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Spermatogonia Stem Cell Dynamics Following Hormonal Induction, Ischemic Disturbance *In Vivo* or Proliferation under *In Vitro* Culture in Pre- and Postpubertal Bulls

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To the Graduate Council:

I am submitting herewith a dissertation written by Gustavo M. Schuenemann entitled "Spermatogonia Stem Cell Dynamics Following Hormonal Induction, Ischemic Disturbance *In Vivo* or Proliferation under *In Vitro* Culture in Pre- and Postpubertal Bulls." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Animal Science.

F. Neal Schrick, Major Professor

We have read this dissertation and recommend its acceptance:

Stephen P. Oliver, J. Lannett Edwards, Tulio M. Prado, Charmindrani Mendis-Handagama

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

To the Graduate Council:

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Accepted for the Council:

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Carolyn R. Hodges

Vice Provost and
Dean of Graduate School

(Original signatures are on file with official student records.)

Spermatogonia Stem Cell Dynamics Following Hormonal Induction, Ischemic Disturbance *In Vivo* or Proliferation under *In Vitro* Culture in Pre- and Postpubertal Bulls

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Gustavo Martin Schuenemann

August 2008

DEDICATION

This dissertation is dedicated to my wife Paula, my daughter Victoria and son Federico, and my parents Carlos and Mirta Schuenemann. From all of you, I draw strength from your love, encouragement, and patience. Paula, Vicky, and Fede are the light of my life. My parents, thanks for your unwavering support no matter what endeavors I embark into.

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First, I would like to thank my advisor, Professor Dr. F. Neal Schrick, for giving me the wonderful opportunity to further my graduate education at the University of Tennessee. He has supported me trough this journey with patience, encouragement, advice, and understanding. This journey has opened new doors for my career.

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ABSTRACT

The overall aim of the studies described herein was to evaluate bovine spermatogonial cell dynamics under various conditions. Results from these experiments will provide the basis for potential production of offspring following spermatogonial stem cell transfer. *Experiment 1* evaluated gonadotropin administration effects at initiation of inhibin passive immunization in Jersey bull calves on testicular morphology and development. Primary treatments consisted of control (KLH) or immunization (INH) plus a combination of saline, FSH, or GnRH. Administration of FSH at the time of initial immunization against inhibin significantly increased number of germ cells (92.2 ± 9 x 10^6 cells) compared to INH-Saline bulls (54.9 ± 10 x 10^6 cells) with INH-GnRH bulls being intermediate (64.5 ± 9 x 10^6 cells; $P < 0.05$). These results suggested that gonadotropin administration at time of inhibin immunization increases number of germ cells in the testis. *Experiment 2* evaluated transiently induced ischemia in testes of Jersey calves on morphology and development. Treatments consisted of control or banding for 2 h, 4 h, and 8 h periods. Transiently induced ischemia significantly decreased number of germ cells in 8 h (12.6 ± 5 x 10^6 cells) compared to 0 (38.1 ± 6 x 10^6 cells), 2 (31.9 ± 6 x 10^6 cells), and 4 h (33.4 ± 5 x 10^6 cells; $P < 0.05$). These results suggested that transiently-induced ischemia significantly decreases number of germ, Sertoli and Leydig cells in the testis. *Experiment 3* evaluated spermatogonial stem cells (SSC) proliferation, isolated from prepubertal and adult bulls, during short term *in vitro* culture. Spermatogonia were cultured in the presence or absence of a feeder monolayer (FL or NF), FBS type (FBS-S or FBS-SF), and media type (ELSC or RSC) treatment combinations. Viable type A

spermatogonia survived under *in vitro* conditions and were able to proliferate and form different types of colonies. Furthermore, co-culture spermatogonial cells with a feeder monolayer plus FBS-S enhanced colony number (may be due to increasing cell viability). At 15 days of culture, colonies from both types of bulls were positive to AP. Therefore, these finding provide the basis for potential production of offspring through *in vitro* genetic manipulation such as intracytoplasmic sperm injection (ICSI), round spermatid injection (ROSI), or following SSC transfer.

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CHAPTER 1 - INTRODUCTION

Spermatogonia stem cells (SSC) are specialized pluripotent cells that have unique capabilities including self-renewal and production of the end product, spermatozoa (de Rooij 2001). The ability to recover these cells from testes of donor animals, perform *in vitro* culture and modification, and transfer these cells to a host testis may provide a valuable tool to transfer economically favorable genes in livestock. Successful spermatogonial transplantation from donor to recipient animals is also a promising technique to preserve genetic material of valuable or endangered males. In the cattle industry, germ cell transfer could be used as an alternative to artificial insemination (AI) in tropical environments, where *Bos indicus* bulls disseminate *Bos taurus* genes.

Male germ cell transplantation in livestock has progressed tremendously in the past few years (Honaramooz *et al.* 2003a, 2003b; Izadyar *et al.* 2003; Joerg *et al.* 2003; Hill *et al.* 2005). However, the efficiency of colonization in seminiferous tubules by transplanted germ cells is variable (Dobrinski *et al.* 2000; Nogano, *et al.* 2002; Izadyar *et al.* 2003; Joerg *et al.* 2003; Hill *et al.* 2005). Depletion of endogenous spermatogonial stem cells in recipient animals using busulfan (Brinster *et al*. 2003) or irradiation (Izadyar *et al*. 2003) have both been used in preparation of recipient animals prior to transplantation. However, both techniques are not without compromises (severe bone marrow depression or specialized radiotherapy equipment required).

Before donor SSC can be transferred to a host testis, these cells must be isolated and maintained *in vitro* with high viability. The ability to culture these cells *in vitro* has the potential to investigate aspects concerning spermatogonial survival and differentiation in long-term cultures (reviewed by Sofikitis *et al*. 2005). The ideal *in vitro* system, one that supports self-renewal or the complete process of spermatogenesis from a population

of SSC, is the goal of several scientists (reviewed by Sofikitis *et al*. 2005). Furthermore, culture systems have been proposed to facilitate propagation of spermatogonia (Izadyar *et al*. 2003a; 2003b), which can then be transferred into recipient testes (Izadyar *et al*. 2003b; Herried *et al*. 2006), genetically modified (Nagano *et al*. 2001), or cryopreserved for future interventions (Oatley *et al*. 2004). However, offspring were not always obtained which may be related to issues with culture systems utilized.

The clinical application of culturing SSC is of great importance in livestock, wildlife, and humans. Animal models may provide new knowledge for therapeutic management in patients with oncological and infertility diseases. Preserving SSC have two advantages (proliferation by mitosis and differentiation to haploid cells by meiosis) as opposed to cryopreservation of spermatozoa (haploid cells cannot undergo mitosis). Therefore, large amounts of genetic material could be preserved through culture of SSC.

