motono *et al.* 2010; Naito *et al.* 2010; Naito *et al.* 2007a; Van De Lavoir *et al.* 2006).

2.7.5 Applications of Germ Cell Transplantation

When the transplanted testis cells contain SSCs/germline stem cells, donor-derived spermatogenesis may arise in the recipient testes allowing the production of donor-derived progeny. Since generation of each individual colony of spermatogenesis represents the product of a single SSC/germline stem cell, this system provides a unique bioassay for quantitative analysis of the number and developmental potential of a given testis cell population (Oatley and Brinster 2008). Several studies in rodents and large animals have used this functional assay as a unique tool for the study of SSCs, and investigation of spermatogenesis and male infertility (Brinster 2002; Brinster 2007; Dobrinski 2006; Franç*a et al.* 1998; Hill and Dobrinski 2006).

Transplantation of germ cells by providing access to germline stem cells, can also lay the foundation for modification of the male germline. Genetic manipulation of farm animals may be aimed at improving production traits/efficiency, disease resistance. The advantage of genetic modification through germline stem cells is that a single genetically-altered stem cell can produce virtually unlimited transgenic spermatozoa without losing its potential. Modification of the male germline by transplantation of genetically-altered SSCs is therefore a novel approach to generation of transgenic animals by fertilization of selected oocytes *in vivo* or *in vitro* (i.e., allow genetic recombination). This approach does not involve extensive micromanipulation and is potentially more efficient, less expensive and less time consuming than the conventional approaches to generation of germline transgenic farm animals (Brinster 2002; Honaramooz *et al.* 2003b; Honaramooz *et al.* 2008; Kim *et al.* 2010; Nagano *et al.* 2002b).

Terminal male gametes (spermatozoa) are commonly used for preservation of male fertility and genetic diversity; however, spermatozoa are not available in prepubertal males. Preservation of progenitor germ cells might be the only option for the continuation of the germline from individual males of rare/endangered species that die prior to reaching maturity age. Preservation of SSCs/germline progenitor cells from prepubertal boys undergoing sterilizing cancer treatments (such as chemotherapy or testicular body irradiation) could also restore the fertility potential after autologous transplantation (Geens *et al.* 2008).

2.8 Hypotheses and Objectives

The general hypothesis of this thesis was that spermatogenesis can be established in recipient testes after transplantation of gonocytes in pigs. The objectives of this thesis

were to specifically identify, and efficiently isolate, purify, preserve and transplant gonocytes in pigs as a farm animal model. Experiments were designed to test the following specific objectives and null hypotheses:

2.8.1 Characterization and Quenching of the Auto-fluorescence in Pig Testis Cells (Chapter 3)

To study and characterize the intrinsic fluorescence found in piglet testis tissue and cells (both *in situ* and *in vitro*), and to develop an effective strategy to mask this auto-fluorescence to facilitate specific identification of piglet gonocytes with DBA immune-fluorescent staining, the following null hypotheses were tested:

A. Pig testis cells do not demonstrate intrinsic fluorescence *in situ* or *in vitro*.
B. If piglet testis cells show auto-fluorescence, it will not be masked using Sudan Black B staining.
C. Piglet gonocytes do not specifically bind lectin Dolichos biflorus agglutinin (DBA).

2.8.2 Development of Novel Strategies for the Isolation of Piglet Testis Cells with High Proportion of Gonocytes (Chapter 4)

To investigate the effects of several factors on piglet testis cell isolation, and to develop novel strategies that can increase the proportion of gonocytes in the freshly isolated piglet testis cells, the following null hypotheses were tested:

A. Erythrocytes will not be eliminated from the freshly isolated piglet testis cells using a NH_4CL-based lysis buffer.
B. Testis cells collected from littermate piglets or piglets of different litters will differ in cell viability or yield.

C. Dissociation of piglet testis tissue using different mechanical methods will not result in collection of cells with differing viability, yield or gonocyte proportions.

D. Collection of piglet testis cells with either one-step or two-step enzymatic digestion methods will not result in different gonocyte proportions.

E. Piglet testis cell viability, yield or proportion of gonocytes will not differ between mechanical dissociation and enzymatic digestion methods of cell isolation.

F. Pre-treatment of piglet testis tissue with cold enzymes or hydrostatic pressurization will not increase the efficiency of enzymatic digestion methods for collection of testis cells.

G. Combination of enzymatic digestion and vortexing will not increase the proportion of gonocytes in the freshly collected piglet testis cells.

2.8.3 Effects of Medium and Temperatures on Preservation of Isolated Porcine Testis Cells (Chapter 5)

To investigate the effects of temperature, solution and preservation duration on different types of cells after short-term preservation of isolated piglet testis cells, the following null hypotheses were tested:

A. The survival rate of piglet testis cells will not change after preservation under room or refrigeration temperatures for up to 6 days.

B. The survival rate of piglet testis cells in different solutions will not be different after hypothermic preservation for up to 6 days.

C. The survival rate of piglet testis cells will not change on different preservation days under room or refrigeration temperatures in different solutions.

D. Piglet testis cells will not survive the short-term culture after 6 days of hypothermic preservation.

E. Proportion of gonocytes, Sertoli and peritubular myoid cells will not change in isolated piglet testis cells after hypothermic preservation.

2.8.4 Effects of Tissue Sample Size and Media on Short-term Hypothermic Preservation of Porcine Testis Tissue (Chapter 6)

To evaluate the effects of tissue size, solution and preservation duration on maintenance of testis cell viability and tissue morphology after short-term preservation of testis tissue, the following null hypotheses were tested:

A. The survival rate of testis cells and the semi-quantitative morphometric characteristics of the preserved testis tissue will not be different after hypothermic preservation of piglet testis tissue.

B. Proportion of gonocytes will not change after hypothermic preservation of piglet testis tissue.

2.8.5 Efficient Purification of Neonatal Porcine Gonocytes with Nycodenz and Differential Plating (Chapter 7)

To study the effects of density gradient centrifugation and differential plating in composition of piglet testis cells, and to develop effective strategies for purification of piglet gonocytes, the following null hypotheses were tested:

A. Proportion of gonocytes will not differ among populations of piglet testis cells collected after density gradient centrifugation using various concentrations of Nycodenz.

B. Proportion of gonocytes will not differ among populations of piglet testis cells collected after differential plating using different extracellular matrix (ECM) coated plates.
C. Proportion of gonocytes will not differ among populations of piglet testis cells by extending culture durations in differential plating.
D. Combination of density gradient centrifugation with Nycodenz and differential plating will not increase the proportion of gonocytes in the resultant piglet testis cells.

2.8.6 Piglet Testis Irradiation and Subsequent Gonocyte Transplantation (Chapter 8)

To examine the effect of local irradiation of piglet testes using different fractionated doses on testis development and progress of endogenous spermatogenesis, and to investigate the potential of piglet gonocytes in initiating spermatogenesis in irradiated recipient pig testes after transplantation, the following null hypotheses were tested:

A. Local irradiation of piglet testes using fractionated doses will not decrease the testis weight.
B. Local irradiation of piglet testes using fractionated doses will not affect the histological characteristics of the seminiferous cords/tubules.
C. Germ cell number (per 1,000 Sertoli cells) will not be decreased in irradiated piglet testes.
D. Post-meiotic germ cells will continue to develop in irradiated piglet testes.

E. Spermatogenesis will not develop in irradiated piglet testis after gonocyte transplantation.

CHAPTER 3 CHARACTERIZATION AND QUENCHING OF AUTO-FLUORESCENCE IN PIG TESTIS CELLS

3.1 Abstract

In searching for a specific bio-marker for identification of piglet gonocytes, a significant intrinsic fluorescence was encountered in the testis tissue and in disassociated testis cells which interfered with immuno-fluorescence. The aim of the present study was to examine this intrinsic fluorescence in both the piglet testis tissue and cells, followed by developing an effective method to block the auto-fluorescence. We found that a number of granules within the testis interstitial cells were inherently fluorescent, detectable using epifluorescent or confocal laser scanning microscope, and flow cytometry. The emission wavelength of the auto-fluorescent substance ranged from 425 to 700 nm, a range that could potentially interfere with the commonly used fluorophores. Following treatment of the testis tissue sections with Sudan Black B for 10-15 min or testis cells for 8 min, the intrinsic fluorescence was completely masked allowing specific staining of gonocytes with lectin Dolichos biflorus agglutinin (DBA). We speculate that the lipofuscin within Leydig cell granules was mainly responsible for the observed intrinsic fluorescence in piglet testes. The method developed in the present study will facilitate the identification and characterization of piglet gonocytes using immuno-fluorescence techniques.

3.2 Introduction

The mammalian testis is composed of seminiferous tubules, primarily containing germ and Sertoli cells, and interstitial tissues containing Leydig cells. As the earliest

identifiable germ cell progenitors, primordial germ cells (PGCs) proliferate and differentiate in the foetal testis gonad into gonocytes (Black and Erickson 1968; Pelliniemi 1974; Pelliniemi 1976). After birth, gonocytes proliferate in the testis and develop into spermatogonial stem cells (SSCs) prior to puberty (De Rooij 1998; Jiang and Short 1998b). In the mature testis, SSCs initiate and maintain the continuity of spermatogenesis through self-renewal, proliferation and differentiation to produce daughter germ cells eventually leading up to spermatozoa (De Rooij 1998; Oatley and Brinster 2008). In the neonatal testis, gonocytes are the only germ cells present (Fran ça *et al.* 2000; Frankenhuis *et al.* 1981; Goel *et al.* 2007; Hughes and Varley 1980; Ryu *et al.* 2004), and although they give rise to SSCs and are considered germline stem cells, there is controversy as to whether gonocytes have the capability to initiate spermatogenesis on their own (McLean *et al.* 2003; Ohbo *et al.* 2003; Ohmura *et al.* 2004; Orwig *et al.* 2002b; Shinohara *et al.* 2002a). Compared with PGCs and SSCs, gonocytes are the least investigated germline progenitor cells (Culty 2009); therefore, obtaining new knowledge about gonocytes may shed light on the germline stem cells as a whole.

Although gonocytes can be identified *in situ* and in histological cross-sections by their distinctive topography within the seminiferous cords/tubules and unique morphological attributes (McGuinness and Orth 1992b; Orwig *et al.* 2002b); specific biomarkers are required for their accurate quantification. The unique expression of bio-markers in/on gonocytes may also indicate specific cellular functions, such as AP-2γ (transcription factor) (Pauls *et al.* 2005), which upon further characterization could uncover important biological information about gonocytes.

Immuno-staining is commonly and widely applied in biomedical investigations, and in laboratory diagnosis to locate specific antigens/biomarkers on/in cells and tissues. Fluorophore-conjugated antibodies enable the quantitative detection of the target antigens, often using multiple fluorescent probes simultaneously (Brandtzaeg 1998; De Matos *et al.* 2010). When multiple fluorescent probes are applied, attention should be given to the interference among fluorophores, especially to the auto-fluorescence (intrinsic fluorescence) present in certain cells or tissues which could interfere with the fluorescence signal of interest by creating false-positive results. Intrinsic fluorescence has been reported in neonatal mouse testis cells, with intensities comparable to the labelling fluorophores (Ohbo *et al.* 2003; Zheng *et al.* 2009), and in neonatal bovine testis cells, interfering with the purification of spermatogonia using fluorescence-activated cell sorting (Herrid *et al.* 2009a). Fluorescent granules were also observed in Leydig cells in testes of mature crossbred boars (Mabara *et al.* 1990).

In search for a specific biomarker for piglet gonocytes, we encountered an intense auto-fluorescence in both neonatal testis tissue and disassociated testis cells. This intrinsic fluorescence was not previously described in neonatal pig testis, and could blur the distinction between specific and non-specific immune-fluorescence signals, interfering with characterization of piglet gonocytes among dissociated testis cells. Therefore, in the present study, we examined the intrinsic fluorescence in piglet testis cells, and evaluated methods to mask such auto-fluorescence.

3.3 Materials and Methods

3.3.1 Testes Collection and Tissue Preparation

Testes were collected after castration of one-week-old, two-month-old and mature

Yorkshire-cross pigs (Camborough-22 × Line 65, PIC Canada Ltd., Winnipeg, MB, Canada) in a university-affiliated swine facility. Testes were then transferred on ice to the laboratory in Dulbecco's phosphate buffered saline (DPBS, cat. # 20-031-CV, Mediatech, Manassas, VA, USA) within 2 h after collection. On arrival, the testes were rinsed three times with DPBS, the tunica albuginea, rete testis and excessive connective tissue were then removed. Experimental procedures involving animals were approved by the University of Saskatchewan's Institutional Animal Care and Use Committee.

3.3.2 *In Situ* Detection of Auto-fluorescence

Small fragments of freshly collected piglet testis tissues in DPBS were gently disassociated into seminiferous cords using fine needles in culture dishes and examined for auto-fluorescence with both epifluorescent (Leica DMI 6000B equipped with A3, I3 and N2.1 filter cubes), and confocal laser scanning microscopes (Leica TCS SP5, Leica Microsystems, Mannheim, Germany) with a 20× objective and an excitation laser of 405 nm, and acquisition of signals from spectrums of 440-490 nm (blue), 495-570 nm (green), 575-620 nm (yellow) and 625-780 nm (red).

Testis tissue samples were also fixed in Bouin's solution, paraffin embedded and processed to prepare tissue sections using standard histological procedures. After deparaffinisation, rehydration, hematoxylin and eosin staining, slides were examined under both epifluorescent and laser scanning microscopes.

3.3.3 Auto-fluorescence Examination of the Isolated Piglet Testis Cells

Testis cells were collected using a two-step digestion method with minor modifications (Honaramooz *et al.* 2002a). Briefly, testis tissue pieces of approximately

100 mg were minced with fine scissors, and digested with 0.2% w/v collagenase IV (cat. # C-5138, Sigma-Aldrich, Oakville, ON, Canada), 0.1% w/v hyaluronidase (cat. # H-3884, Sigma-Aldrich) and 0.01% w/v DNase I (cat. # DN25, Sigma-Aldrich) in Dulbecco's modified Eagle's medium (DMEM, cat. # 10-013-CM, Mediatech) at 37 ℃ for 15 min with agitation every 5 min. After centrifugation at 15*g* at 16 ℃ for 1 min and discarding the supernatant, tissue pellets were further digested with 0.25% w/v trypsin with 2.21 mM EDTA (cat. # 25-053-CI, Mediatech) at 37 ℃ for 5 min. Undiluted foetal bovine serum (FBS, cat. # A15-701, PAA Laboratories GmbH, Etobicoke, ON, Canada) was added to stop the digestion, and the cell suspension triturated with a 1 ml pipette tip before filtration through a 40 μm filter (cat. # 352340, BD Biosciences, Mississauga, ON, Canada). Erythrocytes were then removed with a lysis buffer containing NH_4CL 156mM, $KHCO_3$ 10 mM, Na_2EDTA 0.1mM (Sethu *et al.* 2004; Smith *et al.* 2009) at a ratio of 4:1 (buffer: cell suspension) for 15 min at room temperature. After centrifugation at 600*g* for 4 min and rinsing with 10 mL of 10% FBS-DMEM, newly disassociated cells were suspended in DPBS and smeared onto poly-D-lysine treated slides. A subset of slides were examined immediately using both epifluorescent and laser scanning microscopes, while the remaining slides were dried in air and stored at -20 ℃ for auto-fluorescence blocking and DBA staining. Flow cytometry (Partec CyFlow Space, Partec GmbH, Münster, Germany) was also used to characterize the auto-fluorescence of the freshly isolated testis cells after staining with 4,6-diamino-2-phenyl indole (DAPI, cat. # D-9542, Sigma-Aldrich) for 3 min. To probe the auto-fluorescence spectrum, serial excitation lasers (with wavelengths of 405, 458, 476, 488, 514, 543 or 633 nm) with emission wavelengths ranging from 415 to 800 nm were applied to the freshly isolated testis cells

using a confocal laser scanning microscope. The auto-fluorescence intensity following different excitation and emission wavelengths was subjectively evaluated.

3.3.4 Duration of Auto-fluorescence in Cultured Testis Cells

Freshly isolated testis cells were cultured in 6-well plates with cover slides at the bottom of plates, in DMEM containing 10% FBS at 37 ℃ in a 5% CO_2 humidified atmosphere for 6 days. Every 24 hours, during the culture, cover slides (n = 3/day) with cells on top were collected, rinsed with DPBS, and auto-fluorescence detected using a laser scanning microscope using excitation with a 405 nm laser line.

3.3.5 Elimination of the Auto-fluorescence for Identification of Gonocytes *in Situ*

After deparaffinisation and rehydration, the processed testis tissue sections were rinsed with DPBS and incubated with 5% w/v bovine serum albumin (BSA, cat. # A7638, Sigma-Aldrich) in DPBS at 37 ℃ for 30 min in humidified atmosphere, and stained overnight with a fluorescein-conjugated lectin Dolichos biflorus agglutinin (DBA) (Goel *et al.* 2007) (1:100, cat. # FL-1031, Vector Labs, Burlington, ON, Canada) in humidified atmosphere. After rinsing with DPBS, the sections were incubated with 0.3% w/v Sudan Black B (SBB, cat. # 3545-12, EMD Chemicals, Gibbstown, NJ, USA) in 70% ethanol at 37 ℃ for 0, 3, 5, 8, 10, 12, 15 or 20 min in humidified atmosphere, rinsed with DPBS and stained with DAPI for 3 min. The sections were then mounted (Cat# H-1000, Vector Labs) and examined by a laser scanning confocal microscope for sequential scanning and detection of DAPI and fluorescein, respectively, followed by merging and saving of the images.

3.3.6 Elimination of the Auto-fluorescence for Identification of Gonocytes *in Vitro*

After thawing at room temperature, cell smears were fixed in Bouin's solution for 2-3 min, rinsed in DPBS, blocked using 5% BSA for 15 min at 37 ℃ in humidified atmosphere, rinsed again with DPBS and incubated with a fluorescein-labelled DBA overnight (1:100) in humidified atmosphere. After rinsing with DPBS and incubation with 0.3% SBB in 70% ethanol for 0, 3, 5, 8, 10, 12, 15 or 20 min, cell smears were rinsed in DPBS, stained with DAPI for 2 min, mounted and observed under a laser scanning confocal microscope. DAPI and fluorescein were sequentially scanned using a laser scanning microscope using excitation with a 405 nm laser.

3.4 Results

3.4.1 *In Situ* Auto-fluorescence

An intense fluorescence was consistently detected by both epifluorescence and confocal laser scanning microscopes in testes from neonatal, pre-pubertal and mature pigs (Fig. 3.1-3.3). The auto-fluorescence was exclusively observed in the testis interstitial cells *in situ*, mainly in the cytoplasm of most Leydig cells that contained strongly fluorescent intracellular granules; although some Leydig cells did not demonstrate the auto-fluorescent granules (Fig. 3.1-3.3).

3.4.2 Auto-fluorescence in Disassociated Testis Cells

When freshly isolated testis cells were observed using fluorescent or confocal laser scanning microscope, a strong auto-fluorescence was detected in the cytoplasm of some small and large round cells (~10 μm *vs.* ~20 μm), but the fluorescence intensity differed among the observed auto-fluorescent testis cells (Fig. 3.4). The intrinsic

fluorescence signal was also detectable in testis cells using flow cytometry, with emission wavelengths overlapping those of fluorophores such as FITC, PE and Alexa 647 (Fig. 3.5).

3.4.3 Emission Wavelength of the Auto-fluorescence

Excitation lasers of different wavelengths affected the auto-fluorescence emission wavelengths of the freshly isolated interstitial cells. *In situ* auto-fluorescence was detected with emission wavelengths ranging from 425 to 700 nm with strong signals between 480 and 620 nm, spanning the spectrums of green, yellow and red, thereby interfering with the most commonly used fluorophores (Fig. 3.6).

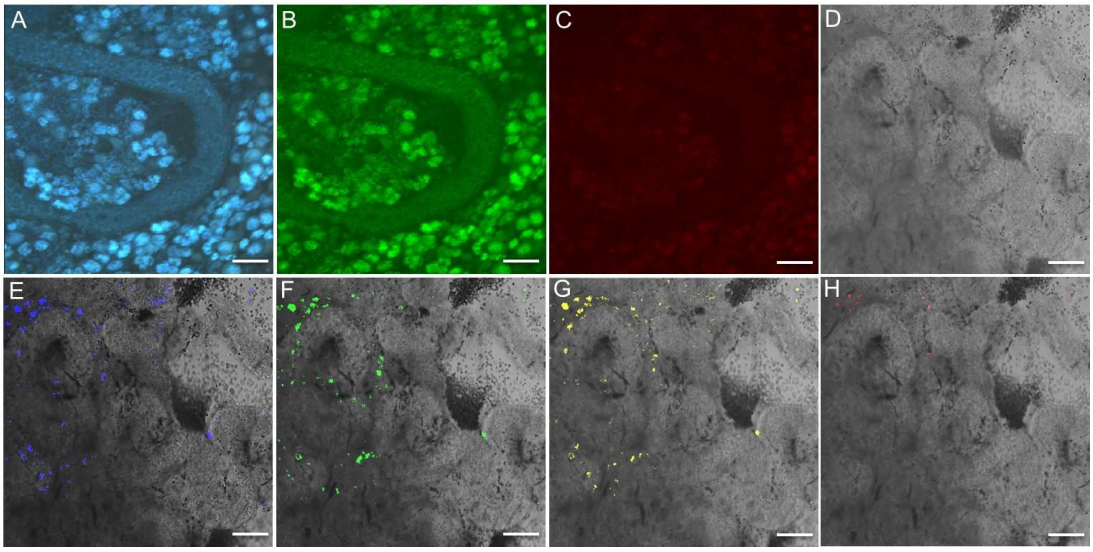


Fig. 3.1. Auto-fluorescence observed in whole-mount seminiferous cords by epifluorescent and confocal laser scanning microscopes. Seminiferous cords were dissociated from 1-wk-old piglet testes and examined under an epifluorescent microscope (A-C) equipped with filters of A (A), I3 (B) and N21 (C). Confocal laser scanning microscopy with excitation laser of 405 nm, brightfield (D) or brightfield overlaid with acquired signal from spectrums of blue (440-490 nm, E), green (495-570 nm, F), yellow (575-620 nm, G) and red (625-780 nm, H). Scale bars, 100 μm.

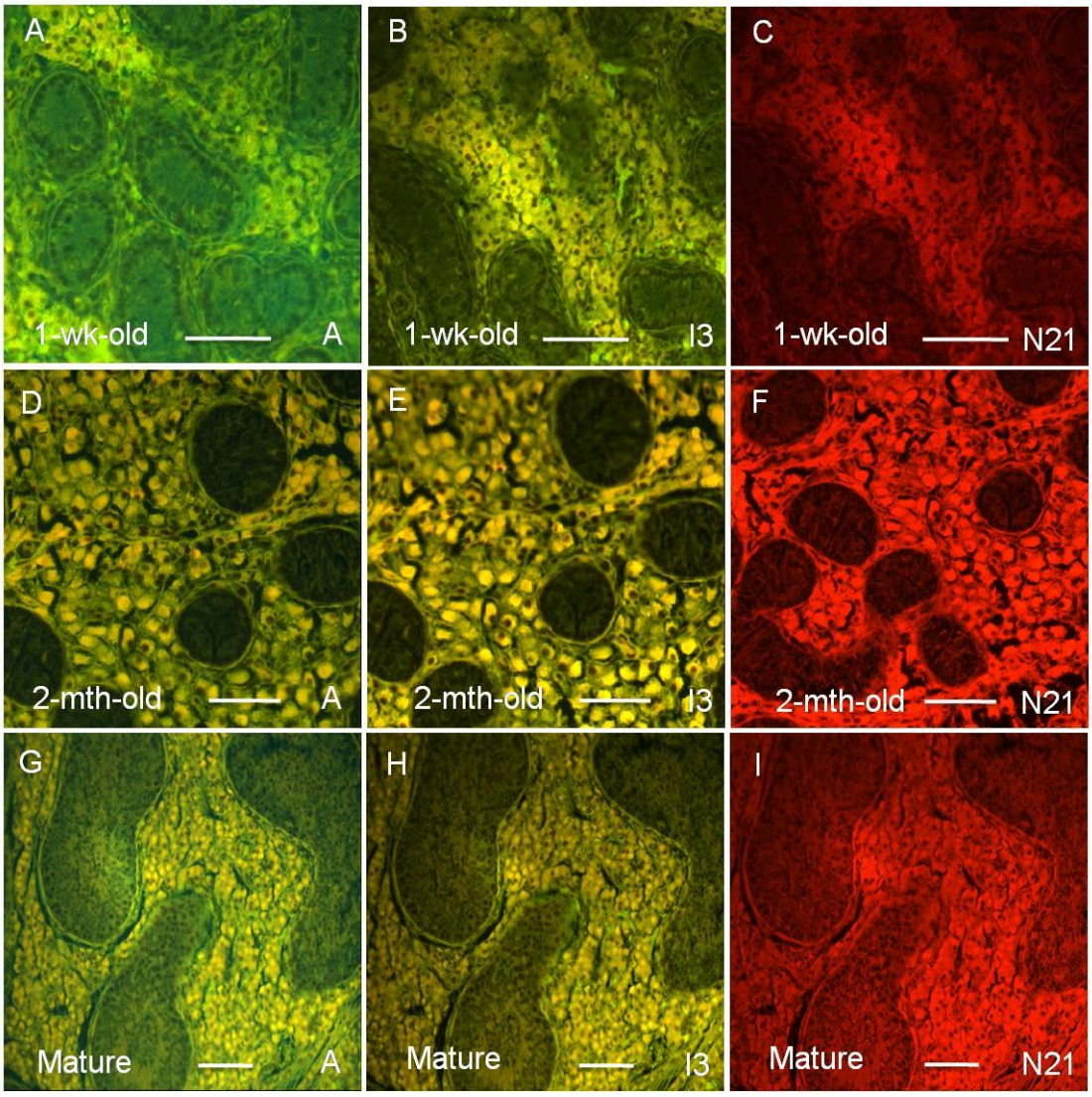


Fig. 3.2. Auto-fluorescence in testis tissue sections from pigs of different ages examined using a epifluorescent microscope. Testis tissues from 1-wk-old (A-C), 2-month-old (D-F) and mature (G-I) pigs were fixed, sectioned and examined for intrinsic fluorescence using an epifluorescent microscope equipped with filters of A (A, D, G), I3 (B, E, H) and N21 (C, F, I). Scale bars, 100 μm.

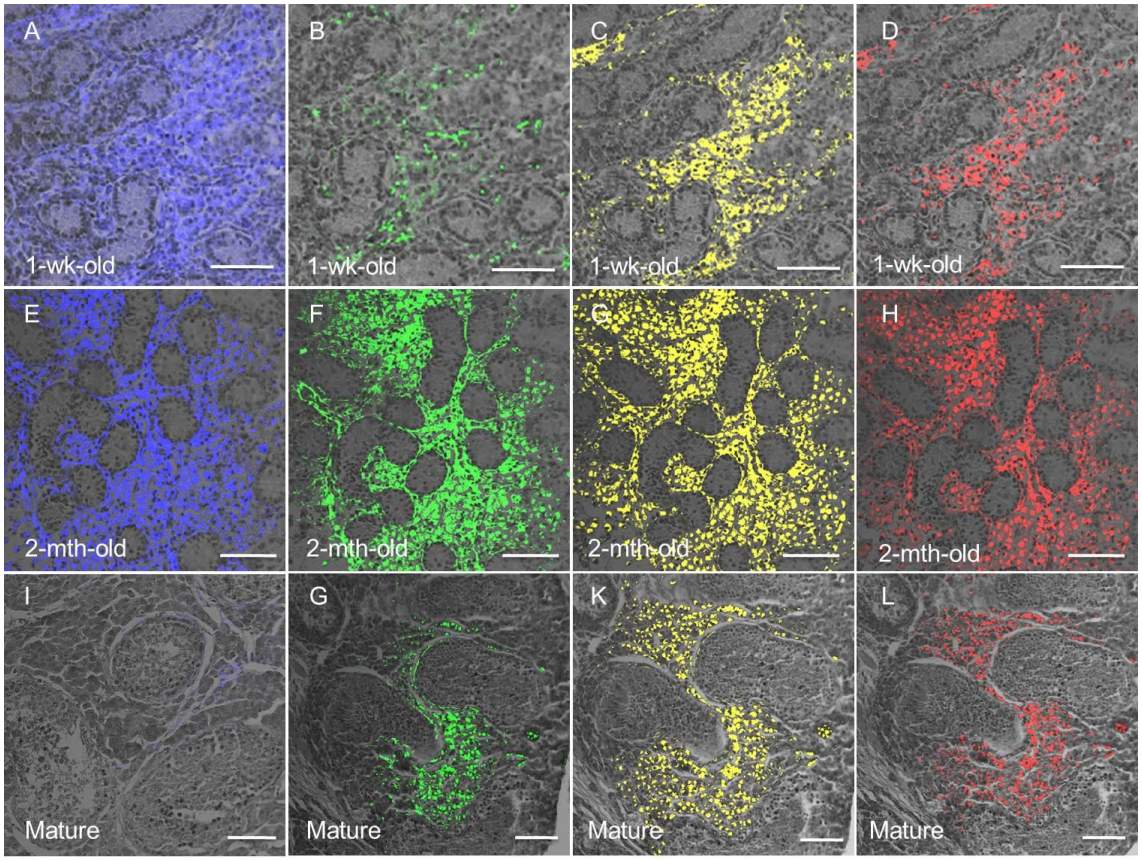


Fig. 3.3. Auto-fluorescence in testis tissue sections from pigs of different ages observed using a confocal laser scanning microscope. Testis tissues from 1-wk-old (A-D), 2-month-old (E-H) and mature (I-L) pigs were fixed, sectioned and examined for intrinsic fluorescence under a confocal laser scanning microscope with excitation by a 405 nm laser and acquisition of signal from spectrums of blue (440-490 nm; A, E, I), green (495-570 nm; B, F, G), yellow (575-620 nm; C, G, K) and red (625-780 nm; D, H, L) with brightfield overlay. Scale bars, 100 μm.

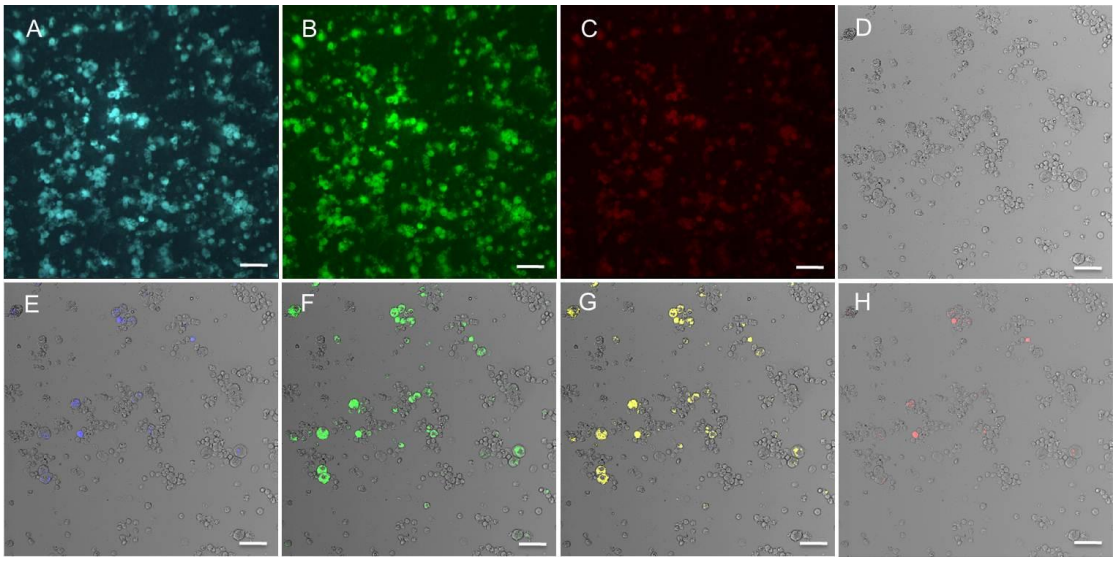


Fig. 3.4. Auto-fluorescence detected in freshly isolated testis cells using epifluorescent and confocal laser scanning microscopes. Testis cells were isolated from 1-wk-old piglet testes and examined under an epifluorescent microscope (A-C) equipped with filters of A (A), I3 (B) and N21 (C). Confocal laser scanning microscopy using an excitation laser of 405 nm, brightfield (D) or brightfield overlaid with acquired signals from spectrums of blue (440-490 nm, E), green (495-570 nm, F), yellow (575-620 nm, G) and red (625-780 nm, H). Scale bars, 50 μm.

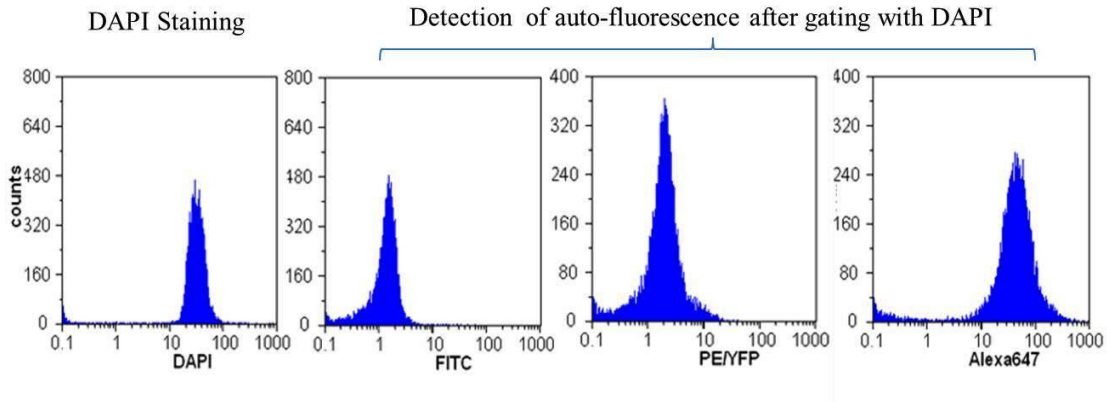


Fig. 3.5. Flow cytometry analysis of auto-fluorescence in freshly isolated testis cells. Testis cells were isolated from 1-wk-old piglet testes, stained with DAPI, and assayed with a flow cytometer equipped with detectors for FITC, PE/YFP and Alexa647 with DAPI gating to specifically detect auto-fluorescence from piglet testis cells.

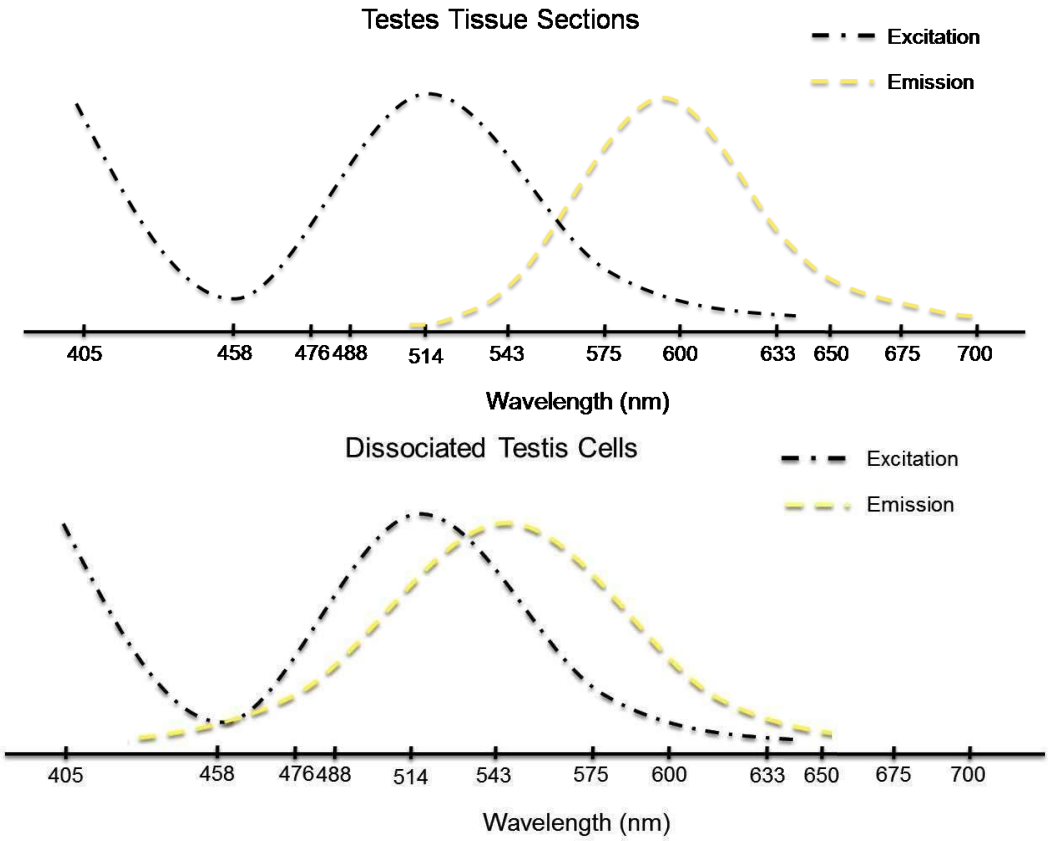


Fig. 3.6. Auto-fluorescence spectrum assessed using a confocal laser scanning microscope. Piglet testis tissue sections and dissociated cells were excited with lasers of 405, 458, 476, 488, 514, 543 and 633 nm. Emission wavelengths excited at 405 nm were probed every 10 nm ranging from 410 to 750 nm and the signal intensity was subjectively evaluated. Intensity of the signal is shown as tendency and may not demonstrate the actual signal strength.

3.4.4 Auto-fluorescence in Cultured Testis Cells

To determine the fate of the auto-fluorescence *in vitro*, testis cells were cultured for 6 days and examined daily with epifluorescent and confocal laser scanning microscopes. Although the auto-fluorescence was consistently observed for at least 6 days, the extent and intensity tended to decrease overtime (Fig. 3.7).

3.4.5 Quenching of the Auto-fluorescence with Sudan Black in Gonocyte Identification

When SBB was applied to testis cells *in situ* and *in vitro*, the expressed auto-fluorescence was completely blocked after staining for approximately 12 or 8 min, respectively (Fig. 3.8). Compared with non-treated testis cells, Sudan Black completely quenched the auto-fluorescence in testis cells both *in situ* and *in vitro*, while not blocking the specific staining of gonocytes with DBA (Fig. 3.9).

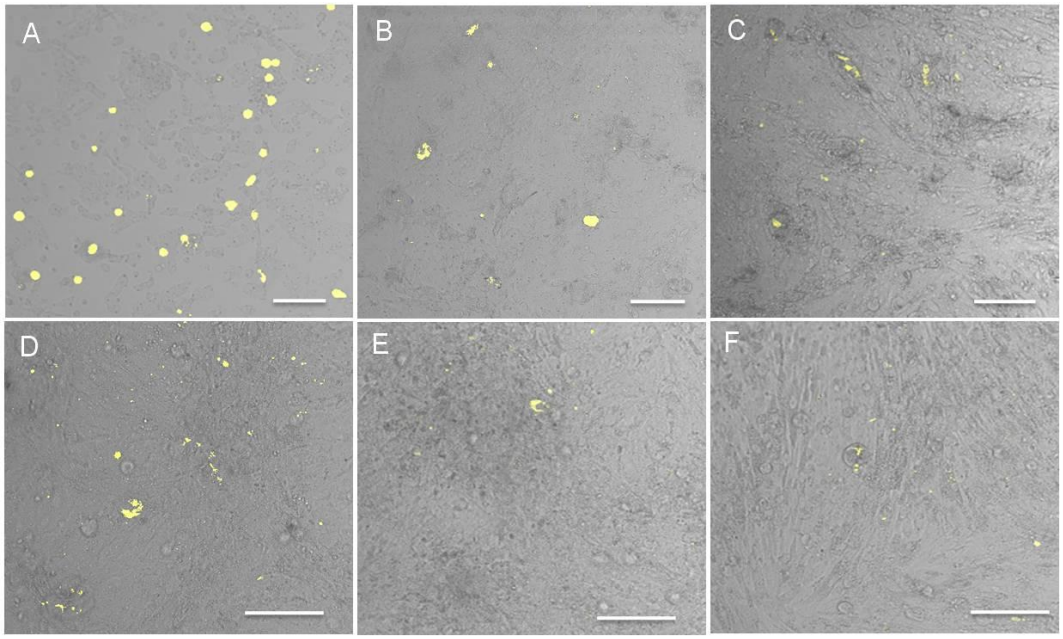


Fig. 3.7. Auto-fluorescence in cultured testis cells. One-wk-old piglet testis cells were cultured *in vitro* for 6 days and examined for auto-fluorescence using a confocal laser scanning microscope, and excited with a 405 nm laser and detection of emissions within 575-620 nm (yellow with brightfield overlay, A-F corresponding to 1-6 days post-culture, respectively). Scale bars, 100 µm.