Overall aims of studies presented herein focussed on the necessity to find practical protocols for: 1) stimulating transplanted germ cells to re-colonize recipient testis through an appropriate testicular environment, 2) depleting endogenous germ cells in recipient animals before transplant, and 3) harvesting and culturing viable SSC. Results from these experiments will provide the basis for potential production of offspring following spermatogonial stem cell transfer.

CHAPTER 2 - LITERATURE REVIEW

1. Development and function of the testis

Early development of the pituitary and the male reproductive tract is a highly coordinated series of events that ends in formation of complete accessory glands and organs (Putney *et al.* 1984; Gorski 1985; reviewed by Ford and D'Occhio 1989). The male reproductive tract consists of two testes, two epididymides (each with its ducts deferens), accessory glands, and penis (Byskov and Hoyer 1994). The male reproductive system has two major functions: 1) exocrine: production of spermatozoa through the process of gametogenesis and fluid secretions from accessory glands, and 2) endocrine: secretion of hormones such as testosterone and inhibin (reviewed by Parker *et al.* 1999). The testicular parenchyma consists of seminiferous tubules compartment (Seminiferous epithelium: Sertoli cells, primordial and developing germ cells and peritubular cells) and interstitial compartment (Leydig cells, immune cells (i.e. macrophages), mesenchimal cells, nerves, blood and lymphatic vessels, and the extracellular matrix; Curtis and Amann 1981; Russell *et al.* 1990; Ariyaratne and Mendis-Handagama 2000). The epididymis provides the environment for final maturation of spermatozoa and serves as a storage organ for these cells (Amann and Almquist 1962; Igboeli and Foote 1968; reviewed by Varner and Johnson 2007). Accessory sex glands produce seminal plasma, and the penis is the copulatory organ (reviewed by Amann *et al.* 1993; Henault *et al.* 1995).

1.1. Fetal and prepubertal testis

Sex determination of offspring occurs at fertilization when the oocyte is penetrated by a sperm delivering either X (female) or Y (male) chromosome (Welshons and Russell 1959; George and Wilson 1994; reviewed by Parker *et al.* 1999). Sexual differentiation occurs during the first trimester of gestation in cattle (George and Wilson 1994; reviewed by Dohle *et al.* 2003). A gene complex located on the short arm of the Y chromosome triggers sexual change in the gonads which then causes differentiation in the fetus (Welshons and Russell 1959; reviewed by Dohle *et al.* 2003). In early embryo development, testes are formed from primordial (primitive) germ cells, which migrate from the yolk sac to the genital ridge of the mesonephros, where they form the primary epithelial cords in association with somatic cells from the genital ridge (George and Wilson 1994; Albrecht and Eicher 2001). Male and female embryos have two sets of paired ducts during this undifferentiated stage: 1) the Müllerian (paramesonephric) ducts and 2) the Wolffian (mesonephric) ducts (reviewed by Jost *et al*. 1970; George and Wilson 1994). Even though the mesonephric and paramesonephric ducts are present, the embryo is in a sexually indifferent stage (reviewed by Jost *et al*. 1970; George and Wilson 1994). Histologically, both the ovaries and testes cell lineages are indifferent in the embryo; thus, termed as bipotential cell precursors (reviewed by Jost *et al*. 1970; Albrecht and Eicher 2001).

In mammals, XY embryos develop testes under the influence of the testis determining gene SRY (sex-determining region), which is present on the Y chromosome (Sinclair *et al.* 1990; George and Wilson 1994; Albrecht and Eicher 2001). On the other

hand, XX embryos develop ovaries in the absence of the Y chromosome (George and Wilson 1994). The sex-determining region (SRY) regulates other genes involved in sex determination (Payen *et al.* 1996). Gonadal precursors present at genital ridges have the potential to further differentiate into testes or ovaries (George and Wilson 1994; Albrecht and Eicher 2001). Common precursors are composed of four bipotential cell lineages that form the gonad: germ cells, connective tissue cells, steroid-producing cells, and supporting cells (Albrecht and Eicher 2001).

The Y chromosome in males influences production of specific hormones during early testes development that lead to male sexual differentiation (George and Wilson 1994; reviewed by Parker *et al.* 1999). Critical hormonal mediators of male sexual differentiation are testosterone (produced by Leydig cells in the interstitial region) and anti-Müllerian hormone (AMH; also called Müllerian-inhibiting substance) produced by Sertoli cells within testicular cords (George and Wilson 1994; reviewed by Parker *et al.* 1999). Testosterone induces Wolffian ducts to differentiate into seminal vesicles, epididymis, vas deferens and ejaculatory ducts (George and Wilson 1994; Drews 2000); whereas, Wolffian ducts regress in the absence of testosterone (George and Wilson 1994; reviewed by Parker *et al.* 1999). On the other hand, regression of Müllerian ducts is exerted by AMH (George and Wilson 1994; reviewed by Parker *et al.* 1999). In the absence of AMH, Müllerian ducts will persist and form the female reproductive tract consisting of oviducts, uterus and upper vagina (George and Wilson 1994; reviewed by Parker *et al.* 1999).

After sex determination in mammals, the testes organize into two different compartments: 1) the testicular cords which are the precursor of the seminiferous tubules,

and 2) the interstitial tissue space (George and Wilson 1994; reviewed by Parker *et al.* 1999). From outside the urogenital ridge originates Sertoli and primordial germ cell precursors which later are present within testicular cords (George and Wilson 1994; reviewed by Parker *et al.* 1999). Located in the interstitial region outside testicular cords are Leydig cells with steroidogenic activity, peritubular myoid cells, and endothelial cell precursors (de Krester and Kerr 1994; reviewed by Parker *et al.* 1999).

The testes first become distinct histologically when the testis cords are formed, which are precursors to seminiferous tubules (George and Wilson 1994; reviewed by Wilhelm *et al.* 2007). In humans, this process begins by approximately the 7^{th} wk of gestation (George and Wilson 1994; Drews 2000). In mice, testis cords are first apparent at embryonic day 12.5 (George and Wilson 1994; Drews 2000). In cattle, formation of the testes is distinct by day 45 of gestation and steroidogenesis activity initiated (Shemesh *et al*. 1978; reviewed by Ford and D'Occhio 1989). In sheep and swine, testicular function and morphology are initiated at 30 days of gestation (reviewed by Ford and D'Occhio 1989). Organization of cords is initiated by pre-Sertoli cells, which express SRY at approximately 6 wk of gestation in humans (George and Wilson 1994). Following onset of SRY expression, these cells undergo a rapid wave of proliferation and exhibit changes in gene expression that denote the onset of the testis pathway (Capel *et al*. 1999; reviewed by Wilhelm *et al.* 2007). Over the next several days, Sertoli cells lay down on the basal membrane that delineates the cords, which contain Sertoli cells and spermatogonia (Yu *et al*. 1998; reviewed by Wilhelm *et al.* 2007). These spermatogonia are germ cells arrested in G1 of the mitotic cycle, presumably due to indirect inhibitory effects of Sertoli cells (reviewed by Wilhelm *et al.* 2007).

Seminiferous cords contain precursors of Sertoli cells of mesenchymal origin (reviewed by Anniballo *et al.* 2000). Primordial germ cells (PGC) of yolk sac mesodermal origin, which will develop later into gonocytes, originate in a distant region from seminiferous cords (reviewed by Anniballo *et al.* 2000). They emerge from the yolk sac and migrate to the final location where seminiferous tubules are forming (McKay *et al.* 1953; reviewed by Parker *et al.* 1999). When embryonic gonocytes are migrating from the yolk sac to their final destination, they are dependent on other cells for a continuous supply of nutrients (Zamboni and Merchant, 1973; reviewed by Parker *et al.* 1999). When PGCs reach seminiferous cords, Sertoli cell precursors maintain them throughout their lifespan (Jost *et al.* 1974; reviewed by Parker *et al.* 1999).

1.2. Sertoli cell

Sertoli cells were first described by Enrico Sertoli (Sertoli 1865) and are the somatic components within the seminiferous epithelium that support developing germ cells throughout spermatogenesis (Wong and Russell, 1983; reviewed by Petersen and Söder 2006). During development of the fetal testis, Sertoli cell defines the initial stage of development in the forming gonad (George and Wilson 1994; reviewed by Petersen and Söder 2006). They express the SRY gene, thus determining sex of the gonad (reviewed by Petersen and Söder 2006). Sertoli cells are the source of anti-Müllerian hormone, responsible for suppressing development of the female reproductive tract (George and Wilson 1994; reviewed by Petersen and Söder 2006). These cells plus peritubular cells are essential during formation of testis cords (Griswold 1998; McLaren 2000). During

testis development, morphological and biochemical differences exist between immature and mature Sertoli cells, indicating that Sertoli cells have a broad spectrum of functions that are of great importance in the physiology of spermatogenesis (Sinowatz and Amselgruber 1986; reviewed by Petersen and Söder 2006). Immature Sertoli cells experience morphological differentiation to mature Sertoli cells during the first 28 wk of proliferative development in postnatal bulls (Sinowatz and Amselgruber 1986). In the bovine, a functional blood-testis barrier (BTB) can be expected at 24 wk of testis development (Sinowatz and Amselgruber 1986). The basal lamina is well developed during early postnatal life which correlates with high secretory activity by the rough endoplasmic reticulum and Golgi apparatus (Sinowatz and Amselgruber 1986; reviewed by Petersen and Söder 2006). Additionally, tight junctions formed by Sertoli cells create the BTB that divide the spermatic epithelium into basal and adluminal compartments (Amann 1983; de Krester and Kerr 1994). Tight junctions seal intercellular space and contribute to the permeability barrier across an epithelium or endothelium (Diamond 1977; Powell 1981). Furthermore, these tight junctions are considered as a major site and platform for vesicle trafficking and signal transduction (Lui *et al.* 2003).

During testicular cord formation, immature Sertoli cells divide constantly; however, this proliferative activity declines in association with onset of puberty (Steinberger and Steinberger 1971). Immediately before puberty, seminiferous fluid is produced by the Sertoli cell transforming the solid testis cords into seminiferous tubules with lumen (reviewed by Petersen and Söder 2006). Mature Sertoli cells produce transferrin (Skinner and Griswold 1980), plasminogen, (Lacroix *et al.* 1977; Skinner and Griswold, 1980) and the inflammatory cytokine IL-1α (Syed *et al.* 1988; Jonsson *et al.*

1999; Sultana *et al.* 2000; Wahab-Wahlgren *et al.* 2000) among other proteins (Table 1). The differentiation process from an immature to a mature Sertoli cell indicates the importance of the functional regulation of mature Sertoli cells during spermatogenesis (reviewed by Petersen and Söder 2006). In fact, the stem cell factor (SCF) produced by Sertoli cells in the tubule directly interact through the c-kit receptor expressed on developing spermatogonia (reviewed by Petersen and Söder 2006). Additionally, functional Sertoli cells have specific FSH receptors and will absorb testosterone secreted by Leydig cells (Silva *et al.* 2002). Androgen-binding protein (ABP), which binds to testosterone and dihydrotestosterone (DHT), are also a product of Sertoli cells (Skinner and Fritz 1985). Sertoli cells also produce inhibin that controls FSH secretion by the pituitary gland (Amann 1983).

Table 1: Summary of proteins expressed by immature and mature Sertoli cells (from Petersen and Söder 2006)*

Protein	Immature cell (prior to puberty)	Mature cell (from puberty onwards)
Cytokeratin	+	-
Transferrin	-	+
Interleukin 1-α	-	+
Androgen receptor	+	+
Anti-Müllerian hormone	+	-
Aromatase	+	-
Inhibin β B-subunit	+	-

*For additional information see (Andersson *et al.* 1998; Sharpe *et al.* 2003)

Sertoli cells play a key role in spermatogenesis, providing a unique environment for germ cell development into functioning sperm (Amann 1983; de Krester and Kerr 1994). Sertoli cells are considered "nurse cells" for this reason and are the only cells inside seminiferous tubules derived from somatic cells of the gonadal ridge (Waites *et al.* 1985; Griswold 1995). Over the course of development, postnatal Sertoli cells inside the testis cord undergo morphological and metabolic changes (Amann 1983; Brehm and Steger 2005). At 4 wk of age, the calf testis cord consists of undifferentiated Sertoli cells and some centrally prepubertal spermatogonia (Sinowatz and Amselgruber 1986). The nuclei of Sertoli cells at this time are round or oval (Sinowatz and Amselgruber 1986). At 8 wk of life, seminiferous tubules have increased in diameter to about 70 μm due to mitotic activity of Sertoli cells (Sinowatz and Amselgruber 1986). A well-developed Golgi apparatus is present in many Sertoli cells at this time, which indicates high secretory activity (Sinowatz and Amselgruber 1986). The Sertoli cell has a more centrally located nucleolus and its ultrastructure has revealed it rests on the basal lamina; this is the identical placement for mature Sertoli cells in functioning testes (Sinowatz and Amselgruber 1986).