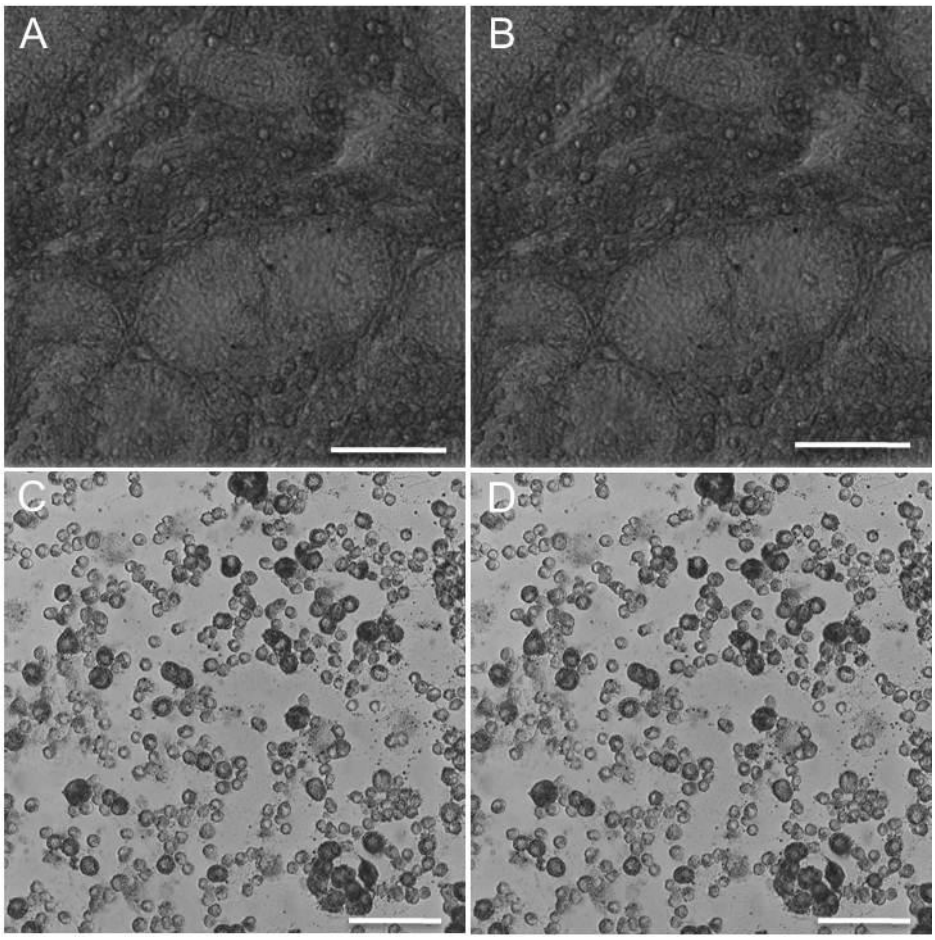


Fig. 3.8. Auto-fluorescence blocked with Sudan Black B staining of testis cells *in situ* and *in vitro*. Piglet testis tissue sections (A, B) and dissociated cells (C, D) were stained with Sudan Black B, examined under a bright-field microscope (A, C), and a confocal laser scanning microscope, excited with a 405 nm laser with detection of emissions within 575-620 nm (B, D) (with brightfield overlay). Scale bars, 100 μm.

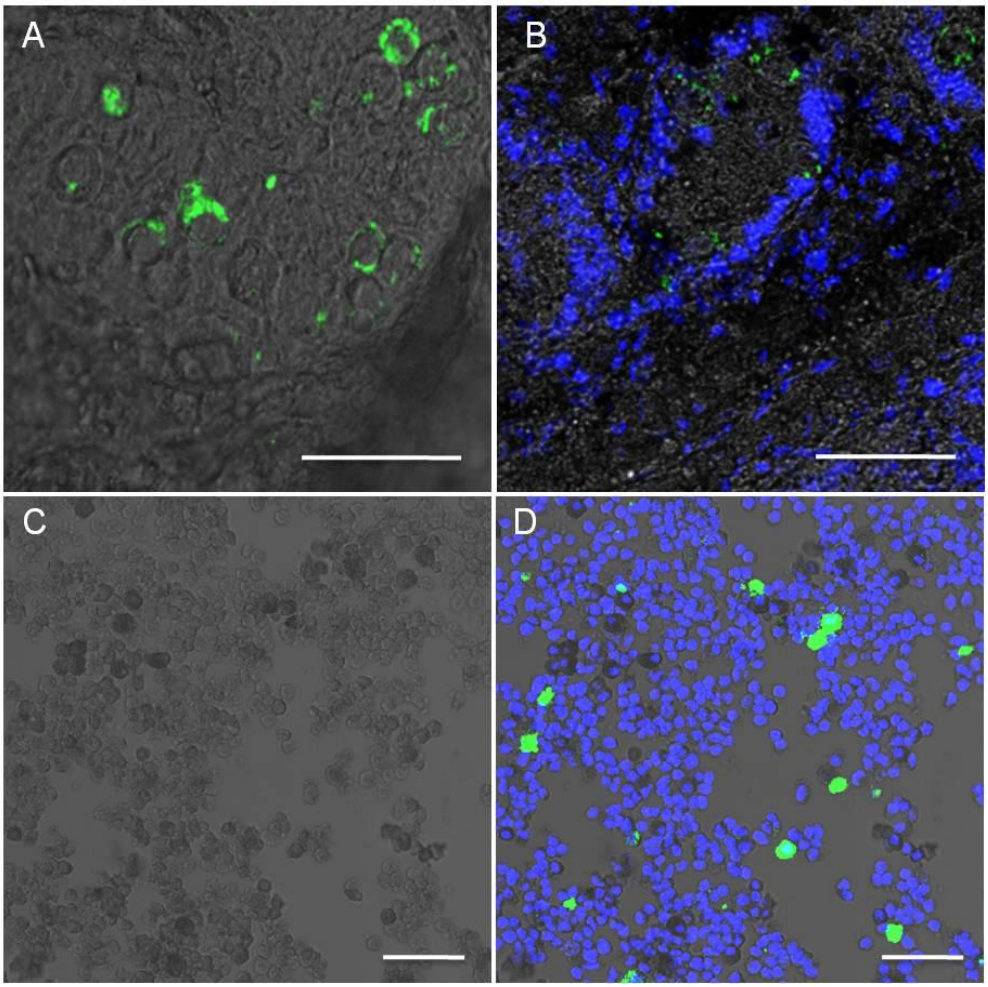


Fig. 3.9. Identification of gonocytes with DBA staining, following the masking of auto-fluorescence by Sudan Black B *in situ* and *in vitro*. Piglet testis tissue sections (A, B) and dissociated cells (C, D) were stained with FITC-labelled lectin DBA and DAPI, followed by Sudan Black B staining and imaging with a confocal laser scanning microscope with brightfield overlay. Scale bars, 100 μm.

3.5 Discussion

Auto-fluorescence within the target tissue or cells could interfere with detection of specific signals from the labelling fluorophores, leading to inaccurate or even false-positive results. In preliminary observations, we noticed that because of the strong innate auto-fluorescence in testis tissue/cells, identifying piglet gonocytes using fluorescence staining was difficult if not impossible. In the present study, we observed that the auto-fluorescence was limited to the interstitial tissue/cells of the testis (Fig. 3.1-3.3), and the source was primarily the intrinsically-fluorescent granules within the cytoplasm of Leydig cells. Gonocytes did not emit fluorescence. The auto-fluorescence had a wide excitation and emission spectrum which decreased in intensity during testis cell culture (Fig. 3.6 and 3.7). Treatment of the testis tissue and cells with the lysochrome SBB completely masked the intrinsic fluorescence while allowing the identification of gonocytes through detection of specific signals from immuno-fluorescence methods (Fig. 3.8 and 3.9).

Throughout our observations, the intrinsic fluorescence was consistently detected in interstitial cells in both *in vitro* and *in situ* (i.e., in freshly disassociated testis cells, tissue whole mounts, tissue sections and testis cell culture). This may imply that the observed auto-fluorescence was indeed intrinsic to the cells and not acquired during the processing.

When mice were under stress treatment (noise exposure), similar granules were reported to form and accumulate particularly in Leydig cells, and lipofuscin was suggested as the fluorescent substance in the granules (Ruffoli *et al.* 2006). Lipofuscin is a non-degradable auto-fluorescent pigment (composed mostly of lipid and protein) which could not be exported from the cells; therefore, accumulates with increasing age in the

cells and is stored in the lysosomes as waste materials (Brunk and Terman 2002; Terman and Brunk 2004). Although lipofuscin was described more than a century ago, its fluorescence properties are not well-defined. Auto-fluorescence with broad excitation and emission wavelengths is considered typical of lipofuscin, and its quenching by lipid staining (e.g., SBB) was suggested to indicate the lipofuscin origin of the auto-fluorescence (Brunk and Terman 2002; Monserrat *et al.* 1995; Schnell *et al.* 1999; Terman and Brunk 2004). Therefore, the fact that the auto-fluorescence in the present study had a broad spectrum (425 to 700 nm with solid signal from 480 to 620 nm, and was observable with widely used filters) and was quenchable with SBB, supports our speculation that lipofuscin is mainly responsible for the auto-fluorescence in the piglet testes.

Staining of neural tissue sections with Sudan Black B was reported to be capable of completely eliminating the auto-fluorescence, without causing the loss of the specific fluorescence signal in immune-staining (Romijn *et al.* 1999; Schnell *et al.* 1999). In the present study, SBB also completely masked the intrinsic fluorescence of pig testis cells both *in situ* and *in vitro*, but allowed the identification of gonocytes with fluorescein-labelled DBA. While degradation and exocytosis do not remove lipofuscin from the cells, mitotic division is the only reported mechanism that can reduce the lipofuscin concentration within cells (Brunk and Terman 2002; Terman 2001; Terman and Brunk 2004). In the present study, decreasing auto-fluorescence was observed over the duration of the testis cells culture. We speculate that the lipofuscin content of the cells was divided into the newly formed cells.

In conclusion, we detected and characterized an intrinsic fluorescence in piglet testes and showed that the use of SBB can completely quench this auto-fluorescence, allowing identification of specific testis cells by immune-fluorescence.

CHAPTER 4 DEVELOPMENT OF NOVEL STRATEGIES FOR THE ISOLATION OF PIGLET TESTIS CELLS WITH HIGH PROPORTION OF GONOCYTES[1]

4.1 Abstract

Gonocytes have germline stem cell potential and are present in the neonatal testis, comprising 5-10% of freshly isolated testis cells. Maximising the number and proportion of gonocytes among freshly isolated testis cells will greatly facilitate their subsequent purification and *in vitro* study and manipulation. Seven experiments were conducted to evaluate the effects of multiple factors on the efficiency of testis cell isolation from neonatal pigs. We found that the use of a lysis buffer led to elimination of erythrocytes without adversely affecting testis cell isolation. Approximately nine-fold as many live cells could be harvested by enzymatic digestion of testis tissues compared with mechanical methods. Digestion with collagenase-hyaluronidase-DNase followed by trypsin resulted in the highest recovery of live cells. However, the proportion of gonocytes (~7%) did not differ between the mechanical and enzymatic methods of testis cell isolation. Pre-treatment of the tissue with cold enzymes increased the recovery of live testis cells. New strategies of combining a gentle enzymatic digestion with two rounds of vortexing resulted in the isolation of testis cells with a very high gonocytes proportion. The efficiency of these novel methods could be further optimised to collect testis cells with a gonocytes proportion of approximately 40%. This novel three-step testis cell

[1]This study has been published. Y. Yang, M. Yarahmadi and A. Honaramooz (2010). *Reprod. Fertil. Dev.* 22(7): 1057-1065. Portions of this study were also presented at the 41st and 42nd annual meetings of the Society for the Study of Reproduction.

isolation strategy can be completed within 1 h and can harvest approximately 17×10^6 live gonocytes per g testis tissue. Therefore, in addition to elucidating the effects of several factors on testis cell isolation, we developed a novel strategy for the isolation of testis cells that yielded approximately 40% gonocytes in the freshly isolated cells (i.e., four- to eightfold higher than the proportions obtained using strategies reported by others). This strategy has instant applications in the purification of gonocytes.

4.2 Introduction

Neonatal testis contains interstitial tissue and seminiferous cords, with gonocytes as the only type of germ cells present (Franca *et al.* 2000; Frankenhuis *et al.* 1981; Goel *et al.* 2007; Hughes and Varley 1980; Ryu *et al.* 2004). Gonocytes give rise to spermatogonial stem cells (SSCs) that form the foundation of spermatogenesis and are responsible for a lifetime supply of spermatozoa (de Rooij 1998; Oatley and Brinster 2008).

In the testis of early postnatal pigs, as in most other domestic species, gonocytes reside mostly in the centre of the seminiferous cords, surrounded by Sertoli cells. Gonocytes then migrate gradually towards the periphery of the cords remaining in close contact with Sertoli cells and peritubular myoid cells at the basement membrane to form the stem cell niche (Pelliniemi 1975; Van Straaten and Wensing 1977; Van Vorstenbosch CJ 1984).

Similar to SSCs, gonocytes have been shown to colonise and generate donor-derived spermatogenesis, after transplantation into seminiferous tubules of recipient mice and rats (Ohbo *et al.* 2003; Ohmura *et al.* 2004; Ohta *et al.* 2004; Shinohara *et al.* 2002b). This supports the notion that gonocytes, along with SSCs, possess germline stem cell

potential (Jiang 2001; Jiang and Short 1998b). Therefore, the isolation of gonocytes has important implications for the *in vitro* study and manipulation of these germline stem cells.

The separation of testis cells is the first step in the subsequent enrichment, modification and transplantation of male germline stem cells (de Rooij and Mizrak 2008; Dobrinski and Travis 2007; Khaira *et al.* 2005; Oatley and Brinster 2008). Transplantation of genetically modified gonocytes and/or SSCs into recipient testes has the potential to modify the future gametes of the individual (Honaramooz *et al.* 2003a; Honaramooz *et al.* 2008).

Depending on the cells of interest or application, a range of strategies has been used to isolate testis cells; however, the efficiency of cell recovery may become a limiting factor. Current cell separation methods usually result in low proportions of gonocytes and/or spermatogonia in freshly isolated testis cells (usually 5-10% of the cell population, (Herrid *et al.* 2009a; Li *et al.* 1997; Lo *et al.* 2005; Luo *et al.* 2006; Orth and Boehm 1990; Orwig *et al.* 2002b; Van Dissel-Emiliani *et al.* 1989). Therefore, the main objectives of the present study were to investigate different approaches to testis cell isolation, and to develop new strategies to maximise the proportion of live gonocytes in freshly isolated porcine testis cells.

4.3 Materials and Methods

4.3.1 Study Design

The effects of several factors on the outcome of testis cell separation were evaluated through seven consecutive experiments with at least seven replicates per

experiment) to develop efficient strategies for maximising the recovery of freshly isolated testis cells with a high proportion of gonocytes. To diminish the effect of erythrocytes that are inevitably isolated along with the cells on the results of testis cell isolation, we initially investigated the use of an erythrocyte lysis buffer. We then tested whether piglet donors from different litters would provide a consistent source for the supply of testis tissue. Thereafter, we studied the application of different mechanical dissociation methods; and enzymatic digestions: (1) one-step *vs.* two-step enzymatic digestion; (2) tissue pre-treatment with cold enzymes; (3) hydrostatic pressurisation. Ultimately, we devised and further optimised a novel strategy for the isolation of testis cells with a high proportion of gonocytes.

4.3.2 Testes Collection and Tissue Preparation

Testes from 1-week-old Yorkshire-cross piglets (Camborough-22 × Line 65, PIC Canada, Winnipeg, MB, Canada) were collected from a University-affiliated swine facility after aseptic castration of the piglets. The testes were transferred to the laboratory on ice in Dulbecco's phosphate buffered saline (DPBS, cat. # 20-031-CV, Mediatech, Manassas, VA, USA) containing 2% w/v antibiotic-antimycotic solution (cat. # 30-004-CI, Mediatech) within 2 h of collection. On arrival, the testes were rinsed three times with DPBS and the tunica albuginea, rete testis and excessive connective tissue removed. The testis parenchyma was then cut into pieces of approximately 100 mg (unless stated otherwise) to be used in each cell isolation method. The experimental procedures in the present study that involved animals were approved by the University of Saskatchewan's Institutional Animal Care and Use Committee.

4.3.3 Experiment 1: Depletion of Erythrocytes

In preliminary experiments, we encountered large numbers of erythrocytes mixed with the isolated testis cells that could interfere with the accuracy of assessing cell isolation results. Therefore, we aimed to remove these blood cells. The efficiency of an erythrocyte depletion lysis buffer (composition: 156 mM NH_4CL, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA; (Sethu *et al.* 2004; Smith *et al.* 2009) was evaluated at different ratios using two different enzymatic digestion methods. The assessment was based on observing the abundance of the remaining erythrocytes, and comparing testis cell viability and or yield after the application of the lysis buffer at different ratios. To reduce variations, pieces of testis tissue used for comparisons were cut from the same testis, minced and digested first with 0.2% collagenase IV (cat. # C-5138, Sigma-Aldrich, Oakville, ON, Canada) and 0.01% DNase I (0.2% w/v, cat. # DN25, Sigma-Aldrich) in Dulbecco's modified Eagle's medium (DMEM, cat. # 10-013-CM, Mediatech) at 37 ℃ for 15 min, with agitation every 5 min. After the removal of the supernatant by brief centrifugation (15*g* for 1 min at 16 ℃), a second round of digestion (0.2% collagenase IV and 0.01% DNase in DMEM) was applied for 30 min with occasional agitation. The duration of these digestion procedures were determined on the basis of our monitoring the progress of digestion under a microscope in preliminary experiments. Digestion was then stopped by the addition of foetal bovine serum (FBS, cat. # A15-701, PAA Laboratories GmbH, Etobicoke, ON, Canada), and the suspension was triturated with 1 mL pipette tips before filtration through a 40 µm nylon filter (cat. # 352340, BD Biosciences, Mississauga, ON, Canada). The volume of the cell suspension was made up to 5 mL by the addition of 10% v/v FBS in DMEM.

Different volumes of the erythrocyte lysis buffer (0 mL, control, (0:1 ratio of lysis buffer: cell suspension; 10 mL (2:1), 20 mL (4:1) and 30 mL (6:1)) were added to the samples and the tubes were then maintained at room temperature for 15 min. After centrifugation at 500*g* for 5 min, the cell suspensions were rinsed with DPBS and made up to a final volume of 5 mL with 10% FBS in DMEM. Cell viability and yield were then assessed with a 0.4% w/v Trypan blue solution (cat. # T8154, Sigma-Aldrich) using a haemocytometer.

To validate the results, a different digestion method (Method 7 as in Table 4.1) was also applied in which, after the first round of digestion, tissue clumps were further digested with 0.25% w/v trypsin with 2.21 mM EDTA, (cat. # 25-053-CI, Mediatech) at 37 ℃ for 5 min. The remaining procedures and application of the lysis buffer were as described above. Throughout the entire study, erythrocytes were not included in any of the cell isolation data, regardless of whether the lysis buffer had been used.

4.3.4 Experiment 2: Variation of Donor Testes

Testis tissues collected from littermate piglets and piglets of different litters were used to determine the potential variation among donor tissues. A two-step enzymatic digestion procedure as in Method 7 described above was applied, with the erythrocyte lysis buffer used at a ratio of 4:1.

4.3.5 Experiment 3: Mechanical Dissociation of Testis Tissue

Mechanical approaches to tissue dissociation are usually less costly, less time-consuming and require less laboratory resources. Therefore, several mechanical strategies were compared for their efficiency and yield of isolated testis cells, as described below:

4.3.5.1 Mincing (Method 1): Testis tissue pieces immersed in 0.5 mL of 10% FBS-DMEM were cut using fine scissors into sizes small enough to pass through a 1 mL pipette tip. An additional volume (up to 4.5 mL) of 10% FBS-DMEM was added to the tissues and the tissue clumps were triturated three to five times with 1 mL pipette tips.

4.3.5.2 Teasing (Method 2): Testis tissue pieces were torn apart into tubular fragments in 1 mL of 10% FBS-DMEM using fine needles (27-gauge) attached to 1 mL syringes. After the addition of 4 mL of 10% FBS–DMEM, the tissue clumps were triturated.

4.3.5.3 Sieving (Method 3): Testis tissue pieces were sieved with a 60 μm mesh (Cell Dissociation Sieve, cat. # CD1, Sigma-Aldrich) according to the manufacturer's instructions. The mesh was then rinsed with 10% FBS-DMEM and the cell suspension triturated.

4.3.5.4 Grinding (Method 4): Testis tissue pieces underwent five rounds of grinding using a tissue grinder (cat. # 7727-7, Corning, Corning, NY, USA), and the grinder was rinsed with 10% FBS-DMEM and the cell suspensions were triturated.

Cell solutions collected using each of the mechanical dissociation strategies described above were made up to a final volume of 5 mL with 10% FBS DMEM. After pipetting with 1mL pipette tips and filtration through 40 μm filters, the erythrocyte lysis buffer was added at a ratio of 4:1. Cell viability and yield were assayed using the Trypan blue exclusion method. Cell smears were prepared routinely from each dissociation strategy, air-dried at room temperature and stored at -80 ℃ for subsequent immunocytochemistry.

4.3.6 Experiment 4: Enzymatic Digestion of Testis Tissue

Testis tissues were cut with fine scissors, and digested with four different enzymatic digestion methods (Methods 5-8) Table 4.1. The common procedures were performed as described above for enzymatic digestions. For methods in which hyaluronidase (0.2% w/v in DMEM, cat. # H-3884, Sigma-Aldrich) was used for tissue digestion, it was added along with collagenase and DNase. Cell viability and yield were determined as described above; in addition, cell smears preparations were prepared as described above.

4.3.7 Experiment 5: Testis Tissue Pre-treatment with Cold Enzymes and/or Hydrostatic Pressurisation

To further enhance the efficiency of enzymatic digestions, we examined the potential effects of incubating minced testis fragments with enzymes at 4 ℃ for 5 h (Methods 9-12, table 4.2). The mixture of enzymes and tissue clumps was then transferred into a 37 ℃ water bath to allow the enzymes to start the digestion.

In addition, a set up as shown in Fig 4.1 was developed with the aim of increasing the hydrostatic pressure and to potentially increase the penetration of enzymes (as listed in Table 4.2) into the tissue. Cell viability and yield were assayed and compared after treatments. Cell smears were also prepared and frozen for immunocytochemistry at a later time.

4.3.8 Experiment 6: Combination of Enzymatic Digestion and Vortexing

After assessing the results of the experiments described above, we devised a new three-step strategy to increase the proportion of gonocytes in the collected testis cells.

Briefly, approximately 600 mg testis parenchyma was cut into small pieces with fine scissors and suspended in 5 mL DPBS. The tissue was then vortexed for 1 min with a test tube shaker (Reax Top, cat. # 541-10000, Heidolph Instruments, Essex, UK) at 500 r.p.m. After allowing the tissue fragments and cell clumps to settle for approximately 30 s, the supernatant was removed, the tissue was rinsed with DPBS, and digested using the methods listed in Table 4.3 (Methods 13-16). Digestion was stopped with the addition of FBS, followed by the addition of DPBS to a final volume of 5 mL, vortexing for a second time, filtration of the cell suspension, and depletion of erythrocytes. Cell viability and yield were then assayed and cell smears were frozen and stored.

4.3.9 Experiment 7: Optimization of the New Three-step Method

After evaluating the results of Experiment 6, one of the methods (Method 13; Table 4.3) was selected and further optimised (Methods 17-19; Table 4.4).

4.3.10 Immunohistochemistry

Fresh testis tissue fragments were fixed in Bouin's solution for 24 h, followed by rinsing with and storing in 70% ethanol, processing for standard histological procedures, embedding in paraffin and sectioned at 4 μm. After deparaffinisation and rehydration, sections were rinsed with DPBS and incubated with 5% w/v bovine serum albumin (BSA, cat. # A7638, Sigma-Aldrich) in DPBS at 37 ℃ for 30 min in humidified atmosphere, and stained with the lectin Dolichos biflorus agglutinin (DBA, Goel *et al.* 2007) conjugated with fluorescein, 1:100, cat. # FL-1031, Vector Laboratories, Burlington, ON, Canada) overnight in humidified atmosphere. After rinsing with DPBS, sections were incubated with 0.3% w/v Sudan Black B (cat. # 3545-12, EMD Chemicals,

Gibbstown, NJ, USA) in 70% ethanol at 37 ℃ for 8 min in humidified atmosphere, rinsed with DPBS and stained with 4,6-diamino-2-phenyl indole (DAPI, cat. # D-9542, Sigma-Aldrich) for 3 min. The sections were then mounted and examined using fluorescent microscope. Laser scanning confocal microscope was also used to sequentially scan DAPI and fluorescein, and fluorescent images were overlaid with brightfield images.

4.3.11 Immunocytochemistry

After thawing at room temperature, the cell smears were fixed in Bouin's solution for 2 to 3 min, rinsed in DPBS, blocked with 5% BSA at 37 ℃ for 15 min in humidified atmosphere, rinsed again with DPBS and incubated with fluorescein labelled lectin DBA (1:100) overnight in humidified atmosphere. After rinsing with DPBS and incubation with 0.3% Sudan Black B for 10-15 min, the cell smears were rinsed and stained with DAPI for 2 min, before being mounted with a mounting medium (Vectashield, cat. # H-1000, Vector Labs) and observed using fluorescent and laser scanning confocal microscope. At least 600 cells were counted for each cell smear to determine the proportion of gonocytes.

Table 4.1. Methods used for enzymatic digestion of the testis tissue.

Method no.	Description
5	Collagenase-DNase 15 min
6	Collagenase-DNase 15 min + collagenase-DNase 30 min
7	Collagenase-DNase 15 min + trypsin 5 min
8	Collagenase-hyaluronidase-DNase 15 min + trypsin 5 min

Table 4.2. Enzymatic digestions after pretreatment of testis tissue with cold enzymes and/or hydrostatic pressurization.

Method no.	Description
9	Cold collagenase-DNase for 5 h & 15 min digestion
10	Cold collagenase-hyaluronidase-DNase for 5 h & 15 min digestion + trypsin 5 min
11	Pressurized-cold collagenase-DNase for 5 h & 15 min digestion
12	Pressurized-cold collagenase-hyaluronidase-DNase 5 h & 15 min digestion + trypsin 5 min

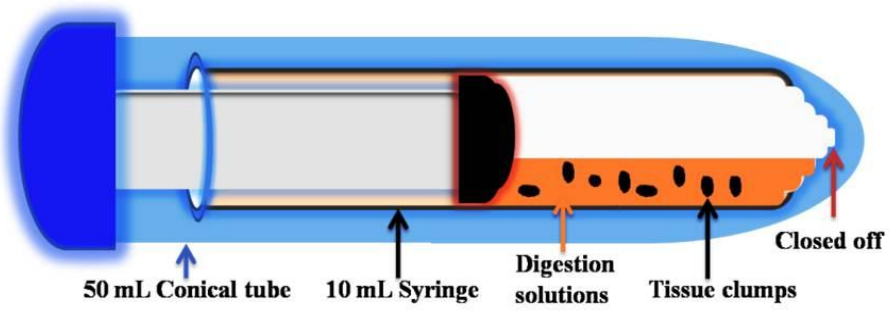


Fig. 4.1. Schematic overview of the set up used for increasing hydrostatic pressure in the digestion solution. The hub of a 10 mL syringe was closed off using flames and the syringe was filled with 1 mL digestion solution containing 100 mg minced testis tissues. The plunger was then inserted and pressed down to the 5 mL level, and the syringe was fitted inside a 50 mL conical tube and capped to maintain pressure.

Table 4.3. Combination of vortexing and enzymatic digestion to improve gonocytes recovery

Method no.	Description
13	Vortexing 1 min + collagenase-hyaluronidase-DNase 10 min + vortexing 10 s
14	Vortexing 1 min + collagenase-hyaluronidase-DNase 10 min + vortexing 10 s & collagenase-hyaluronidase-DNase 10 min + vortexing 10 s
15	Vortexing 1 min + cold collagenase-hyaluronidase-DNase for 5 h & 10 min digestion + vortexing 10 s
16	Vortexing 1 min + cold collagenase-hyaluronidase -DNase for 5 h & 10 min digestion + vortexing 10 s & collagenase-hyaluronidase-DNase 10 min + vortexing 10 s

Table 4.4. Optimisation of the newly developed three-step isolation method.

Method no.	Description
17	Vortexing 1 min + collagenase-hyaluronidase-DNase 10 min + vortexing 20 s
18	Vortexing 1 min + collagenase-hyaluronidase-DNase 10 min + vortexing 30 s
19	Vortexing 1 min + collagenase-hyaluronidase-DNase 10 min + vortexing 40 s

4.3.12 Statistical Analysis

Data from different groups were compared with one-way ANOVA, followed by a post hoc Tukey's HSD test using SPSS (Version 17.0; SPSS, Chicago, IL, USA). Unless stated otherwise, data are presented as the mean ± s.e.m.. For cell numbers, the values are given per g fresh tissue. Differences were considered significant at $P < 0.05$.

4.4 Results

4.4.1 Experiment 1: Effects of Depleting Erythrocytes

Different ratios of a lysis buffer were compared for their efficiency in depleting erythrocytes found among testis cells isolated by two enzymatic digestion methods. Testis cell viability and yield did not differ between control (with no lysis buffer) and treated groups (with the lysis buffer used at different ratios), or among different ratios of the lysis buffer (range 94-95% and 93-96% for cell viability, and 110-118 $\times 10^6$ and 218-239 $\times 10^6$ /g for yield, in each of the two digestion methods applied; $P > 0.05$). Although the addition of the lysis buffer had no adverse effects on the viability or yield of testis cells, it facilitated the assessment of results by greatly reducing the number of contaminating erythrocytes, especially at a ratio of 4:1. Therefore, for the remaining experiments in the present study we used lysis buffer at ratio of 4:1.

4.4.2 Experiment 2: Effects of Variations in Donor Testes

Cell viability and yield did not differ between testis tissues collected from littermate piglets and piglets from different litters (range 92-94% for cell viability and 214-247 $\times 10^6$ /g for cell yield; $P > 0.05$).

4.4.3 Experiment 3 & 4: Mechanical Dissociation *vs.* Enzymatic Digestion

Overall, enzymatic digestion methods yielded about 9-fold more live cells than mechanical methods ($206 \pm 5 \times 10^6$ *vs.* $22 \pm 7 \times 10^6$ /g respectively; $P < 0.05$; Fig. 4.2). For mechanical disassociations, different methods did not differ in cell viability or total live cells recovered ($P > 0.05$). Cell viability did not differ among different enzymatic digestion methods. The use of two rounds of digestion with collagenase and DNase resulted in less total cells and live cells than digestion with collagenase and DNase followed by trypsin ($P < 0.05$; Fig. 4.2).

4.4.4 Experiment 5: Effects of Testis Tissue Pre-treatment with Cold Enzymes and/or Hydrostatic Pressurisation

Pre-treatment of the testis tissue with cold enzymes and/or hydrostatic pressurisation did not affect the viability of isolated cells. However, pre-treatment of tissues with cold enzymes increased the recovery of live cells digested with collagenase-hyaluronidase-DNase followed by trypsin (Method 10), with an average of $260 \pm 7 \times 10^6$ /g ($P < 0.05$). There were no differences in cell viability, yield or live cell number among groups of tissues undergoing cold enzyme pre-treatment with or without pressurisation ($P > 0.05$; Fig. 4.2).

4.4.5 Gonocytes Quantification

Lectin DBA was uniquely expressed on the cell membranes of gonocytes (Goel *et al.* 2007) both as isolated cells and in tissue sections (Fig. 4.3). The proportion of gonocytes, with an average yield of approximately 7%, did not differ among mechanical disassociation and enzymatic digestion methods (Fig. 4.4).

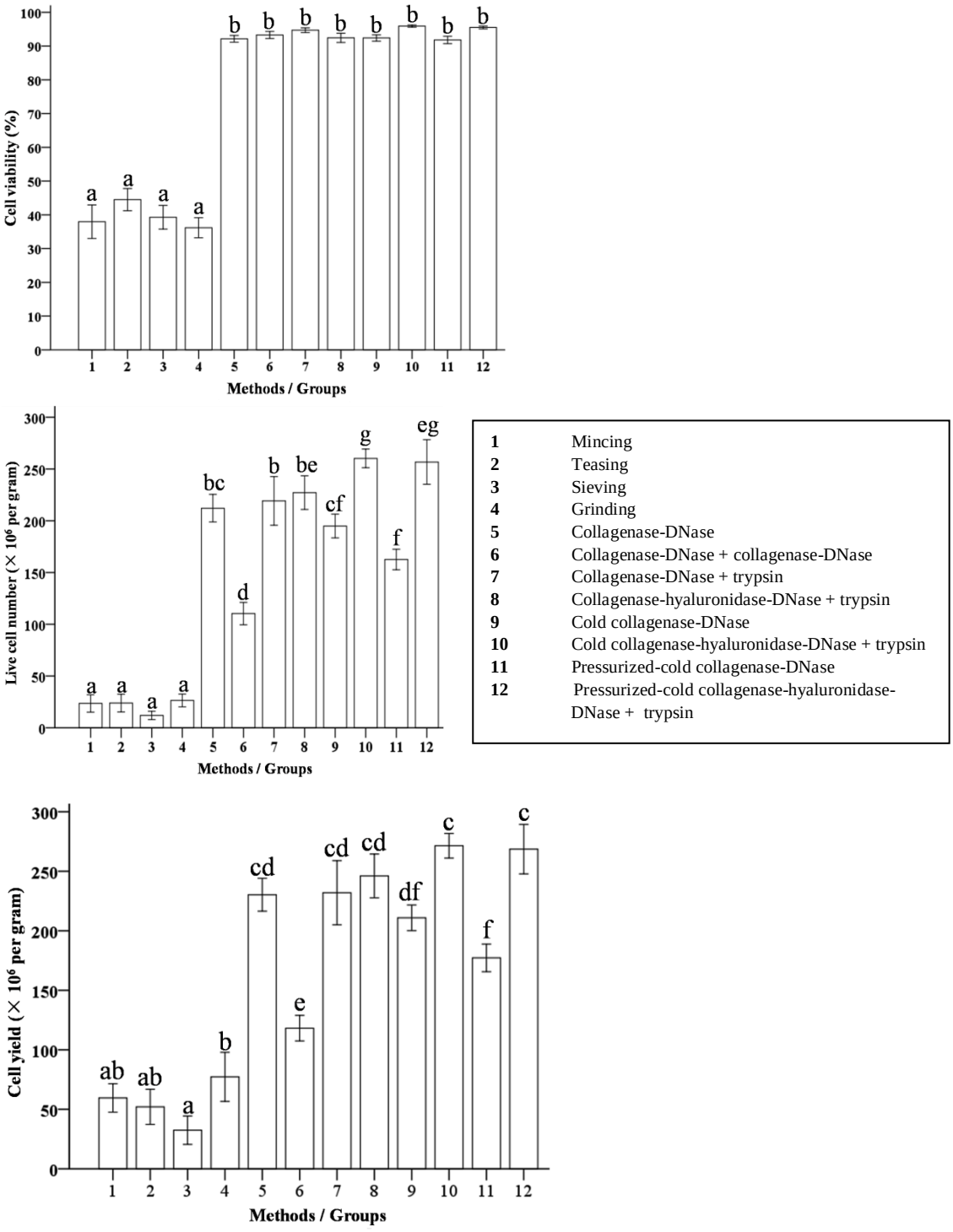


Fig. 4.2. Cell viability and yield after testis cell isolation using different methods. Cell viability, yield and total number of live cells ($\times 10^6$ /g testis tissue) are shown for different mechanical dissociation and enzymatic digestion methods. Groups of testis tissue pretreated with cold enzymes and/or hydrostatic pressurisation were also compared. Data are the mean ± s.e.m. Columns without a common letter differ significantly ($P < 0.05$).

4.4.6 Experiment 6: Effects of Combining Enzymatic Digestion and Vortexing on Gonocytes Recovery

There were no differences in the percentage of DBA positive cells, cell viability, or total number of live cells ($P > 0.05$) after vortexing, and one or two rounds of enzymatic digestion, with or without cold enzyme pre-treatment (Methods 13-16). However, vortexing with one round of enzymatic digestion without cold enzyme pre-treatment (three steps; vortexing-digestion-vortexing; Method 13) resulted in maximum recovery of live gonocytes, higher than combining vortexing with two rounds of enzymatic digestion (Method 14, $P < 0.05$, Fig. 4.5). Therefore, this novel three-step approach (Method 13) was selected for optimisation.

4.4.7 Experiment 7: Optimization of the Newly Developed Three-step Method

Comparison of the duration of vortexing after enzymatic digestion showed that vortexing for 30 s resulted in higher cell viability and live testis cell recovery than vortexing for 20 s (90 ± 1 *vs.* 82 ± 2; and $42 \pm 2 \times 10^6$ *vs.* $33 \pm 3 \times 10^6$ /g for yield; respectively, $P < 0.05$). Vortexing for 30 s also resulted in the recovery of more live gonocytes than did vortexing for 20 or 40 s ($17 \pm 1 \times 10^6$ *vs.* $11 \pm 2 \times 10^6$ and $11 \pm 2 \times 10^6$/g, respectively, $P < 0.05$). This strategy resulted in the recovery of 40% ± 2% gonocytes (Fig. 4.3F and 4.6).

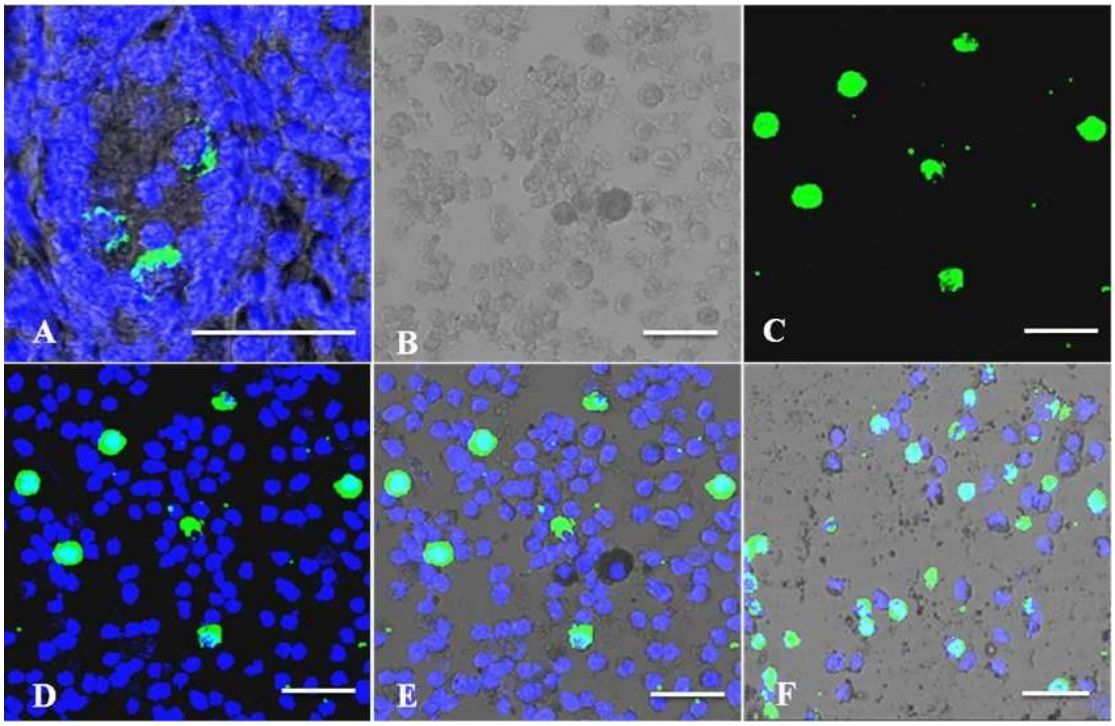
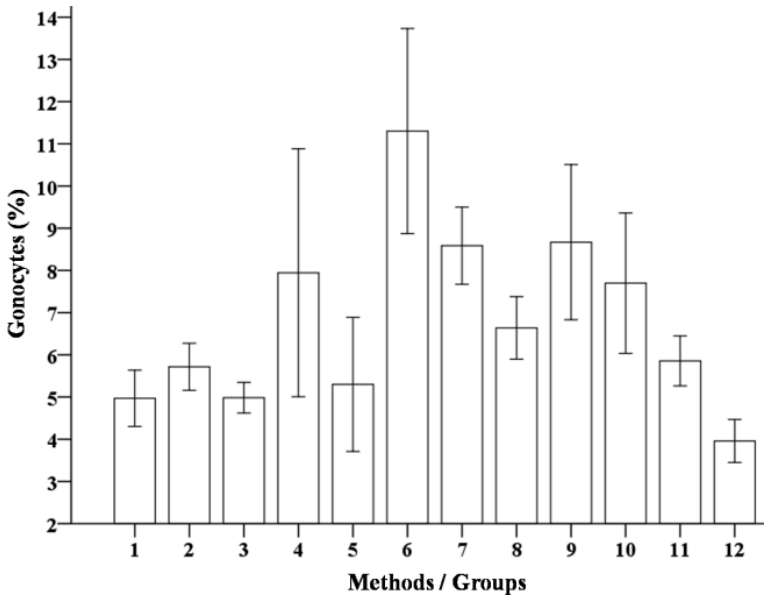


Fig. 4.3. Immunohistochemical and immunocytochemical detection of gonocytes. Confocal scanning images of donor pig testis tissue and in isolated testis cells to showing labelling with either a fluorescein conjugated lectin Dolichos biflorus agglutinin (DBA, green) to detect gonocytes, or DAPI to show all cell nuclei (blue). A: Merged image of the donor testis tissue with transmitted light as well as staining for DBA and DAPI. B: Transmitted light image of testis cells separated using a two-step enzymatic process. C: Isolated testis cells stained with DBA (gonocytes). D: Merged image of isolated testis cells stained with DBA and DAPI. E: Merged image of isolated testis cells stained with DBA and DAPI and the transmitted light image. F: Merged image of isolated testis cells using the novel three-step process, stained with DBA and DAPI and the transmitted light image. Scale bars, 50 µm. Note the strong green fluorescent signal that is restricted to the gonocytes, and the high percentage of DBA positive cells (gonocytes) among cells isolated using the novel method.



1	Mincing
2	Teasing
3	Sieving
4	Grinding
5	Collagenase-DNase
6	Collagenase-DNase + collagenase-DNase
7	Collagenase-DNase + trypsin
8	Collagenase-hyaluronidase-DNase + trypsin
9	Cold collagenase-DNase
10	Cold collagenase-hyaluronidase-DNase + trypsin
11	Pressurized-cold collagenase-DNase
12	Pressurized-cold collagenase-hyaluronidase-DNase + trypsin

Fig. 4.4. Proportion of gonocytes obtained after testis cell isolation using different methods. The proportion of gonocytes is compared among different methods of mechanical dissociation, enzymatic digestion, and testis tissue pretreatment with cold enzymes and/or hydrostatic pressurisation.