The tubular diameter increases rapidly from 8 (60-80 μm) to 20 wk (100-140 μm) of age in bulls due in part to mitotic division of Sertoli and spermatogonia cells (Sinowatz and Amselgruber 1986; Wrobel 2000). Seminiferous tubules are in close proximity with each other, exhibit a reduction in interstitum space, and tubules switch from solid to the presence of a small lumen (Sinowatz and Amselgruber 1986). During this time, Sertoli cells send out lateral processes containing rough endoplasmic reticulum

(RER) that surround spermatogonial cells in close proximity (Sinowatz and Amselgruber 1986).

At 24-30 wk of age, tubules continue to increase in size to 170 μm in diameter and primary spermatocytes in different stages of meiosis can be present (Sinowatz and Amselgruber 1986). At 40 wk of age, which is the approximate age of puberty in the bull, the average seminiferous tubule diameter has reached 190-200 μm (Amann 1983; Curtis and Amann 1981; Sinowatz and Amselgruber 1986). For the most part, Sertoli cells have become differentiated, well developed, and positioned in the basal part of the tubular epithelium with a nucleus of irregular shape (Sinowatz and Amselgruber 1986; Wrobel 2000). At this point in development, Sertoli cells have formed the blood testis barrier, which divide the spermatic ephitelium into the basal compartment containing spermatogonia and adluminal compartment with more advanced stages of spermatogenesis (Sinowatz and Amselgruber 1986). Mitochondria are located in the basal region of the Sertoli cell as well as both smooth endoplasmic reticulum (SER) and RER located in the cytoplasm and in the basal lateral processes of the Sertoli cell (Sinowatz and Amselgruber 1986).

Testicular size is regularly used as an indicator of Sertoli cell number and spermatogenesis (reviewed by Petersen and Söder 2006). Furthermore, daily sperm production (DSP) in bulls correlates with Sertoli cell number (Berndtson *et al.* 1987a; 1987b). Additionally, DSP and testis weight in grams correlates positively to total Sertoli cells (Curtis and Amann 1981; Berndtson *et al.* 1987a). Therefore, factors that modify Sertoli cell number may influence potential sperm output (Curtis and Amann 1981; Berndtson *et al.* 1987a).

1.1. Sertoli-germ cell interactions

During spermatogenesis, the spermatogonia translocation is facilitated by Sertoli cells, which participate in movement of developing germ cells from the basal to the adluminal compartment (de Krester and Kerr 1994; reviewed by Mruck and Cheng 2004). Therefore, there is a close interaction between Sertoli cells and developing germ cells throughout the spermatogenesis process (Curtis and Amann 1981, Rusell *et al.*, 1990; reviewed by Mruk and Cheng 2004). The translocation movement of developing germ cells may be associated to Rho GTPase family members (Lau and Mruk 2003; Lui *et al.* 2003). Furthermore, translocation of elongating spermatids across the seminiferous epithelium is conferred by ectoplasmic specialization, a modified adherens junction type (de Kretser 1990; Vogl *et al.* 2000), microtubules, several motor proteins such as ATPases, GTPases (Lui *et al.* 2002), dynein (Gutmann *et al.* 2000), and myosin VIIa (Velichkova *et al.* 2002) present in the testis seminiferous epithelium. These ectoplasmic specializations allow translocation of spermatids and spermiation process, along with tight and desmosome-like junctions between Sertoli and germ cells (de Kretser and Kerr 1994; reviewed by Vogl *et al.* 2000; reviewed by Mruck and Cheng 2004). Spermatic epithelium formed by Sertoli cells, developing germ cells, and extracellular matrix connections inside the tubuli constantly change during spermatogenesis (de Krester and Kerr 1994; reviewed by Mruk and Cheng 2004). However, the precise mechanisms that govern ectoplasmic specializations between Sertoli-germ cells interactions still need to be elucidated (reviewed by Mruk and Cheng 2004).

Sertoli cells have a fine developed cytoskeleton that supports the progressive spermatic epithelium during spermatogenesis (reviewed by Volg *et al.* 2000; reviewed by Mruk and Cheng 2004). Translocation of developing germ cells from the basal to the adluminal compartment of the seminiferous epithelium is assisted by Sertoli cells during spermatogenesis (reviewed by Mruk and Cheng 2004). Additionally, Sertoli cells actively participate in spermiation (release of mature spermatids to the lumen of the seminiferous epithelium) and phagocytosis of degenerated developing germ cells (reviewed by Mruk and Cheng 2004; Nakagawa *et al.* 2005). Sertoli cells secrete a variety of compounds such as proteases, proteases inhibitors, hormones, energy substrates, growth factors, and extracellular matrix components (Bardin *et al*. 1994; reviewed by Mruk and Cheng 2004), with autocrine and paracrine effects within the seminiferous epithelium (Table 2).

However, unanswered questions remain regarding the signal(s) that dictates the precise movement of spermatocytes across the spermatic epithelium during spermatogenesis (reviewed by Mruk and Cheng 2004). What is the mechanism(s) that triggers signaling to start selecting germ cells from the basement membrane and enter into epithelial cycle? How can some committed germ cells progress to spermatids while others remain undifferentiated at the basal compartment of the seminiferous epithelium? What is the signal(s) that triggers germ cells to be committed and start differentiating to become a specialized cell "spermatozoa"?

Nutrients for spermatogonial, spermatocytes, and spermatids need to be synthesized in sufficient quantities and efficiently delivered by Sertoli cells (Bardin *et al.* 1994; reviewed by Mruk and Cheng 2004). Additionally, a mature Sertoli cell has an

Table 2. Compounds secreted by Sertoli cells to the surrounding environment and their functions (from Bardin *et al*. 1994; reviewed by Mruk and Cheng 2004).

Compounds secreted by Sertoli cells	Compounds	Functions
Proteases	Cathepsin D, L, and S	Tissue maintenance, repair, and growth, germ cell movement, and development
	Plasminogen activator (PA)	
Proteases inhibitors	Cystatin C, Cathepsin L	germ cell movement
	α_2-macroglobulin	
	Urokinase-type Plasminogen activator (u-PA)	
Growth, autocrine, and paracrine factors	Transforming growth factor-α (TGF-α)	Proliferation of prepubertal Sertoli cells
	TGF-β1, β-2, and -β3	Cell division and prevent apoptosis
	Stem cell factor (SCF)	Junction dynamic
	Tumor necrosis factor-α (TNF-α)	
	Sertolin	
Extracellular matrix components	Type IV collagen	Sertoli cell tight junctions integrity Structural integrity
	Heparan sulfate Proteoglycans	
	Entactin	Blood-testis barriers integrity
	Collagen	
	Laminin	
Hormones	Inhibin	Negative feedback on FSH
	Dihydrotestosterone	
	Estradiol	Enhance germ cell survival
	Androgen Binding Protein	
Energy	Lactate	Enhance germ cell survival
	Pyruvate	
	Carbohydrate	
	Amino acids	
	Lipids	
Others	Vitamins	Enhance germ cell survival
	Metal ions (transferrin)	

extensive surface area in proximate contact with approximate 50 developing germ cells in mammals (Russell *et al.* 1990). These cellular structures give an idea of the enormous amount of energy needed during germ cell development and progression through the spermatogenesis cycle.

Sertoli cells support germ cells throughout development as evident by supplying energy substrates such as glucose, lactate, and pyruvate (Setchell 1993; reviewed by Griswold 1995; Biellia 1997). Furthermore, it was suggested that germ cells use lactate as the primary source for energy production (Jutte *et al.* 1983; reviewed by Mruk and Cheng 2004). Interestingly, Sertoli cells have aromatase and 5-α reductase enzymes responsible for conversion of testosterone secreted from Leydig cells to 17β-estradiol and dihydrotestosterone (reviewed by Silva *et al.* 2002; reviewed by Petersen and Söder 2006).

1.4. Leydig cell

Originally discovered by Franz Leydig (1850), Leydig cells are found adjacent to seminiferous tubules in the testis. Differentiation of Leydig cells is a multi-step process that can be divided in fetal and postnatal development (reviewed by Mendis-Handagama and Ariyaratne 2001). In the fetal testis, it is proposed that these somatic cells originate from undifferentiated mesenchymal cells in the mesonephros (reviewed by Byskov 1986). Postnatal differentiation of Leydig cells is crucial in male mammals to establish adult Leydig cell populations and development of male characteristics (reviewed by Dohle *et al*. 2003). Leydig cell proliferation is divided into several steps, from

mesenchymal cells (precursor cells) to mature Leydig cells (Figure 1; reviewed by Mendis-Handagama and Ariyaratne 2001, Ge *et al.* 2006).

During gestation, precursors of Leydig cells differentiate and develop to adult Leydig cells in the postnatal testis (reviewed by Byskov 1986; Mendis-Handagama *et al*. 1987; reviewed by Mendis-Handagama and Ariyaratne 2001). Mesenchymal cells are spindle-like shape, contain little cytoplasm, absent of steroidogenic activity, and lack LH receptors (Hardy *et al.* 1990; reviewed by Mendis-Handagama and Ariyaratne 2001; Teerds *et al.* 2007). Progenitor cells aquire steroidogenic enzymes, 3β-hydroxysteroid dehydrogenase (3β-HSD), 5-α reductase, cytochrome P450 cholesterol side-chain cleavage ($P450_{scc}$), and the P450 17α-hydroxylase/C17-20 lyase ($P450_{c17}$) needed for androgen production (Hardy *et al.* 1990; Haider and Servos 1998; Ariyaratne *et al.* 2000; Ge *et al*. 2005; Teerds *et al.* 2007). At the same time, LH receptors become functional

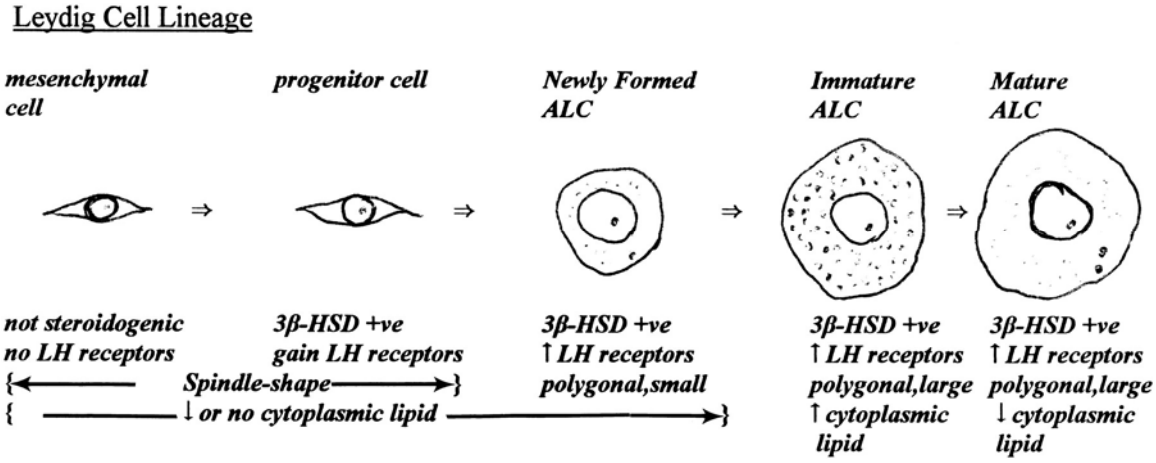

Figure 1. Proliferation and differentiation of Leydig cell lineage (Extracted from Mendis-Handagama and Ariyaratne 2001).

in these type of cells (Teerds *et al.* 2007). The final step in Leydig cell differentiation is disappearance of cytoplasmic lipids and a significant increase in average cell size (Mendis-Handagama *et al*. 1987; Ariyatatne *et al*. 2000).

Fetal testes cells produce two major hormones: anti-müellerian hormone (AMH) and testosterone (de Kretser and Kerr 1994; reviewed by Dohle *et al.* 2003). Secretion of AMH causes regression of Müellerian ducts; thus, regressing the female reproductive tract (George and Wilson 1994). On the other hand, testosterone enhances the development of Wolffian ducts, which results in formation of the epididymis, vas deferens, and seminal vesicles (George and Wilson 1994). Additionally, testosterone can be converted by 5-α reductase to dihydrotestosterone (DHT), a more potent metabolite needed during spermatogenesis (Singh *et al*. 1996; reviewed by Dohle *et al*. 2003). Beside testosterone, Leydig cells produce 17β-estradiol (E_2), dihydroepiandrosterone (DHEA), and androstenedione (Eckstein *et al*. 1987; reviewed by Payne and Youngblood 1995).