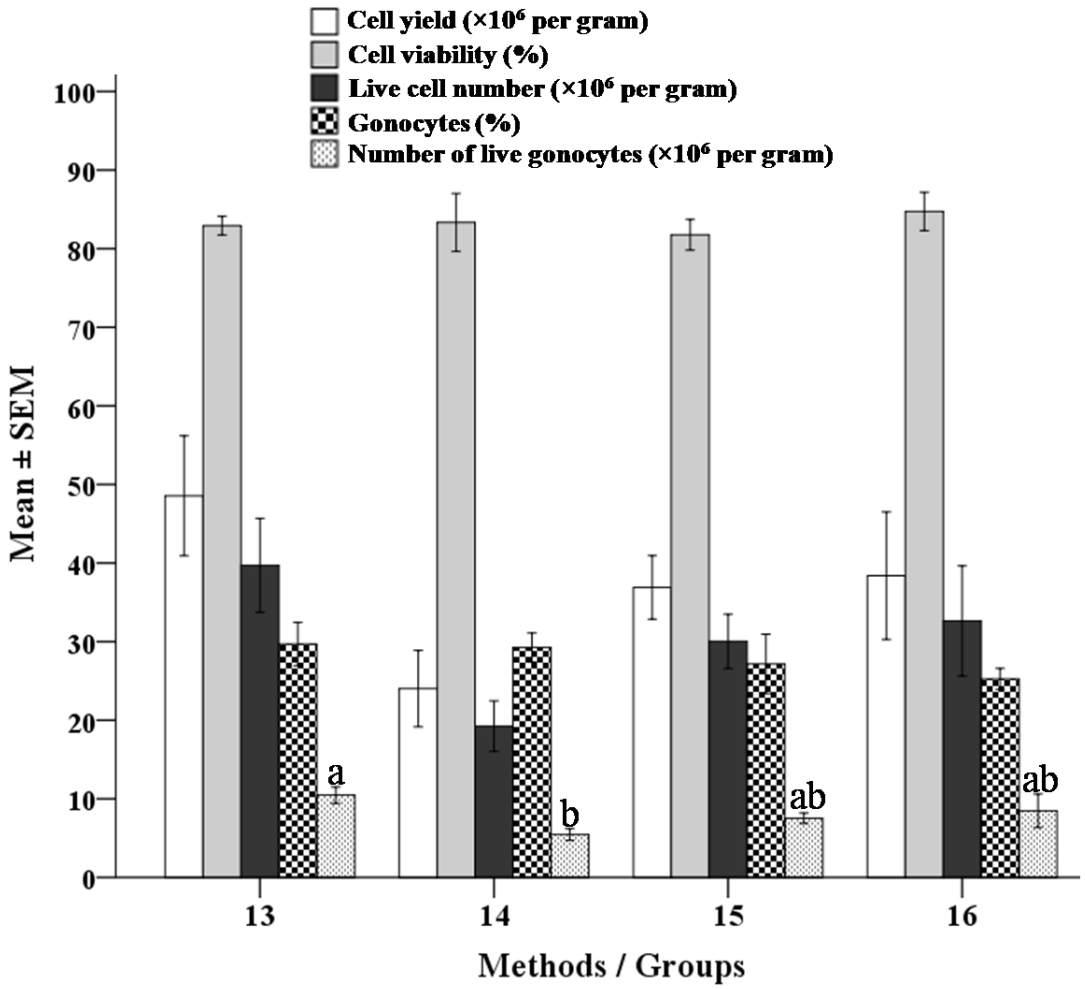


Fig. 4.5. Comparison of new three-step strategies for the isolation of testis cells. Cell viability (%), yield, the total number of live cells ($\times 10^6$ /g testis tissue, mean ± s.e.m.), proportion of gonocytes (% ± s.e.m.), and the number of live gonocytes ($\times 10^6$ per gram testis tissue) are shown for different combinations of vortexing and enzymatic digestions with or without pretreatment of the tissue with cold enzymes. Data are the mean ± s.e.m. Of the same texture, columns without a common letter differ significantly ($P < 0.05$).

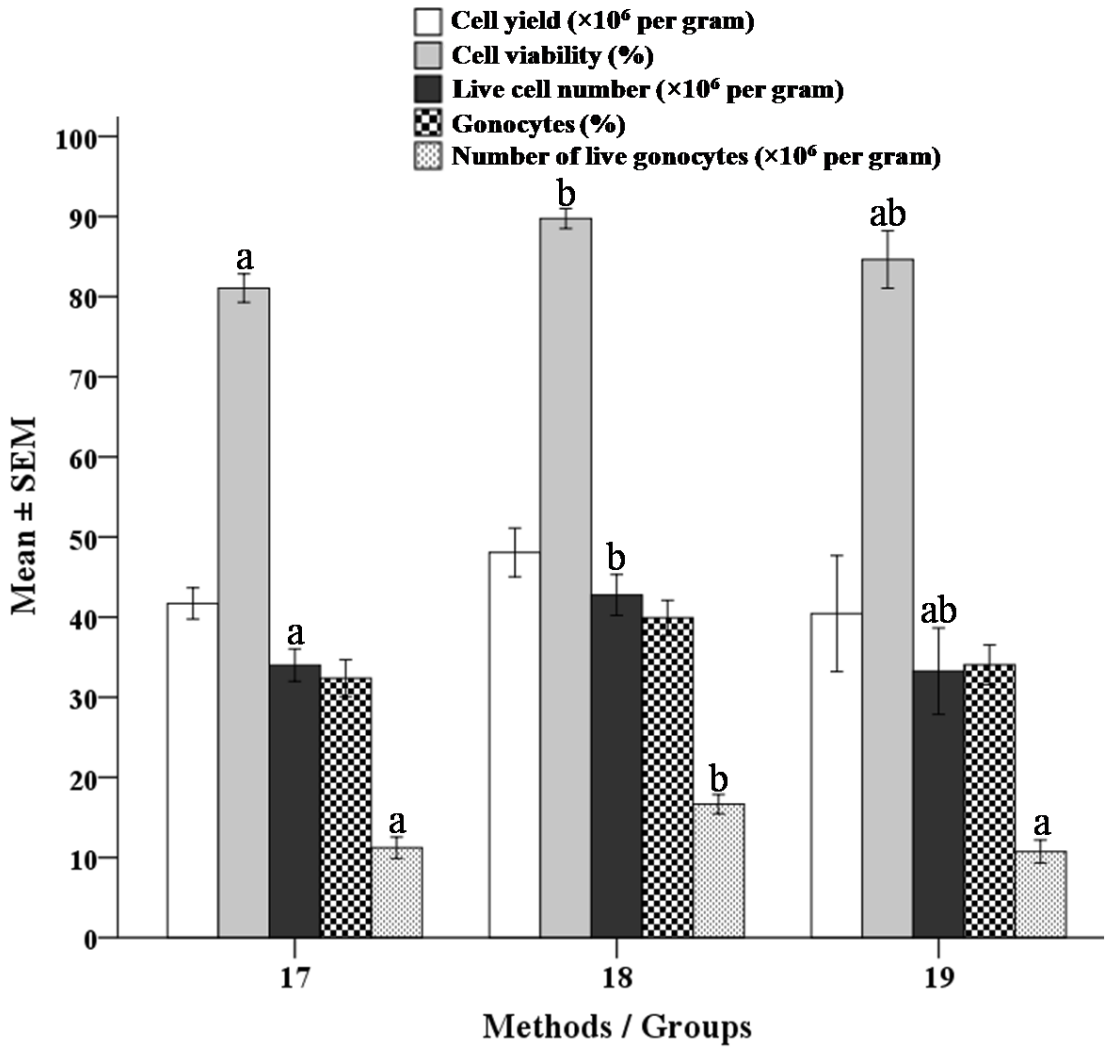


Fig. 4.6. Optimization of the new three-step testis cell isolation method. The duration of vortexing following enzymatic digestion was compared among different groups. Cell viability (%), yield, the total number of live cells ($\times 10^6$ /g testis tissue, mean ± s.e.m.), gonocytes proportion and the number of live gonocytes ($\times 10^6$ /g testis tissue) were compared among groups vortexed for different durations. Data are the mean ± s.e.m. Of the same texture, columns without a common letter differ significantly ($P < 0.05$).

4.5 Discussion

The isolation of testis cells containing a high proportion of gonocytes is the first fundamental and potentially limiting step in the study and manipulation of these important germ cells. Subpopulations of gonocytes have been demonstrated to have stem cell potential, similar to SSCs (Jiang 2001; Jiang and Short 1995; Jiang and Short 1998a; Jiang and Short 1998b; Orwig *et al.* 2002b; Ryu *et al.* 2003). Starting with higher proportion of gonocytes in the freshly isolated testis cells will increase the efficiency of the subsequent purification of gonocytes which is still challenging. In the present study, we examined a wide range of approaches to isolation of testis cells and tested the effects of several factors that could potentially affect the outcome. Our investigations elucidated these effects and also led to the development of new strategies for testis tissue digestion, providing very high percentages of gonocytes (~40%). To our knowledge, no other tissue digestion strategy has yielded such a high percentage of gonocytes.

Procedures used for testis cell isolation vary among laboratories and depend on the type of target cells and the species from which the testes are collected. Little research has compared the virtues of different methods and procedures. In preliminary experiments, we compared the digestion efficiency of two types of collagenase (I *vs.* IV). Although there were no differences between the two types, we chose to use collagenase IV for the experiments in the present study because its use led to fewer cell aggregates. However, in both groups, large numbers of erythrocytes were isolated along with testicular cells, which could cause variations in testis cell counting by aggregating in the haemocytometer. Lysis buffers have been used to deplete erythrocytes during leukocyte and bone marrow cell preparations (Erdmann *et al.* 2004; Klein *et al.* 2006; Lal *et al.* 2006; Leng *et al.* 2006; Mustafa *et al.* 2008; Sethu *et al.* 2004), but there are no reports

on their application in testis cell isolation. We found that an NH_4Cl-based lysis buffer efficiently eliminated erythrocytes without compromising testis cell viability or yield.

In the present study, testis tissues collected from littermate piglets and those of different litters did not differ in cell viability, yield or live cell number, indicating that such variations in the source of testis tissue do not affect the results of cell isolation.

Our results clearly showed that enzymatic digestion of the testis tissue was superior to mechanical dissociation methods in terms of cell viability, yield and live cells recovered, which is in agreement with a previous observation (Crabbé *et al.* 1997). Overall, enzymatic digestion of the testis tissue resulted in the recovery of many more (nine-fold) live testis cells than mechanical dissociation methods. Interestingly, there was no difference in the proportion of gonocytes isolated between these two approaches (~7%), indicating that the current standard strategies for testis cell isolation do not necessarily enrich for gonocytes.

Most enzymes used in cell isolations target specific components within the tissue and have optimal working temperatures; however, prolonged exposure of the tissue to these enzymes could damage surface cells while the tissue core may not be fully exposed to the enzymes. Therefore, shortening the duration of digestion, while increasing the access of enzymes into the tissue, could reduce the risk of over-digestion damage to the cells and increase the efficiency of cell isolation. We hypothesised that the testis tissue could be more efficiently digested if it were initially exposed to enzymes at cold temperature, allowing the enzymes to penetrate deeper into the tissue before an optimal temperature was provided to initiate digestion. We were also interested to investigate whether hydrostatic pressurisation facilitated the penetration of enzymes into the tissue.

In the present study, although testis cell viability and yield did not differ between the groups, pre-treatment of tissue with cold enzymes, but not with pressurisation, followed by a two-step enzymatic digestion protocol (collagenase-hyaluronidase-DNase plus trypsin) increased the total number of live cells and therefore the overall number of gonocytes harvested.

It has been reported that two-step enzymatic digestions can effectively eliminate disassociated interstitial cells (Bellve *et al.* 1977), thereby increasing the proportion of germ cell recovery. However, in the present study, additional digestion with collagenase-DNase or trypsin as a second step did not increase the yield of testis cell or live cells, and in fact, two rounds of digestion with collagenase-DNase reduced the yield of testis cell and live cells. Furthermore, there was no significant difference in the percentage of gonocytes isolated between the one and two-step enzymatic digestion methods.

To increase the proportion of gonocytes we developed a novel strategy to use an initial period of vortexing, followed by gentle digestion of the ECMs, and further vortexing. This new approach yielded a surprisingly high proportion of gonocytes (~40%) with high cell viability (~90%) in a short period of time (< 1 h). This is four to eightfold the proportion of gonocytes and/or spermatogonia obtained using other approaches (Herrid *et al.* 2009a; Li *et al.* 1997; Lo *et al.* 2005; Luo *et al.* 2006; Orth and Boehm 1990; Orwig *et al.* 2002b; Van Dissel-Emiliani *et al.* 1989). Interestingly, although other mechanical methods in the present study had very low cell isolation efficiency compared with enzymatic digestion, it was the combination of a mechanical method (vortexing) and enzymatic digestion that resulted in the highest gonocytes proportion.

Prior to digestion, the *in situ* proportion of gonocytes in tubular sections of neonatal pig testis tissue is approximately 7% (Honaramooz *et al.* 2005). Therefore, although the conventional strategies for enzymatic digestion of testis tissue do not result in gonocytes proportions that are higher than those of the intact seminiferous tubules, our novel three-step digestion and vortexing strategy results in significantly higher proportion of gonocytes . At the end of digestion using our new three-step procedure, a large number of small fragments of the seminiferous cords remain. Given the high proportion of gonocytes harvested, we speculate that our initial vortexing of the minced testis tissue followed by a gentle enzymatic digestion may have largely dissociated the interstitial cells from the seminiferous cords. The application of a second round of vortexing may have led to disproportional separation of gonocytes that are located at or near the broken ends of tubule fragments. It was interesting that extending the duration of the second round of vortexing by only 10 s (from 20 to 30 s) could improve cell viability and total number of harvested live cells including that of gonocytes. The reasons for these observations and the underlying mechanisms for obtaining such high proportions of gonocytes will need to be examined in further studies.

In conclusion, we systematically investigated and elucidated the effects of several factors on testis cell isolation with a focus on gonocytes. More importantly, a novel strategy of combining gentle enzyme digestion with two rounds of vortexing was developed for harvesting testis cells that can be completed within 1 h, with the highest proportion of gonocytes in freshly isolated cells reported to date (40% or four- to eightfold higher than current strategies). The resultant testis cells have a cell viability of > 90%, resulting in the harvest of approximately 17×10^6 live gonocytes per g testis tissue.

This high proportion of gonocytes among freshly isolated testis cells, as a starting point, could greatly facilitate their subsequent purification and the *in vitro* study and manipulation of these germline stem cells.

CHAPTER 5 EFFECTS OF MEDIUM AND TEMPERATURES ON PRESERVATION OF ISOLATED PORCINE TESTIS CELLS[1]

5.1 Abstract

The effects of medium and hypothermic temperatures on testis cells were investigated to develop a strategy for their short-term preservation. Testes were collected from 1-week-old piglets and enzymatically dissociated for cell isolation. In Experiment 1, testis cells were stored at either room (RT) or refrigeration (RG) temperature for 6 days in one of thirteen media. Live cell recovery was assayed daily using trypan blue exclusion. In Experiment 2, three media at RG were selected for immunocytochemistry and *in vitro* culture. Live cell recovery was also assayed daily for 6 days using both trypan blue exclusion and a fluorochrome assay kit. For all media tested, significantly or numerically more live cells were maintained at RG than RT. On preservation Day 3 at RG (cell isolation day as Day 0), 20% FBS-L15 resulted in the highest live cell recovery (89.5% ± 1.7, mean ± s.e.m.) and DPBS in the lowest (60.3% ± 1.9). On Day 6 at RG, 20% FBS-L15 also resulted in the best preservation efficiency with 80.9% ± 1.8 of Day 0 live cells recovered. There was no difference in live cell recovery detected by the two viability assays. After preservation, the proportion of gonocytes did not change, whereas that of Sertoli and peritubular myoid cells increased and decreased, respectively. After 6 days of preservation, testis cells showed similar culture potential to fresh cells. These

[1] This study has been published. Yang, Y. and A. Honaramooz (2010). *Reprod. Fertil. Dev.* 22(3): 523-532. Portions of this study were also presented at the 41st and 42nd annual meetings of the Society for the Study of Reproduction.

results show that testis cells can be preserved for 6 days under hypothermic conditions with a live cell recovery of more than 80% and after-storage viability of 88%.

5.2 Introduction

Recent breakthroughs in the study and manipulation of spermatogonial stem cells (SSCs) have highlighted the importance and unique potential of these cells in male fertility and animal transgenesis and conservation (Dobrinski and Travis 2007; Ehmcke and Schlatt 2008; Oatley and Brinster 2008). These SSCs reside at the basement membrane of seminiferous tubules and have the ability to both self-renew and to finally differentiate into virtually unlimited numbers of spermatozoa throughout adulthood. However, they need to interact with other testis cells, especially cells within the seminiferous tubules to form the foundation of spermatogenesis.

One of the fundamental steps in the study and manipulation of testis cells, as with any cell type, is to preserve them for various length of time *in-vitro*. Current options for maintaining live isolated testis cells are usually limited to culturing or freezing them. Cell culture could be used for short-term storage but it may affect the characteristics of the cells to a degree that they may no longer represent the in-situ population. On the other hand, whereas cryopreservation is used to preserve cells for extended periods of time, it is not appropriate for short-term storage, as cells may undergo considerable damage during freezing and thawing. For instance, current cryopreservation techniques result in testis cell viability of 30% to 82% after preservation depending on the species and methods used (Geens *et al.* 2008), and even lower efficiency for SSCs (Izadyar *et al.* 2002a).

There are situations where short-term storage of testis cells is required and seems more appropriate. This includes various steps in preparation for male germ cell

transplantation, in which testis cells from a donor individual are to be transplanted into seminiferous tubules of recipients. Other circumstances include maintenance of cells during routine cell manipulation intervals and ease of transporting cell samples between collaborating laboratories.

Hypothermic preservation maintains biological samples (cells, tissues or organs) below normal mammalian body temperatures but above the freezing point, in order to slow down the cellular metabolism and to minimise oxygen and intracellular energy consumption (Belzer and Southard 1988; de Perrot *et al.* 2003; Taylor 2000). Hypothermic preservation may lead to prolonged cell viability while reducing the unwanted consequences of culturing or cryopreserving cells for short-term storage. Our objective, therefore, was to test hypothermic preservation as an alternative strategy for short-term maintenance of cells.

5.3 Materials and Methods

5.3.1 Experimental Design

The present study was undertaken in two phases. In the first phase, testis cells were maintained in 13 different media at room and refrigeration temperatures for up to 6 days. In the second phase, the most promising media from the first phase were further evaluated for their cell preservation efficiency.

5.3.2 Testis Tissue Preparation

Testes were collected after aseptic castration of one-week-old piglets (Camborough-22 × Line 65, PIC Canada Ltd., a hybrid of Yorkshire, Large White and Landrace, Winnipeg, MB, Canada) at a university-affiliated swine facility. The testes

were transferred to the laboratory within 2 h after excision in Dulbecco phosphate buffered saline (DPBS, cat. # 20-031-CV, Mediatech, Manassas, VA, USA) containing 2% antibiotic-antimycotic solution (cat. # 30-004-CI, Mediatech). In the laboratory, the testes were immediately rinsed three times with DPBS and the tunica albuginea, rete testis and overt connective tissue were removed. Experimental procedures involving animals were approved by the University of Saskatchewan's Institutional Animal Care and Use Committee.

5.3.3 Isolation of Testis Cells

Trimmed testis parenchyma was minced with fine scissors, and dissociated using a two-step enzymatic digestion as previously described (Honaramooz *et al.* 2002a), with minor modifications. Briefly, the testis tissue was digested with 0.2% collagenase IV (cat. # C-5138, Sigma-Aldrich, Oakville, ON, Canada), 0.1% hyaluronidase (cat. # H-3884, Sigma-Aldrich) and 0.01% Dnase I (cat. # DN25, Sigma-Aldrich) in Dulbecco modified Eagle medium (DMEM, cat. # 10-013-CM, Mediatech) at 37 ℃ for 15 min, with agitation every 5 min. After sedimentation for 6 min at room temperature and removal of the supernatant, tissue clumps were further digested with 0.25% trypsin with 2.21 mM EDTA (cat. # 25-053-CI, Mediatech) for 5 min at 37 °C, the digestion was stopped by adding foetal bovine serum (FBS, cat. # A15-701, PAA Laboratories GmbH, Etobicoke, ON, Canada), and the suspension was triturated with 1 mL pipette tips before filtration through a 40 μm filter (cat. # 352340, BD Biosciences, Mississauga, ON, Canada). Erythrocytes were depleted with the addition of a lysis buffer (156mM NH_4CL, 10 mM $KHCO_3$, 0.1mM Na_2EDTA) (Sethu *et al.* 2004; Smith *et al.* 2009) at a ratio of 1:4 (cell suspension : lysis buffer) for 15 min at room temperature, followed by centrifugation at

600*g* for 4 min at 4 ℃ and rinses with 10% FBS-DMEM and DPBS, respectively. The resultant cells were resuspended at a concentration of 2×10^6/mL in the various preservation media being tested.

5.3.4 Short-term Storage of Testis Cells

Isolated cells were stored at room (RT: 22 ± 1 ℃) or refrigeration (RG: 4 ± 1 ℃) temperature in 2 mL polypropylene tubes in one of the following 13 media: DPBS, DMEM, DMEM with 5% ethylene glycol (5% EG-DMEM), DMEM with FBS (10%, 20% or 50% FBS-DMEM), FBS, Leibovitz L15 (L15), L15 with 5% EG (5% EG-L15), L15 with FBS (10%, 20% or 50% FBS-L15) or HypoThermosol-FRS solution (HTS-FRS, cat. # 609144, Biolife Solutions, Bothell, WA, USA).

5.3.5 Evaluation of Cell Viability

In the first phase of the present study, viability and concentration of cells were assayed daily (at similar times ± 2 hr) with a haemocytometer using the trypan blue exclusion method (0.4%, cat. # T8154, Sigma-Aldrich) for 6 consecutive days, and the day of cell isolation was considered as Day 0 (control). Cell viability was defined as the percentage of live cells remaining at each time point, compared with the original number of live cells on Day 0. On Days 3 and 6, data were analysed to select the most promising protocols for the second phase of the study.

There is an array of methods available for assessment of cell viability, of these trypan blue exclusion is one of the most widely used. However, using this method might create variation in results depending on the operator and the procedures applied. Therefore, in the second phase, for each sample of the selected protocols, cell viability

was assayed both with the trypan blue exclusion method and with a fluorochrome assay (Live/Dead Viability/Cytotoxicity Kit, cat. # L-3224, Invitrogen, Carlsbad, CA, USA) to validate the cell viability data. On preservation Days 0, 3, and 6, cells were also smeared onto slides coated with poly-L-lysine (cat. # P-8920, Sigma-Aldrich) and stored at -80 ℃ for use in immunocytochemistry analysis.

5.3.6 Immunocytochemistry

Seminiferous tubules in the one-week-old piglet testis contain gonocytes (as the only germ cell type present), Sertoli cells and to a lesser degree, peritubular myoid cells. Therefore, immunocytochemistry was performed to identify and quantify these cellular subpopulations using specific antibodies on cell smears from preservation Days 0, 3 and 6.

5.3.6.1 Gonocytes

Cell smears were fixed in Bouin's solution for ~2 min, rinsed in DPBS, blocked with 3% H_2O_2 for 10 min and 5% BSA for 15 min, and treated with a peroxidase-conjugated lectin from Dolichos biflorus (1:100, cat. # L1287, Sigma-Aldrich) for 1 h at 37 ℃ (Goel *et al.* 2007). Slides were then incubated with DAB enhanced liquid substrate system (cat. #D3939, Sigma-Aldrich) for 3-5 min, counterstained with hematoxylin, mounted, and observed under a light microscope.

5.3.6.2 Sertoli Cells and Peritubular Myoid Cells

Cell smears were fixed in methanol for 4 min and acetone for 4 min at -20 ℃, rinsed with DPBS, and incubated with the first antibodies (1:40, mouse anti-vimentin for

Sertoli cells, cat. # V6630, Sigma-Aldrich; or 1: 75, mouse anti-alpha smooth muscle actin for peritubular cells, cat. # Ab7817, Abcam, Cambridge, MA, USA) for 1 h at 37 ℃ in humidified atmosphere (Dufour *et al.* 2005; Dufour *et al.* 2003; Goel *et al.* 2007; Tung and Fritz 1990). This was followed by incubation with the second antibody (1:600, donkey anti-mouse conjugated with Texas Red, cat. # Ab6818, Abcam) at room temperature for 1 h, and rinsing in DPBS. Slides were then counterstained with 4, 6-diamino-2-phenyl indole (DAPI, cat. # D-9542, Sigma-Aldrich), mounted with a fluorescence anti-fading medium (Vectashield, cat. # H-1000,Vector Laboratories, Burlingame, CA, USA), and observed under a fluorescent microscope.

5.3.7 Cell Culture

Samples of testis cells from preservation Days 0 and 6 of the selected protocols were cultured in 6-well plates with DMEM containing 10% FBS at 37 ℃, in a 5% CO_2 humidified atmosphere for 3 days. On the last day of culture, the cells were observed, then re-suspended using 0.25% trypsin with 2.21 mM EDTA and smeared on slides to be further examined under a microscope.

5.3.8 Statistical Analysis

For general trends, data from each medium were analysed using two-way repeated measures ANOVA, using days as within-factor and storing temperatures (room *vs.* refrigeration) as between-factor variables. For *post-hoc* analysis, a Holm-Sidak test was used. Data are expressed as percentage of live cells compared with the number of live cells present at the corresponding Day 0 ($n \geq 8$ replicates per group). For each of the preservation Days 3 and 6, data were separately analysed using one-way ANOVA for the

effects of media and temperature, and Holm-Sidak test for post-hoc analysis ($n \geq 8$ replicates per group). The two methods of assessing cell viability were compared using a paired-sample *t*-test ($n \geq 5$ replicates per group). Two-way repeated measures ANOVA was also used for the analysis of cellular subpopulations with days as within-factor and storage temperatures (room *vs.* refrigeration) as between variables, followed by a Holm-Sidak test for *post-hoc* analysis ($n \geq 6$ replicates per group). Data are presented as mean ± s.e.m.. Differences were considered to be statistically significant when $P < 0.05$.

5.4 Results

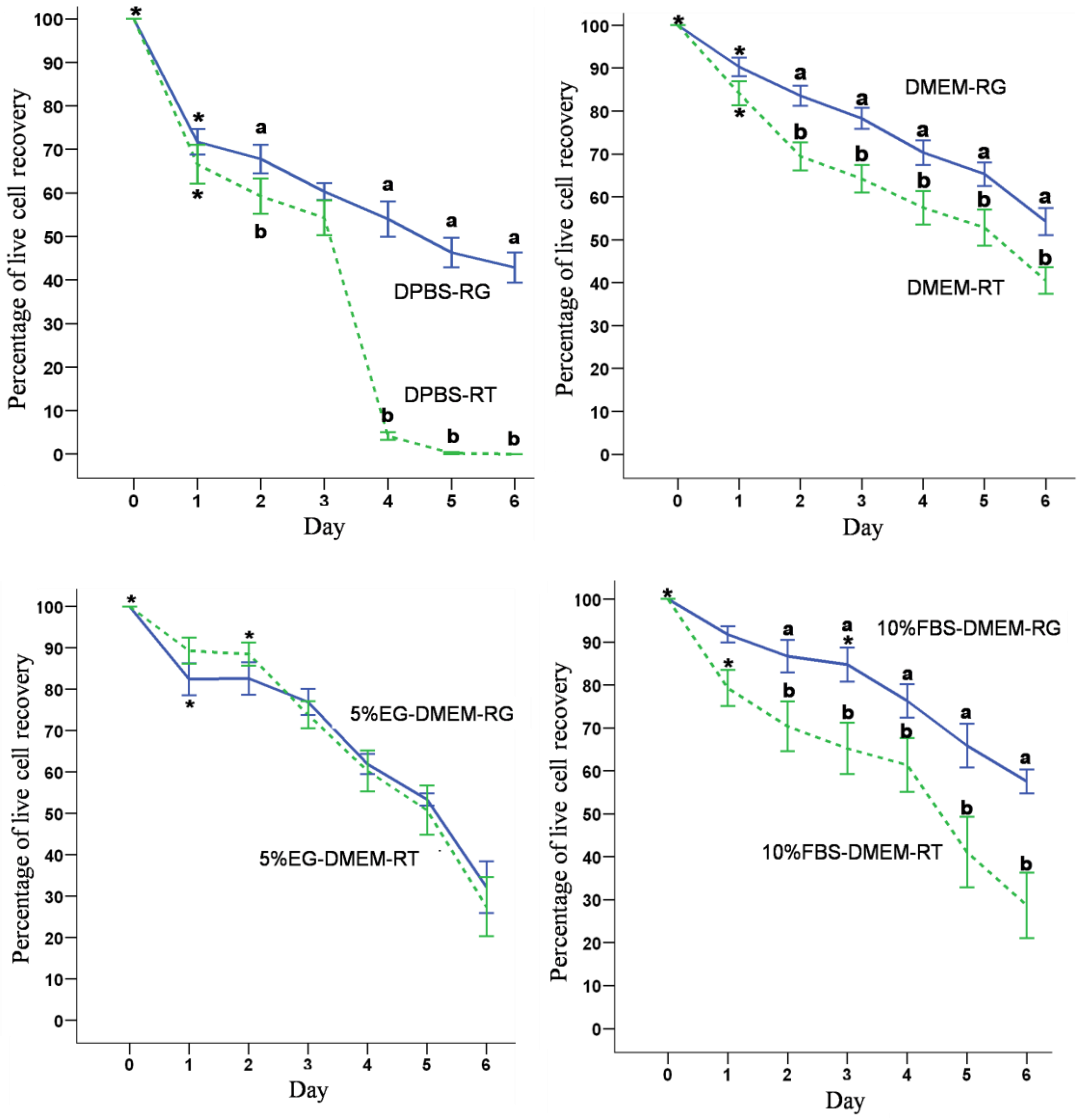
5.4.1 Temperature Effect

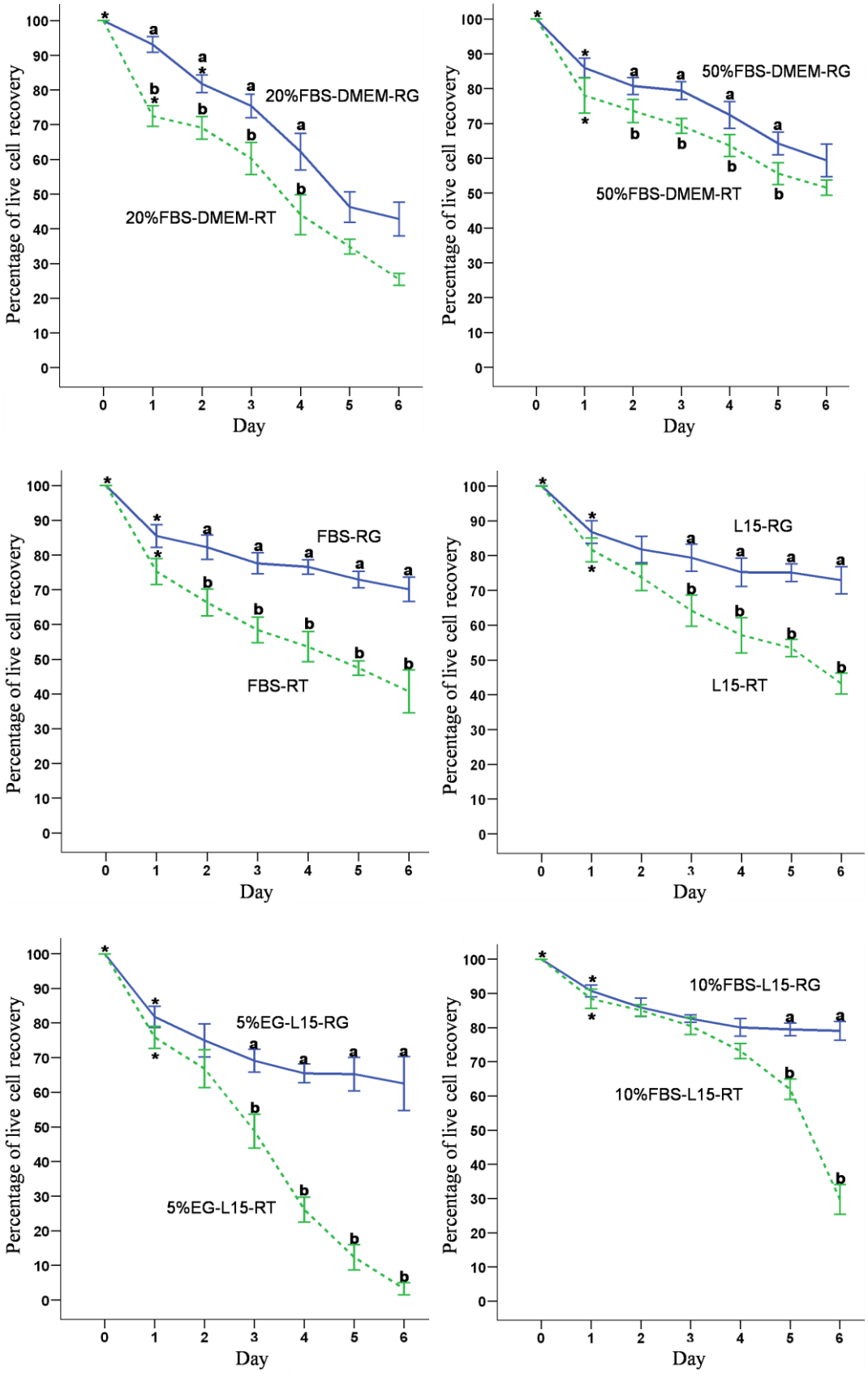
Fig. 5.1 displays the summary of data from comparison of 13 different media at room and refrigeration temperatures for their efficiency in short-term preservation of testis cells. By preservation Day 6, in most media (except DMEM with 20% or 50% FBS and 5% EG), a higher percentage of cells survived storage at refrigeration temperature than at room temperature ($P < 0.01$). For DPBS, FBS and DMEM-based media (except 5% EG-DMEM), the storage temperature started to cause differences in cell viability as early as preservation Day 1-2 ($P < 0.05$), while for L15-based and HTS-FRS media, these differences started from preservation Day 3-5 ($P < 0.003$, Fig. 5.1). The beneficial effect of FBS on cell viability did not seem to increase in proportion to its concentration for either DMEM- or L15-based media; however, 10% FBS postponed differences for L15-based media from Day 3 to Day 5 onward ($P < 0.001$, Fig. 5.1). Ethylene glycol was examined for its potential effects at refrigeration temperature. There were no benefits in adding EG to the media and it appeared deleterious to cells when used with DMEM or

L15 at room temperature, but the harmful effects were lessened when added to L15 at refrigeration temperature (Fig. 5.1).

5.4.2 Day Effect

We preserved testis cells for up to 6 days to simulate short-term storage or long-distance shipment of cell suspensions. When different media were compared at room temperature, there was a drop in the percentage of live cells on preservation Day 1 compared to Day 0 ($P < 0.005$), with the exception of 20% FBS-L15 and 5% EG-DMEM for which the drop was observed on Day 2 ($P < 0.001$, Fig. 5.1). For RG, most media resulted in lower cell viability rates on Day 1 or Day 2 compared with Day 0 control ($P < 0.005$), whereas 10%-FBS-DMEM and 20%-FBS-L15 showed this pattern on Day 3 ($P < 0.005$, Fig. 5.1). Supplementation of EG made no improvement when added to either DMEM or L15, and was in fact, generally deleterious to cells especially at RT. However, when compared with RG on Day 6, EG showed less harmful effects in L15 than in DMEM ($P < 0.05$, Fig. 5.3). At RG, the addition of FBS at 10% and 20% into DMEM, and at 20% and 50% into L15 delayed the drop in cell viability, compared with Day 0 control ($P < 0.05$, Fig. 5.1).


Percentage of live cell recovery
Day
DPBS-RG
DPBS-RT
DMEM-RG
DMEM-RT
5%EG-DMEM-RG
5%EG-DMEM-RT
10%FBS-DMEM-RG
10%FBS-DMEM-RT


Percentage of live cell recovery
Day
20%FBS-DMEM-RG
20%FBS-DMEM-RT
50%FBS-DMEM-RG
50%FBS-DMEM-RT
FBS-RG
FBS-RT
L15-RG
L15-RT
5%EG-L15-RG
5%EG-L15-RT
10%FBS-L15-RG
10%FBS-L15-RT

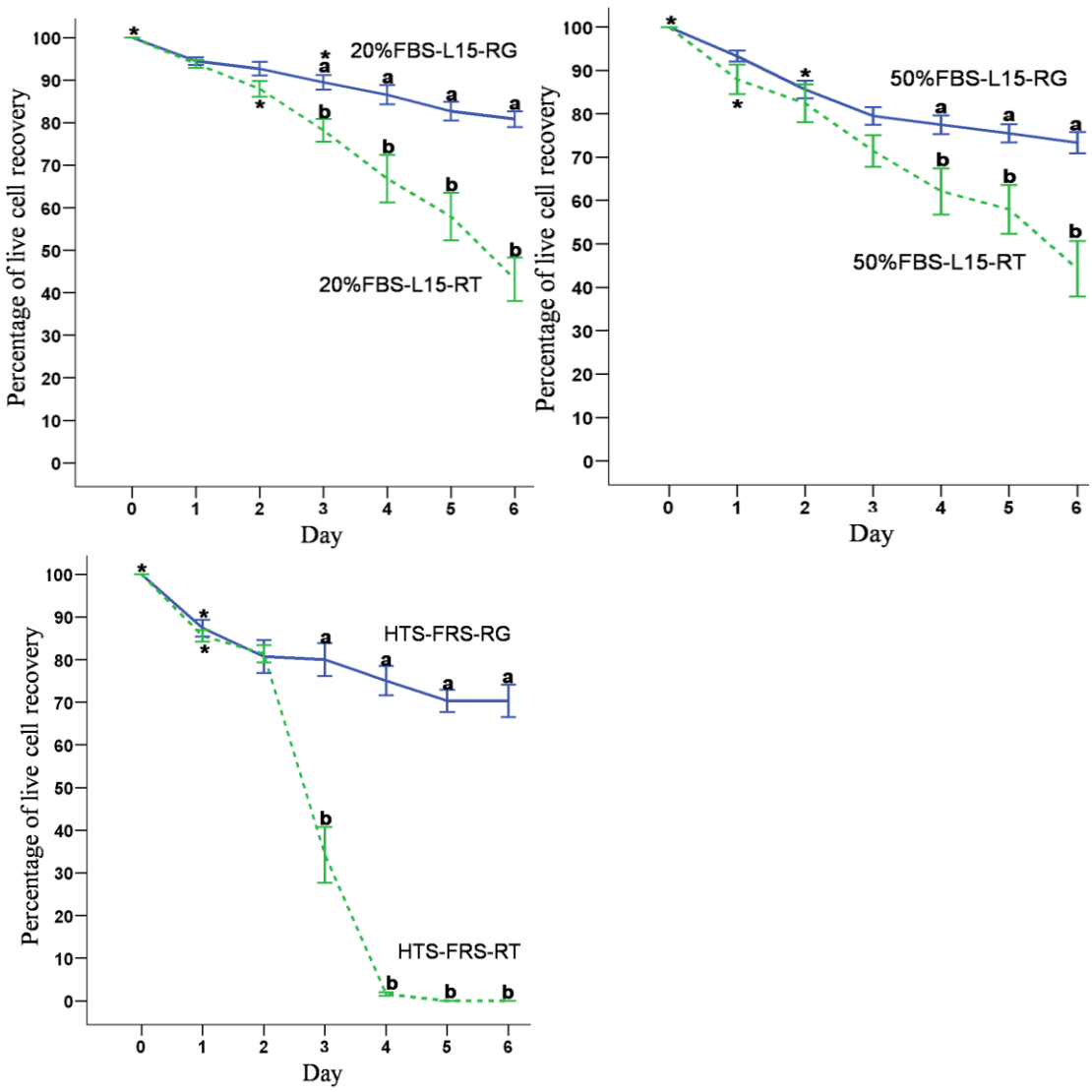


Fig. 5.1. Percentage of live cell recovery in different media at room (RT) and refrigeration (RG) temperatures. Cell samples were assessed daily for 6 days, and the percentage of live cells remaining at each time point was compared to the original number of live cells on Day 0 (fresh/control). Asterisks represent the day from which onward, the number of live cells significantly decreased compared with Day 0. Different letters between RT and RG represent significant differences ($P < 0.05$).

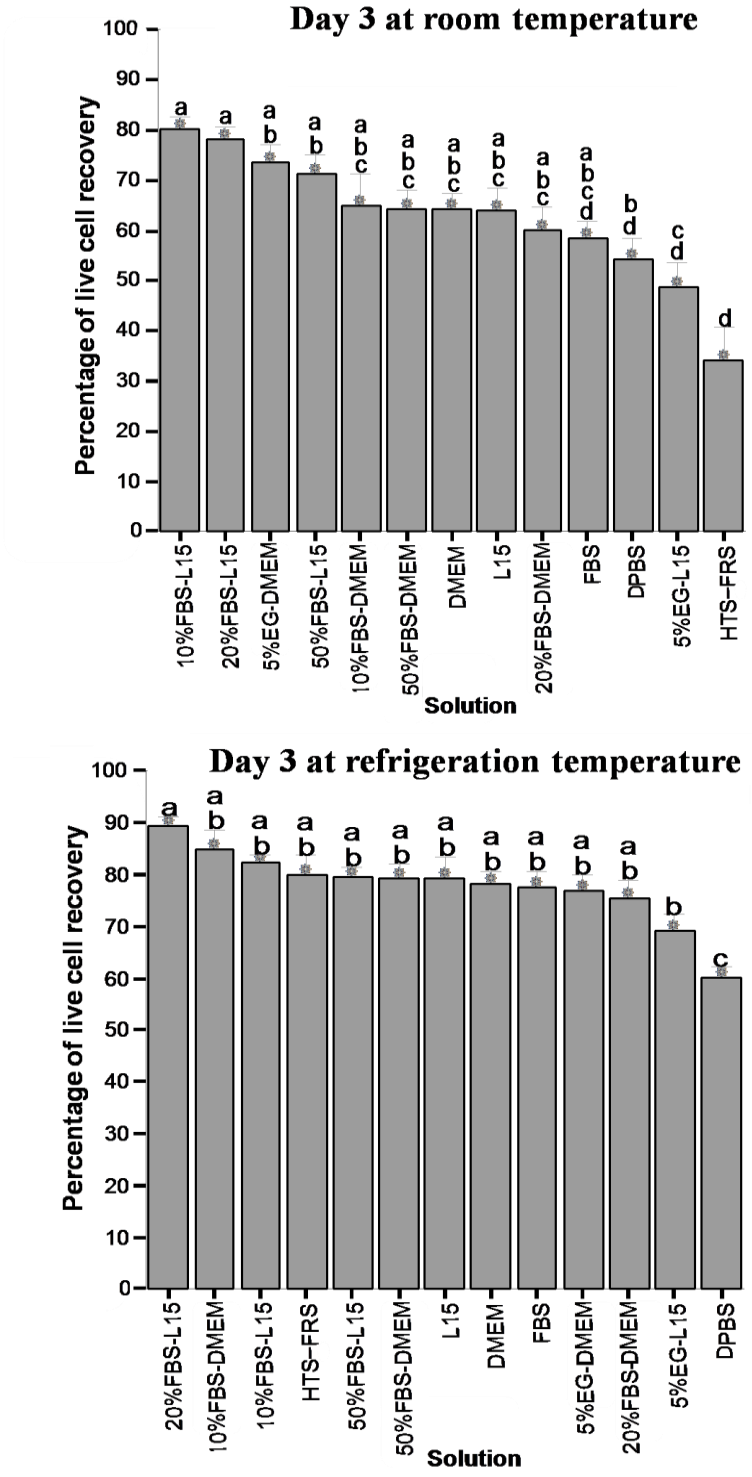


Fig. 5.2. Comparison of different preservation media on day 3 at room (RT) and refrigeration (RG) temperatures. On day 3, thirteen media were compared for the percentage of live cells compared to Day 0. Columns without a common letter are significantly different ($P < 0.05$).