Leydig cells are found in the testicular interstitial space in proximity of seminiferous tubules (Ariyaratne and Mendis-Handagama 2000). Development of organelle components and LH receptors increases steroidogenic activity in adult Leydig cells; thus, testosterone biosynthesis (Eckstein *et al*. 1987; Hardy *et al.* 1989; Shan and Hardy 1992; Teerds *et al.* 2007). The increase of testosterone production leads to onset of puberty (Amann 1983; reviewed by Mendis-Handagama and Ariyaratne 2001).

1.5. Structure and components of the testis at puberty

Puberty in male is generally defined as the age at which reproductive function, hormone levels, and presence of motile sperm are found in mammals in order to reproduce (Amann 1983). In terms of sperm output, puberty is defined as the age at which a bull is first able to produce an ejaculate containing 50 million sperm with a minimum of 10% motility (Wolf *et al.* 1965; Lunstra *et al.* 1978; Amann 1983). Testicular cell development, hormones (LH and testosterone), and sperm production of bulls at puberty is related to age, body weight (nutrition), breed, and testes weight (Lunstra *et al.* 1978; Curtis and Amann 1981; Pruitt and Corah 1985; Evans *et al.* 1995). The first sexual interest is exhibited approximately 3 wk prior to puberty and reach mating ability about 6 wk after puberty in bulls (Lunstra *et al.* 1978; Hamilton 2006).

An increase in gonadotropin releasing hormone (GnRH) secretion is associated with the early rise in gonadotropins secretion (FSH and LH) in bull calves (Evans *et al*. 1995). Additionally, the early rise in LH and FSH secretion is followed by proliferation and differentiation of Leydig and Sertoli cells in prepubertal calves (Curtis and Amann 1981; Amann and Walker 1983; Amann *et al*. 1986; Evans *et al.* 1995). Furthermore, secretion of LH between 10 and 20 wk of age has been shown to stimulate development of testicular components in prepubertal bulls (Curtis and Amann 1981; Amann and Walker 1983; Evans *et al.* 1995).

Based on histological evaluations, testis weight increases 10 fold and seminiferous tubules double their presence in the parenchyma from 12 to 32 wk of age in bulls (Curtis and Amann 1981). Sertoli cells differentiation starts at 20 wk and formation

of adult Sertoli cells was completed near 28 wk of age (Curtis and Amann 1981). Additionally, gonocytes are present at 12 wk and were replaced by prespermatogonia and A-spermatogonia at 20 wk of age, indicating a transition period when meiosis has begun (Curtis and Amann 1981). Furthermore, preleptotene to diplotene spermatocytes appear for the first time at 16 wk and their number increase through 32 wk of age when spermatids appear (Curtis and Amann 1981).

A complete blood testis barrier appears only at puberty in which the primary function must be to allow for conditions favorable for meiosis and to allow secretion of fluid that transports spermatozoa out of seminiferous tubules (Setchell *et al*. 1969; Setchell 1980). Appearance of the tubule lumen indicates fluid secretion and completion of the blood testis barrier between 24 and 28 wk of age in bulls (Setchell 1980; Curtis and Amann 1981). Therefore, transformation from prepubertal to pubertal testis containing Sertoli cells, developing spermatogia, and spermatozoa occurred at approximately 16 wk in bulls (Curtis and Amann 1981). Functional normalization of the spermatic epithelium (Sertoli and germ cells) is a prolonged process that attains efficient sperm production approximately 6 mo after initiation of spermatogenesis (Amann 1983).

Anatomically, the testicular parenchyma at puberty consists of: 1) seminiferous tubules compartment composed of Sertoli cells, developing germ cells, and peritubular cells; and 2) interstitial compartment composed of Leydig cells, immune cells (i.e. macrophages), mesenchymal cells, nerves, blood and lymphatic vessels, and connective tissue (Curtis and Amann 1981; Russell *et al.* 1990; Mullins and Saacke 2003). The mediastinum is the central connective tissue core of the testis that houses ducts called rete testis that connect seminiferous tubules and epididymal duct through the efferent ducts

(Mullins and Saacke 2003). These interconnected channels allow spermatozoa and fluid originating in the spermatic epithelium to move freely out of the testis (Mullins and Saacke 2003). Functions of the epididymis are to provide the environment for final maturation of spermatozoa resulting in acquisition of motility and potential fertility, and serve as the storage reservoir of spermatozoa (Amann 1987; reviewed by Amann *et al.* 1993).

1.6. Process of spermatogenesis

Spermatogenesis is a progressive and complex multi-day process in mammals (Curtis and Amann 1981, Rusell *et al.* 1990). The Sertoli cell aids in developing germ cells from A spermatogonia to functioning sperm, a continuous process in the adult male (Amann, 1983; Rusell *et al.* 1990). Spermatogonial multiplication (spermatocytogenesis), meiosis (genetic material exchange and reduction division producing haploid spermatids), and spermiogenesis (differentiation process) are the three primary phases in spermatogenesis that occur inside the seminiferous tubule (Curtis and Amann 1981; Amann 1983; Russell *et al.* 1990; Johnson *et al.* 1997).

To study molecular aspects of type A_S spermatogonia, stem cells of spermatogenesis (Huckins 1971; de Rooij and Grootegoed 1998), in an active spermatic epithelium is difficult because the complexity of the seminiferous tubules (reviewed by de Rooij 2001). Purification of type A_S spermatogonia cells is difficult in adult males; thus, prepubertal bulls (Izadyar *et al*. 2003a; Herrid *et al*. 2006), pigs (Luo *et al*. 2006), goats (Honaramooz *et al.* 2003a), or transgenic mice (Brinster and Zimmermann 1994)

are used in an attempt to isolate type A_S spermatogonia cells. It is difficult to make comparison between studies because the pattern of type A_S spermatogonia cells development is species-specific. For instance, spermatogenesis start immediately after birth in mice and is completed at 20 days of age (Baker and O'Shaughnessy 2001) while in bulls is delayed several months (Wrobel 2000; Aponte *et al*. 2005). Because is difficult to stablish the exact moment when germ stem cells start differentiating *in vivo*, the most effective way to isolate pure populations of spermatogonial stem cells is to collect all forms of type A spermatogonia (A_s, A_{pr}, A_{al}, and A_1-A_4) from prepubertal males.

To understand the process of spermatogenesis, the arrangement of developing type A spermatogonial cells (including stem cell, Type A-single; reviewed by de Rooij 2001) and spermatocytes inside the seminiferous tubules needs to be described. Primordial germ cells from an early stage of seminiferous tubules formation differentiate and become gonocytes until birth when they differentiate to form type A_S spermatogonia (reviewed by Parks *et al.* 2003). In non-primate mammals, A-single (A_S) spermatogonia is the stem cell that undergo mitosis to ensure the continuous supply of stem cells throughout spermatogenesis (Huckins 1971; de Rooij and Grootegoed 1998). A-paired (A_{pr}) spermatogonia divides into daughter cells and remain connected by an intercellular bridge that further develop into chains of four A-aligned (A_{al}) spermatogonia (Figure 2, Russell *et al.* 1990; reviewed by de Rooij 2001). The chains of A_{al} spermatogonia can

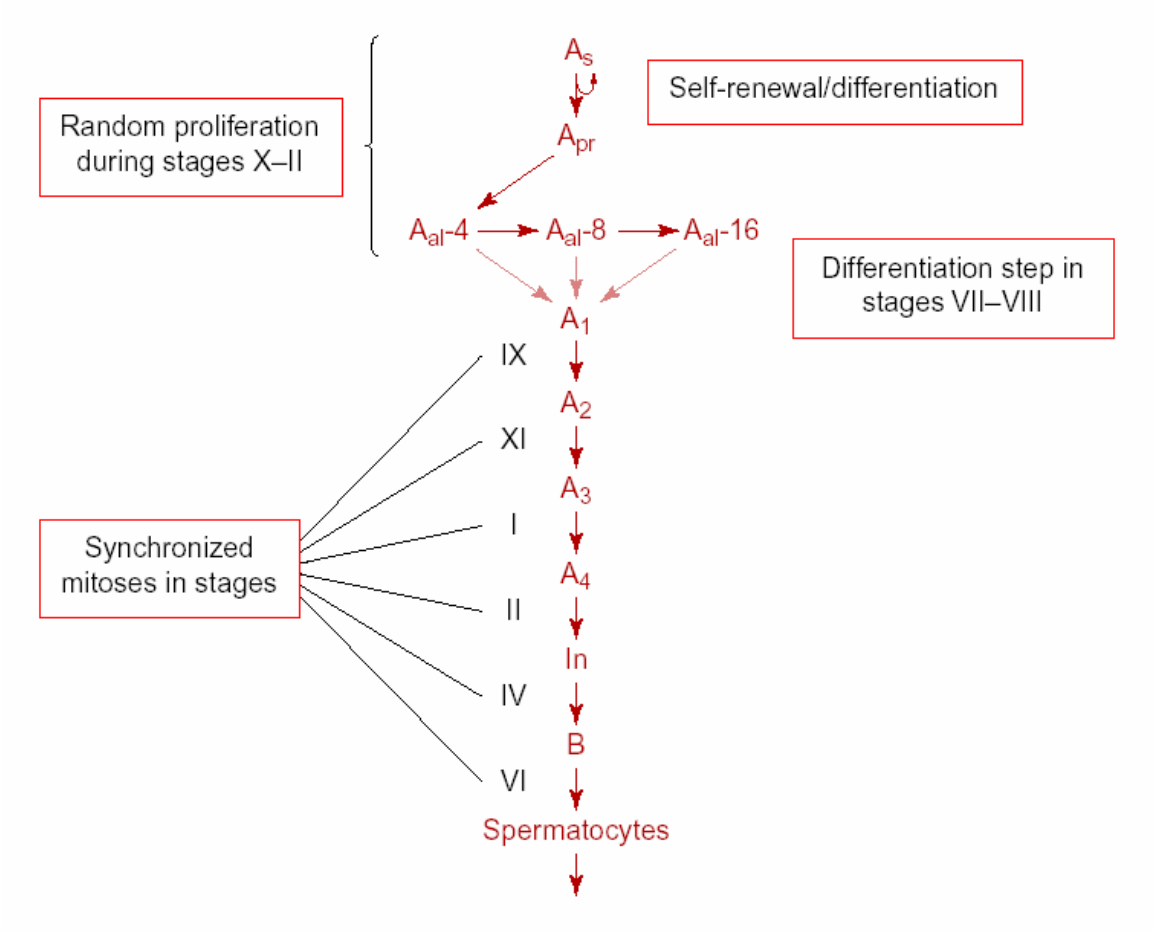

Figure 2. Sequence of spermatogenesis in mice. Spermatogonia (A1-A4, In and B) undergo a series of mitotic divisions before they enter meiosis. This series of mitotic divisions allows for continuous proliferation and replacement of spermatogonia stem cells (Extracted from de Rooij 2001).

divide further into chains of 8 and 16 cells (Figure 3). The spermatogenic cycle can be divided into 12 stages (I–XII) based on germ cell development (Curtis and Amann 1981). In the different stages of spermatogenesis, the A_s, A_{pr}, and A_{al} spermatogonia progress and differentiate into A1, A2, A3, A4, In, B spermatogonia, and primary spermatocytes (Curtis and Amann 1981; reviewed by de Rooij 2001). The A1, A2, and A3 cell types have unique nuclei stereological characteristic from small-flattened nuclei to larger nuclei that contain only one large nucleolus or multiple large fragments of nucleoli (Curtis and Amann 1981; Russell *et al.* 1990; Johnson *et al.* 1997). The In and B spermatogonia have large oval to spherical nuclei that contain large chromatin flakes (Curtis and Amann 1981; Russell *et al.* 1990).

At the end of the proliferation phase, primary spermatocytes enter into the meiotic phase and give rise to secondary spermatocytes (Russell *et al*. 1990; Johnson *et al*. 1997). During this phase, genetic diversity is guaranteed by DNA recombination and four unique haploid spermatids are produced (Russell *et al*. 1990). The final phase of spermatogenesis is the differentiation phase, commonly referred to as spermiogenesis (Johnson *et al*. 1997). During this phase, the spherical spermatid undergo a remarkable morphological transformation that results in a fully differentiated highly specialized spermatozoon containing a head (nuclear material and acrosome), mid piece (mitochondrial helix), and tail (Russell *et al.* 1990; Johnson *et al*. 1997).

Finally, spermiation is the process where spermatids are released as spermatozoa into the lumen of the seminiferous tubule (Johnson *et al.* 1997). The complete process of spermatogenesis from A_s-spermatogonia to the formation of fully differentiated spermatozoa takes 61 d in bulls (Amann 1983). In the bull, the spermatogenic cycle lasts

13.5 days, which originate from spermatogonia that differentiate 4.5 cycles earlier for a total of 61 d on average (Amann 1983; de Kretser and Kerr 1994). Sertoli cell plasticity supports and maintains the spermatogenesis process; thus, these cells are correlated positively to daily sperm production in bulls (Berndtson *et al.* 1987a; 1987b). Additionally, the process of spermatogenesis at puberty is associated with endocrine changes such as hypothalamic discharge of gonadotropin (GnRH), discharge of LH and FSH by the pituitary gland, and testosterone secretion by Leydig cells in response to LH secretion (Amann 1983; Amann and Walker 1983; Amann *et al*. 1986).