5.4.3 Comparison on Day 3

As shown in Fig. 5.1, cell viability decreased for all protocols no later than preservation Day 3; therefore, data for Day 3 were compared among different preservation conditions. The use of different media resulted in a wide range of cell viability from 34 to 80% at RT and 60 to 90% at RG (Fig. 5.2). After 3 days of preservation, 10% FBS-L15 at RT and 20% FBS-L15 at RG showed the highest cell viability values, while HTS-FRS and DPBS maintained the lowest cell viability values for RT and RG, respectively (Fig. 5.2).

5.4.4 Comparison on Day 6

For all media, on Day 6, there was a drop in viability rates (0 to 52%) for cells maintained at RT; however, at RG, this drop was not as obvious (32 to 81%). Moreover, 50% FBS-DMEM (51.6 ± 5.2%) and 20% FBS-L15 (80.9 ± 1.8%) resulted in the highest viability rates and HTS-FRS and 5% EG-DMEM had the lowest rates for RT and RG, respectively (Fig. 5.3).

5.4.5 Selection of Protocols for Further Analysis

For both preservation Days 3 and 6, we obtained statistically or numerically higher cell viability rates at RG than at RT. Therefore, for the second phase of the study we chose three media at RG for immunocytochemical analysis and *in vitro* culture. For both Days 3 and 6, 20% FBS-L15 at RG consistently resulted in the highest percentage of live cells among all media (Fig. 5.2 and 5.3) and therefore was selected for further analysis. We also selected 50% FBS-DMEM at RG because it resulted in an overall

average viability rate (Fig. 5.2 and 5.3). HTS-FRS was included because it is a commercially available medium for hypothermic preservation of cells and tissues.

5.4.6 Comparison of Trypan Blue Exclusion and a Fluorochrome Assay for Assessment of Cell Viability

To validate the results obtained from the trypan blue assays, in the second phase of the study we performed the trypan blue method, alongside a fluorochrome assay (Live/Dead Viability/Cytotoxicity Kit). There was no difference between these two methods for the selected media on any preservation day (Days 0, 3 or 6, $P > 0.5$, Fig. 5.4).

5.4.7 Subpopulations of Preserved Cells

To examine whether different media favour particular types of testis cells during storage, the final cellular subpopulations were identified and quantified. The cellular subpopulations included gonocytes, Sertoli and peritubular myoid cells that make up seminiferous tubules. The proportion of gonocytes did not differ among the three selected media on preservation Days 0, 3, or 6 ($P > 0.05$, Fig. 5.5). The percentage of Sertoli cells increased from preservation Day 0 to Day 3 in the three selected media ($P < 0.01$). However, there was no difference in the proportion of Sertoli cells among the three media on any given preservation day ($P > 0.05$, Fig. 5.5). On the other hand, the percentage of peritubular myoid cells decreased from preservation Day 0 to Day 3 in the three selected media ($P < 0.01$), and dropped further from Day 3 to Day 6 in 50% FBS-DMEM and HTS-FRS ($P < 0.01$, Fig. 5.5). In addition, the proportion of peritubular myoid cells did not differ among the three media on any preservation day ($P > 0.05$, Fig. 5.5).

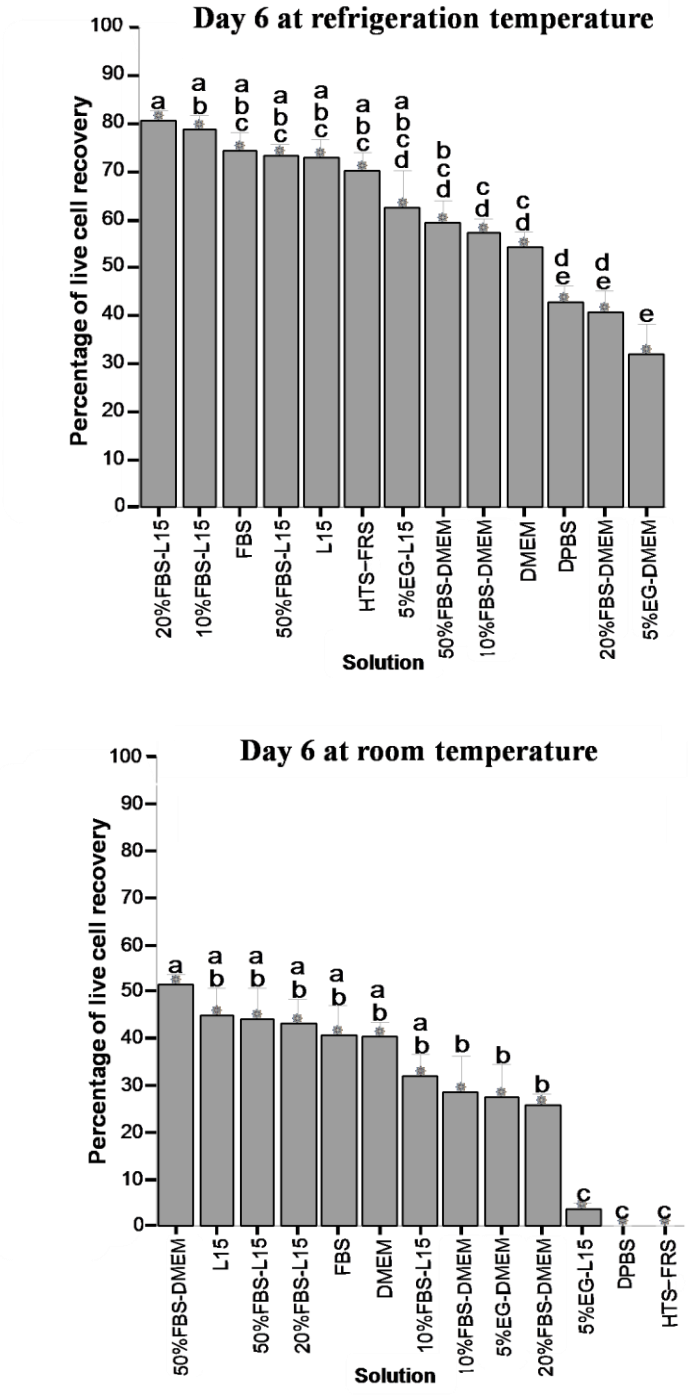


Fig. 5.3. Comparison of different preservation media on day 6 at room (RT) and refrigeration (RG) temperatures. On day 6, thirteen media were compared for the percentage of live cells compared to Day 0. Columns without a common letter are significantly different ($P < 0.05$).

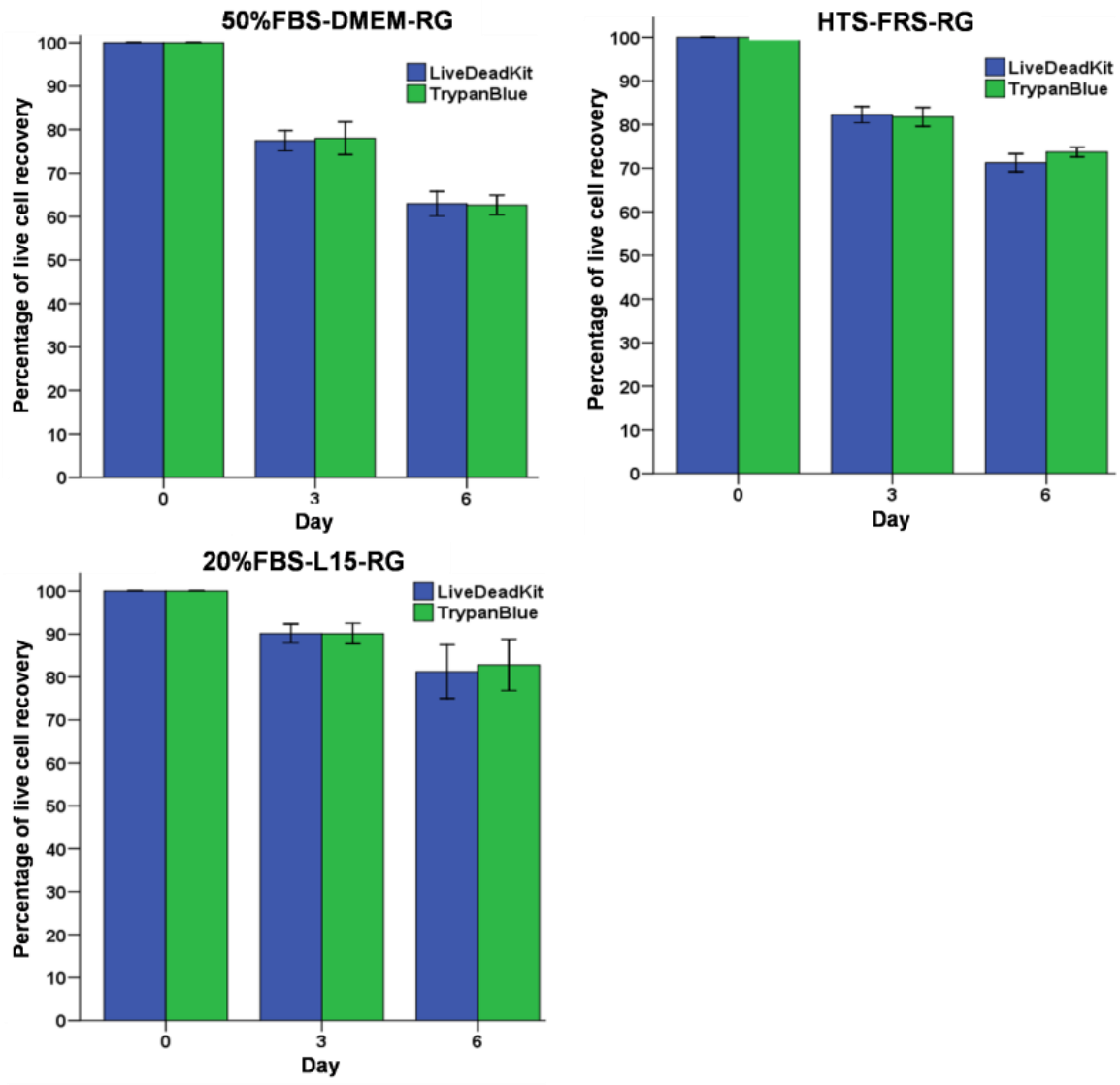


Fig. 5.4. Comparison of cell viability results assayed using trypan blue, or live/dead viability/cytotoxicity kit for selected protocols. Percentage of lives cells obtained from the two assays were compared for preservation Days 0, 3, and 6 for three selected protocols (50% FBS-DMEM, 20% FBS-L15 and HTS-FRS at refrigeration temperature). No significant difference was found between the two methods for any of the protocols or days.

5.4.8 *In Vitro* Culture of Preserved Cells

After 6 days of preservation, the remaining cells were cultured for 3 days to evaluate their developmental potential. In culture, preserved cells from the selected media showed no obvious morphological changes, compared with fresh cells (Fig. 5.6). A confluent cellular layer was formed at the bottom of the plate with single round cells or colony-like 3-dimentional structures on top, for cultures of both preserved and fresh cells (Fig. 5.6).

5.5 Discussion

To our knowledge, this is the first study of testis cell preservation under hypothermic conditions (for up to 6 days with up to 81% viability at RG). It should be emphasised that our cell viability data actually represent the cell survival rate over time, since it is expressed as the percentage of remaining live cells, compared with the number of live cells before preservation. This differs from most reports where viability of cells after preservation is provided without cell recovery information. Without such information, it is difficult, if not impossible, to evaluate the actual cell viability/survival, because dead cells may disintegrate and therefore cannot be detected using viability assays, resulting in seemingly higher viability rates. Here, testis cells were repeatedly measured daily for 6 days and on each day live cell number was compared with the pre-storage total live cell number; therefore, cell recovery information is also incorporated in the reported viability data.

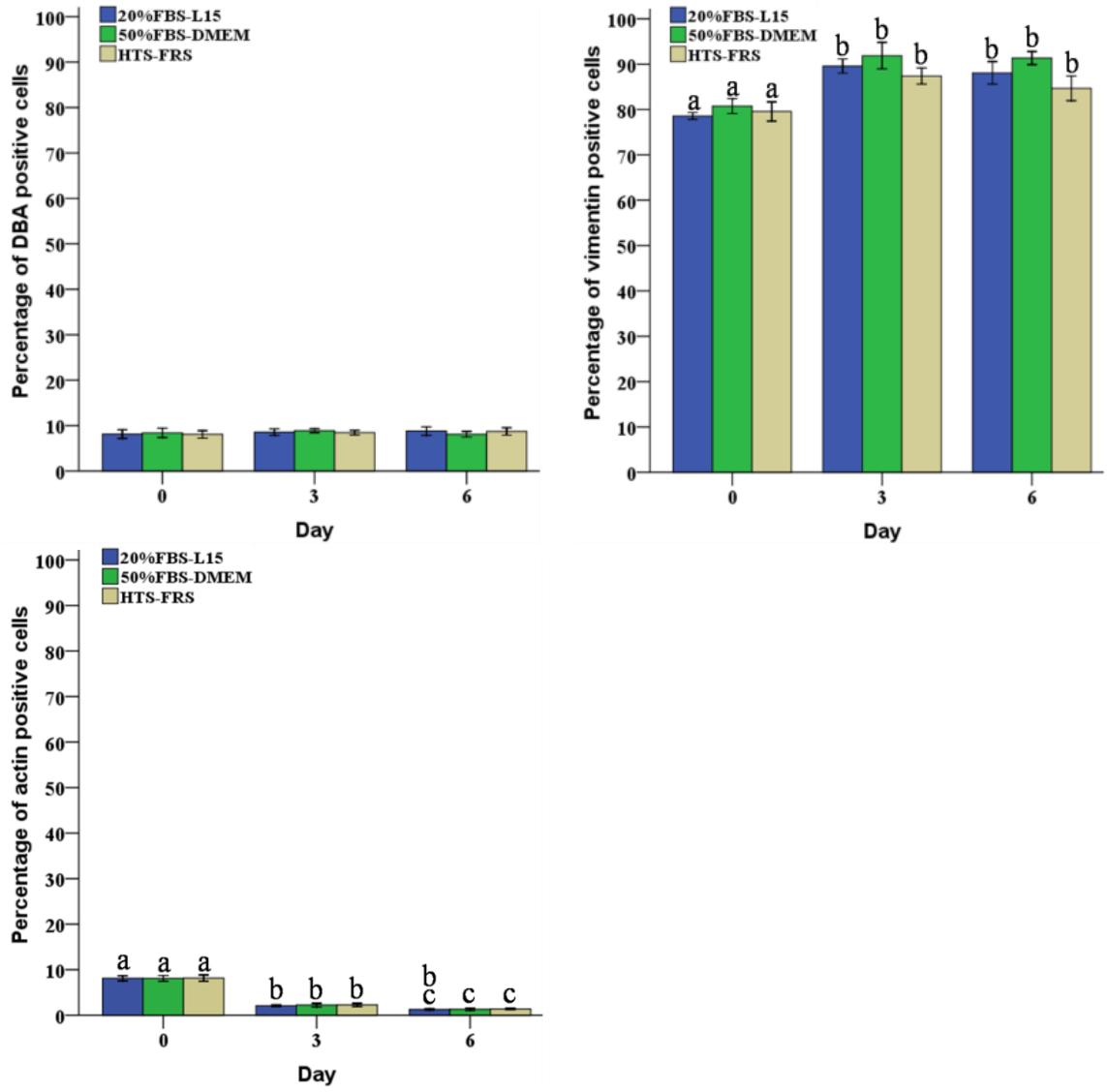


Fig. 5.5. Cellular sub-populations of testis cells on days 0, 3, and 6 preserved with three selected protocols. Preserved cells underwent immunocytochemistry to identify gonocytes, Sertoli and peritubular myoid cells on preservation Days 0, 3, and 6 for the three selected protocols (50% FBS-DMEM, 20% FBS-L15 and HTS-FRS at refrigeration temperature). Data were analyzed for the effects of media and preservation day. Columns without a common letter are significantly different ($P < 0.05$).

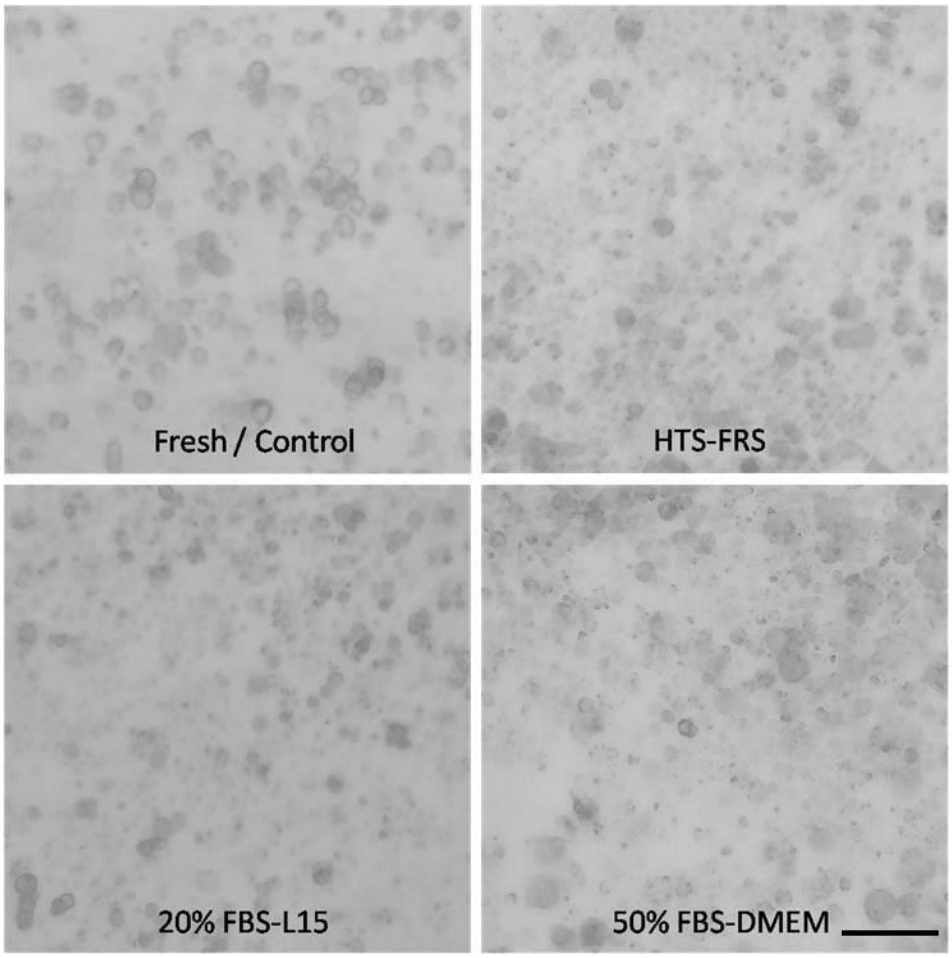


Fig. 5.6. Appearance of cultured cells from preservation days 0 and 6 for three selected protocols. Day 0 (fresh) testis cells as well as those preserved for 6 days in one of three selected protocols (50% FBS-DMEM, 20% FBS-L15 and HTS-FRS at refrigeration temperature) were cultured *in vitro* for 3 days. No obvious changes were observed between fresh and preserved cells in their appearance after 3 days in culture. In each case, they displayed confluent layers of cells at the bottom of plates and colony-like structures formed by round cells on top. Scale bar, 60 μm.

Hypothermic conditions can be classified into four levels, namely mild (35 to 32 ℃), moderate (32 to 27 ℃), profound (27 to 10 ℃), and ultra-profound (10 to 0 ℃) (Taylor 2000), with profound temperatures being mostly used for organ preservation. For hypothermic preservation of cells, two rather distinct cell damage mechanisms have been reported to occur at above 10 ℃ and below 5 ℃ (Kruuv *et al.* 1995); therefore, we compared testis cell storage at room and refrigeration temperatures. All tested media showed higher cell viability rates after preservation at RG than at RT, although the use of certain media and additives narrowed this difference. These results collectively indicate that it is feasible to preserve testis cells with high cell survival rate for at least 6 days at RG.

In the present study, a range of media from DPBS to a specialised hypothermic preservation product (HTS-FRS) were compared for their efficiency in short-term storage of testis cells. Interestingly, even saline at RT appeared capable of maintaining more than half of testis cells for up to 3 days, while refrigeration and the use of complex media may be necessary to preserve testis cells for 6 days or possibly longer.

There were significant interactions between temperature and media ($P < 0.001$). Compared with DPBS, viability of cells maintained in DMEM and L15 tended to be in a closer range when kept at RT and RG. The use of HTS-FRS at RT resulted in very low cell viability (Fig. 5.1- 5.3), probably because this solution is designed for use at 2 to 8 ° C.

Surprisingly, under refrigeration conditions, L15 was comparable to HTS-FRS in maintaining cell viability on both preservation Days 3 and 6 (Fig. 5.2 and 5.3). Most culture media such as DMEM are buffered with a bicarbonate system requiring an

atmosphere with 5% CO_2 in order to maintain a physiological pH (Dontchos *et al.* 2008). However, L15 has been developed with a phosphate buffer system for CO_2 independent usage (Barngrover *et al.* 1985; Leibovitz 1963), and this may have helped to maintain high cell viability by stabilising pH in ambient air in our study.

Hypothermic preservation may induce cell injury and death due to apoptosis and necrosis. Therefore, inhibition of these processes could improve the efficiency of hypothermic preservation. FBS is commonly used as an additive in cell culture and preservation protocols. Therefore, we supplemented media (DMED and L15) with serial concentrations of FBS. Although 20% and 50% FBS resulted in the highest cell viability in L15 and DMEM, respectively, the beneficial effect of FBS did not seem to be proportional to its concentration. Of many undefined components in FBS, serofendic acid has been found to be able to attenuate cell injures caused by necrotic and apoptotic changes (Kume *et al.* 2006). These positive effects of FBS have also been observed in hypothermic preservation of osteochondral allografts (Pennock *et al.* 2006).

The cryoprotective agent polyethylene glycol has been used in hypothermic media and was shown to protect the renal medulla during kidney preservation (Faure *et al.* 2004). We tested the addition of EG since it has advantages over most permeating cryoprotectants such as DMSO, glycerol and propanediol including low toxicity and high permeability (Davidson and Gerald 1977; Massip 2001; Moore and Bonilla 2006). In DMEM, EG showed an obvious deleterious effect on cell viability under both RT and RG. Surprisingly, however, EG in L15 maintained more live cells after 6 days at RG than RT, to levels that were even higher in value than those obtained in DMEM with FBS at all concentrations tested (Fig. 5.1 and 5.3).

Testis cells used in the present study were a heterogeneous mixture mainly of gonocytes, Sertoli and peritubular myoid cells. The ratio of cell subpopulations after preservation has important implications, for example in subsequent enrichment of gonocytes, or for the study of these cell types, their culture or interactions. After 6 days of preservation, the percentage of Sertoli cells increased, whereas that of peritubular myoid cells decreased (Fig. 5.5). This increase in the proportion of Sertoli cells might be due to their tolerance for hypothermic conditions (Young *et al.* 1988). Peritubular myoid cells in humans and rats are smooth muscle cells (Virtanen *et al.* 1986), and smooth muscle cells show high apoptosis rates in response to low temperatures (Yiu *et al.* 2007). This may explain why in the present study peritubular myoid cells were more vulnerable to hypothermic conditions than Sertoli cells. Therefore, depending on the cell type of interest, hypothermic preservation conditions may need to be optimized to obtain maximum efficiency (Caputo *et al.* 1998; Hendry *et al.* 1990; Mathew *et al.* 2004; Wu *et al.* 2006).

In conclusion, testis cells could be successfully preserved under hypothermic conditions, for at least 6 days with ~80% survival, and 88% after-storage viability (comparable to fresh cells). Cells could be efficiently preserved at room temperature for 3 days. Leibovitz L15 alone could be used as an effective defined preservation medium, the effects of which can be further increased with FBS supplementation. After 6 days of hypothermic preservation, testis cells could still be cultured without obvious morphological changes.

CHAPTER 6 THE EFFECTS OF TISSUE SAMPLE SIZE AND MEDIA ON SHORT-TERM HYPOTHERMIC PRESERVATION OF PORCINE TESTIS TISSUE[1]

6.1 Abstract

The objective of this study was to develop effective strategies for hypothermic preservation of immature porcine testis tissue to maintain structural integrity and cell viability. In Experiment 1, testes from 1-wk-old piglets were used to study the effects of tissue sample size (as intact testis or fragments of 100- or 30 mg) and the use of one of 9 different media on hypothermic preservation of the testis tissue for 6 days. The examined media included: Dulbecco phosphate buffered saline (DPBS), Dulbecco modified Eagle medium (DMEM), Leibovitz L15 (L15), L15 with foetal bovine serum (FBS, at 10%, 20% or 50%), HypoThermosol-FRS solution (HTS-FRS), Ham's F12, and Media 199. On Day 0, 3 and 6, testis tissues were digested to compare the cell survival rates. Tissue sections were also semi-quantitatively assessed to determine the efficiency of different preservation strategies. There was no effect of testis sample size ($P > 0.05$), but cell survival rates of testis cells isolated from preserved testis tissues changed depending on the medium and day ($P < 0.05$). Testis tissue within HTS-FRS did not show morphological changes after 6 days. In Experiment 2, two of the top performing media (20% FBS-L15 and HTS-FRS) were selected for immunocytochemical detection of gonocytes. Proportions of gonocytes (%) in isolated testis cells, however, did not differ

[1] This study has been published. Y. Yang, J. Steeg and A. Honaramooz (2010). Cell Tissue Res. 340(2): 397-406. Portions of this study were also presented at the 42nd annual meeting of the Society for the Study of Reproduction.

between the two media on Days 0, 3 or 6. These results show that testis tissue can be maintained for 3 days at 4 ℃ with high cell survival rate, and tissue morphology can be preserved for at least 6 days in HTS-FRS.

6.2 Introduction

The functional components of the early postnatal testis include seminiferous cords surrounded by the interstitial cells responsible for releasing androgens. Within the seminiferous cords, Sertoli cells provide support for gonocytes which are progenitors for spermatogonial stem cells (França *et al.* 2000; Frankenhuis *et al.* 1981; Goel *et al.* 2007; Hughes and Varley 1980; Ryu *et al.* 2004). Spermatogonial stem cells are unique among adult stem cells in that they possess the unique capability to both self renew and to contribute genes to the next generation through differentiation to give rise to a lifetime supply of gametes (de Rooij 1998; Oatley and Brinster 2008). Recent breakthroughs in the study and manipulation of these cells have highlighted their important potential for overcoming male infertility as well as for animal transgenesis and conservation (Dobrinski and Travis 2007; Ehmcke and Schlatt 2008; Oatley and Brinster 2008).

Effective preservation of testis tissue and cells is therefore essential for most downstream applications. In many instances, the immediate use of the testis tissue or its cryopreservation is neither practical nor desired and where short-term storage of the tissue is required and seems more appropriate. Such instances include collection of testes sent to collaborating laboratories for testis tissue xenografting (Honaramooz *et al.* 2004; Honaramooz *et al.* 2002b), or various steps in preparation for male germ cell

transplantation, where testis cells from a donor individual are to be transplanted into seminiferous tubules of recipients (Honaramooz et al. 2002b; 2003; 2008).

We recently found that isolated testis cells could be preserved for at least 6 days at 4 ℃ with 88% after-storage cell viability. Furthermore, testis cells preserved for 6 days could develop in culture with no obvious morphological changes (Yang and Honaramooz 2010). These promising results encouraged us to preserve testis tissue.

The preservation of intact or fragmented testis tissue, as opposed to preservation of isolated cells, has certain advantages. Preserved testis tissue could maintain the *in situ* relationship of structures, including spatial orientation, stem cell niche and functional interplay, between live somatic cells and germ cells essential for the study of spermatogenesis or testis function. Preserved tissue could also provide each type of testis cell for later isolation. For example, preserved testis tissue could be xenografted to produce donor-derived spermatogenesis in recipient laboratory mice (Goossens *et al.* 2008; Honaramooz *et al.* 2002b; Shinohara *et al.* 2002a; Song and Silversides 2007; Zeng *et al.* 2009). The efficiency of frozen-thawed testis tissue for such applications is quite low, while that of testis tissue preserved hypothermically for up to 2 days is not much different from fresh tissue (Abrishami *et al.* 2010; Zeng *et al.* 2009). However, storage longer than 2 days could be beneficial.

Preservation of intact testes or testis tissue fragments is especially practical in field situations where tissues are to be collected, requiring considerably less laboratory resources, expertise and facilities than for cell isolation. Furthermore, testis tissue amounts used for cryopreservation are usually about 3-5 mg, and the recovery of sufficient number of cells from such small samples as well as the preservation of cell

viability after thawing are currently challenging (Jahnukainen *et al.* 2007a; Keros *et al.* 2007; Milazzo *et al.* 2008; Wyns *et al.* 2007). Therefore, short-term storage of chilled testis tissue may offer an alternative to cryopreservation.

The objective of this study was to investigate the effect of tissue amount, intact or fragmented, and media on cell recovery and testicular morphology after short-term hypothermic preservation.

6.3 Materials and Methods

6.3.1 Experimental Design

In order to develop strategies for short-term hypothermic preservation of testis tissue we undertook this study in two phases. In the first phase, testis tissue samples of different sizes were maintained in 9 different media at refrigeration temperatures for 6 days. In the second phase, the two most promising media in the first phase were further evaluated for their tissue and cell preservation efficiency.

6.3.2 Collection of Testes and Preparation of Testis Tissue

Testes were collected after aseptic castration of 1-wk-old Yorkshire-cross piglets (n = 228 piglets, Camborough-22 × Line 65, PIC Canada Ltd., Winnipeg, MB, Canada) at a university-affiliated swine facility. Testes were transferred to the laboratory within 2 h in ice-cold Dulbecco phosphate buffered saline (DPBS, cat. # 20-031-CV, Mediatech, Manassas, VA, USA) containing 2% w/v antibiotic-antimycotic solution (cat. # 30-004-CI, Mediatech). In the laboratory, the testes were immediately rinsed three times with DPBS, the epididymis removed and the testes were either used intact or were further trimmed by removing the tunica albuginea, rete testis and overt connective tissue. The

testis parenchyma was then cut into pieces of either 100 or 30 mg. Experimental procedures involving animals were approved by the University of Saskatchewan's Institutional Animal Care and Use Committee.

6.3.3 Hypothermic Storage of Testis Tissue

Intact testes and testis fragments of 100 or 30 mg were stored for 6 days at 4 ℃ in polypropylene tubes containing one of the following media: DPBS, Dulbecco modified Eagle medium (DMEM, cat. # 10-013-CM, Mediatech), Leibovitz L15 (L15, cat. # 95016-512, VWR International, Mississauga, ON, Canada), L15 with foetal bovine serum at 10%, 20% or 50% (FBS, cat. # A15-701, PAA Laboratories GmbH, Etobicoke, ON, Canada), HypoThermosol-FRS solution (HTS-FRS, cat. # 609144, Biolife Solutions, Bothell, WA, USA), Ham's nutrient mixture F12 with L-glutamine, 25 mM HEPES (F12, cat. # 09321, ScienCell Research Laboratories, Carlsbad, CA, USA), and Media 199 with Hank's salts and L-glutamine (M199, cat. # 09121, ScienCell).

6.3.4 Isolation of Testis Cells

On preservation Days 0, 3 and 6 after collection, the tissues were digested to assess testis cell recovery using a previously described two-step digestion method with minor modifications (Honaramooz *et al.* 2002a). Testis tissues were minced with fine scissors, and digested with 0.2% w/v collagenase IV (cat. # C-5138, Sigma-Aldrich, Oakville, ON, Canada), 0.1% w/v hyaluronidase (cat. # H-3884, Sigma-Aldrich) and 0.01% w/v DNase I (cat. # DN25, Sigma-Aldrich) in DMEM at 37 ℃ for 15 min with agitation every 5 min. After centrifugation at 15*g* at 16 ℃ for 1 min and discarding of the supernatant, tissue pellets were further digested with 0.25% w/v trypsin with 2.21 mM

EDTA (cat. # 25-053-CI, Mediatech) at 37 ℃ for 5 min. FBS was then added to stop the digestion and the suspension was triturated with a 1 ml pipette tip before filtration through a 40 μm filter (cat. # 352340, BD Biosciences, Mississauga, ON, Canada). The erythrocytes that were inevitably collected along with testis cells and can interfere with cell counting, were then removed with a lysis buffer (NH_4CL 156mM, $KHCO_3$ 10 mM, Na_2EDTA 0.1mM (Sethu *et al.* 2004; Smith *et al.* 2009)) at ratio of 4:1 (buffer: cell suspension) for 15 min at room temperature. The suspension was then centrifuged at 600 $\times g$ at 4 ℃ for 4 min and rinsed twice with 10 mL of 10% FBS-DMEM. Cell viability and concentration were assayed with a hemocytometer using the trypan blue exclusion method (0.4% w/v, cat. # T8154, Sigma-Aldrich).

Cell survival rate was defined as the percentage of live cells isolated from preserved tissues on Days 3 or 6, compared to the original number of live cells on Day 0 ($n \geq 6$ replicates per group). To reduce potential between-sample variations, the intact testis and tissue pieces (≥2 of each 100- and 30 mg pieces) used for comparison of different tissue sizes for each medium were from the testes of the same donor piglet, for each preservation Day 3 and 6. Cell survival rates were compared among preservation strategies, and based on these data, cell smears were prepared from fresh samples, as well as from the two best preservation media, and stored at -80 ℃ for subsequent immunocytochemistry.

6.3.5 Semi-Quantitative Morphometric Analyses

Samples of fresh and preserved testis tissue were fixed in Bouin's solution for 24 h, rinsed with and stored in 70% ethanol, processed for standard histological

preparations, embedded in paraffin and sectioned at 4 μm thicknesses. The sections were then prepared and stained with haematoxylin and eosin.

The histological attributes of testis tissue were then evaluated using transmitted light microscope as previously described (Milazzo *et al.* 2008) with minor modifications as follows. Slides were coded for blinded analysis and a minimum of 30 seminiferous cords were randomly selected from different areas within each sample ($n \geq 4$ replicates/medium/tissue size). The structural and cellular degenerative changes of the tissues were semi-quantitatively scored at magnifications of ×400 and ×1000. The evaluations were based on the assessment of nucleic and epithelial morphology and included: **1)** Nucleic distinction between Sertoli cells and gonocytes was scored 0 if easy, 1 if difficult or 2 if impossible; **2)** observation of nucleoli in Sertoli cells and gonocytes was scored as either 0 if easy (visible in >40% of cells) or 1 if indistinguishable (when pyknotic nuclei present in large numbers and very condensed); **3)** nucleic condensation for Sertoli cells and gonocytes was scored 0 where absent, 1 where <40% of nuclei were condensed or 2 where >40% were pyknotic; **4)** detachment of cells from the basement membrane was scored 0 if absent, 2 if partial or 3 if total or observed on >75% of the circumference; **5)** fragmentation of the basement membrane was scored either as 0 if absent and as 1 if obvious; and **6)** Leydig cells nuclei condensation was also scored as either 0 if <40% were condensed or 1 if >40% were condensed. The sum of the above scores was calculated as the degeneration score for each seminiferous cord and its surrounding Leydig cells. The average for all measurements in the slides was referred as the global degeneration score for that testis sample. Therefore, the degeneration score could range from 0 to a maximum of 10 per sample, where 0 represents ideal normal

morphology and an absence of any of the measured aberrations, and 10 indicates the worst morphometric score for the sample.

6.3.6 Immunohistochemistry

Tissue sections (4 μm) were prepared as described above, deparaffinised, rehydrated, rinsed with DPBS and incubated with 5% w/v bovine serum albumin (BSA, cat. # A7638, Sigma-Aldrich) at 37 ℃ in humidified atmosphere for 30 min. Samples were then stained with the lectin Dolichos biflorus agglutinin (DBA, (Goel *et al.* 2007) conjugated with fluorescein (1:100 v/v (50 μg/mL), cat. # FL-1031, Vector Laboratories, Burlington, ON, Canada) overnight in humidified atmosphere. The sections were then rinsed with DPBS, incubated with 0.3% Sudan Black B (cat. # 3545-12, EMD Chemicals, Gibbstown, NJ, USA) in 70% ethanol at 37 ℃ in humidified atmosphere for 8 min, followed by rinsing and staining with 4, 6-diamino-2-phenyl indole (DAPI, cat. # D-9542, Sigma-Aldrich) for 3 min. The sections were subsequently mounted with a mounting medium (Vectashield, cat. # H-1000, Vector Laboratories) and examined using fluorescent microscope. Laser scanning confocal microscope was also used to sequentially scan DAPI and fluorescein.

6.3.7 Immunocytochemistry

After thawing at room temperature, the cell smears from the two best preservation media were fixed in Bouin's solution for 2 to 3 min, rinsed in DPBS, blocked with 5% BSA at 37 ℃ in humidified atmosphere for 15 min ($n \geq 4$ replicates/medium/tissue size). The smears were then rinsed with DPBS and incubated with fluorescein labelled lectin DBA (1:100) overnight in humidified atmosphere. After further rinsing with DPBS and

incubation with 0.3% Sudan Black B for 10-15 min, the cell smears were rinsed and stained with DAPI for 2 min, mounted and observed under fluorescent and laser scanning confocal microscopes. At least 600 cells were counted for each cell smear to determine the proportion of gonocytes.

6.3.8 Statistical Analysis

Cell survival rates (% ± s.e.m.) are expressed as the percentage of live cells isolated from preserved tissue samples on Days 3 or 6, compared to the original number of live cells on Day 0. For comparison of cell survival rates, three-way ANOVA was used with preservation day, media and tissue sample sizes as independent factors. There were significant effects of the day and medium but not tissue sample size, and no interactions were found for the tissue sample size. Therefore, the data across all tissue sizes were pooled and the analysis was repeated using two-way ANOVA, with the day and medium as main factors, followed by Holm-Sidak tests. Semi-quantitative morphometric data were transformed and analyzed using three-way ANOVA as above. Again, there was no effect of testis sample size or interactions with it; therefore, the data were pooled and reanalyzed using two-way ANOVA for the day and medium. Spearman's rank correlation coefficient was performed to test the relationship between the preserved testis cells survival rates and morphometric data. Similarly, three-way ANOVA was performed on gonocytes proportion data, with the day, medium and tissue sample size as the main factors. Data are expressed as mean ± s.e.m. and differences were considered statistically significant when $P < 0.05$.

6.4 Results

6.4.1 Effects of Different Preservation Conditions on Cell Survival Rates

The actual average number of cells resulting from digestion of fresh testis tissue (Day 0) was 235×10^6 per gram tissue with a cell viability of 92%. However, the cell survival rates were expressed as the percentage of remaining live cells on Days 3 or 6, compared to the number of live cells prior to preservation (Day 0); therefore, the survival rate of fresh control was considered as 100%. Survival rates for cells isolated after enzymatic digestion of preserved testis tissues changed depending on the main factors of day and media, without an interaction between them. These rates, however, did not vary based on the main effect of tissue sample size (intact testes, 100, or 30 mg pieces), and no interactions were found for the tissue size ($P > 0.05$). Therefore, tissue sample size data were pooled. For preservation Day 3, the cell survival rates differed among media groups ($P < 0.001$), where HTS-FRS group preserved the greatest number of live cells and DMEM preserved the least number of cells (50% ± 3.5 and 18% ± 3.9, mean ± s.e.m., respectively, Fig. 6.1A). Similarly, when compared on preservation Day 6, the cell survival rates changed among different media groups ($P < 0.001$), with HTS-FRS maintaining the greatest cell survival rate and DMEM resulting in the least cell survival rate (26% ± 2.5 and 5% ± 3.3, respectively, Fig. 6.1B). When compared among days, cell survival rates dropped from Day 0 to 3, and from Day 3 to 6 in testis tissues within all media tested ($P < 0.001$), with the majority of the media including the specialized preservation solution HTS-FRS maintaining this rate at about 50% on Day 3, and 25% or less on Day 6, compared to that of control fresh cells (Fig. 6.1AB).

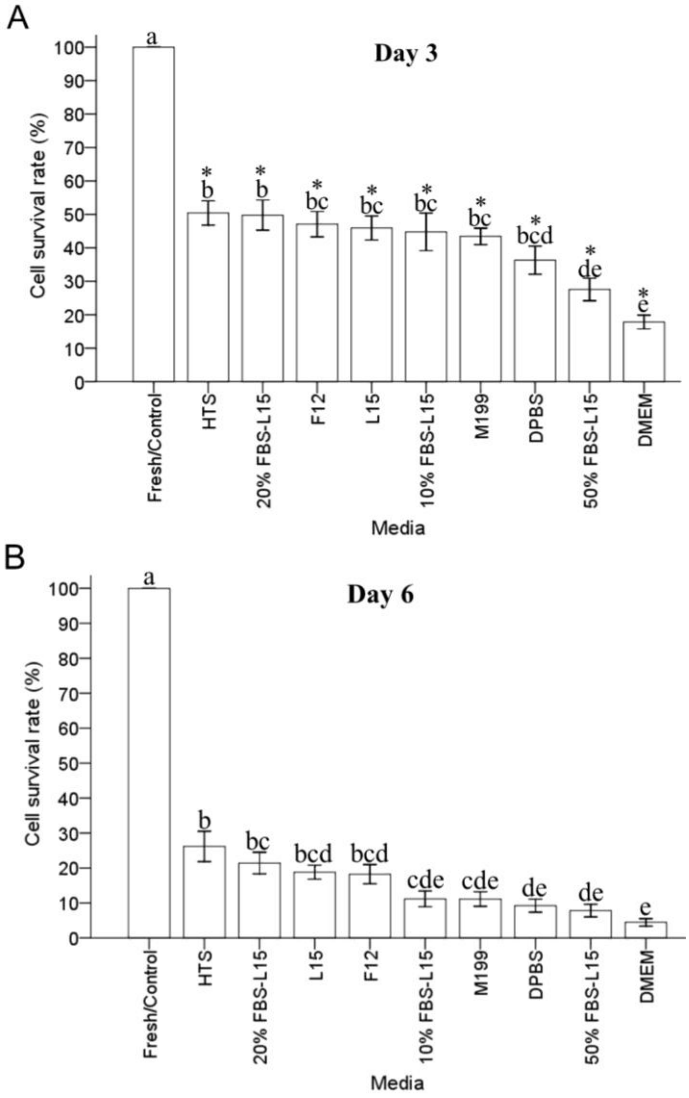


Fig. 6.1. The effect of media on cell survival rates of preserved testis tissue samples. Nine media were evaluated for the preservation of testis tissue samples at 4 ℃ for 6 days. On preservation Days 0, 3 or 6, testis samples were digested. The percentage of live cells (mean% ± s.e.m.) remaining on Day 3 or 6, were compared to the original number of live cells on Day 0 and defined as the cell survival rate. Columns without a common letter within each day are significantly different ($P < 0.05$). Columns for Day 3(A) with asterisks are significantly different from the corresponding columns for Day 6(B) ($P < 0.05$).

6.4.2 Semi-quantitative Evaluations of Tissue Morphology

Global degeneration scores for preserved testis tissues changed depending on the main effects of day and media, without interactions. These scores, however, did not vary based on the tissue sample size as a main effect and no interactions were found ($P > 0.05$); therefore, data were pooled.

For Day 3, the results of morphological evaluations for global degeneration varied among different media groups ($P < 0.001$). Compared with the fresh testis tissue (0.5 ± 0.2, mean global degeneration scores ± s.e.m.), HTS-FRS preserved the tissue with minimal degenerative changes (0.7 ± 0.2, $P > 0.05$) while DMEM resulted in the maximum morphometric changes (2.8 ± 0.2, $P < 0.05$, Fig. 6.2A and Fig. 6.3A-J). Compared to the fresh control testis tissue, nuclei differentiation between Sertoli cells and gonocytes in tissues within DMEM was not as easily performed (0.0 ± 0.4 *vs.* 1.4 ± 0.2, $P < 0.01$). When compared to the controls, the basement membrane of seminiferous cords were partially detached in tissues maintained in DMEM (0.05 ± 0.27 *vs.*1.3 ± 0.2, respectively, $P < 0.01$), L15 (1.2 ± 0.1, $P < 0.01$) and 50% FBS-L15 (1.1 ± 0.18, $P < 0.01$, Fig. 6.2A and Fig. 6.3A-J).

On Day 6, the global degeneration scores again differed among the media groups ($P < 0.001$). Compared with the fresh testis tissue, the minimum morphological changes were observed in tissues within HTS-FRS (0.5 ± 0.23 *vs.*1.1 ± 0.38, respectively, $P > 0.05$) while once more the maximum changes were observed with preservation in DMEM (7 ± 0.38, $P < 0.05$, Fig. 6.2B and Fig. 6.4A-J). There was a negative correlation between the cell survival rates and tissue degeneration scores ($r = -0.47$, $P < 0.01$).

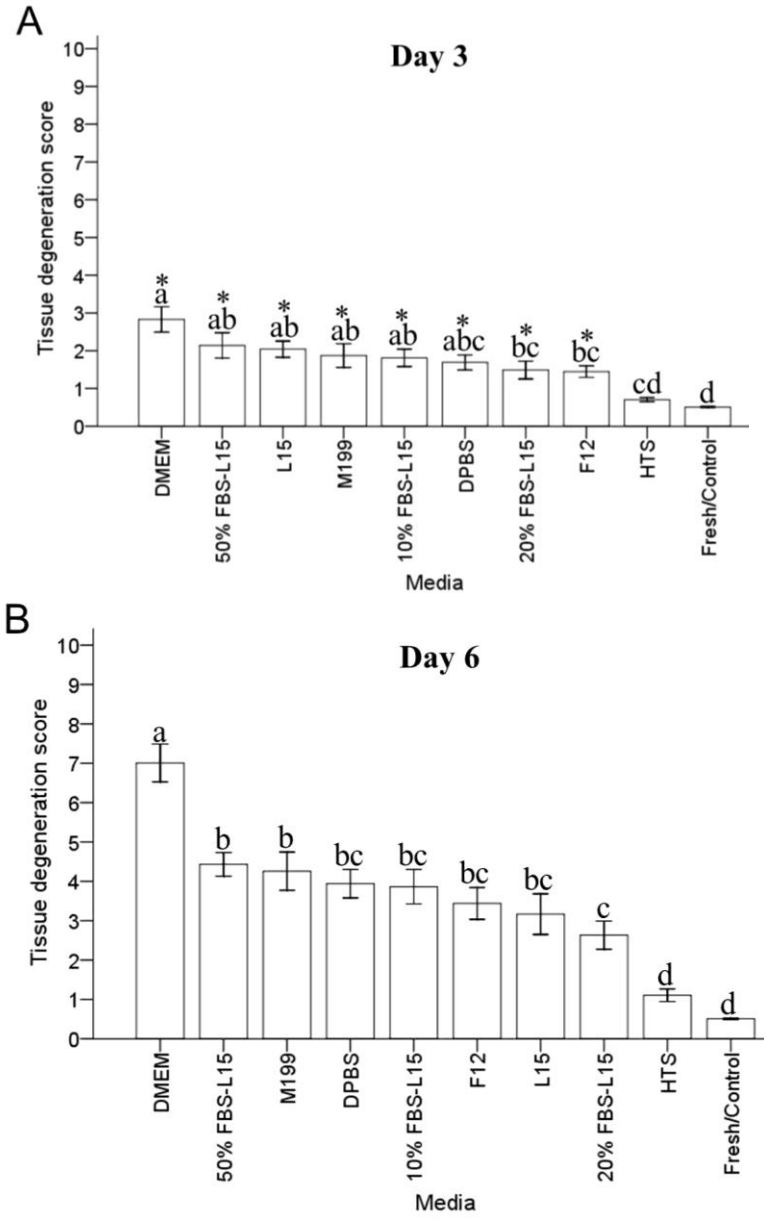


Fig. 6.2. The effect of media on morphological degeneration scores of preserved testis tissue samples. Different media were examined for preserving testis morphology at 4 ℃ for 6 days. On preservation Days 0, 3 or 6, testis tissue samples were fixed, processed, sectioned, and semi-quantitatively analyzed. The tissue degeneration scores (mean ± s.e.m.) were calculated based on scores given to the degree of nucleic distinction between Sertoli cells and gonocytes, observation of nucleoli, nucleic condensation of Sertoli cells and gonocytes, detachment of the basement membrane, fragmentation of the basement membrane, and nucleic condensation of Leydig cells. The lower the score, the less morphological abnormalities were observed. Columns without a common letter within each day are significantly different ($P < 0.05$). Columns for Day 3 (A) with asterisks are significantly different from the corresponding columns for Day 6 (B) ($P < 0.05$).

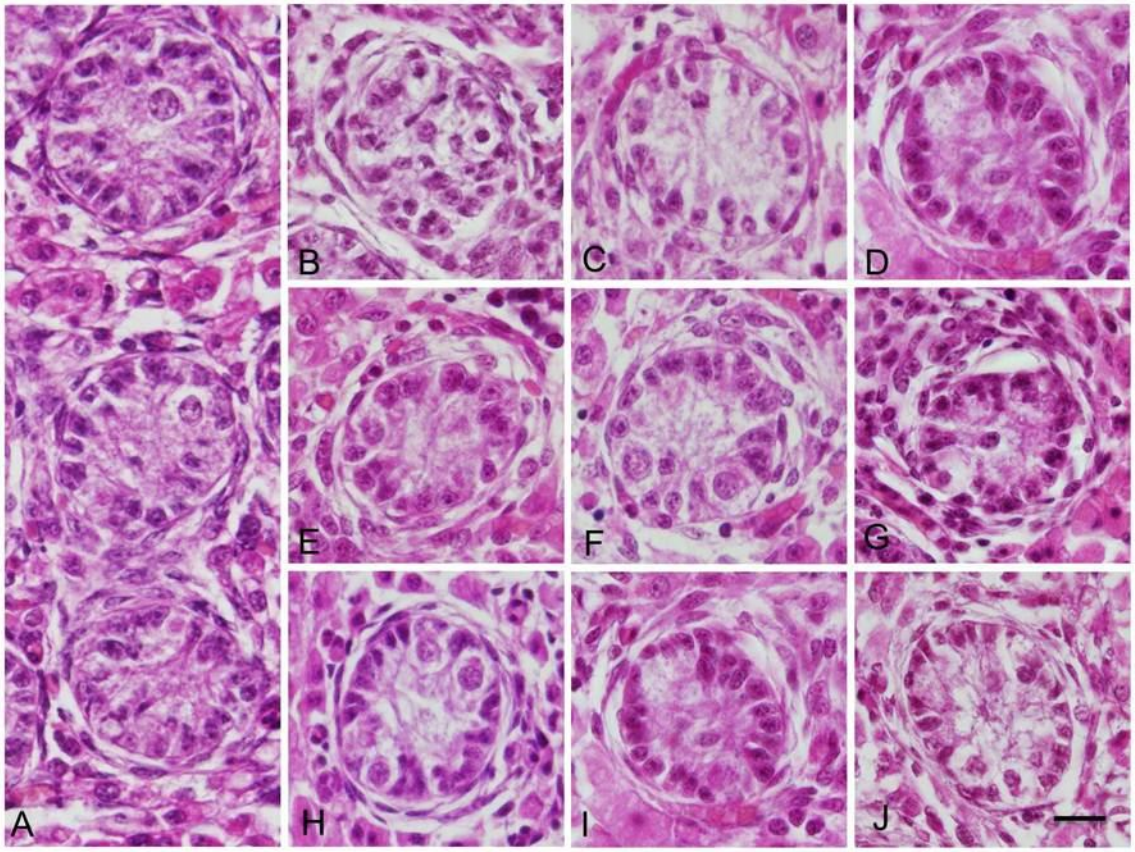


Fig. 6.3. Morphology of preserved testis tissue samples on day 3. Photomicrographs of representative seminiferous cords after storage in different media at 4 ℃ for 3 days. Semi-quantitative morphometric analysis was performed to assess the changes in the appearance of the nuclei, nucleoli, as well as the epithelial integrity in preserved testis tissues, compared to the fresh control samples (A). The examined media included Dulbecco modified Eagle medium (DMEM, B), Dulbecco phosphate buffered saline (DPBS, C), Leibovitz L15 (L15, D), L15 with foetal bovine serum (FBS, at 10%, 20% or 50%) (E, F, G), HypoThermosol-FRS solution (HTS-FRS, H), Ham's F12 (F12, J), and Media 199 (M199, I). Scale bars, 15 µm.

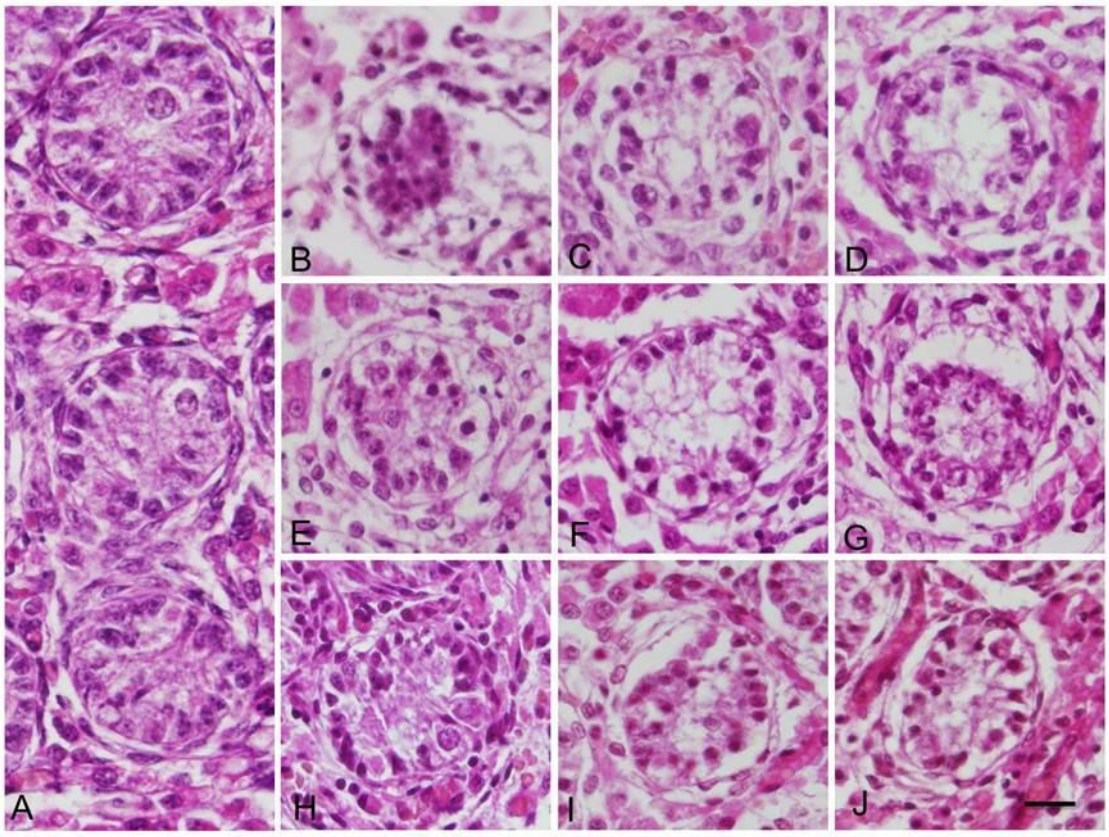


Fig. 6.4. Morphology of preserved testis tissue samples on day 6. Photomicrographs of representative seminiferous cords after storage in different media at 4 ℃ for 3 days. Semi-quantitative morphometric analysis was performed to assess the changes in the appearance of the nuclei, nucleoli, as well as the epithelial integrity in preserved testis tissues, compared to the fresh control samples (A). The examined media included Dulbecco modified Eagle medium (DMEM, B), Dulbecco phosphate buffered saline (DPBS, C), Leibovitz L15 (L15, D), L15 with foetal bovine serum (FBS, at 10%, 20% or 50%) (E, F, G), HypoThermosol-FRS solution (HTS-FRS, H), Ham's F12 (F12, J), and Media 199 (M199, I). Scale bars, 15 μm.

When compared to the control tissue, and based on nucleic observations, gonocytes were less easily distinguishable from Sertoli cells ($P < 0.01$) in tissues within 50% FBS-L15 (0.7 ± 0.12), or DPBS (0.8 ± 0.12). It was very difficult to discern nucleoli in cells of tissues within DMEM (1.6 ± 0.13, $P < 0.05$). Nucleic condensation occurred in cells within the seminiferous cords as well as in Leydig cells for tissues within DMEM (1.7 ± 0.13 and 0.5 ± 0.03, respectively, $P < 0.01$). Tissues maintained in 50% FBS-L15 also showed nucleic condensation in gonocytes and Sertoli cells (0.9 ± 0.13), and those of DPBS in Leydig cells (0.4 ± 0.02) ($P < 0.01$). Detachments of the seminiferous basement membrane were frequently encountered in tissues maintained in DMEM (2.2 ± 0.17), M199 (1.9 ± 0.17), 50% FBS-L15 (1.8 ± 0.17), L15 (1.8 ± 0.19) and 10% FBS-L15 (1.7 ± 0.17) ($P < 0.01$). Broken basement membranes were also observed in tissues within DMEM (0.6 ± 0.04), M199 (0.5 ± 0.04) and 10% FBS-L15 (0.3 ± 0.04) ($P < 0.01$, Fig. 6.4A-J).

6.4.3 Proportion of Gonocytes

Based on cell survival rates and semi-quantitative morphological assessments of the preserved tissues on both preservation Days 3 and 6, two media that were consistently among the top performing media (HTS-FRS and 20% FBS-L15, Fig. 6.1AB and Fig. 6.2AB) were selected and used to determine the proportion of gonocytes among a minimum of 600 cells in each cell smear using immunocytochemical detection of DBA.

The proportion of gonocytes did not differ between cells collected from fresh tissues and those collected from presevered testis tissues within either media (HTS-FRS and 20% FBS-L15) for either preservation Day 3 or 6 ($P > 0.05$). Similarly, the gonocyte

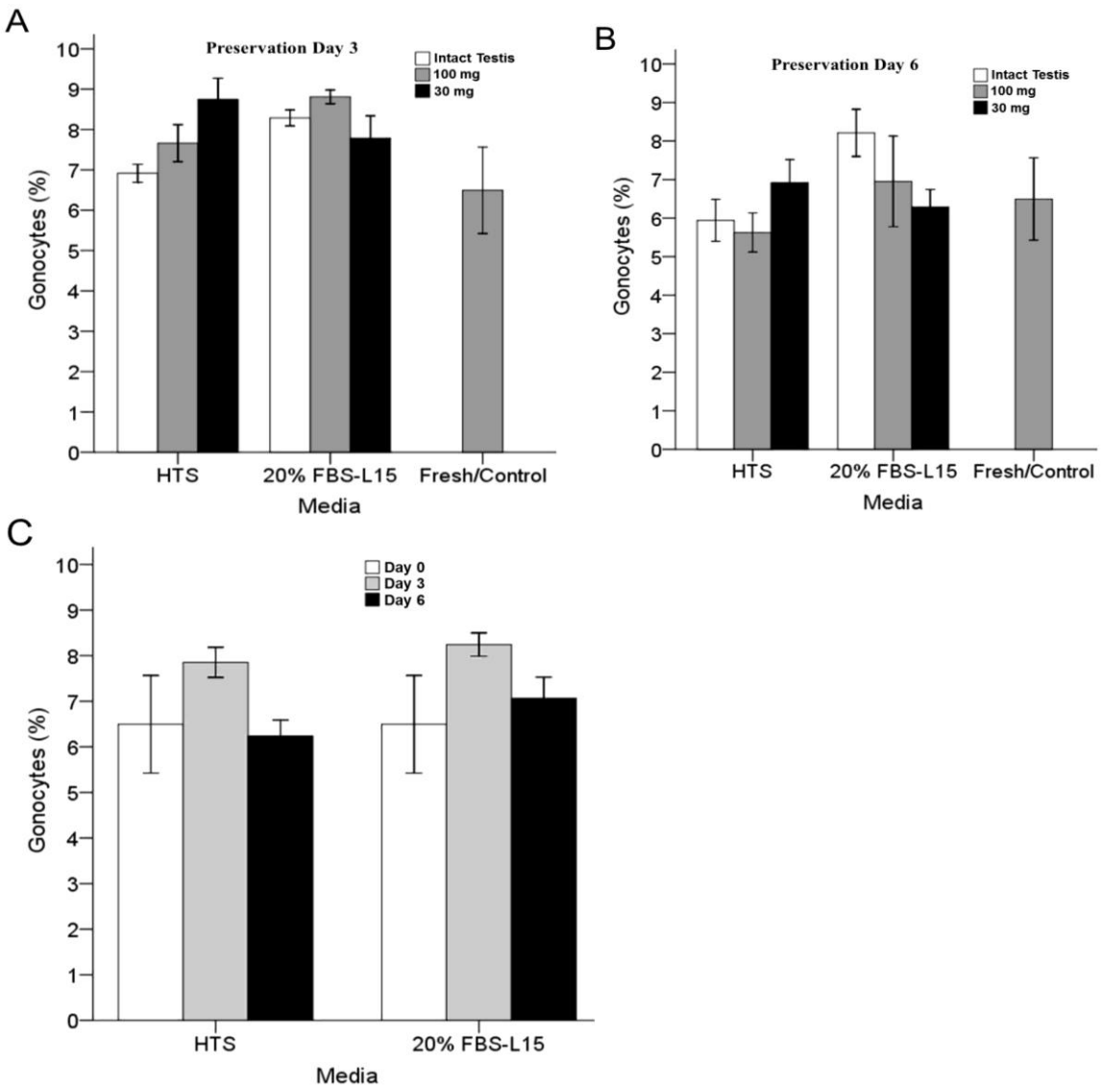


Fig. 6.5. Proportion of gonocytes remaining after storage of testis tissue samples in two select media at 4 ℃ for 3 or 6 days. In the second phase of the study, out of the nine media tested, two top performing media, HypoThermosol-FRS solution (HTS-FRS) and 20% foetal bovine serum-Leibovitz L15 (20% FBS-L15), were selected for this comparison. On preservation Days 0, 3 or 6, testis samples were digested and the proportion of gonocytes (mean% ± s.e.m.) among the testis cells were determined through immunocytochemistry with lectin Dolichos biflorus agglutinin (DBA) (A, B, C).

proportions did not change between the two media for either preservation Day 3 or 6 ($P > 0.05$, Fig. 6.5A-C).

6.5 Discussion

The current study investigated the effects of tissue sample amount and media on survival rates of testis cells in tissues preserved up to 6 days in various media. The size of the tissue during preservation had no effects on the survival or morphology of the tissue and cells. However, media composition had significant effects on both cell survival rates and testis morphology. Compared to fresh tissues, up to half of the testis cells could survive in preserved tissues after 3 days of storage at 4 ℃. Even after 6 days of preservation in these conditions, about a quarter of testis cells survived in certain media and displayed morphology similar to that of the fresh tissue. It is generally believed that hypothermic temperatures (4 ℃) used for preservation of biological samples suppress the metabolism and reduce the activity of catabolic enzymes. Metabolic rate is shown to be reduced by half for every 10 ℃ drop in the temperature, resulting in a remaining 10–12% metabolism at 4 ℃ (Southard and Belzer 1995). This may partially explain the survival of cells in simple media for days.

We expected to have higher number of cells survive in smaller size testis fragments compared to larger fragments or intact testes. It has been reported that spermatozoa could be better cryopreserved in minced tissues than in larger tissue biopsies (Crabbé *et al.* 1999). Tissue sizes ranging from 0.5 to 1.5 mm^3 are routinely used for testis cryopreservation (Abrishami *et al.* 2010; Zeng *et al.* 2009). The absence of differences among tissue sizes in our study could be partly due to the fact that the three tissue sizes we used are still much bigger than those in the above mentioned reports.

Significant differences also exist between the ways in which tissue responds to hypothermic and cryogenic storage. It should be emphasized that in the current study, in order to compare different preservation conditions we used the survival rates of isolated cells and not their actual cell viability. Cell survival rates are expressed as the percentage of remaining live cells, compared to the number of live cells prior to preservation. This differs from most reports where only viability of cells after preservation is provided without further information on cell recovery. Lack of such information makes it difficult, if not impossible, to evaluate the actual cell viability/survival rate, because dead cells may disintegrate during digestion and not be detected using viability assays, resulting in seemingly higher viability rates. Nevertheless, it appears that tissue size per se may not be an important factor in determining the outcome of hypothermic preservation of testis tissue. For short-term preservation of testis tissue in refrigeration temperatures, it may be more practical to store the testes as intact or in larger tissue pieces because this may reduce the risk of contamination and maintain the tissue for a wider range of future applications.

Our results showed that the specialized tissue preservation solution (HTS-FRS) was able to preserve the greatest testis morphology and numerically highest cell survival rates after 6 days of preservation. However, in many cases, the preservation efficiency of HTS-FRS was not significantly different from non-specialized media (e.g., 20% FBS-L15, or even L15). When small fragments (0.5 to 1 mm^3) of primate testis tissues were stored in 10% FBS-L15 for one day, the *in vitro* cell survival rates and *in vivo* ability for spermatogenic development after xenografting did not differ from those of the fresh testis fragments. This led the authors to conclude that the testis tissue was not harmed from

ischemia (Jahnukainen *et al.* 2007a). L15 has also been reported to be suitable as a base medium for use in cryopreservation of immature mouse testis tissues (Milazzo *et al.* 2008). We had previously observed that L15 can efficiently maintain isolated testis cells at hypothermic temperatures for over 6 days, especially when used in combination with 20% FBS (Yang and Honaramooz 2010). Effects of L15 might be mainly due to its content of a CO_2-independent buffer system to maintain pH constant under open-air conditions. Therefore, in the present study, we also included other pH fluctuation-resistant media such as F12 with HEPES and M199. Rather similar performances of these media (i.e., L15, M199 and F12), as compared to DMEM and DPBS which have a less efficient CO_2-independent buffer system, confirmed the importance of providing a proper pH buffer systems in the media used for hypothermic preservation of testis tissue. Similar observations have also been made for hypothermic preservation of other types of tissues and cells (Baicu and Taylor 2002; Bonventre and Cheung 1985; Bronk and Gores 1993; Fuller *et al.* 1988; Hochachka and Mommsen 1983; Lindell *et al.* 1998).

In our study, cell survival rates dropped from Day 0 to 3, and from Day 3 to 6 in testis tissues within all media tested, with the majority of the media including the specialized preservation solution HTS-FRS maintaining this rate at about 50% on Day 3, and about 25% on Day 6.

Significant decreases in cell viability have also been observed in testis tissues within DPBS at 4 ℃ for 3 days (Zeng *et al.* 2009). However, hypothermically-preserved testis tissues were also shown to have higher spermatogenic developmental potential when grafted into recipient mice than that of cryopreserved testis fragments (Jahnukainen *et al.* 2007a). In fact, short term ice-cold storage of testis tissues has been suggested to

improve donor-derived spermatogenesis after xenografting (Jahnukainen *et al.* 2007a; Zeng *et al.* 2009). Therefore, shorter term (3 days) refrigeration temperature preservation is preferred for testis tissue when the situation permits. These observations may indicate that such drops in cell viability may not completely prevent future *in vivo* development likely because the tissue is able to rebound upon transplantation into a suitable environment. In the current study, our observations were based on morphology, and could be further confirmed using transplantation studies. Although, our previous data on xenografting of neonatal porcine testis tissue indicate that *in vitro* cell viability of preserved tissue could be used as a relatively reliable predictor of testis tissue potential for development (Abrishami *et al.* 2010).

Gonocytes constitute the only type of germ cells in the neonatal testis and as such their abundance within the preserved tissue is of interest for those involved in the study and manipulation of the male germline. Therefore, in this study, gonocytes proportions were evaluated in select preservation conditions. The percentages of gonocytes did not differ between two of the most promising media, or among preservation Days 0, 3 and 6. This is in agreement with the reported viability of recovered gonocytes which did not differ from that of other testis cells isolated after short-term hypothermic preservation of testis tissues (Zeng *et al.* 2009). These findings also indicate that selection of the most promising preservation media based on total testis cell survival rates may be sufficient for future comparisons. This conclusion was further supported by the presence of correlation between the results of testis cell survival rates and the semi-quantitative evaluations of the tissue morphology.

The same two selected media in the current study of tissue preservation had resulted in even higher preservation efficiency of isolated testis cells in a previous study, where approximately 80% of testis cells survived hypothermic preservation for 6 days (Yang and Honaramooz 2010). This indicates that if the goal of tissue preservation is to use the cells, it might be advisable to preserve cells rather than tissue when possible.

In conclusion, for short-term hypothermic preservation of testis tissues, the use of HTS-FRS or 20% FBS-L15 for 3 days was feasible, while preservation for longer-term (up to 6 days) resulted in considerable drop in cell survival rates. However, compared to fresh tissues, testis morphology did not show severe degenerative changes even after 6 days in HTS-FRS as preservation medium.

CHAPTER 7 EFFICIENT PURIFICATION OF NEONATAL PORCINE GONOCYTES WITH NYCODENZ AND DIFFERENTIAL PLATING[1]

7.1 Abstract

Gonocytes are the only type of germ cells present in the postnatal testis and give rise to spermatogonial stem cells. Purification of gonocytes has important implications for the study and manipulation of these cells may provide insights for ongoing investigation of the male germline stem cells. To obtain a pure population of gonocytes from piglet testis cells, a wide range of Nycodenz concentrations were investigated for density gradient centrifugation. We also examined differential plating of testis cells for various culture durations with different extracellular matrix (ECM) components (fibronectin, poly-D-lysine, poly-L-lysine, laminin, collagen type I and collagen type IV). Gonocytes were highly enriched in pellets of testis cells after using 17% Nycodenz centrifugation to a purity of 81 ± 8.5%. After culturing testis cells on plates pre-coated with different ECMs for 120 min, the proportion of gonocytes increased among non-adherent cells (suspended in the medium), with fibronectin or poly-D-lysine resulting in the greatest (up to 85%) and laminin in the lowest (54%) gonocyte proportion. Combining the most promising ECM coatings (fibronectin and poly-D-lysine) and further extension of their culture duration to 240 min did not improve the final gonocytes purity. Centrifugation with 17% Nycodenz followed by differential plating with fibronectin and ploy-D-lysine coating; however, further purified gonocytes among the collected cells to more than 90%. These results provide a simple, quick and efficient approach for

[1] This study has been accepted for publication in *Reproduction, Fertility and Development*.

obtaining highly enriched populations of piglet gonocytes for use in the study and manipulation of these germline stem cells.

7.2 Introduction

Germline stem cells can be categorized into the primordial germ cells (PGCs), gonocytes and spermatogonial stem cells (SSCs) (Jiang 2001; Jiang and Short 1998b). The PGCs are the first traceable germline-directed progenitors for both male and female germ cells, initially distinguishable in the 18 dpc pig embryos as elongated cells or with distinct pseudopods. Upon transplantation into the recipient testis of rats or mice, the PGCs have been demonstrated to successfully colonize and initiate donor-derived spermatogenesis (Chuma *et al.* 2005; Jiang and Short 1995; Jiang and Short 1998a; Ohta *et al.* 2004). However, the use of the PGCs as a model for the study and manipulation of the male germline is less advantageous than their progenies, because they need to be collected from early embryos at relatively limited numbers, and also because they are not gender-specific (Adams and McLaren 2002; Chuma *et al.* 2005; Jiang and Short 1998a; Ohta *et al.* 2004; Wilhelm *et al.* 2007).

Gonocytes are a transitional population of germline stem cells after the mitotic arrest of the male PGCs and before their differentiation into SSCs (de Rooij 1998; Jiang 2001; Jiang and Short 1998b). Unlike SSCs, gonocytes can be morphologically identified as distinctively large round cells that are not yet fully migrated into the basement compartment of the seminiferous cords of immature testes (McGuinness and Orth 1992b). Before or soon after birth, some gonocytes migrate to the basement membrane and develop into SSCs, while others degenerate as a result of apoptosis (Coucouvanis *et al.* 1993). Despite some differences with SSCs (Forand *et al.* 2009a; Hasthorpe 2003;

McLean *et al.* 2003; Meehan *et al.* 2000; Ohbo *et al.* 2003; Shinohara *et al.* 2001; Van Den Ham *et al.* 2002), gonocytes transplanted to the recipient testes have been demonstrated to colonize the recipient seminiferous tubules in mice (McLean *et al.* 2003; Ohbo *et al.* 2003; Ohmura *et al.* 2004; Ohta *et al.* 2004; Shinohara *et al.* 2002a) and generate donor-derived spermatogenesis in rats (Jiang and Short 1995; Jiang and Short 1998a; Orwig *et al.* 2002b; Ryu *et al.* 2003). Therefore, gonocytes may provide an easily collectable stem cell source for the study and manipulation of the male germline.

Transplantation of male germ cells in large animals has provided a functional assay for the study of SSCs in these species (Herrid *et al.* 2006b; Hill and Dobrinski 2006; Honaramooz *et al.* 2003a; Honaramooz *et al.* 2002a). With progress in manipulation and modification of these male germline stem cells, germ cell transplantation can also offer an alternative approach for the production of transgenic farm animals where the current methodologies are inefficient, costly and time-consuming (Bacci 2007; Honaramooz *et al.* 2003b; Honaramooz *et al.* 2008; Lee and Piedrahita 2003).

A major step toward improving the efficiency of germ cell transduction and transplantation is the ability to select germline stem cells or at least to enrich them in the population of donor cells (Honaramooz and Yang 2010). The SSCs comprise about 0.02% of all cells in an adult testis (Kanatsu-Shinohara *et al.* 2005c; Tegelenbosch and de Rooij 1993). After depositing the mixed population of donor testis cells into the lumen of the recipient seminiferous tubules, Sertoli cells recognize and allow the stem cells access to the niche at the basement compartment of the tubule (Chuma *et al.* 2005; Hasthorpe *et al.* 1999; Jiang and Short 1995; Jiang and Short 1998a; Nagano *et al.* 1999; Ohta *et al.*

2000; Shinohara *et al.* 2001; Yuji Takagi 1997). Furthermore, the extent of colonization has been shown to be directly proportional to the relative abundance of the SSCs/germline stem cells (Dobrinski *et al.* 1999b; Shinohara *et al.* 1999; Shinohara *et al.* 2000), emphasizing the importance of selecting the target cells.

Although neonatal gonocytes could be distinguished based on their unique morphology from other cells of the seminiferous cords (McGuinness and Orth 1992b; Orwig et al. 2002b), specific bio-markers are required for their accurate selection. In rodents, several markers including NANOG and OCT4 were shown to be expressed by gonocytes, but their expression was not limited to gonocytes or they were not expressed by all gonocytes (Culty 2009). Some of these markers, may indicate the pluripotency of certain sub-populations of gonocytes, although probably not all gonocytes have stem cell ability (Goel *et al.* 2008). Recently, lectin Dolichos biflorus agglutinin (DBA) was found to have specific affinity for piglet gonocytes, and it was proposed to provide a bio-marker for *in vitro* identification of porcine gonocytes (Goel *et al.* 2007), although DBA binding could not be detected in the testis of mice or bulls (Goel *et al.* 2008; Izadyar *et al.* 2002b). To date, few studies have investigated purification of gonocytes, especially from large animals. In pigs, gonocytes were enriched by combining Percoll density gradient centrifugation and differential plating, with a reported purity of up to 80% as identified by DBA staining (Goel *et al.* 2007; Kim *et al.* 2010); however, further purification of gonocytes would be beneficial for their *in vitro* characterization, study and application as a model for germ cell transplantation in farm animals (Honaramooz and Yang 2010).

As the progenitors of gonocytes, PGCs have been enriched to a purity of more than 90% with a Nycodenz gradient centrifugation in mice, quails and chicks (Mayanagi

et al. 2003; Zhao and Kuwana 2003). The objective of the present study was to develop a practical and reliable approach for the purification of piglet gonocytes using gradient centrifugation and differential plating.

7.3 Materials and Methods

7.3.1 Experimental Design

This study was performed through multiple advancing stages, where the outcome of each experiment was used to design the next experiment until satisfactory results were obtained. To control for potential variations among individual experiments, groups within experiments occasionally overlapped. In separate experiments, we assessed the gonocyte enrichment efficiency of various concentrations of Nycodenz for density gradient centrifugation, and different extracellular matrix components for differential plating. Culture durations for differential plating were then compared for select extracellular matrix components. The top-performing density gradient centrifugation and differential plating strategies were consequently combined and evaluated for improved gonocyte enrichment.

7.3.2 Testes Collection and Preparation

Testes were collected after aseptic castration of 180 one-wk-old Yorkshire-cross piglets (Camborough-22 × Line 65, PIC Canada Ltd, Winnipeg, MB, Canada) at a university-affiliated swine facility and within 2 h transferred to our laboratory in ice-cold Dulbecco's phosphate buffered saline (DPBS, cat. # 20-031-CV, Mediatech, Manassas, VA, USA) containing 2% w/v antibiotic-antimycotic solution (cat. # 30-004-CI, Mediatech). The testes were rinsed three times with DPBS, and the tunica albuginea, rete

testis and excess connective tissue were removed. The testis parenchyma was then used for collection of testis cells. Experimental procedures involving animals were approved by the University of Saskatchewan's Institutional Animal Care and Use Committee.

7.3.3 Isolation of Testis Cells

A three-step strategy, involving vortexing and gentle digestion which we have recently developed (Yang *et al.* 2010b), was used to isolate testis cells resulting in very high proportion of gonocytes in freshly isolated testis cells. Briefly, testis parenchyma was minced with fine scissors, and the tissue was vortexed for 1 min with a test tube shaker (Reax Top, cat. # 541-10000, Heidolph Instruments, Essex, UK) at a vibration frequency of 500 rpm. After rinsing, the testis tissue was digested with 0.2% w/v collagenase IV (cat. # C-5138, Sigma-Aldrich, Oakville, ON, Canada), 0.1% w/v hyaluronidase (cat. # H-3884, Sigma-Aldrich) and 0.01% w/v DNase I (cat. # DN25, Sigma-Aldrich) in Dulbecco modified Eagle medium (DMEM, cat. # 10-013-CM, Mediatech) at 37 ℃ for 10 min. Digestion was stopped by adding 100% foetal bovine serum (FBS, cat. # A15-701, PAA Laboratories GmbH, Etobicoke, ON, Canada), and followed by another round of vortexing for 30 s, and filtration of the resultant cell suspension through a 40 μm filter (cat. # 352340, BD Biosciences). The erythrocytes were then removed with a lysis buffer as previously described (Yang *et al.* 2010b). When large numbers of testis cells were needed for investigation of several concentrations of Nycodenz for density gradient centrifugation, we combined testis cells from different donor piglets; otherwise, testes from each donor piglet were used as a replicate within each experiment. We have previously examined potential variations among testis donors and shown that there are no significant differences among the tissues collected from our

source swine herd (Yang *et al.* 2010b). The cell viability was assayed with a 0.4% w/v trypan blue solution (cat. # T8154, Sigma-Aldrich) using a haemocytometer. Cell smears were prepared from collected cells, allowed to be air-dried at room temperature and stored at -80 ℃ for immunocytochemical study at a later time.

7.3.4 The Effect of Density Gradient Centrifugation Using Various Concentrations of Nycodenz

In preliminary experiments, various amounts of Nycodenz (cat. # D2158, Sigma-Aldrich) were dissolved in DPBS to find concentrations that form cell layers after centrifugation. Out of 41 different concentrations of Nycodenz tested (range, 10-30%, w/v), those below 11.5% did not form visible cell layers (data not shown). Among those resulting in formation of visible cell layers, 11 concentrations (15.5-30%) were selected for examination on their potential for enrichment of gonocytes.

Three mL of each Nycodenz concentration was placed at the bottom of a 15 mL graduated conical tube and 2 mL of the testis cell suspension was gently placed on top, and the tubes were centrifuged at 500*g* at 4 ℃ for 15 min. The cells in the cell pellet and in the interface between Nycodenz and the cell suspension were gently collected and rinsed with DPBS ($n \geq 5$ replicates per group). The assessment of the cell viability and the storage of cell smears for immunocytochemistry were performed as described above.

7.3.5 Gonocytes Quantification

After thawing at room temperature, the cell smears were fixed in Bouin's solution for 2-3 min, rinsed in DPBS, and blocked with 5% BSA at 37 ℃ for 15 min in humidified atmosphere. The cell smears were then rinsed with DPBS and incubated

overnight with a fluorescein-labelled lectin Dolichos biflorus agglutinin (DBA, (Goel *et al.* 2007; Kim *et al.* 2010), 1:100, v/v, cat. # FL-1031, Vector Laboratories, Burlington, ON, Canada) in humidified atmosphere. After rinsing with DPBS and incubation with 0.3% w/v Sudan Black B (cat. # 3545-12, EMD Chemicals, Gibbstown, NJ, USA) in 70% alcohol for 10-15 min, the cell smears were rinsed and stained with 4, 6-diamino-2-phenyl indole (DAPI, cat. # D-9542, Sigma-Aldrich) for 2 min, mounted using a mounting media (cat. # H-1000, Vector Laboratories) and observed using a fluorescent microscope. DAPI and fluorescein were also sequentially scanned using a laser scanning confocal microscope (TCS SP5, Leica Microsystems, Richmond Hill, ON, Canada), and the obtained images were merged. More than 600 cells were counted for each cell smear to determine the gonocyte proportion.

7.3.6 Comparison of Different Concentrations of Nycodenz

The percentages of gonocytes, as identified by DBA staining, were determined in cell populations collected from the pellets and interfaces in different concentrations of Nycodenz. The Nycodenz concentrations resulting in the highest proportion of gonocytes were then used in further experiments to be combined with the differential plating method.

7.3.7 Comparison of Different ECM Coatings for Differential Plating

In order to screen for the most effective extracellular matrix (ECM) components for gonocyte enrichment, commercially available pre-coated 6-well culture plates (cat. # 354431, BD Biosciences) were seeded with freshly isolated testis cells at a concentration of $0.25 \times 10^6/cm^2$. Each plate well was either a non-coated control or coated with one of

the following ECMs: fibronectin, poly-D-lysine, laminin, collagen type I or collagen type IV. The cells were fed with 10% FBS-DMEM and cultured at 37 ℃ in humidified atmosphere with 5% CO_2 for 15 or 120 min. Cells non-adherent to the plate bottom (suspended in the culture medium) were then collected by brief centrifugation of the culture media. Adherent cells were also harvested by digestion with trypsin-EDTA for up to 3 min, while being observed under an inverted microscope for appropriate digestion (i.e., 30-50% of cells dissociated). This was followed by agitation using pipetting of the remaining adherent cells, centrifugation at 500*g* at 16 ℃ for 5 min and rinsing with DPBS ($n \geq 3$ replicates per group). The assessment of cell viability and the preparation of cell smears for immunocytochemistry were performed as described above.

7.3.8 Combining the Most Promising ECM Coatings for Differential Plating

After comparison of different ECM components, fibronectin and poly-D-lysine were the most effective ECMs and were selected for further investigation. We hypothesized that combining these ECM components may increase the efficiency of selection; therefore, plates were coated in our laboratory as follows. Each well in a non-coated 6-well culture plate (cat. # 353046BD Biosciences) was covered with 1 mL of either poly-D-lysine (cat. # 477743-736, VWR International, Mississauga, ON, Canada) or fibronectin (cat. # 477743-728, VWR) at concentrations of 50 or 10 μg/mL in DPBS, respectively, or with 1 mL combination of poly-D-lysine and fibronectin. Other wells in the plate were covered with 1 mL of DPBS to serve as control, or with 1 mL of poly-**L**-lysine (cat. # P8920, Sigma-Aldrich), to test another variant of poly-lysine. The plates were maintained at 37 ℃ in humidified atmosphere with 5% CO_2 for 1 h, allowed to dry in air in a biosafety cabinet and rinsed twice with DPBS. Freshly isolated testis cells were

then seeded onto the plates and cultured as above for 120 min ($n \geq 5$ replicates per group). Cell smears were prepared for immunocytochemistry as described above.

7.3.9 Optimization of Culture Duration for Gonocytes Enrichment

From the differential plating experiments to this point, we observed that extending the culture duration from 15 to 120 min could improve the final gonocytes purity in non-adherent cells. In order to optimize the duration of culture for gonocyte enrichment, separate groups of isolated testis cells were cultured in plates coated with a combination of fibronectin and poly-D-lysine as described above, for 30, 60, 90, 120 or 240 min ($n \geq 8$ replicates per group). After culture, non-adherent cells were collected and smeared onto slides for subsequent immunocytochemistry.

7.3.10 Combining Nycodenz Centrifugation and Differential Plating

At this stage and based on the results from above experiments, the most effective methods of gradient centrifugation and differential plating were combined to determine if the efficiency can be improved further. Among the 11 concentrations tested, 17% Nycodenz resulted in the highest enrichment of gonocytes from the cell pellets and therefore was chosen. In order to determine whether culture duration for differential plating can affect the results, two culture durations from the previous experiment were included for comparison in this experiment. The cell pellets obtained from a 17% Nycodenz gradient centrifugation were then rinsed with 10% FBS-DMEM and cultured for 120 or 240 min in culture plates coated with the combination of fibronectin and poly-D-lysine, which had also resulted in the highest enrichment efficiency ($n \geq 8$ replicates per group). The cell viability of non-adherent cells was assessed as described above with

the evaluation of the gonocyte recovery rates. Smears were made from non-adherent cells and stored at -80 ℃ for immunocytochemistry.

7.3.11 Statistical Analysis

Analysis of variance was performed on the data to analyze differences between groups, followed by Tukey's HSD tests. Data are presented as mean ± s.e.m., and differences were considered statistically significant when $P < 0.05$.

7.4 Results

7.4.1 Comparison of Different Concentrations of Nycodenz for Density Gradient Centrifugation

Immunocytochemical analysis using DBA showed that after centrifugation with Nycodenz, gonocytes could be more efficiently enriched in cell pellets than in cell layers, with the final gonocyte proportion in the ranges of 28-81% and 21-50%, respectively (Fig. 7.1). The gonocyte proportion in testis cells prior to centrifugation was ~38%. Centrifugation with 17% Nycodenz resulted in the collection of the highest proportion of gonocytes in cell pellets (81 ± 9%, mean ± s.e.m.) and that of 15.5% Nycodenz resulted in the lowest gonocyte proportion in cell layers (21 ± 4%, Fig. 7.1). The cell viability rates after Nycodenz centrifugation were more than 80%. Therefore, we selected the 17% concentration of Nycodenz to collect cell pellets for gonocyte enrichment in the remaining experiments.

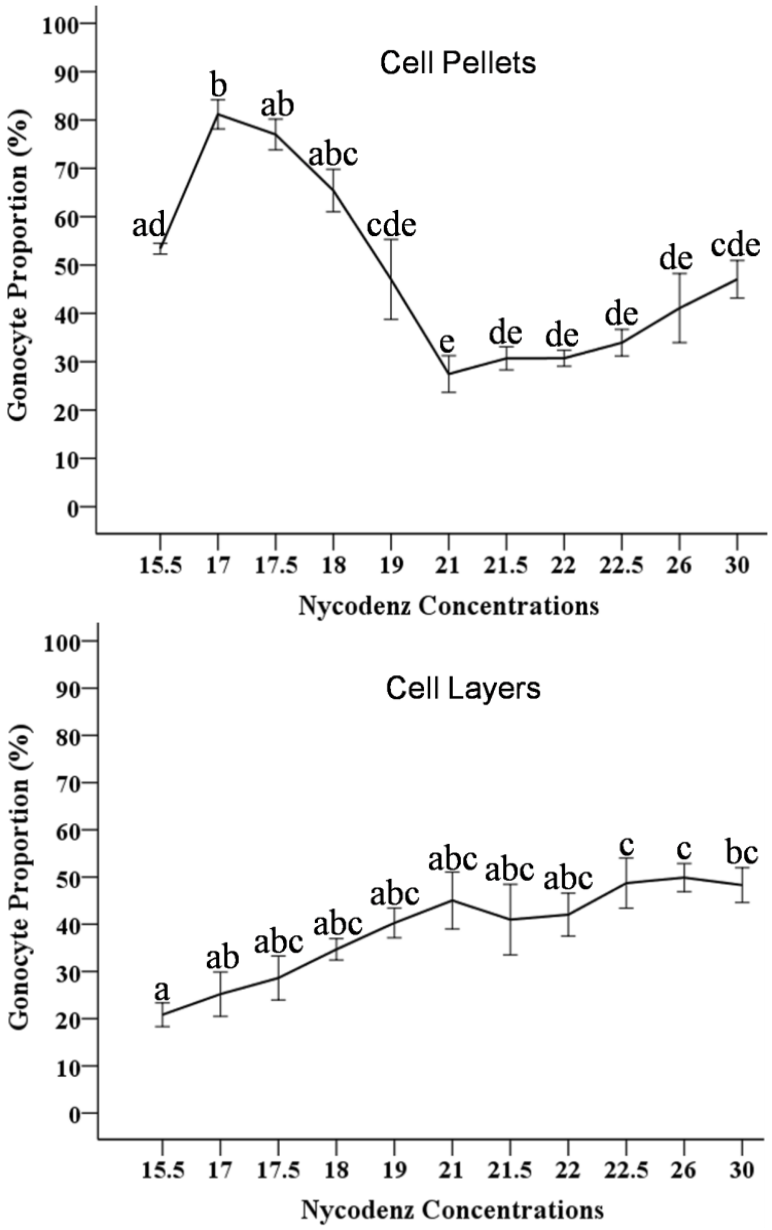


Fig. 7.1. Gonocyte proportion in cell layers and cell pellets after density gradient centrifugation of neonatal porcine testis cells with Nycodenz at different concentrations. Nycodenz density gradient centrifugation was applied at 11 increasing concentrations to enrich for porcine gonocytes. After centrifugation at each concentration, cells in the layers and cell pellets were separately collected and assayed for the percentage (± s.e.m.) of gonocytes. Different superscript letters among Nycodenz concentrations represent significant differences ($P < 0.05$, $n \geq 5$ replicates per group).

7.4.2 Comparison of Different ECMs Coatings for Differential Plating

Gonocyte proportion among non-adherent testis cells (suspended in the medium) cultured for 15 min varied among groups ($P < 0.05$), but did not differ between ECM-coated and non-coated plates or compared with those in non-cultured testis cells (31-39% *vs.* 37% or 38%, respectively, $P > 0.05$, Fig. 7.2). Subsequently, additional groups of plates were cultured for 120 min with the adherent and non-adherent cells examined. Compared with the freshly isolated (non-cultured) cells, the proportion of gonocytes among testis cells adherent to the plates dropped in all groups of cultured cells, regardless of the plate (from 38% to 15-23%, $P < 0.05$). The percentage of gonocytes in these non-adherent cells varied among groups ($P < 0.05$) and increased among cells in all ECM-coated and non-coated plates, compared with those of non-cultured testis cells (54-85% and 65% *vs.* 38%, respectively, $P < 0.05$, Fig. 7.2). Among the groups of non-adherent cells, those collected from the fibronectin-coated plates had the greatest proportion of gonocytes (85 ± 3%, with a cell viability of 79 ± 2%) and those of the laminin-coated plates had the lowest (54 ± 3%, with a cell viability of 81 ± 2%).

7.4.3 Combining the Most Promising ECM Coatings for Differential Plating

Combining fibronectin and poly-D-lysine in coating the plates did not significantly improve the proportion of gonocytes among the non-adherent cells over the levels obtained by using these ECMs separately (83 ± 3% *vs.* 80 ± 3% and 80 ± 3%, respectively, $P > 0.05$, Fig. 7.3). Coating the plates with poly-L-lysine did not enrich gonocytes when compared with that of non-coated plates (62 ± 3% *vs.*60 ± 3%, $P > 0.05$, Fig. 7.3). Furthermore, the results obtained from differential plating using commercially-

coated plates were not different from those obtained using plates that were coated in our laboratory ($P > 0.05$, Fig. 7.2 and 7.3).

7.4.4 Optimization of Culture Duration for Gonocyte Enrichment in Differential Plating

The percentage of gonocytes among non-adherent cells collected after 240 min of culturing in plates coated with a combination of fibronectin and poly-D-lysine were as high as 86 ± 3%, which was different from those after 30 min (57 ± 3%, $P < 0.05$). However, gonocyte proportion did not statistically differ among culturing durations of 60, 90,120 and 240 min groups ($P > 0.05$, Fig. 7.4).

7.4.5 Combining Nycodenz Centrifugation and Differential Plating

Cells obtained after density gradient centrifugation with 17% Nycodenz were subsequently subjected to differential plating with a mixed fibronectin and poly-D-lysine coating for 120 or 240 min, to maximize the purity of resultant gonocytes. Compared with the gonocyte percentage of freshly isolated testis cells (40 ± 2%, Fig. 7.5 and 7.6), combination of the two approaches increased gonocyte purity for both 120 and 240 min culture durations (90 ± 3% and 92 ± 3%, respectively, $P < 0.05$, Fig. 7.5 and 7.6), which were also higher than the two approaches used separately (80 ± 2%, 80 ± 2%, or 83 ± 2% for 17% Nycodenz, mixed fibronectin and poly-D-lysine for 120 or 240 min, respectively, $P < 0.05$). The cell viability after combination of the two approaches was 81 ± 2% or 76 ± 2%,with gonocyte recovery rates (compared to freshly isolated cells) of 11 ± 2.4% or 6 ± 3.2% (for 120 or 240 min, respectively).

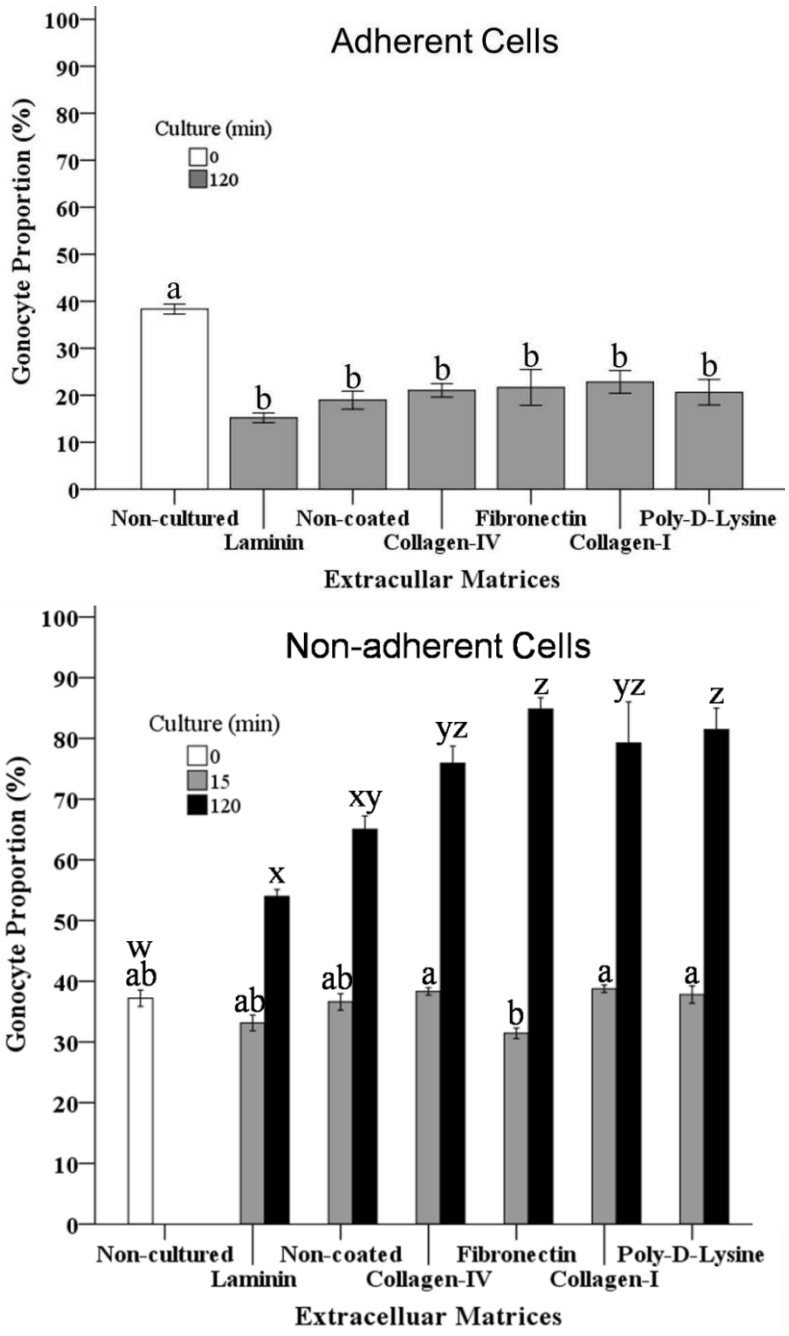


Fig. 7.2. Gonocyte proportion in adherent and non-adherent cells after culturing neonatal porcine testis cells on commercially-available plates pre-coated with different extracellular matrix (ECM) components. After culturing neonatal porcine testis cells on non-coated (control) plates or those coated with different ECMs for 15 min, cells remaining non-adherent (suspended in the medium) were collected and assayed to determine gonocyte percentage (± s.e.m.). Additional groups of testis cells were cultured for 120 min and the proportion of gonocytes was compared among adherent and non-adherent cells. Within each culture duration, columns with different superscript letters (a, b) or (w-z) are significantly different ($P < 0.05$, $n \geq 3$ replicates per group).

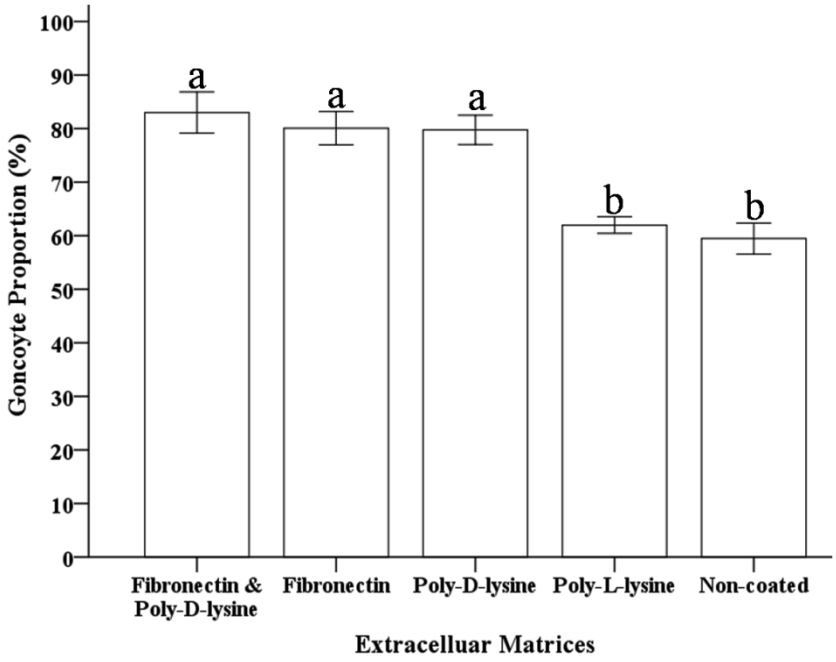


Fig. 7.3. Gonocyte proportion in non-adherent cells after culturing neonatal porcine testis cells on plates coated in the laboratory with single extracellular matrix (ECM) components or their combination for 120 min. After culturing neonatal porcine testis cells on plates coated with different ECMs (as single or in combination) for 120 min, non-adherent cells were collected and the percentage (± s.e.m.) of gonocytes determined. Columns without a common superscript letter are significantly different ($P < 0.05$, $n \geq 5$ replicates per group).

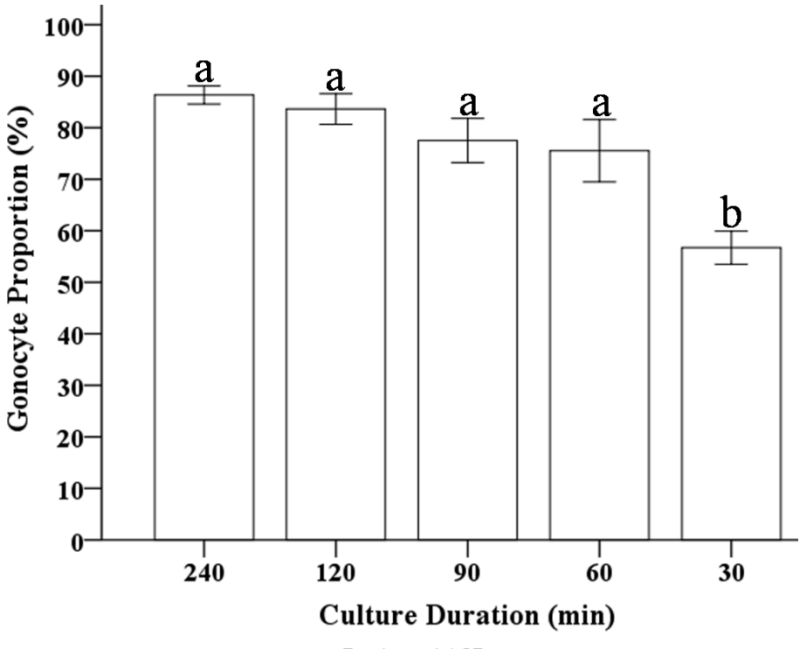


Fig. 7.4. Comparison of different durations for culturing neonatal porcine testis cells on plates coated in the laboratory with combined extracellular matrix (ECM) components for gonocytes enrichment. After culturing neonatal porcine testis cells on plates coated with a combination of fibronectin and poly-D-lysine for different durations, non-adherent cells were collected and the percentage (± s.e.m.) of gonocytes compared. Columns with different superscript letter are significantly different ($P < 0.05$, $n \geq 8$ replicates per group).

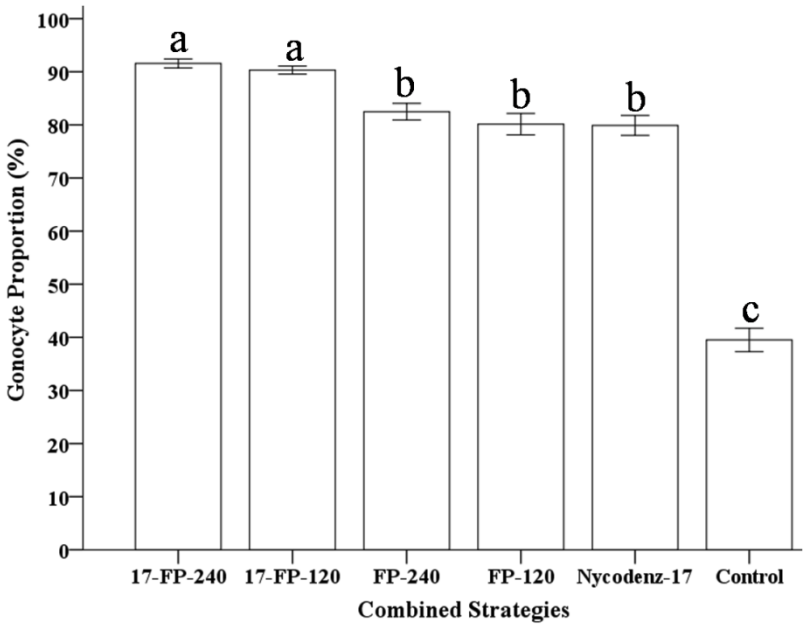


Fig. 7.5. Combination of optimized strategies for Nycodenz density gradient centrifugation and differential plating for further purification of porcine neonatal gonocytes. After centrifugation of neonatal porcine testis cells with 17% Nycodenz (17/Nycodenz-17), cells in pellets were cultured on plates coated with a combination of fibronectin (F) and poly-D-lysine (P) for 120 or 240 min, and the percentage (± s.e.m.) of gonocytes determined in non-adherent cells. Columns with different superscript letters are significantly different ($P < 0.05$, $n \geq 8$ replicates per group).

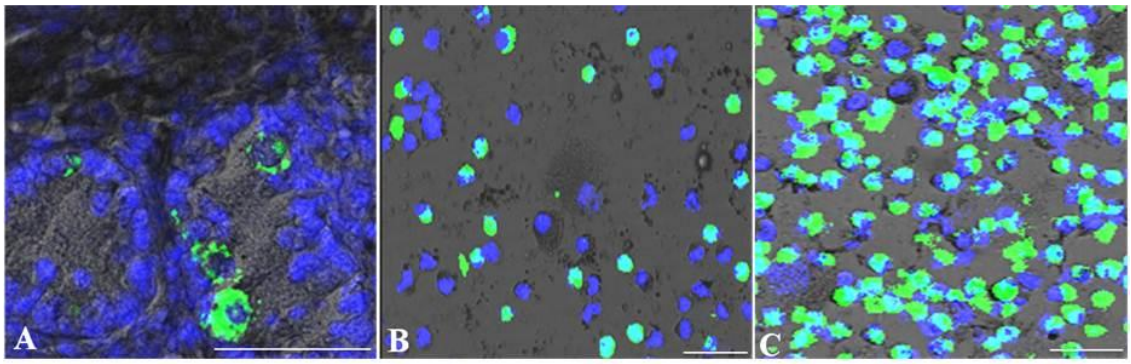


Fig. 7.6. Detection of gonocytes with immunostaining. Confocal scanning images of the donor pig (1-wk-old) testis tissue isolated testis cells before, and after enrichment for gonocytes show labelling with either a fluorescein-conjugated antibody against the lectin Dolichos biflorus agglutinin (DBA, green) to detect gonocytes, or DAPI to show all cell nuclei (blue). A: Merged image of the donor testis tissue with transmitted light as well as staining for DBA and DAPI. B: Merged image of the freshly isolated testis cells with transmitted light as well as staining for DBA and DAPI. C: Merged image of the isolated testis cells stained with DBA and DAPI as well as the transmitted light image after enrichment for gonocytes with a combination of Nycodenz density gradient centrifugation and differential plating. Scale bars, 50 µm.

7.5 Discussion

Several strategies have been applied to enrich spermatogonial stem cells (SSCs) of different species (Khaira *et al.* 2005). These approaches could be generally categorized into those with or without using antibodies. Specific antibodies may be used against the target or contaminating cells using fluorescent activated cell sorting (FACS) (Herrid *et al.* 2009a; Izadyar *et al.* 2002b; Kubota *et al.* 2004a; Lo *et al.* 2005; Moudgal *et al.* 1997; Shinohara *et al.* 2000) and magnetic activated cell separation (MACS) methods (Gassei *et al.* 2009; Giuili *et al.* 2002; Herrid *et al.* 2009a; Kubota *et al.* 2004a; Schönfeldt *et al.* 1999). Strategies that do not rely on the application of antibodies, employ the innate cellular characteristics that differentiate the target and contaminant cells, include forward and side scatter measurements using FACS (Kubota *et al.* 2003; Lo *et al.* 2005; Shinohara *et al.* 2000), density gradient centrifugation (Dirami *et al.* 1999; Herrid *et al.* 2009a; Izadyar *et al.* 2002b; Luo *et al.* 2006; Marret and Durand 2000; Rodriguez-Sosa *et al.* 2006) and differential plating (Dirami *et al.* 1999; Herrid *et al.* 2009a; Izadyar *et al.* 2002b; Luo *et al.* 2006; Rodriguez-Sosa *et al.* 2006). Using the aforementioned approaches, SSCs have been enriched to levels as high as 75% among testis cells from large animals (Herrid *et al.* 2009a; Izadyar *et al.* 2002b; Luo *et al.* 2006; Rodriguez-Sosa *et al.* 2006). In contrast, as the progenitors of SSCs, enrichment of gonocytes or pre-spermatogonia was only reported in a few studies in rodents (Moore *et al.* 2002; Van Den Ham *et al.* 1997; Van Dissel-Emiliani *et al.* 1989), and more recently in pigs (Goel *et al.* 2007; Kim *et al.* 2010). In the present study, density gradient centrifugation using different concentrations of Nycodenz, and differential plating with different ECM coatings were investigated for their efficiency in enriching porcine gonocytes. Using either strategy, we were able to efficiently enrich testis cells for gonocytes to more than

80%, and using their combination to more than 90%. This is up to 45 fold higher than the gonocyte proportion found in the freshly isolated piglet testis cells using conventional methods of cell separation (with only 2% gonocyte yield, Kim *et al.* 2010).

Although target cells could potentially be highly enriched or virtually purified using FACS and MACS, specific cell markers especially those on the cell surface need to have been identified and fluorophore-conjugated antibodies made. These cell markers could include antigens/receptors on/in cells and upon blocking by the antibodies used for sorting, the behaviour or fate of germ cells may alter in response to manipulations (Bashamboo *et al.* 2006; Bendel-Stenzel *et al.* 2000; Gilner *et al.* 2007; Yan *et al.* 2000).

A variety of cell types have been enriched by density gradient centrifugation with Percoll (Pertoft 2000) and attempts were made to isolate spermatogonia from boar, bull and ram testis cells (Izadyar *et al.* 2002b; Marret and Durand 2000; Rodriguez-Sosa *et al.* 2006). Since gonocytes are different from testis somatic cells in size, shape and sedimentation velocity (Orwig *et al.* 2002b; Van Dissel-Emiliani *et al.* 1989), they are also likely to be enriched with a density gradient centrifugation method (Van Dissel-Emiliani *et al.* 1989). In the few studies on enrichment of piglet gonocytes, conflicting results were obtained (70% vs. 5% gonocyte purity) after using similar Percoll density gradient centrifugation protocols, possibly because of technical and handling differences, and it was noted that an easier and more reliable method is still required (Goel *et al.* 2009; Goel *et al.* 2007; Kim *et al.* 2010). Nycodenz is a non-ionic iodinated gradient medium which can be dissolved readily in water. Compared with Percoll, gradients of Nycodenz are easier to prepare and sterilize, as the solutions are autoclavable and non-toxic to cells. Nycodenz gradients have been successfully utilized in collection of

primordial germ cells (PGCs) with more than 90% purity in cell layers from mice, quails and chicks using 8% Nycodenz at a density of 1.035 g/mL (Mayanagi *et al.* 2003; Zhao and Kuwana 2003). However, to our knowledge, there are no reports on its application in enrichment of SSCs or gonocytes in farm/large animals. In the present study, interestingly, no cell layer formation was visible after centrifugation of cells with Nycodenz gradients below 11.5% (w/v), and the greatest gonocyte purity (>80%) was collected from cell pellets at a gradient of 17% (density ~1.089 g/mL). For ram and bull testis cells, Percoll was used at densities ranging from 1.0542 to 1.0654 g/mL to obtain SSCs enriched to 65% and 38%, respectively (Herrid *et al.* 2009a; Rodriguez-Sosa *et al.* 2006), whereas for porcine gonocytes, a purity of up to 70% was possible at 50-60% fractions (Goel *et al.* 2007), comparable to a density range of 1.060 to 1.075 g/mL (Semple and Szewczuk 1986). These studies may collectively suggest that gonocytes differ from PGCs and SSCs in the optimal density gradients for enrichment, with gonocytes possibly requiring a density of 1.060 to 1.089 g/mL of either Percoll or Nycodenz (i.e., PGCs < SSCs < gonocytes). Nevertheless, species differences should also be considered, and suitable gradients established for gonocyte enrichment in each species.

Testis cells from mice, rats, pigs and bulls have been enriched for SSCs by differential plating using laminin-coated plates; however, the results differed depending on species, with the highest enrichment at 30% (Hamra *et al.* 2004; Herrid *et al.* 2009a; Luo *et al.* 2006; Orwig *et al.* 2002c; Shinohara *et al.* 1999). Because gonocytes are progenitors of SSCs and may share common adhesion properties (Hasthorpe *et al.* 1999; Li *et al.* 1997), one might expect that they would respond similarly to differential plating.

For coating plates in the present study, we used some of the major ECM molecules found in the basement membrane of seminiferous tubules. Interestingly, among adherent testis cells, gonocyte proportion decreased after incubation for 120 min for all coated and non-coated plates. On the other hand, whereas among non-adherent cells gonocyte proportion did not change in any of the coated or non-coated plates when incubated for 15 min, it increased significantly when incubated for 120 min, and this proportion was greatest in fibronectin and poly-D-lysine coated plates (to >80% gonocyte purity). These results are in contrast to reports showing the affinity of rodent SSCs for binding to laminin during short-term culture, or those showing that bovine type A spermatogonia could be enriched among both adherent and non-adherent testis cells using laminin-coated flasks (Hamra *et al.* 2004; Orwig *et al.* 2002c; Shinohara *et al.* 1999). However, our observations are in agreement with the recent reported use of laminin-coated plates for selection of porcine gonocytes among non-adherent cells (Kim *et al.* 2010; Luo *et al.* 2006). The results from these studies may collectively indicate that, at least during short-term culture conditions and compared with SSCs, gonocytes have little affinity for adhering to ECM molecules or even to non-coated plates. Therefore, in the present study, the application of some of the tested ECM components effectively decreased the contamination by testis somatic cells, indicating that in differential plating for enrichment of gonocytes, negative selection may be more efficient and practical than potential positive selection. If these observations accurately reflect the *in vivo* adherence property of gonocytes and SSCs, they may point to the possibility of fundamental differences between these two cell populations, and that gonocytes change their binding behaviour after developing into SSCs. This may also explain why in the neonatal testis, gonocytes remain in the center of

the seminiferous cords and only show affinity for the basement membrane at later developmental stages when they are closer to becoming pre-spermatogonia and SSCs.

It is not completely clear as to why in the present study some of the outcomes of differential plating changed as the duration of culture increased. For instance, testis somatic cells seemed to show preferential affinity for ECM components, particularly for fibronectin and poly-D-lysine, and this process took 30 min or longer. Similarly, laminin-coating of culture plates improved gonocyte purity through negative selection after incubation for 120 min but not for 15 min. Whether the longer culture durations merely increased the chance of more somatic cells coming in contact with the plate surface and binding to it, or in fact gonocytes/somatic cells changed their adherence behaviour toward each other or toward the plate coating during this period, reflecting a fundamental change in their biology, may need further investigation. In a recent study, significant improvement in piglet gonocyte purity was observed after negative selection using laminin-coated plates for 20 min (Kim *et al.* 2010). The differences between our observations and those of the latter study could be due to differences in plate coating procedures and the fact that we started with much higher gonocyte purity than in that report (38% vs. 2%).

Here, we identified fibronectin and poly-D-lysine as more efficient ECM molecules for negative selection of piglet gonocytes than laminin. In another study where improvement in piglet gonocyte purity was observed after incubation of testis cells with laminin-coated plates, no significant effect of fibronectin was noted (Kim *et al.* 2010). In two relevant previous studies, although both differential plating and gradient centrifugation strategies were tested for enrichment of porcine gonocytes, in one study it

was concluded that gradient centrifugation, but not differential plating, is effective in enrichment of gonocytes up to 70% (Goel *et al.* 2007), and in another study it was concluded that gonocytes can be enriched up to 80% using a differential plating protocol that required culturing the cells for 12 h, while using gradient centrifugation only 5% gonocyte enrichment could be achieved (Kim *et al.* 2010). In conclusion, in the present study, we showed that gonocyte proportion among testis cells can be increased to more than 80% using either a simple Nycodenz gradient centrifugation or differential plating (incubated for only 2 h), and to more than 90% when the two strategies are combined. Findings in this study, therefore, provide a simple, quick and efficient approach for obtaining highly enriched populations of piglet gonocytes which could also be applicable for purification of gonocytes in other species. These results will be valuable for the study and manipulation of gonocytes, as a transient population of germline stem cells.

CHAPTER 8 PIGLET TESTIS IRRADIATION AND SUBSEQUENT GONOCYTE TRANSPLANTATION

8.1 Abstract

Preparation of the recipient testes can enhance the outcome of germ cell transplantation. Whether neonatal pig gonocytes have stem cell potential has not been shown. Therefore, the objectives of the present study were 1) to examine the effects of neonatal piglet testis irradiation on testis development and subsequent endogenous spermatogenesis, and 2) to investigate the potential of donor neonatal piglet gonocytes in establishing spermatogenesis after homologous transplantation into irradiated recipient testes. Nine-day-old piglets underwent daily local irradiation of testes using fractionated gamma-rays at doses of 0 (control), 1, 2 or 3 Gy (n = 6 piglets/group) for 3 consecutive days. Two months after irradiation, half of the pigs in each group were sacrificed and the testes examined. At the same time, the remaining pigs in each group were used as recipients for donor neonatal piglet testis cells (with ~38% gonocytes). The donor cells were injected, through the rete testis under ultrasound guidance, into one testis of each irradiated and non-irradiated recipient animal, and the contra-lateral testis was injected with saline as an internal control. Two months after transplantation (i.e., 4 months after irradiation), pigs were sacrificed and the testes collected for examination. Compared with the control (0 Gy), at both 2- and 4 months post-irradiation, the testis weight indices from the group of pigs undergoing daily irradiation doses of 3 Gy were smaller, seminiferous tubule density from 2- and 3-Gy groups were lower, and tubule diameter in all irradiated testes were also lower ($P < 0.05$). Two months after irradiation with doses of 2 or 3 Gy,

the relative number of germ cells per 1,000 Sertoli decreased ($P < 0.05$). At 4 months post-irradiation, spermatogenic differentiation up to elongating spermatids was observed in all non-irradiated testes, and while no spermatogenic development was found in any of the irradiated testes injected with only saline; after gonocyte transplantation, a small number of tubules in the groups of 1- and 2-Gy contained spermatogenesis up to elongating and round spermatids. In conclusion, local irradiation of recipient piglet testes with as low as 1 Gy (for 3 days) could completely suppress the endogenous spermatogenesis, and gonocyte transplantation into irradiated recipients led to initiation of spermatogenesis.

8.2 Introduction

Proliferation and differentiation of spermatogonial stem cells (SSCs) in the testis maintain a life-long supply of male gametes in an adult. With support from the recipient testis somatic cells, SSCs are capable of generating donor-derived spermatogenesis in recipient testes after germ cell transplantation (GCT) (Clouthier *et al.* 1996; França *et al.* 1998; Nagano *et al.* 1999; Parreira *et al.* 1998; Russell and Brinster 1996). GCT has been used as a bioassay in the assessment of SSC potential of a given population of testis cells. GCT has also led to the introduction of an alternative strategy for producing transgenic animals (Nagano *et al.* 2001a; Nagano *et al.* 2000a). Furthermore, GCT provides a potential approach for preservation and propagation of the genetic potential of individual animals of high value or endangered species, especially if they are prepubertal. Despite their great potential and wide applications, SSCs are rare in the testis and it is difficult if not impossible to unequivocally identify them by morphological or biochemical characteristic (Oatley and Brinster 2008).

In the neonatal testis, gonocytes are the only type of germ cells present (de Rooij 1998; Jiang 2001; Jiang and Short 1998b). Before or soon after birth, gonocytes resume their proliferation, migrate to the basement membrane and eventually develop into SSCs (Coucouvanis *et al.* 1993). Populations of gonocytes were reported to express multiple pluripotency markers, and produce teratomas after subcutaneous transplantation into mice (Goel *et al.* 2009; Hoei-Hansen *et al.* 2005; Niu and Liang 2008; Tu *et al.* 2007). Gonocytes were also observed to differentiate directly into somatic cells of the three germ layers (Simon *et al.* 2009), attesting to the likelihood that populations of gonocytes retain pluripotency.

The distinctive morphological features and unique positioning of gonocytes within the seminiferous cords/tubules facilitate their identification (McGuinness and Orth 1992b; Orwig *et al.* 2002b). Unlike in rodents, gonocytes remain in the neonatal testis of large/farm animals for a number of months after birth, providing a window of opportunity for their study and manipulation. In rats, neonatal gonocytes have been shown to produce complete donor-derived spermatogenesis after homologous transplantation (Jiang and Short 1995; Jiang and Short 1998a; Orwig *et al.* 2002b; Ryu *et al.* 2003). However, transplantation of gonocytes from donor mice did not generate spermatogenesis in recipient mouse testes, suggesting that spermatogenesis-potent pseudopoded gonocytes may be typical for rats (McLean *et al.* 2003; Ohbo *et al.* 2003; Ohmura *et al.* 2004; Shinohara *et al.* 2002a). Interestingly, both the progenitors and -descendants of gonocytes (i.e., primordial germ cells and SSCs) produced donor-derived spermatozoa after homologous transplantation in rats and mice (Chuma *et al.* 2005; Jiang and Short 1995; Jiang and Short 1998a; Ohta *et al.* 2004). Whether gonocytes are indeed capable of

producing donor-derived spermatogenesis after transplantation to recipients is therefore controversial, and homologous transplantations of gonocytes has not been investigated in large animals.

For SSC transplantation, colonization of the recipient testes and donor-derived spermatogenesis could be improved if the recipient's endogenous germ cells were depleted due to genetic mutations (Boettger-Tong *et al.* 2000; Geissler *et al.* 1988; Ogawa *et al.* 2000; Ohta *et al.* 2001; Shinohara *et al.* 2001) or following ablative treatments such as the use of chemotherapeutic drug busulfan (Brinster 2002; Brinster and Avarbock 1994; Brinster and Zimmermann 1994; Ogawa *et al.* 1999b; Okabe *et al.* 1997). Due to the lack of proper large animal models that genetically lack germ cells, busulfan injection was used to prepare recipient pigs for GCT (Honaramooz *et al.* 2005). However, when busulfan was applied at sufficient doses to eliminate endogenous spermatogenesis in postnatal piglets, significant side-effects were also observed and in some cases mortality made the approach unacceptable (Honaramooz *et al.* 2005). To reduce the lethal toxicity, pregnant females especially in multiparous species can be injected with busulfan to wipe out the endogenous germ cells in the male offspring. However, this *in utero* approach can also lead to unwanted side effects in the dam and female littermates (Brinster *et al.* 2003; Hemsworth and Jackson 1963; Honaramooz *et al.* 2005; Moisan *et al.* 2003).

Compared with busulfan treatment, local irradiation of testes has less systematic toxicity and is not lethal at doses used for causing germ cell depletion; therefore, may be more practical for use in large animals (Creemers *et al.* 2002; Giuili *et al.* 2002; Herrid *et al.* 2009a; Herrid *et al.* 2009b; Honaramooz *et al.* 2005; Izadyar *et al.* 2003b; Kim *et al.*

2008; Oatley *et al.* 2005a; Schlatt *et al.* 2002; Zhang *et al.* 2006). It was reported that testis irradiation with a single dose of 15 Gy improved the donor SSC-derived spermatogenesis in sheep (Herrid *et al.* 2009b).

Gonocytes were reported to be more sensitive to irradiation than SSCs/spermatogonia in rats, pigs and bulls (Erickson 1964; Erickson *et al.* 1972; Forand *et al.* 2009a; Hughes 1962). However, irradiation has not been studied for eliminating endogenous germ cells for germ cell transplantation in pigs.

Pigs are important models for biomedical research and are considered as a potential source of 'humanized' tissues/organs for xenotransplantation into humans (Arundeii and McKenzie 1997; Cooper *et al.* 2008; Klymiuk *et al.* 2010; Kuwaki *et al.* 2004; Sachs 1994). The objectives of the present study were to evaluate the effectiveness of local irradiation of piglet testes in eliminating endogenous germ cells, and to investigate the effects of gonocyte transplantation into testis-irradiated recipients.

8.3 Materials and Methods

8.3.1 Experimental Design

To study the effects of early postnatal irradiation on piglet testis development and spermatogenic progress, piglets were subjected to 0, 1, 2, or 3 Gy of local irradiation of testes (n = 6 piglets/group), followed by sacrifice to retrieve testes for semi-quantitative morphological analysis at two months post-irradiation. Additionally, to assess the potential of donor gonocytes in establishing spermatogenesis in recipient testes, isolated piglet testis cells were transplanted into testes of the remaining pigs at two months post-irradiation, followed by sacrifice and examination at two months after transplantation.

8.3.2 Animals and Donor Testes

Nine-day-old Yorkshire-cross piglets (n = 24, Camborough-22 × Line 65, PIC Canada Ltd., Winnipeg, MB, Canada) were acquired from a university-affiliated swine facility, and maintained at the animal care unit of the University of Saskatchewan College of Veterinary Medicine from three days before and up to four months after-irradiation. Testes for donor cell preparation were collected after routine aseptic castration of 1-week-old piglets from the same swine facility, transferred to the laboratory within 2 hours after excision in ice-cold Dulbecco phosphate buffered saline (DPBS, cat. # 20-031-CV, Mediatech, Manassas, VA, USA) containing 2% antibiotic-antimycotic solution (cat. # 30-004-CI, Mediatech). Experimental procedures involving animals were approved by the University of Saskatchewan's Institutional Animal Care and Use Committee.

8.3.3 Irradiation of Neonatal Porcine Testes

Animals were randomly assigned into groups ensuring that littermate piglets were distributed across groups. Following acclimation to the new facility for three days and overnight withdrawal of feed and water, piglets were sedated with Azaperone through intramuscular injections (Stresnil, 2.2 mg/kg, NAC No. 11820822, Merial Canada Inc, Baie d'Urfé, QC, Canada), placed in dorsal recumbency with their scrotums exposed, and anaesthesia maintained using isoflurane inhalation during the entire irradiation process. A Theratron 780 Cobalt therapy unit (Best Theratronics Ltd., Ottawa, ON, Canada) was used to deliver gamma rays to the testes from a ^{60}Co source with doses of 0, 1, 2 or 3 Gy (n = 6 piglets each) and the procedure repeated for three consecutive days (corresponding to 9, 10 and 11 days of age). Ketoprofen was injected intramuscularly (Anafen, 3 mg/ kg, NAC No.: 11820042, Merial Canada Inc) following the procedure to alleviate any

potential pain. Piglets were monitored until fully recovered from the anaesthesia before returning to their pens.

8.3.4 Preparation of Donor Testis Cells with High Proportion of Gonocytes

The collected testes were immediately rinsed three times with DPBS, and the tunica albuginea, rete testis and overt connective tissue were removed. Testis cells with a high proportion of gonocytes were prepared using a previously-described approach (Yang *et al*. 2010b). Briefly, testis parenchyma of approximately 600 mg was cut into small pieces with fine scissors, vortexed for 1 min, rinsed with DPBS and digested with 0.2% collagenase IV (cat. # C-5138, Sigma-Aldrich, Oakville, ON, Canada) plus 0.1% hyaluronidase (cat. # H-3884, Sigma-Aldrich) and 0.01% Dnase I (cat. # DN25, Sigma-Aldrich) in Dulbecco modified Eagle medium (DMEM, cat. # 10-013-CM, Mediatech) for 10 min at 37 ℃. Foetal bovine serum (FBS, cat. # A15-701, PAA Laboratories GmbH, Etobicoke, ON, Canada) was added to stop the reaction, followed by another round of vortexing for 30 seconds. The resultant testis cell suspension was filtered through a 40 μm filter (cat. # 352340, BD Biosciences, Mississauga, ON, Canada) and depleted of erythrocytes with a lysis buffer (156mM NH_4CL, 10 mM $KHCO_3$, 0.1mM Na_2EDTA). The collected testis cells from multiple isolation procedures were pooled and stored at 4 ℃ overnight in 20% FBS-Leibovitz L15 (L15, cat. # 95016-512, VWR International, Mississauga, ON, Canada). Cell viability and yield were examined using a 0.4% trypan blue solution (cat. # T8154, Sigma-Aldrich) prior to transplantation. Cell smears were also prepared, air-dried at room temperature and stored at -80 ℃ for immunocytochemistry at a later time.

8.3.5 Immunocytochemistry

After thawing at room temperature, donor testis cell smears were fixed in Bouin's solution for 2 to 3 min, rinsed in DPBS and blocked with 5% bovine serum albumin (BSA, cat. # A7638; Sigma-Aldrich) at 37 ℃ in humidified atmosphere for 15 min. The smears were subsequently rinsed with DPBS and incubated with fluorescein labelled lectin Dolichos biflorus agglutinin (DBA, 1∶100; cat. # FL-1031; Vector Laboratories, Burlington, ON, Canada) overnight in humidified atmosphere. Following rinsing and incubation with 0.3% w/v Sudan Black B in 70% ethanol (cat. # 3545-12; EMD Chemicals, Gibbstown, NJ, USA) for 10-15 min, the cell smears were rinsed and stained with 4′,6′-diamidino-2-phenylindole (DAPI; cat. # D-9542; Sigma-Aldrich) for 2 min, and mounted (Vectashield; Vector Laboratories) for observation under fluorescent and laser scanning confocal microscopes. More than 600 cells from each smear slide were counted for quantification of gonocyte proportion.

8.3.6 Gonocyte Transplantation

Two months after testis local irradiation, half of the pigs in each group were randomly-selected and transplanted with piglet donor testis cells through rete testis injection as previously described (Honaramooz *et al.* 2002a), with minor modifications. Briefly, overnight-stored testis cells were rinsed twice and the volume adjusted with DPBS to a final concentration of 25×10^6/mL (cells had an overall viability of $\geq$ 95%). Recipient pigs were off-feed overnight, sedated and anaesthetised using isoflurane inhalation throughout the transplantation process. With piglets placed in lateral recumbency, the scrotal skin and surrounding areas were cleaned and disinfected, a 5-cm

linear incision was made along the median raphe of the scrotum to expose the testis (one at a time and enclosed in the parietal layer of tunica vaginalis). Under guidance of a portable B-mode ultrasound scanner (equipped with a 7.5-MHz linear-array probe, Aloka SSD 900; Aloka Co. Ltd., Tokyo, Japan), the rete testis were located and inserted with a Teflon i.v. catheter (20G × 1 1/4" , SR-OX2032CA, Terumo Medical Corporation, Somerset, NJ, USA) through the tail of the epididymis. A small drop of a tissue adhesive solution (cat. # 1469SB, 3M, St. Paul, MN, USA) was applied to temporarily affix the catheter in place. The testis cell suspension was then gradually infused through a connecting tube into the left testis of all animals, while DPBS was infused into the right testis as an internal control within each animal. After the infusion and removal of the catheter, a drop of the tissue adhesive solution was applied to the insertion site to block the leakage of the infused solution. Once the infusion of both testes was complete, the testes were returned in position, the scrotal skin incision sutured and covered with OpSite dressing spray (cat. # 66004978, Smith & Nephew Inc., St-Laurent, QC, Canada). Pigs were injected intramuscularly with Ketoprofen and intensively cared for until fully recovered before returning to their pens.

8.3.7 Histological Analysis

At two months after irradiation, half of the pigs in each group were sacrificed and the testes analyzed, while the remaining pigs were sacrificed at two months after gonocytes transplantation (i.e., four months post-irradiation) for retrieval of testes. At the time of sacrifice, pigs were sedated and euthanized using an intracardiac injection of sodium pentobarbital (Euthanyl Forte; 0.2 mL/kg, Bimeda-MTC Animal Health Inc.,

Cambridge, ON, Canada). Testes were weighed and tissue samples obtained from the same topographic areas of each testis for histological analysis.

Briefly, testis tissue fragments were fixed in Bouin's solution for 24 h, rinsed and processed with standard histological procedures, embedded in paraffin, sectioned and stained with haematoxylin and eosin. The slides were then randomly coded for blinded histological analysis under a transmitted light microscope equipped with digital photomicrography (Image-Pro Express, version 6.3, Media Cybernetics Inc., Bethesda, MD, USA) as previously described (Abrishami *et al.* 2010; Honaramooz *et al.* 2005). The numbers of seminiferous tubules per unit of surface (tubule density) was quantified in 12 random transverse sections in each testis sample (comprising 576,346 μm^2/transverse section). A minimum of 200 tubules in each testis sample were measured for the outer diameter as well as for the lumen diameter and epithelium thickness (Image Pro Plus, version 7.0, Media Cybernetics). Testes collected from pigs at two months post-irradiation were considered immature; therefore, the slides were analyzed for the total number of gonocytes/spermatogonia located in the seminiferous cords/tubules per 1,000 Sertoli cells. Cells on the slide were counted in random fields with > 25% coverage of the tissue cross-section.

For testes collected at four months post-irradiation (i.e., two months after gonocytes transplantation), the seminiferous tubules were scored for the presence of the most advanced germ cell types classified as (i) Sertoli-cell-only (no germ cells present); (ii) gonocytes or spermatogonia as the only germ cells; (iii) primary or secondary spermatocytes as the most advanced germ cells; (iv) round spermatids as the most advanced germ cells; (v) elongating or elongated spermatids as the most advanced germ

cells; or (vi) spermatozoa present in the lumen of the tubule, as previously described (Abrishami *et al.* 2010). Presence of germ cells was examined in all seminiferous tubules in randomly selected fields, covering > 25% of the tissue sections.

8.3.8 Statistical Analysis

We observed intra- and inter-group variations in body weights among pigs which is known to be correlated with testis weights (Fran ça *et al.* 2000; Van Straaten and Wensing 1977); therefore, to minimize the confounding effects of body weights on testis weights, we used testis weight index (% of testis weight/body weight) to compare the effects of irradiation on testis development among pigs. One-way ANOVA was performed for comparison of the effects of local testis irradiation, followed by a post-hoc Tukey's HSD test. Chi-square test was used for comparison of expected *vs.* observed presence of spermatogenesis following gonocyte transplantation. Kruskal Wallis test was also applied on non-parametric data using SPSS (Version 17.0; SPSS, Chicago, IL, USA) and SigmaStat (Version 3.5; Aspire Software International, Ashburn, VA, USA). Data are expressed as mean ± s.e.m. and differences were considered statistically significant when $P < 0.05$.

8.4 Results

8.4.1 Effect of Irradiation on Testis Weight Indices

Due to individual variations in body and testis weights (Fig. 8.1B), we used testis weight indices for comparing the groups. At two months post-irradiation, the testis weight index was reduced in the group of animals receiving daily testis irradiation doses of three Gy, compared with the non-irradiated control pigs ($P < 0.05$, Fig. 8.1A). At four

months post-irradiation, the same pattern of testis weight index was observed for the irradiated testes which were subsequently injected with DPBS ($P < 0.05$, Fig. 8.1A, B).

8.4.2 Effect of Irradiation on Testis Histology

Compared with non-irradiated testes, fewer seminiferous tubules/mm^2 were observed in testes collected at two months post-irradiation with either two or three Gy daily doses ($P < 0.05$, Fig. 8.2). The same pattern of seminiferous tubule density was observed at 4 months post-irradiation, except the values were even lower in the three Gy dose group than the two Gy group ($P < 0.05$, Fig. 8.2). Seminiferous tubule diameter was reduced in all irradiated testes (1, 2 or 3 Gy at both two and four months after irradiation ($P < 0.05$, Fig. 8.3). By four months post-irradiation, virtually all the tubules had formed a visible lumen; therefore, we also compared the lumen diameter and epithelial thickness in testes from different groups. Both the seminiferous epithelium thickness and lumen diameter were reduced in all irradiated testes at four months post-irradiation ($P < 0.05$, Fig. 8.4).

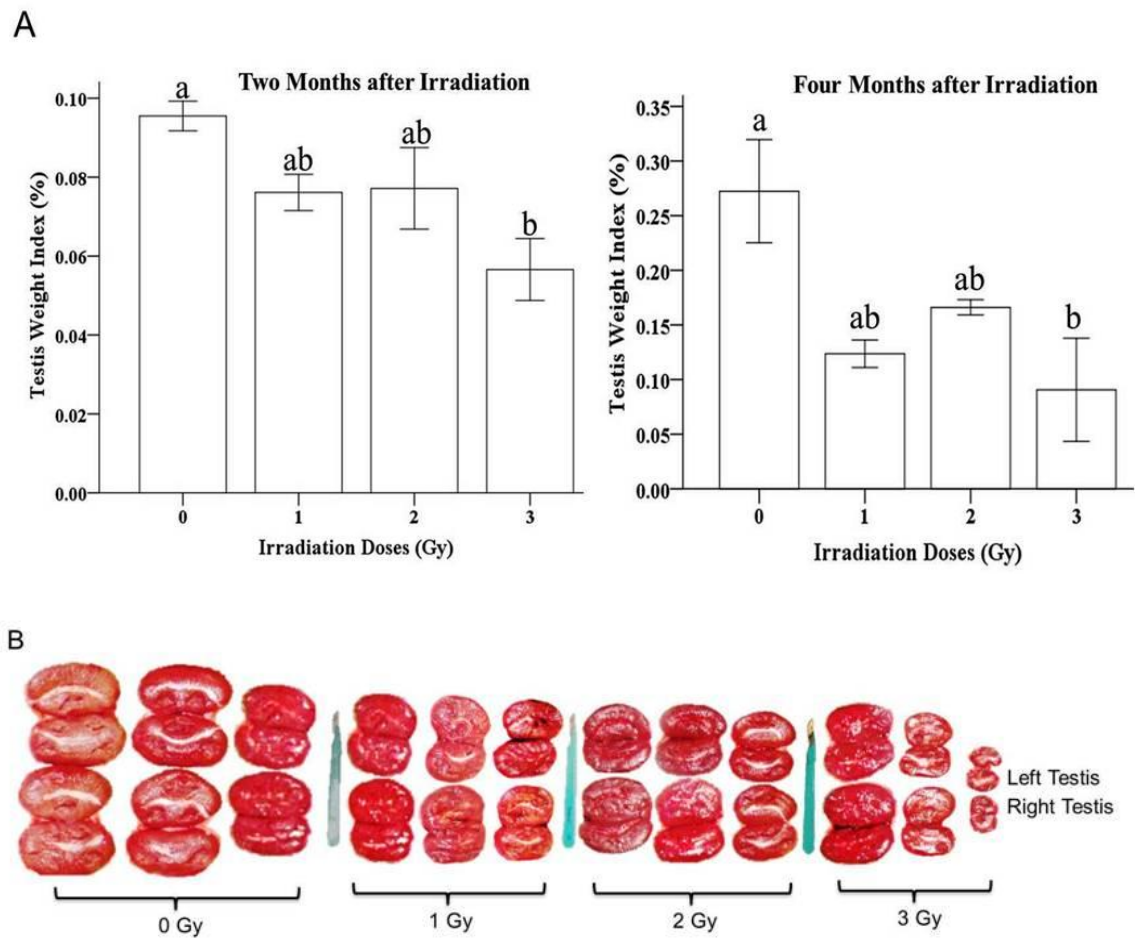


Fig. 8.1. Gross testis development following local irradiation of piglet testes using different irradiation doses. Piglet testes were irradiated using daily doses of 0, 1, 2 or 3 Gy for three consecutive days (6 pigs/group). Testes were collected from half of pigs in each group at two months after irradiation for assessment of the testis weight index (% of testis weight/body weight). For the remaining pigs at this age, one testis in each pig was infused with saline, and collected at four months post-irradiation for compassion of gross testis development. Data are the mean ± s.e.m. Columns without a common letter differ significantly ($P < 0.05$) (A). Images of the longitudinally-sectioned testes collected at four months after irradiation are also shown (B).

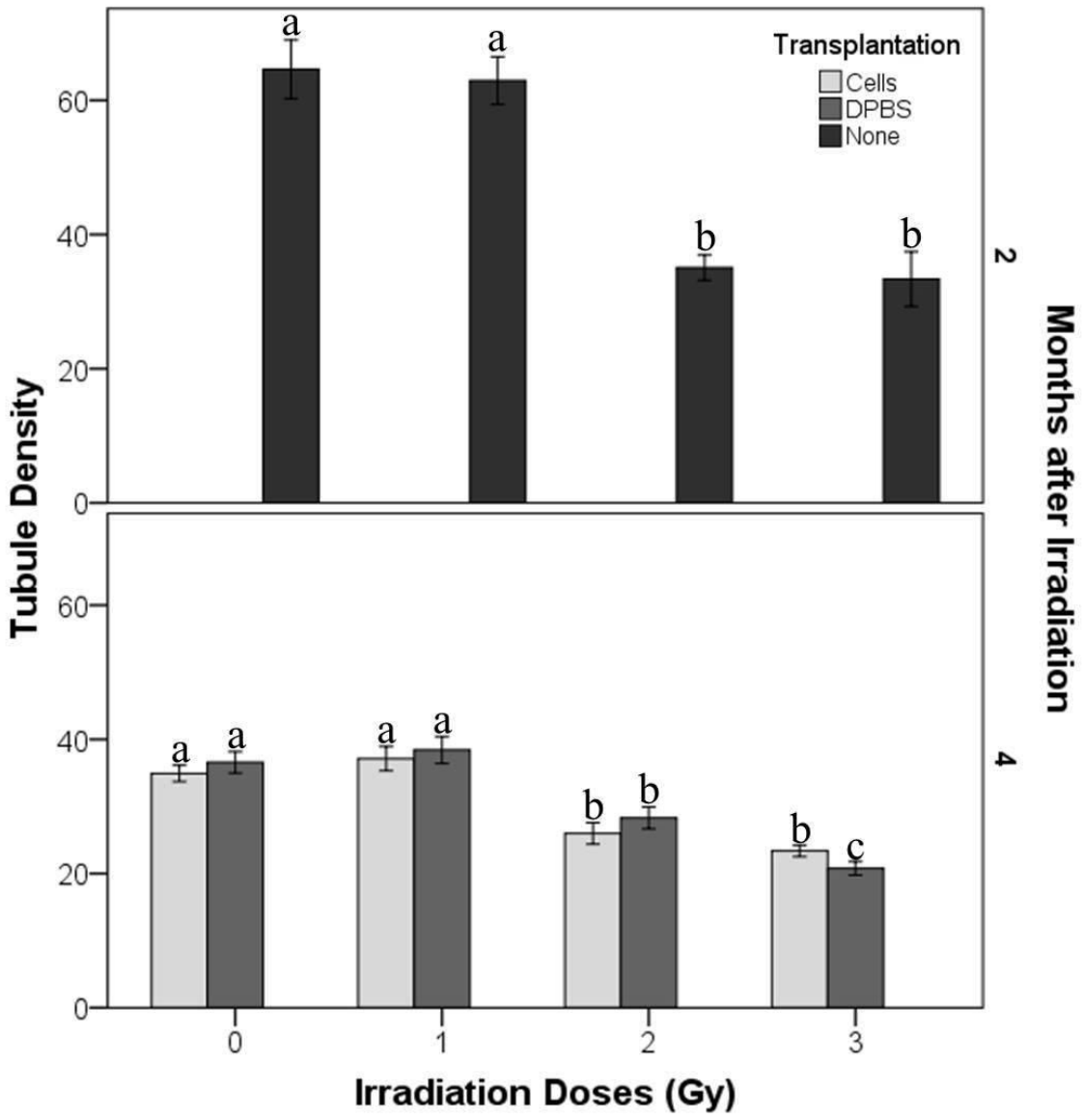


Fig. 8.2. Seminiferous tubule density after testis irradiation and transplantation of gonocytes or DPBS. Early postnatal recipient piglets underwent local irradiation of testes (using daily doses of 0, 1, 2 or 3 Gy for three consecutive days) in preparation for germ cell transplantation. At 2 months post-irradiation, testes were collected for assessment of seminiferous tubules from half of the pigs in each group, while in the remaining pigs, one testis in each animal was infused with saline (DPBS), and the contra-lateral testis was infused with donor testis cells, and testes retrieved at 4 months after irradiation. Seminiferous tubule density was expressed as the number of tubules per mm^2 of testis tissue cross-sections and compared among groups. Data are the mean ± s.e.m. Of the same texture, columns without a common letter differ significantly ($P < 0.05$).

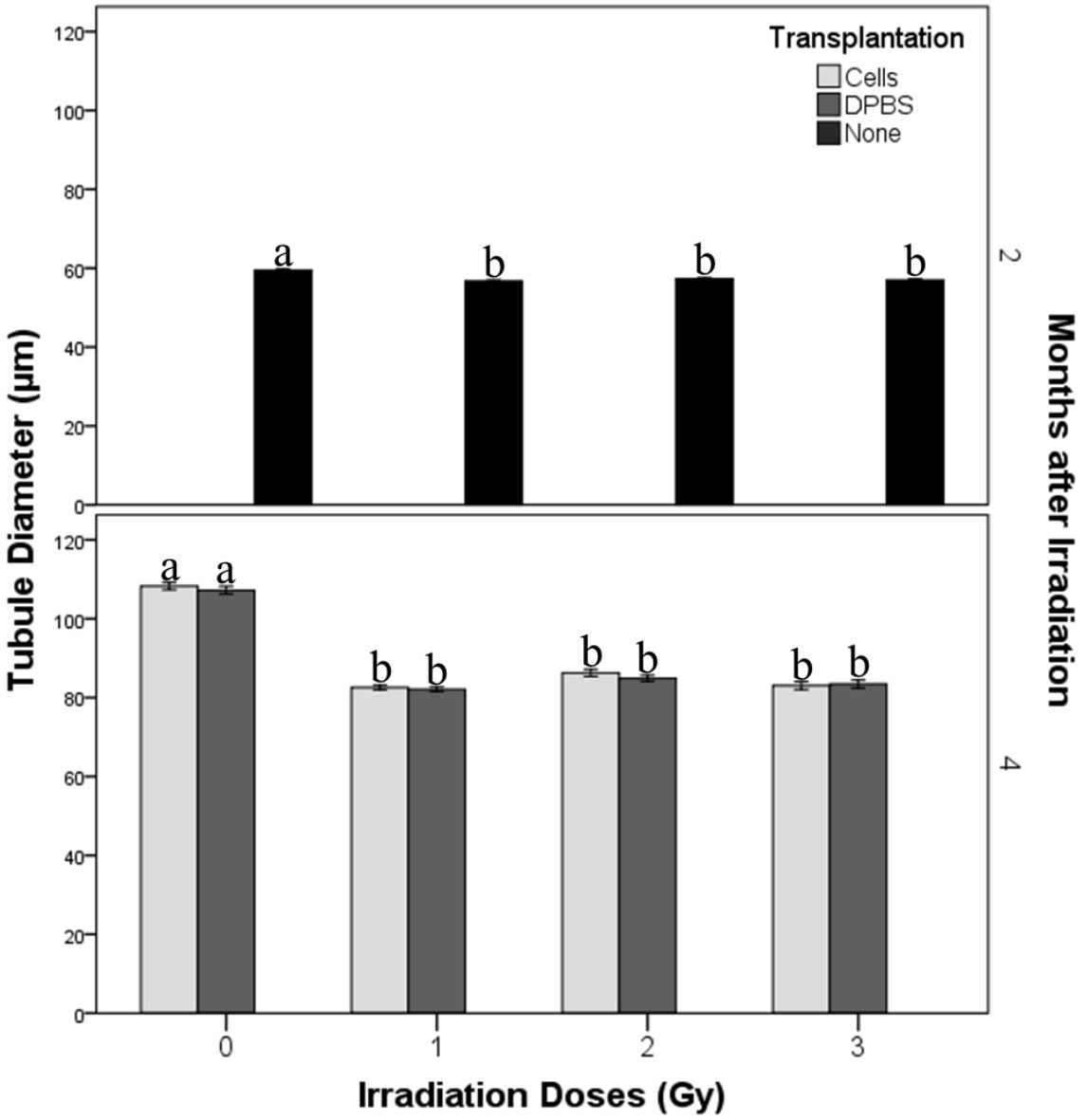


Fig. 8.3. Seminiferous tubule diameter after testis irradiation and transplantation of gonocytes or DPBS. Early postnatal recipient piglets underwent local irradiation of testes (using daily doses of 0, 1, 2 or 3 Gy for three consecutive days) in preparation for germ cell transplantation. At 2 months post-irradiation, testes were collected for assessment of seminiferous tubules from half of the pigs in each group, while in the remaining pigs, one testis in each animal was infused with saline (DPBS), and the contra-lateral testis was infused with donor testis cells, and testes retrieved at 4 months after irradiation. Seminiferous tubule outer diameter (µm) was evaluated and compared among groups. Data are the mean ± s.e.m. Of the same texture, columns without a common letter differ significantly ($P < 0.05$).

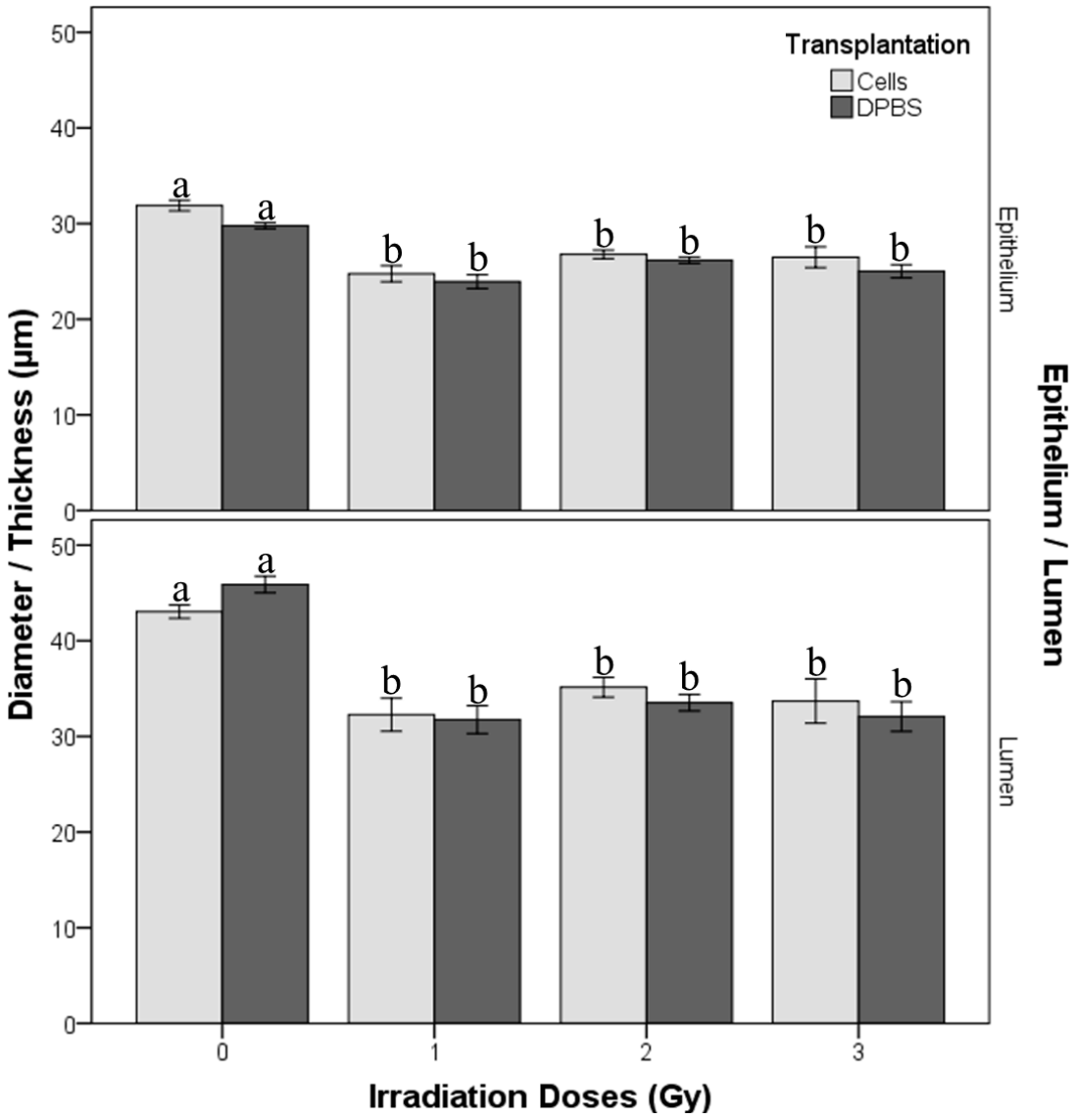


Fig. 8.4. Diameter of the tubular lumen and thickness of the seminiferous epithelium after testis irradiation and transplantation of gonocytes or DPBS. Early postnatal recipient piglets underwent local irradiation of testes (using daily doses of 0, 1, 2 or 3 Gy for three consecutive days) in preparation for germ cell transplantation. At 2 months post-irradiation, testes were collected for assessment of seminiferous tubules from half of the pigs in each group, while in the remaining pigs, one testis in each animal was infused with saline (DPBS), and the contra-lateral testis was infused with donor testis cells, and testes retrieved at 4 months after irradiation. Seminiferous tubule epithelium thickness (µm) and lumen diameter (µm) were measured and compared among groups. Data are the mean ± s.e.m. Of the same texture, columns without a common letter differ significantly ($P < 0.05$).

8.4.3 Effect of Irradiation on Germ Cell Development

At two months post-irradiation, gonocytes/spermatogonia were the only germ cell types present in all testes. Therefore, for the evaluation of testis irradiation effects on germ cell development, we compared germ cell numbers as per 1,000 Sertoli cells. Compared with the non-irradiated control testes, germ cell numbers decreased in the groups of testes receiving three daily doses of two or three Gy ($P < 0.05$, Fig. 8.5A, B). At four months post-irradiation, all stages of germ cell development up to elongating or elongated spermatids were present in the non-irradiated control testes (Fig. 8.6A, B). However, no differentiated germ cells were present in any irradiated testes injected only with DPBS (Fig. 8.6A, B).

8.4.4 Spermatogenesis in Recipient Testes after Gonocyte Transplantation

At 4 months post-irradiation (i.e., 2 months post-transplantation), while no differentiated germ cells were present in any irradiated testes injected only with DPBS, spermatogenesis was observed after transplantation of gonocytes into irradiated testes (Fig. 8.7). Spermatids were observed in two (out of three) recipient animal testes receiving three doses of one Gy irradiation, although in only a few tubules accounting for 0.6% of seminiferous tubules, and one (out of three) recipient animal testis receiving three doses of two Gy irradiation, representing 1% of seminiferous tubules ($P < 0.05$, Fig. 8.7).

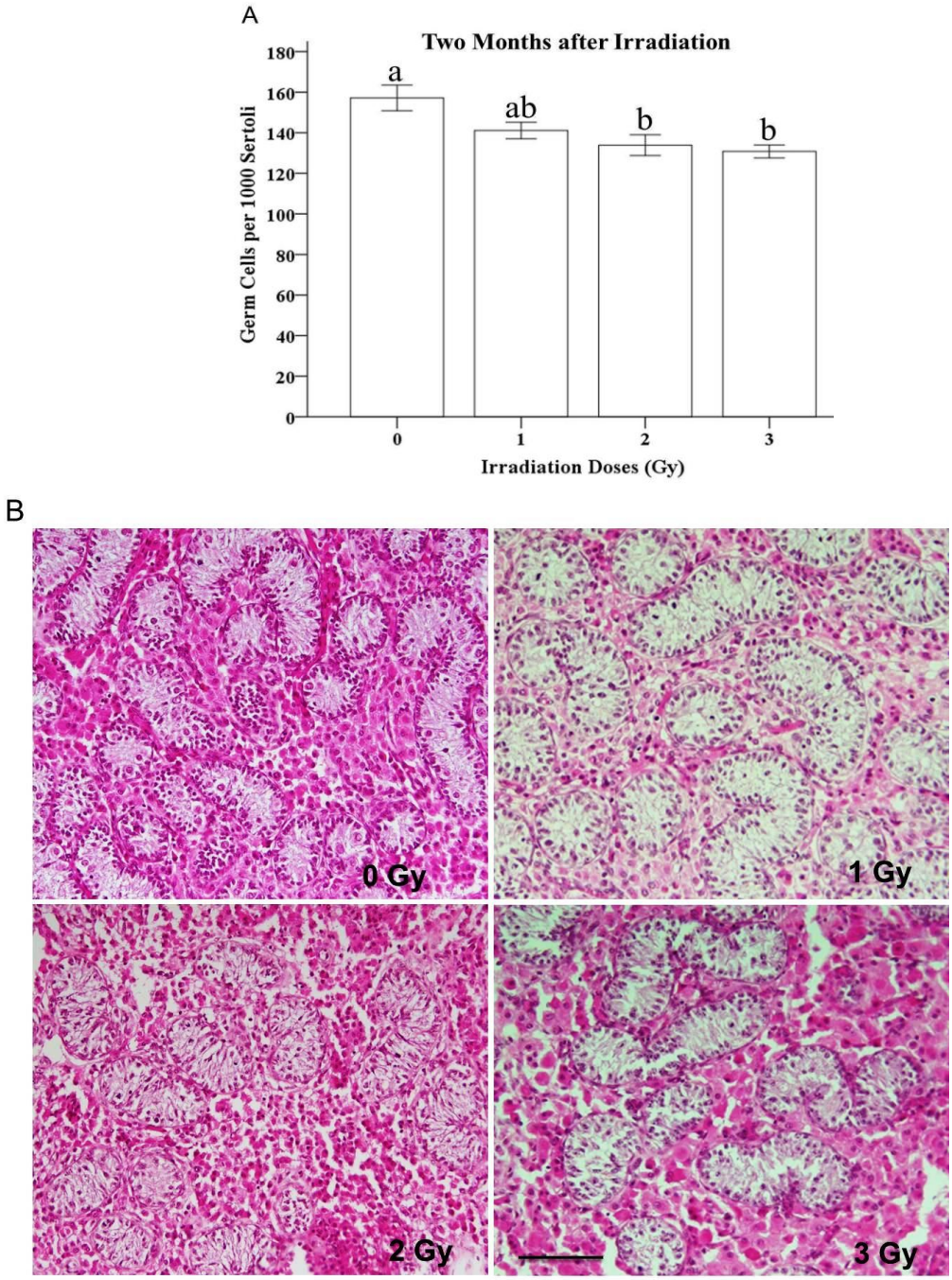


Fig. 8.5. Examination of endogenous germ cell development at two months after irradiation of piglet testes. Early postnatal recipient piglets underwent local irradiation of testes (using daily doses of 0, 1, 2 or 3 Gy for three consecutive days) in preparation for germ cell transplantation. At 2 months post-irradiation, testes were collected for assessment of seminiferous tubules from half of the pigs in each group. Germ cell number per 1,000 Sertoli cells was determined and compared among different doses (A). Data are the mean ± s.e.m. Columns without a common letter differ significantly ($P < 0.05$). Representative photomicrographs of testis tissue (stained with hematoxylin and eosin) from an animal in each group are also shown (B). Scale bar, 100 μm.

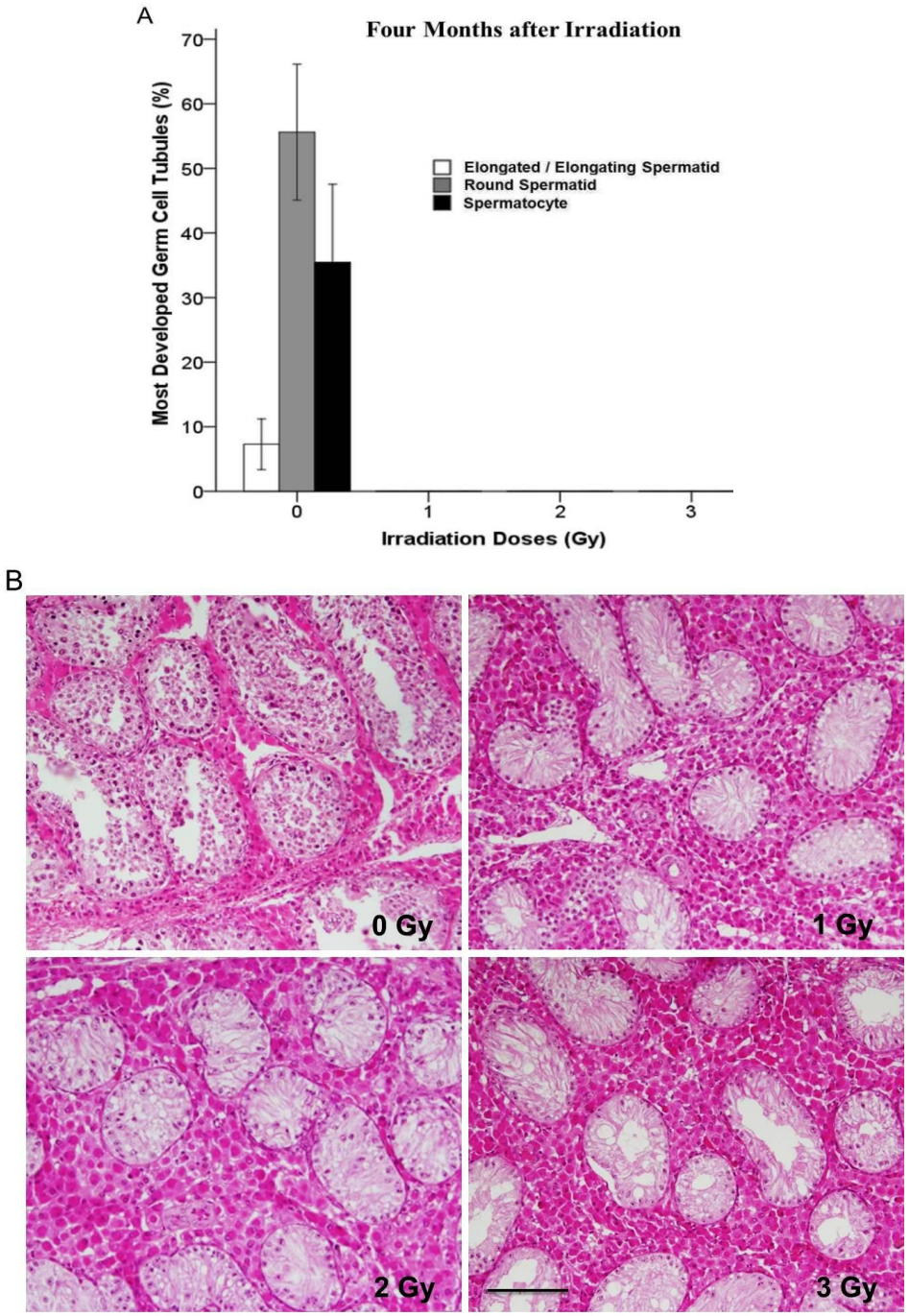


Fig. 8.6. Germ cell development four months after irradiation of piglet testes. Early postnatal recipient piglets underwent local irradiation of testes (using daily doses of 0, 1, 2 or 3 Gy for three consecutive days) in preparation for germ cell transplantation. At 4 months post-irradiation, percentage of tubules containing spermatocytes, round or elongating/elongated spermatids as the most advanced germ cells, were determined for comparison among different groups (A). Data are the mean ± s.e.m. Representative photomicrographs of testis tissue (stained with hematoxylin and eosin) from an animal in each group are also shown (B). Scale bar, 100 μm.

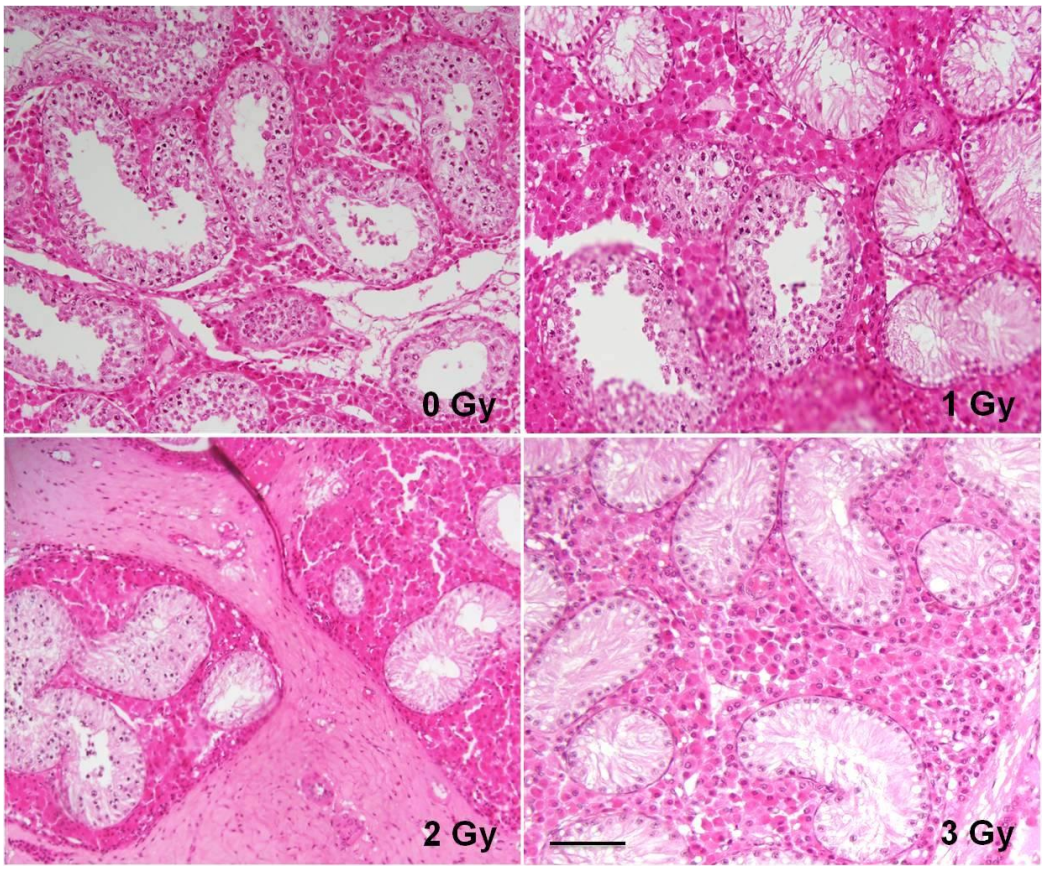


Fig. 8.7. Spermatogenesis in the irradiated recipient pig testes at two months after gonocyte transplantation. Early postnatal recipient piglets underwent local irradiation of testes (using daily doses of 0, 1, 2 or 3 Gy for three consecutive days) in preparation for germ cell transplantation. At 2 months post-irradiation, one testis in each animal was infused with saline (DPBS) and the contra-lateral testis was infused with donor testis cells, and testes retrieved at 4 months after irradiation. Although no spermatogenesis was observed in DPBS-injected recipient testes, limited spermatogenesis was evident in the recipient testes injected with donor gonocytes, with elongating spermatids as the most advanced germ cells observed by the end of the experiment at 2 months post-transplantation. Scale bar, 100 μm.

8.5 Discussion

Upon transplantation, SSCs can generate donor-derived spermatogenesis in recipient testes; however, it is not clear whether gonocytes also possess such potential in all species. Successful gonocyte transplantation could rescue the fertility potential of animals that die at early neonatal stages or that of prepubertal boys who receive sterilizing treatments for cancer treatment. In the present study, we found that fractionated daily irradiation of piglet testes with gamma-ray doses as low as 1 Gy for three consecutive days completely eliminated endogenous spermatogenesis, when examined four months post-irradiation (Fig. 8.6). Gonocyte transplantation initiated the spermatogenesis in the irradiated pig testes.

To prepare recipients for SSC/gonocyte transplantation, busulfan injection could be used, but can potentially cause systematic toxicity in recipient animals (Honaramooz *et al.* 2005; Ogawa *et al.* 1999a; Savchenkova *et al.* 2006; Shinohara *et al.* 2002a; Udagawa *et al.* 2001). Therefore, in the present study, we investigated irradiation of testes as an alternative approach to busulfan treatment. We observed that in early postnatal piglets, local irradiation of testes with daily doses of 3 Gy for three consecutive days significantly reduced the testis weight. Piglet testes grow considerably during the perinatal period, mainly due to the increasing volume of Leydig cells, and after 7 postnatal weeks, mainly due to the extension and widening of the seminiferous tubules (Erickson 1964; Franca *et al.* 2000; Van Straaten and Wensing 1977). It has been suggested that germ cells are susceptible to irradiation damage, whereas the testis somatic cells (e.g., Leydig cells) may not be as severely affected (Erickson 1964; Lambrot *et al.* 2007; Oatley *et al.* 2005b; Vergouwen *et al.* 1994). Therefore, the decreased number of

germ cells (gonocytes/spermatogonia) in the present study may be a reason for the lower testis weight in the irradiated testes, compared with the controls. During the first postnatal month, the increase in the pig testis weight has been shown to correlate with the length and width of the seminiferous cords, and the cord lengths extended more than 7-fold (Franҫa *et al.* 2000). Whether the lowered number of gonocytes (and likely Sertoli cells) decreased the growth of irradiated piglet testes by restricting the expansion of the seminiferous tubules needs to be investigated. The fact that in the present study both the density and diameter of tubules were reduced in irradiated pig testes, may indicate that irradiation of neonatal testis may affect both the length and width of the tubule expansion.

Ionizing radiation could cause DNA lesions, with double-strand breaks and cross-links as the most crucial damages; unrepaired breaks could lead to cell death (apoptosis or necrosis) through different pathways (Hamer *et al.* 2003; Jeggo and Lavin 2009). In the present study, 9-day-old piglets were selected for gamma-ray irradiation to eliminate endogenous germ cells. During the first couple of weeks postpartum, pig gonocytes and Sertoli cells proliferate actively as the testis weight doubles (Erickson 1964; Franҫa *et al.* 2000). Mammalian cells exhibit highest sensitivity to irradiation when undergoing mitosis, with cell cycles lasting 8-30 hours (~24 hours for neonatal germ cells) (Forand *et al.* 2004; Pawlik and Keyomarsi 2004; Sinclair and Morton 1966; Sinclair 1968; Sinclair and Morton 1963). Sensitivity of gonocytes to irradiation was demonstrated to increase before birth in rodents, and decrease with age in neonatal calves and piglets (Erickson and Martin 1972; Erickson 1963; Erickson *et al.* 1972; Hughes 1962; Moreno *et al.* 2001; Moreno *et al.* 2002; Vergouwen *et al.* 1995). Therefore, neonatal piglet testes at earlier

ages are likely more sensitive to irradiation, and elimination of the endogenous germ cell progenitors at such ages may be more efficient in preparing them as recipients for germ cell transplantation.

Testes from animals of different species may have different levels of sensitivity to irradiation, requiring the establishment of a specific threshold irradiation dose for complete and permanent removal of endogenous spermatogenesis. In addition to the irradiation dose, the application strategy (e.g., single dose *vs.* fractionated doses) could also affect the expected results from testis radiation. A single irradiation dose of 1.5 Gy for foetal and neonatal rat gonocytes was reported to cause complete and permanent infertility of male offspring (Hughes 1962; Moreno *et al.* 2001); however, a 3 Gy dose was required for the similar effects on mouse gonocytes (Moreno *et al.* 2002). When fractionated gamma-rays were delivered to adult mice using two doses of 1.5 and 12 Gy, 24 hours apart, germ cells were eliminated in more than 95% of seminiferous tubules without obvious side effects (Creemers *et al.* 2002). In humans, the sensitivity of germ cells to X-ray irradiation has been described as being more than 3-fold higher than that of mice (Clifton and Bremner 1983). Irradiation of human foetal testes with doses as low as 0.1 Gy of gamma-rays was reported to cause a decrease in gonocyte numbers without affecting testosterone production (Lambrot *et al.* 2007). Oligospermia was observed after irradiation of human adult testes with doses from 0.1 to 1.2 Gy (Centola *et al.* 1994; Clifton and Bremner 1983; Howell and Shalet 1998; Rowley *et al.* 1974), and fractionated irradiation with doses more than 2 Gy resulted in permanent sterility (Ash 1980; Howell and Shalet 1998; Speiser *et al.* 1973). In contrast, complete eradication of germ cells in non-human primates was observed only with single doses of more than 8

Gy or with two fractionated doses of 3 Gy (de Rooij *et al.* 2002; Jahnukainen *et al.* 2007b). Spermatogonia in the young mouse testes were described to be ~7-fold more susceptible to fractionated- than single-dose irradiation, and it was suggested that their sensitivity increases ~24 hours after the first irradiation (De Ruiter Bootsma *et al.* 1977; Van der Meer *et al.* 1993; Van der Meer *et al.* 1992). Similar observations were made in humans and non-human primates in which fractionated irradiation was more effective in cell death than single-dose irradiation with doses up to 6 Gy (Ash 1980; de Rooij *et al.* 2002; Speiser *et al.* 1973). In the present study, we irradiated neonatal piglet testes 3 times (24 hours apart) with fractionated gamma-rays of 1, 2 or 3 Gy (3, 6 or 9 Gy in total). At 4 months post-irradiation, spermatogenesis was evident in virtually all seminiferous tubules in control animals. However, no differentiated germ cells (spermatocytes or later stages) were present in any tubule in irradiated pigs. Therefore, it appears that fractionated irradiation of neonatal piglet testes for 3 consecutive days with even 1 Gy should be sufficient in depleting endogenous spermatogenesis.

Single- and fractionated irradiation have been applied in several species in preparing recipients for germ cell transplantation with varying results. A single dose of 12 Gy for irradiation of ram testes was reported to result in ~78% seminiferous tubules with spermatogenesis (Oatley *et al.* 2005b), and irradiation of bull calf testes with a similar dose (10-14 Gy) also maintained spermatogenesis in 60% of tubules (Izadyar *et al.* 2003b). In comparison, fractionated irradiation resulted in higher efficiency in eliminating endogenous germ cells. When testes from goats of different prepubertal ages were irradiated with daily doses of 2 Gy for 3 days, only 2-4% of seminiferous tubules contained spermatocytes two months after irradiation (Honaramooz *et al.* 2005).

Spermatocytes were also present in 4-9% of seminiferous tubules after fractionated irradiation of testes in peripubertal cats with daily doses of 3 Gy for 3 consecutive days; however, less than 1% of tubules eventually contained spermatozoa (Kim *et al.* 2006). When the same irradiation strategy used in cats in the latter study was applied in 5-month-old dogs, spermatogenesis was depleted at two months post-irradiation in all seminiferous tubules, with less than 5% of tubules containing spermatogonia as the only germ cells (Kim *et al.* 2008). When applied within the first postnatal month, whole-body irradiation of 6- and 12-day-old piglets with a single dose of 2 Gy resulted in the lowest testis weight, semen volume and spermatozoa number. Up to 90% of gonocytes were eliminated 40 days after irradiation, with ~25% of tubules showing no spermatogenesis in the mature testes (Erickson 1964). Based on these evidence, we speculate that neonatal piglet testes may be more sensitive to (fractionated) irradiation than other species such as goats and cats.

In the present study, at two months post-irradiation, we observed significant decreases in germ cells indices (number of gonocytes per 1,000 Sertoli cells) in testes of piglets in the 2- and 3-Gy groups but not in the 1-Gy group, in comparison with non-irradiated testes. Apoptosis of irradiated spermatogonia was reported to happen when they start the mitosis divisions (Van der Meer *et al.* 1992), and it was suggested that the death of gonocytes after whole-body irradiation of neonatal calves needed at least 1 month to manifest itself (Erickson 1963). In neonatal testes, gonocytes were reported to be the most sensitive cells to gamma irradiation (Erickson and Blend 1976), and while most Sertoli cells survived testis irradiation (Forand *et al.* 2009b; Oatley *et al.* 2005b; Vergouwen *et al.* 1994), a decrease in Sertoli cell number was observed following high

doses of irradiation for neonatal rats and non-human primates without affecting the levels of testosterone or estradiol production (Allan *et al.* 1988; de Rooij *et al.* 2002; Erickson and Blend 1976; Jahnukainen *et al.* 2007b). The sensitivity of gonocytes to irradiation in the present study was probably dose-dependent, because even though all doses eventually led to complete depletion of germ cells (at 4 months post-irradiation), it took the higher doses (2 and 3 Gy) less time than the lowest dose to cause significant germ cell losses. This observation may also indicate that there are different populations of gonocytes in the neonatal pig testes, and that some gonocytes may be more resistant to lower doses of irradiation and/or take longer period of time to show apoptosis. Furthermore, since Sertoli cells would be actively proliferating in the early neonatal pig testes (when the irradiation was applied in the present study), it is feasible to assume their number was also decreased. If this is indeed the case, then the efficiency of testis irradiation on gonocyte depletion may be underestimated for the two-month post-irradiation analysis in the present study, because the deceased Sertoli cell numbers would have resulted in artificially higher germ cell indices for irradiated testes (de Rooij *et al.* 2002; Honaramooz *et al.* 2005). Nevertheless, no germ cell differentiation could be identified four months after irradiation using any of the irradiation doses tested.

To date, transplantation of neonatal gonocytes has only been investigated in a few studies using rodent models, leading to contradictory conclusions about the spermatogenic capacity of gonocytes (Jiang and Short 1995; Jiang and Short 1998a; McLean *et al.* 2003; Ohbo *et al.* 2003; Ohmura *et al.* 2004; Orwig *et al.* 2002b; Ryu *et al.* 2003; Shinohara *et al.* 2002a). To evaluate the stem cell potential of piglet gonocytes, we transplanted neonatal pig testis cells with high proportion of gonocytes into one testis of

both irradiated and non-irradiated animals and used the infusion of DPBS into the contra-lateral testis of the same animals as an internal control. Complete spermatogenesis was observed in a limited number of tubules only after transplantation of gonocytes into irradiated testes (that had received daily doses of either 1 or 2 Gy), at the time when complete spermatogenesis was evident in all seminiferous tubules of non-irradiated testes.

In the present study, piglets of ~10 weeks of age were used as recipients for germ cell transplantation, because at this stage the lumen of the seminiferous tubules has just formed, the tubules are expanding steadily in length and new germline stem cell niches are being shaped by the proliferation of Sertoli cells (Fran ça *et al.* 2000; Van Straaten and Wensing 1977). Additionally, gonocytes are migrating to the basement membranes (Kohler *et al.* 2007), and younger recipient testes were reported to provide more suitable microenvironments for colonization of donor germline stem cells (Brinster *et al.* 2003; Ogawa *et al.* 1999b; Ryu *et al.* 2003; Shinohara *et al.* 2001). After transplantation, neonatal donor rat gonocytes have been reported to establish contact with the host Sertoli cells and generate complete spermatogenesis with up to 5% of donor gonocytes colonizing ~80% of recipient tubules. It was also suggested that from the two populations of gonocytes, those with pseudopods were actually the putative germ cell progenitors capable of initiating spermatogenesis after transplantation (Jiang and Short 1995; Jiang and Short 1998a; Orwig *et al.* 2002b; Ryu *et al.* 2003), whereas pseudopoded gonocytes could not be identified in mice, and neonatal mouse gonocytes did not generate spermatogenesis after transplantation (McLean *et al.* 2003). The extent of DBA staining has been reported to vary among piglet gonocytes, (Goel *et al.* 2007), suggesting that different populations of gonocytes may exist. Given our results, we speculate that

endogenous germ cells were totally wiped out after local irradiation of piglet testes, and that some of the transplanted piglet gonocytes were capable of colonizing and generating spermatogenesis in the irradiated recipient testes.

High-dose irradiation of testes has been reported to cause reduction of Sertoli cell numbers (Allan *et al.* 1988; de Rooij *et al.* 2002; Erickson and Blend 1976; Jahnukainen *et al.* 2007b). The reduction of Sertoli cells prior to puberty could adversely affect spermatogenesis in adults (Orth *et al.* 1988), and severe damage to the intra-testicular environment could result in lack of support for spermatogenesis (Kangasniemi *et al.* 1996; Meistrich and Shetty 2003; Zhang *et al.* 2007; Herrid, *et al.* 2010). In the present study, the low doses of fractionated testis irradiation allowed some degree of spermatogenesis after gonocytes transplantation; however, the intra-tubular microenvironment might have been too impaired (especially with higher doses) to allow spermatogenesis to be readily established by the transplanted gonocytes (by 2 months post-transplantation). It has been suggested that the presence of some level of spermatogenesis in the recipient testis is beneficial for germ cell transplantation as it indicates the capability of the host testis environment in providing support for the donor germ cells (Brinster *et al.* 2003; Ryu *et al.* 2003). Therefore, for application in preparation of neonatal piglets for subsequent germ cell transplantation it might be useful not to exceed the lower dose (1 Gy daily for three consecutive days) as established in this study.

In conclusion, in the present study, we showed that fractionated irradiation of testes in neonatal piglets with doses as low as 1 Gy (for 3 consecutive days) is effective in depleting the endogenous spermatogenesis. Using piglets as a non-rodent animal model, we also provided support for the notion that gonocytes possess stem cell potential,

and that gonocyte transplantation may lead to donor-derived spermatogenesis in irradiated recipient testes.

CHAPTER 9 GENERAL DISCUSSION AND FUTURE DIRECTIONS

9.1 General Discussion

The studies presented in this thesis were designed to focus on the study and manipulation of gonocytes in neonatal pigs, as a large animal model. To follow is a discussion of common themes and findings among different experiments, and general conclusions that can be drawn from them.

9.1.1 Lectin DBA Binds to Piglet Gonocytes

Based on the expression in PGCs and SSCs, several bio-markers may initially appear as suitable candidates for identification of piglet gonocytes. For instance, Vasa - a general germ cell marker (Castrillon *et al.* 2000; Gab Sang Lee 2005; Tanaka *et al.* 1997), Nanog - a transcriptional factor in certain germ cell populations (Chambers *et al.* 2003; Hoei-Hansen *et al.* 2005; Rajpert-De Meyts 2006; Yamaguchi *et al.* 2005) and AP-2γ - a transcription factor activator protein (Pauls *et al.* 2005) are specifically expressed in both PGCs and gonocytes. Additionally, Oct 3/4 (also known as POU5F1) - a POU-family transcription factor detected in all three germ cell progenitors (Ohbo *et al.* 2003; Ohmura *et al.* 2004), PGP9.5 – a ubiquitin-C-terminal hydrolase (Kon *et al.* 1999; Luo *et al.* 2006; Tokunaga *et al.* 1997; Tokunaga *et al.* 1999; Wrobel 2000; Wrobel *et al.* 1996) and lectin Dolichos biflorus agglutinin (DBA) (Aponte *et al.* 2006; Herrid *et al.* 2007; Izadyar *et al.* 2003a; Izadyar *et al.* 2002a; Izadyar *et al.* 2002b; Suda *et al.* 1998) are primarily used in SSC/spermatogonia identification. However, most of these bio-markers are found within the cell nucleus or cytoplasm; thereby, could not be utilized in selection

of live gonocytes in preparation for transplantation. Therefore, after preliminary experiments we chose the cell-surface marker lectin DBA.

Studies presented in Chapters 3, 4 and 7 (Fig. 3.9, 4.3 and 7.6) demonstrated that in the neonatal pig testis, DBA specifically binds to gonocytes, allowing it to be used as a bio-marker for pig gonocytes. Our observations were subsequently validated by another group (Goel *et al.* 2007). We observed that an intense non-specific fluorescence (most likely due to lipofuscin in Leydig cells) was interfering with accurate immune-staining identification of gonocytes *in vitro* (Fig. 3.1-3.7), which could be completely masked by Sudan Black B staining (Fig. 3.8 and 3.9).

Each type of lectin has affinity for a specific type of carbohydrate (Gupta *et al.* 2010; Katrlík *et al.* 2010; Kurmyshkina *et al.* 2010; Sharon 2008), and DBA was reported to recognize and specifically bind to the terminal N-acetylgalactosamine residues (GalNAc) (Hammarström *et al.* 1977; Imberty *et al.* 1994; Muramatsu 1988); however, the role of GalNAc in cell activities of gonocytes is unknown. The expression patterns of DBA binding in the testis are quite likely species-specific. For instance, while DBA was found to specifically bind to pre-spermatogonia in prepubertal human and bull testes (Aponte *et al.* 2006; Ertl and Wrobel 1992; Gheri *et al.* 2004; Herrid *et al.* 2007; Izadyar *et al.* 2003a; Izadyar *et al.* 2002a; Izadyar *et al.* 2002b; Suda *et al.* 1998), it did not show affinity to any germ cell type in sheep or goat testes (Borjigin *et al.* 2010; Rodriguez-Sosa *et al.* 2006; Kurohmaru *et al.* 1991). Even though rat and hamster spermatogonia were reported to react with DBA (Arya and Vanha-Perttula 1984; Ballesta *et al.* 1991), the results in mice were contradictory (Izadyar *et al.* 2002b; Arya and Vanha-Perttula

1986), and in cats, DBA stained both spermatocytes and round spermatids (Desantis *et al.* 2006).

It is not clear whether the specific affinity to DBA has any potential role in gonocyte development. In our experiments, some gonocytes showed intense (dark) DBA staining, while others showed weak (light) staining. The reason for the differential staining with DBA is not completely understood, but it has been reported that the number of DBA-positive cells decreased with the transition of gonocytes into SSCs, and spermatogonia did not bind DBA (Goel *et al.* 2007). Because the observed DBA binding pattern closely corresponds to the migration pattern of gonocytes to the basal membrane of the seminiferous cord, we can speculate that the binding between DBA-like molecules and N-acetylgalactosamine on gonocyte surface may be involved in gonocyte migration. Whether such a relationship indeed exists or is important for gonocyte migration, can be the subject of an interesting investigation.

9.1.2 Preparation of Gonocytes for Transplantation

Effects of several factors on piglet gonocyte isolation were systematically studied in Chapter 4. Abundance of erythrocytes collected while separating testis cells was considered a source of cell contamination; therefore, in experiments described in Chapter 4, an erythrocyte lysis buffer was identified to be capable of eliminating virtually all red blood cells from the cell isolates without adversely effecting testis cells. Although in a side-by-side comparison, we showed that enzymatic digestion methods were superior to mechanical dissociation methods, the resultant piglet gonocyte proportion from either strategy did not differ from that *in situ* (i.e., ~7%) (Fig. 4.2 and 4.4). However, using our

novel three-step strategy, piglet testis cells could be collected with considerably higher gonocyte proportions (~40%) than any other reported method thus far (Fig. 4.5 and 4.6). The purity of the harvested piglet gonocytes were further increased as described in Chapter 7, by investigating the efficiency of density gradient centrifugation using Nycodenz and differential plating. Piglet gonocytes with > 80% purity were obtained using either 17% Nycodenz centrifugation or differential plating with a combination of fibronectin and poly-D-lysine coating (Fig. 7.1-7.4). The gonocyte purity could be further increased to > 90% (the highest ever reported) by combining the two purification strategies (Fig. 7.5 and 7.6). One of the reasons why we chose to use gradient centrifugation and differential plating was to provide an alternative for purification of gonocytes using FACS or MACS which require binding of antibodies to the cell surface epitopes which may affect the cell behaviour or fate (Bashamboo *et al.* 2006; Bendel-Stenzel *et al.* 2000; Gilner *et al.* 2007; Yan *et al.* 2000).

In addition to improving donor cell purification for applications in gonocyte transplantation or *in vitro*/culture studies, the results of Chapter 7 also pointed out some previously unknown biological features of piglet gonocytes. For instance, we learned that piglet gonocytes do not preferentially adhere to fibronectin or poly-D-lysine (Fig. 7.2-7.4). Recently, differential plating with laminin, fibronectin and collagen-IV coatings were reported capable of enriching piglet gonocytes with negative selection (Kim *et al.* 2010), which validated our observations. During development of male germline progenitor cells, they may alter their affinity for ECM components. Mouse PGCs were reported to adhere to both laminin and fibronectin during migration, but after arrival in the genital ridge they tend to lose their affinity only for fibronectin (Garc á-Castro *et al.*

1997). ECMs have been reported to promote colonization of mouse gonocytes (Hasthorpe *et al.* 1999), and laminin (but not fibronectin) was observed to enhance the migration of rat gonocytes in culture (Orth and McGuinness 1991; Orth *et al.* 1998). In our study (Chapter 7), coating with laminin resulted in the maximum adhesion of gonocytes (minimum number among non-adherent cells) (Fig. 7.2). We speculate that piglet gonocytes may selectively decrease/cease the expression of certain ECM adhesion molecules (e.g., fibronectin) when residing in the central part of the seminiferous cords, but increase the accumulation of certain ECM receptors (e.g. laminin) which promote their migration into and residency in the basement membrane.

9.1.3 Prospects of Hypothermic Conditions on Gonocytes Survival

We investigated a number of factors involved in hypothermic preservation of piglet testis cells and tissue in chapters 5 and 6, and studied their effects on gonocyte survival and development. Lower temperatures (4 ℃ *vs.* 20-25 ℃) were found to considerably extend the maintenance of testis cells viability (Fig. 5.1). Media components also significantly affected the testis cells survival rates (Fig. 5.1-5.3), where > 80% of piglet testis cells could survive at least 6 days of hypothermic preservation in 20% FBS-L15 resulting in 88% after-storage cell viability (Fig. 5.1-5.4), with no obvious changes in germ cell proportions or their morphology in culture (Fig. 5.5 and 5.6). However, for hypothermic preservation of the testis tissue, similar media resulted in the survival of approximately half of testis cells after 3 days, and a quarter after 6 days (Fig. 6.1). Media components, but not tissue sizes, affected gonocyte survival and tissue morphology during hypothermic preservation of the testis tissue (Fig. 6.1-6.5).

Leibovitz L15 plus 20% FBS was identified to be comparable to a specialized product (HTS-FRS solution) in maintaining the viability of disassociated testis cells (Fig. 5.1-5.5). The effectiveness of L15 in persevering testis cells could be due to its unique buffering system in maintaining media pH in ambient air (Barngrover *et al.* 1985; Leibovitz 1963), whereas, DMEM, for instance, requires CO_2 in providing the physiological pH (Dontchos *et al.* 2008). Additionally, certain elements in the media may also enhance the survival of testis cells. Antioxidants in HTS-FRS such as Trolox and lactobionate may have protected testis cells from the free radical damage (McAnulty and Huang 1996; Ostrowska *et al.* 2009; Tanaka *et al.* 2006). Serofendic acid in FBS (Kume *et al.* 2006; Pennock *et al.* 2006) may also inhibit the hypothermia-induced cell injury and death due to apoptosis and necrosis. Given the equal efficiency of L15 and HTS-FRS in maintaining testis cells at refrigeration temperature, higher survival rates may be expected with L15 as a defined base media by supplementing it with protectants against hypothermia-induced damages. Comparable performance of L15, M199 and F12 media in maintaining testis cell viability and tissue integrity, further confirmed the importance of proper pH buffering systems in the media (Baicu and Taylor 2002; Bonventre and Cheung 1985; Bronk and Gores 1993; Fuller *et al.* 1988; Hochachka and Mommsen 1983; Lindell *et al.* 1998). However, the testis tissue/organ was best preserved in the specialized media (HTS-FRS), with a morphology not different from that in fresh control tissues (Fig. 6.1-6.5), implying that the preservation of tissue morphology requires much more than pH stability from the media. Inadequate oxygenation could cause diminishing ATP, followed by disintegration of cellular membrane and tissue injury (Belzer and Southard 1980; Brinkkoetter *et al.* 2008; Goujon *et al.* 2000; Southard *et al.* 1987), and it

was estimated that 5-10% of renal oxygen consumption had remained at 5 ℃ (Fuller and Lee 2007). Oxygenation has been reported capable of decreasing tissue damage while maintaining cell metabolism during prolonged organ hypothermic preservation (Berkowitz *et al.* 1976; Kuhn-Régnier *et al.* 2000; Manekeller *et al.* 2007; Minor and Kötting 2000; Minor *et al.* 2005; Okada *et al.* 1995). Compared with hypothermic preservation of testis cells, probably the immersion of testis tissue/organ in media severely deprived the supply of oxygen to the residing cells (especially those within the tissue core). Although necrosis has been observed in primary culture of the testis tissue with plasma clot (Steinberger and Steinberger 1970), development of the testis tissue has been reported in organ culture at the interface between air and media (Oatley *et al.* 2004b; Roulet *et al.* 2006; Steinberger and Steinberger 1970), highlighted the importance of oxygen and nutrients for survival of the testis tissue/organ. Nevertheless, short term ice-cold storage of the testis tissue has been suggested to improve donor-derived spermatogenesis after xenografting (Jahnukainen *et al.* 2007a; Zeng *et al.* 2009); therefore, the *in vivo* spermatogenesis potential of hypothermically-preserved testis tissue (even with decreased cell viability) may not be completely impaired and the tissue is likely to rebound upon transplantation into a suitable environment.

The proportion of gonocytes did not change following preservation of testis cells at refrigeration temperature, whereas, that of Sertoli cells increased and peritubular myoid cells decreased (Fig. 5.5). The increased proportion of Sertoli cells could be explained by their tolerance of damage induced by hypothermic conditions (Young *et al.* 1988), and the high apoptotic rate of peritubular myoid cells under hypothermic temperatures may have caused their declining number (Virtanen *et al.* 1986; Yiu *et al.*

2007). Interestingly, the proportion of gonocytes after 6 days of testis tissue preservation also did not change (Fig. 6.5), but the survival rate of testis cells dropped by a minimum of 74% (Fig. 6.1); therefore, it appears that the hypothermic conditions were not appropriate for testis cells within the tissue to survive, regardless of cell types. Retrograde persufflation of testis with Perfluorocarbons (PFCs, with extremely high oxygen solubility) may help oxygenate cells within the tissue and enhance the survival of testis cells (Berkowitz *et al.* 1976; Kuhn-Régnier *et al.* 2000; Minor and Kötting 2000; Okada *et al.* 1995). Alternatively, disassociated testis cells, rather than testis tissue, could be preserved at refrigeration temperature.

9.1.4 Gonocyte Transplantation using Recipient Testis Irradiation

It is generally agreed that elimination of endogenous spermatogenesis can improve donor-derived spermatogenesis, after germ cell transplantation (Honaramooz and Yang, 2011). As shown in Chapter 8, complete eradication of the endogenous spermatogenesis was observed at least up to four months after fractionated gamma-ray irradiation of neonatal piglet testes using dose as low as 1 Gy daily for three consecutive days (Fig. 8.6). Initiation of full spermatogenesis was observed following gonocyte transplantation into the irradiated testes (Fig. 8.7).

Given the systematic toxicity of busulfan injection (Honaramooz *et al.* 2005; Ogawa *et al.* 1999a; Savchenkova *et al.* 2006; Shinohara *et al.* 2002a; Udagawa *et al.* 2001), we investigated testis irradiation for preparing pig recipients for gonocyte transplantation. Irradiation of testes at earlier development stages may be more efficient in eliminating potential endogenous spermatogenesis, and gonocytes were reported to be

more sensitive to irradiation damage than SSCs and spermatogonia in different species (Erickson 1964; Erickson *et al.* 1972; Forand *et al.* 2009a; Hughes 1962). Gonocytes resume active proliferation in the newborn testis (Erickson 1964; França *et al.* 2000), and therefore are most vulnerable to irradiation as they go through mitosis (Forand *et al.* 2004; Pawlik and Keyomarsi 2004; Sinclair and Morton 1966; Sinclair 1968; Sinclair and Morton 1963). At least part of the high efficiency of suppressing the endogenous spermatogenesis in our study could be due to the irradiation strategy of using fractionated, rather than single doses. Spermatogonia have been reported to be more susceptible to fractionated irradiation, and five-fold more stem spermatogonia (SSCs) were eliminated after using fractionated irradiation, compared with single dose radiation (De Ruiter Bootsma *et al.* 1977; Van der Meer *et al.* 1993; Van der Meer *et al.* 1992). It has been suggested that the surviving SSCs/gonocytes from one round of irradiation become more sensitive to the following rounds of radiation (Ash 1980; de Rooij *et al.* 2002; De Ruiter Bootsma *et al.* 1977; Speiser *et al.* 1973; Van der Meer *et al.* 1993; Van der Meer *et al.* 1992).

We observed spermatids at two months post-transplantation of donor gonocytes in the testes that received lower doses of irradiation (either 1 × 3 or 2 × 3 Gy) but not in those that received the high dose (3 × 3 Gy, Fig. 8.7). Testis growth was also severely affected with the high irradiation dose, with effects on the tubular density and diameter (Fig. 8.1 -8.4). It is likely that the testis intratubular microenvironment may have been too impaired to support spermatogenesis after the high dose, as suggested for rodents (Kangasniemi *et al.* 1996; Meistrich and Shetty 2003; Zhang *et al.* 2007).

Gonocytes with pseudopods have been observed in rats and suggested to be capable of initiating full spermatogenesis after homologous transplantation (Jiang and Short 1995; Jiang and Short 1998a; Orwig *et al.* 2002b; Ryu *et al.* 2003); however, such gonocytes were not identified in mice, and spermatogenesis was not initiated after gonocyte transplantation (McLean *et al.* 2003). In the present study (Chapter 8), piglet gonocytes with pseudopods were indeed observed both among the freshly isolated testis cells and during their culture (data not shown), and sub-populations of gonocytes could be recognized based on their DBA staining intensity (DBA-biding molecules) (Goel *et al.* 2007). Pseudopods are cellular processes directed toward and motivate the gonocyte migration to the basement membrane (McGuinness and Orth 1992a; Orwig *et al.* 2002b). In our study, it is not clear whether gonocytes possessing pseudopods had indeed better chances to survive, migrate and develop following transplantation. In fact, a sub-population of gonocytes was found able to directly initiate full spermatogenesis, while other gonocytes transformed into SSCs (Yoshida *et al.* 2006).

9.1.5 Establishing a System for Gonocyte Transplantation with the Pig as a Model

Even though the existence of SSCs has been speculated for many decades, it was only recently verified when the technique of SSC transplantation was developed. However, the efficiency of SSC transplantation needs to be improved before it can be widely used in practice. Additionally, specific identification of SSCs is very difficult if not impossible; however, gonocytes are the only germ cell type present in the neonatal testis and can be identified based on their *in situ* morphology. Transplantation of gonocytes was only investigated in a few studies in mice and rats, with conflicting results as to the potential of gonocytes in establishing spermatogenesis in recipient testes. It is

unclear whether gonocytes indeed have spermatogenic capability; therefore, using pigs as an important large/farm animal model, the present studies tested the capability of gonocytes in establishing spermatogenesis in recipient testes after transplantation. Evidence was presented in support of our hypothesis that gonocytes can generate spermatogenesis in recipient testes after transplantation. In the meantime, a functional system was established for the study and manipulation of gonocytes.

9.1.6 Applications

Gonocytes are the least investigated germline progenitor cells (Culty 2009), and their study can also shed light on PGCs and SSCs. During the last decade, investigation of SSCs made rapid progress, largely because of the availability of SSC transplantation. The present studies paved way for piglet gonocyte identification, isolation, short-term preservation, and transplantation. Therefore, these studies contributed to development of a key system for further analysis of gonocyte developmental potential (Oatley and Brinster 2008).

Another important application of gonocyte transplantation is its potential as a novel means for manipulation and propagatin of genetic modification in domestic animlas. Integration of exogenous genes into gonocytes *in vitro* prior to transplantation can result in transgene expression by the progeny. Genetically modified stem cells can produce virtually unlimited numbers of transgenic spermatozoa without losing their potential. This approach can be more efficient and less expensive than the conventional methods of farm animal transgenesis (Brinster 2002; Honaramooz *et al.* 2003b; Honaramooz *et al.* 2008; Kim *et al.* 2010; Nagano *et al.* 2002b).

In the neonatal testis, SSCs and/or differentiated germ cells do not exist, and gonocytes are the only available germ cell type. Therefore, preservation of gonocytes is the only option for preservation of the male germline from individual males of rare/endangered species that die shortly after birth. We have now shown that testis cells/tissue can be temporarily preserved under hypothermic conditions, which can then be used for restoration of the germline through gonocyte transplantation or tissue xenografting. Autologous gonocyte transplantation could also be applied in fertility preservation of prepubertal boys undergoing sterilizing cancer treatments (Geens *et al.* 2008).

9.2 Future Directions

9.2.1 Gonocyte Development

Do germline progenitor cells modify their migration behaviour during development? Is the modification of migratory patterns specific to the germ cell function/stage of development (PGCs, Gonocytes, SSCs)?

PGCs elongate and actively migrate through the embryo into the gonads (Anderson *et al.* 2000; Black and Erickson 1968; Pelliniemi 1974; Pelliniemi 1976), and gonocytes relocate from the central part of the seminiferous cords into the basement membrane. Although SSCs residing at the basement membrane show little mobility, they can also move onto the basement membrane after germ cell transplantation when they are deposited in the tubular lumen (Brinster and Avarbock 1994; Brinster and Zimmermann 1994; Chuma *et al.* 2005; Jiang and Short 1995; Jiang and Short 1998a; Ohta *et al.* 2004; Orwig *et al.* 2002b; Ryu *et al.* 2003). While some common surface adhesion substances may exist and mediate the migration, other surface molecules may

constitute the profile of the germ cells at specific development stages. PGCs, gonocytes and SSCs could be analyzed with newly developed lectin (glycan) microarrays for their identification and characterization during male germline development (Gupta *et al.* 2010; Katrl ƙ *et al.* 2010; Tateno *et al.* 2007). This assay has been successfully applied in characterizing ESCs (Muramatsu and Muramatsu 2009), and other cell types (He *et al.* 2010a; He *et al.* 2010b; Miyagawa *et al.* 2010; Song *et al.* 2009; Tao *et al.* 2008). It is possible that Sertoli cells may also produce chemokine substances actively guiding the migration of gonocytes into niches in the basement membrane of seminiferous cords, and facilitate the transformation of gonocytes into SSCs (Simon *et al.* 2010).

9.2.2 Gonocyte Sub-populations

Are gonocytes a homogenous/heterogeneous population? Why do some gonocytes have pseudopods but not others? Is there an overlap between PGCs, gonocytes and SSCs in potency, leftover from the previous progenitor cells? Are these cells essentially the same but in different stages or status?

Transition from PGCs to gonocytes, and from gonocytes to SSCs is mostly defined based on phenotypes, since no unequivocal functional distinctions have been identified to date. These germline progenitors were all observed to be capable of initiating full spermatogenesis after transplantation, with PGCs and SSCs giving rise to multi-potent stem cells. However, populations of cells within each cell type may have differential potential. PGCs contain sub-populations with ESCs-like cell potential (Labosky *et al.* 1994; Matsui and Tokitake 2009; Matsui *et al.* 1992; Morita-Fujimura *et al.* 2009; Pease and Williams 1990; Resnick *et al.* 1992; Shim *et al.* 2008; Smith *et al.* 1988; Stewart *et al.* 1994; Williams *et al.* 1988). Similarly, stem cell potential has been

attributed to certain sub-populations of SSCs (Conrad *et al.* 2008; Golestaneh *et al.* 2009; Guan *et al.* 2006; Huang *et al.* 2009; Kanatsu-Shinohara *et al.* 2004; Ko *et al.* 2009; Kossack *et al.* 2009; Mizrak *et al.* 2010; Nakagawa *et al.* 2010; Ning *et al.* 2010; Seandel *et al.* 2007). While some gonocytes can directly initiate spermatogenesis, gonocytes without pseudopods are not likely to generate spermatogenesis after transplantation in rat. Recently, a number of SSC identity and self-renewal genes were also found to be conserved in gonocytes (Wu *et al.* 2009a). It is quite likely that gonocytes are also heterogeneous and contain sub-populations with different potential.

Lectin DBA specifically identified piglet gonocytes in our studies, and pure populations of gonocytes may be prepared using negative selection in differential plating pre-coated with fibronectin and poly-D-lysine, followed by positive selection with DBA- or its analogues-coated plates. Alternatively, flow cytometry could be utilized in preparation and characterization of pure gonocyte sub-populations with co-staining of Leydig cells and/or labelling of gonocytes with far-red/near-infrared fluorophores to avoid the auto-fluorescence. The sorted gonocyte sub-populations may be examined in culture for colonization, expression of stem cell markers or their differentiation capability in different tissue reconstruction studies (co-culture with cells from the same or different tissues, e.g. testis cells from primates, cells from ovary and kidney). Similar investigations could also be performed *in vivo*, for example, to characterize subcutaneous re-construction of testis tissue in nude mice (in a controlled manner) to uncover the mechanisms regulating testis morphogenesis, and spermatogenesis by germ and somatic cells from different species/sex or cell types (e.g. macrophages (Bukovsky *et al.* 1995; Chiquoine 1954; Dirami *et al.* 1999; Yao *et al.* 2006)).

9.2.3 'Stemness' of Neonatal Gonocytes

In long-term culture of SSCs *in vitro*, colonies with morphologies similar to ESCs start to appear, leading to derivation of pluripotent stem cells in neonatal and adult mice (Guan *et al.* 2006; Huang *et al.* 2009; Kanatsu-Shinohara *et al.* 2004; Ko *et al.* 2009; Seandel *et al.* 2007), and adult humans (Conrad *et al.* 2008; Golestaneh *et al.* 2009; Kossack *et al.* 2009; Mizrak *et al.* 2010). LIF has been used to obtain and maintain the ESC-like cells from cultured mouse PGCs (Pease and Williams 1990; Smith *et al.* 1988; Williams *et al.* 1988), or with a combination of other growth factors (Labosky *et al.* 1994; Matsui *et al.* 1992; Resnick *et al.* 1992; Shim *et al.* 2008; Stewart *et al.* 1994). The addition of basic fibroblast growth factor (bFGF) in culture media also stimulated the transformation of PGCs into embryonic germ cells (EGCs) (Kawase *et al.* 1994; Matsui *et al.* 1992; Resnick *et al.* 1992), which share many morphological, cell phenotypical and pluripotent characteristics with ESCs (De Felici *et al.* 2009; Laible and Alonso-González 2009; Shamblott *et al.* 1998; Solter and Knowles 1978; Stewart *et al.* 1994).

Currently, pluripotency has not been reported/demonstrated for gonocytes in any species; however, multiple pluripotency markers were observed on some gonocyte sub-populations, and teratomas were formed after subcutaneous transplantation of the cultured piglet gonocytes (Goel *et al.* 2009; Hoei-Hansen *et al.* 2005; Niu and Liang 2008; Tu *et al.* 2007).

9.2.4 Gonocyte Self-Renewal with Seminiferous Cord Expansion and Vasculature Development

PDGF was shown to promote gonocyte proliferation (Li *et al.* 1997; Wang and Culty 2007), and transplanted spermatogonia tend to relocate into areas of seminiferous

tubules close to vasculature (Yoshida *et al.* 2007). There might be positive correlations between gonocyte proliferation, seminiferous cord elongation and vasculature development within the neonatal testis. Gonocytes were reported to develop specialized cytoplasmic processes and form colonies in culture mimicking their *in vivo* behaviour in mice and rats (Hasthorpe *et al.* 1999; McGuinness and Orth 1992a; Orth and Boehm 1990; Orth and Jester Jr 1995; Orth *et al.* 2000; Orth and McGuinness 1991; Orth *et al.* 1998). Glial cell line derived neurotrophic factor (GDNF) was identified as an essential SSC regulating factor that effectively promoted SSC proliferation but not differentiation in mice, rats and bulls (Hobbs *et al.* 2010; Kubota *et al.* 2004b; Oatley and Brinster 2008). However, the potential action of GDNF on gonocytes is unknown. Gonocyte purification usually results in low total cell recovery rates, whereas *in vitro* transduction requires large numbers of gonocytes, and the number of transplanted gonocytes can be proportional to the extent of colonization in recipients. Therefore, propagation of gonocytes in culture is expected to be an area of interest in the future. In addition to PDGF and GDNF, the effects of estrogen and progesterone could be investigated for potential roles in regulating gonocyte proliferation and development (Kohler *et al.* 2007; Kubota *et al.* 2004b; Oatley and Brinster 2008; Thuillier *et al.* 2010).

9.2.5 Gonocyte-Mediated Transgenesis

One of the most important applications of germ cell transplantation is to introduce genes of interest into the germline stem cells, with permanent integration. Gonocytes could be transfected prior to transplantation to initiate spermatogenesis in recipients and express the transgene. Labelling gonocytes by inserting marker/functional genes allows the efficient identification of donor gonocytes and facilitate the investigation of

mechanisms regulating gonocyte development in recipients. Despite failure of *in vivo* electroporation in transfecting germ cells in mice (Yamazaki *et al.* 1998), electroporation of bovine testis tissue resulted in stable *in vitro* transfection of SSCs (Oatley *et al.* 2004a). Successful gene transfer to SSCs has also been achieved using lentiviral and recombinant adeno-associated viral (rAAV) vectors (Hamra *et al.* 2002; Honaramooz *et al.* 2008; Honaramooz *et al.* 2003c; Nagano *et al.* 2000a; Nagano *et al.* 2002b; Orwig *et al.* 2002a; Ryu *et al.* 2006). While viral vectors are clearly more efficient in transferring genes into target cells than non-viral systems, they usually can only carry small foreign genes and have high bio-safety risks. Piglet gonocytes were reported to result in ~11% transduction efficiency with lentiviral vectors, and colonization in mouse testes after transplantation (Kim *et al.* 2010), demonstrating that gonocytes can be transducted without sacrificing the development potential. Further investigations on gonocyte transduction with non-viral methods, improving the efficiency of viral vector transduction, and production of transgenic animals after transplantation of genetically modified gonocytes are anticipated.

9.2.6 Gonocyte Transplantation Efficiency

The establishment of spermatogenesis in recipient testes after germ cell transplantation is a systematic process, where several steps including preparation of donor germline stem cells and recipients could potentially affect the success rate. It was reported that full spermatogenesis was predominantly initiated by gonocytes with pseudopods, suggesting that not all rat gonocytes were capable of generating spermatogenesis after transplantation (Orwig *et al.* 2002b). Cytoplasmic processes were also observed on piglet gonocyte populations, showing different DBA staining intensity.

Sub-populations of gonocytes were also reported to directly initiate full spermatogenesis, whereas other gonocytes transformed into SSCs before being able to start spermatogenesis (Yoshida *et al.* 2006). In either case, the spermatogenic efficiency of transplanted gonocytes needs to be improved from what we observed in the present study (Chapter 8). The putative sub-populations of gonocytes that directly generate full spermatogenesis may be identified, studied and eventually purified, which may improve the spermatogenic development efficiency following transplantation. Additionally, freshly isolated gonocytes may be cultured *in vitro* for a few days before transplantation, not only to increase the number of gonocytes, but also to potentially enhance the migration and colonization capability of resultant gonocytes, although some will likely transform into SSCs. Improved donor-derived spermatogenesis has been observed after xenografting of short-term preserved testis tissues (Jahnukainen *et al.* 2007a; Zeng *et al.* 2009); however, it is unknown whether donor gonocyte-derived spermatogenesis can also be enhanced with short-term hypothermic preservation before transplantation. Furthermore, growth/pseudopod-inducing factors may be transplanted along with donor cells to promote the migration of gonocytes into the basement membrane, because gonocytes were reported to undergo apoptosis unless they relocated into the basement membrane of the cord (Coucouvanis *et al.* 1993; Orwig *et al.* 2002b).

Fractionated irradiation of piglet testes in our study (Chapter 8) completely eliminated the endogenous spermatogenesis with dose as low as 1 Gy (daily for three consecutive days); however, further investigation with lower dose will be beneficial. The less damage is inflicted on the intratubular microenvironment, the better are chances of gonocytes initiating spermatogenesis. However, specialized facility and expertise are

required for irradiation, and as an alternative strategy especially for field applications, busulfan injection might be easier. To avoid systemic toxicity, we propose to use local injection of busulfan into the testis via the internal testicular artery.

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