2. Endocrine function associated with testicular function

2.1. Gonadotropin-releasing hormone and gonadotropins

Testicular development depends greatly upon maturation of the hypothalamus-pituitary-testis axis. Gonadotropin-releasing hormone (GnRH) secreted by the hypothalamus and gonadotropins (FSH and LH) secreted by the anterior pituitary in response to GnRH dictate a unique pattern of testicular development and function before and after puberty in bulls.

2.1.1. Gonadotropin-releasing hormone

One of the most important hormones to achieve proper testicular development is GnRH, a neuropeptide that originates in the hypothalamus (Silverman *et al.* 1994). The hypothalamo-hypophyseal-portal system allows minute quantities of GnRH to cause a release primarily of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary gland (Amann 1983; Sharpe 1994). Before puberty in the male, GnRH neurons in the tonic center of the hypothalamus release low amplitude and low frequency pulses of GnRH (Foster 1994). The onset of puberty is due to a decreased hypothalamic sensitivity to the negative feedback to testosterone and estradiol (Amann 1983; Amann *et al*. 1986). Therefore, more pituitary LH is secreted; thus, Leydig cells became more responsive and produce more testosterone until puberty is reached (Amann and Walker 1983; Amann *et al.* 1986).

2.1.2. Follicle-stimulating hormone

Follicle-stimulating hormone (FSH), a glycoprotein produced and secreted by the anterior pituitary, is the major endocrine hormone known to regulate Sertoli cell function (reviewed by Silva *et al.* 2002; reviewed by Petersen and Söder 2006). In prenatal and newborn animals, FSH plays an important role in controlling Sertoli cell proliferation (reviewed by Griswold 1998; reviewed by Silva *et al.* 2002). After birth (from 4 to 32 wk), FSH remains relatively low (Amann and Walker 1983); then, a peak in FSH

concentration occurs that is believed to be related to cell proliferation and differentiation in the bull testis (Evans *et al.* 1993). After puberty, FSH regulates steroidogenesis (conversion of testosterone to estradiol by Sertoli cells) and gametogenesis (Sharpe 1994; reviewed by Silva *et al*. 2002).

Sertoli cells respond through its receptor (G protein-coupled receptor) to FSH and stimulates production of inhibin, androgen receptor (AR), androgen binding protein, and transferrin (Verhoeven and Cailleau 1988). Continuous availability of FSH is essential during spermatogenesis (Amann 1983). It has been suggested that FSH and testosterone play an active role initiating and maintaining spermatogenesis (Sharpe 1994). The spermatogenic epithelium also depends on discharges of LH by the pituitary gland that stimulate Leydig cells to produce testosterone; thus, establishing a negative feedback on production of GnRH, FSH, and LH at the hypothalamus and pituitary gland (Amann 1983; Sharpe 1994).

2.1.3. Luteinizing hormone

Luteinizing hormone (LH), a glycoprotein secreted by the anterior lobe of the pituitary in response to GnRH (Sharpe 1994), is the major hormone known to regulate steroidogenic function of Leydig cells (Hardy *et al.* 1989; Shan and Hardy 1992; reviewed by Mendis-Handagama and Ariyaratne 2001). In the bull, apparent circadian rhythm does not exist for the release of LH; however, it has been reported that episodic release does occur (Schanbacher and Echternkamp 1978; Amann 1983; Amann and

Walker 1983). Following release of LH, blood concentrations of testosterone increase through the steroidogenic response of Leydig cells (Schanbacher and Echternkamp 1978; Amann *et al.* 1986; Evans *et al.* 1993; Jimenez-Severiano *et al.* 2005).

In postnatal bull calves, an early (between 6 and 24 wk of age) but transient increase in LH concentration occurs (Amann 1983; Evans *et al.* 1995; 1996; Rawlings and Evans 1995; Chandolia *et al.* 1997; Aravindakshan *et al.* 2000). The early increase in frequency of GnRH pulses during this prepubertal period may be associated with pulsatile discharges of LH, characteristic of the early prepubertal phase in bulls (Amann and Walker 1983; Evans *et al*. 1996; 1993). An early increase of LH occurs parallel to testicular growth and establishment of spermatogenesis (Amann 1983; Evans *et al.* 1996; Aravindakshan *et al.* 2000). The peripubertal period, from 25 wk of age until puberty, is characterized by rapid testicular growth and high levels of testosterone in bull calves (Lunstra *et al*. 1978; Evans *et al.* 1993; 1995). However, blood levels of testosterone increase to an amount that is capable of suppressing LH pulses immediately before puberty (Amann 1983; Coulter 1986; MacDonald *et al.* 1990; Evans *et al.* 1993), suggesting gonadotropin-independent mechanisms may contribute in regulating testicular development in bulls (Barth and Ominski 2000; Brito *et al.* 2007).

2.2. Inhibin

In the male, inhibin is produced by Sertoli cells (Cuevas *et al*. 1987; de Krester *et al*. 2000; Kaneko *et al.* 2003) under the control of FSH by cyclic adenosine 3’ and 5’-

monophosphate pathways (de Kretser and Robertson 1989). Inhibin, a glycoprotein, is composed of α- and β-subunits (α-β_A is inhibin-A or α-β_B is inhibin-B) in the bovine (Sugino *et al*. 1992). Inhibin plays a physiological role on FSH secretion from infancy to puberty in bulls (Kaneko *et al*. 2001). Moreover, it was suggested that inhibin may have an inhibitory effect on spermatogenesis (Hakovirta *et al.* 1993; Kaneko *et al*. 2003). In general, the primary role of inhibin is an endocrine negative feedback hormone on FSH secretion at the pituitary gland (Franchimont *et al.* 1979; Ying 1987; Bame *et al.* 1999; Kaneko *et al.* 2001; Kaneko *et al.* 2003). Moreover, this negative feedback is disrupted in castrated bulls that exhibited low concentrations of inhibin and high concentrations of FSH (MacDonald *et al*. 1991). Additionally, an autocrine and paracrine role for inhibin in regulation of testis function has been suggested, reversing the inhibitory action of activin on testosterone production by Leydig cells in culture (Lin *et al.* 1989). It has been shown that inhibin-A and -B are found in the Sertoli cells of the prepubertal and postpubertal bulls and production of inhibin decreases as a bull ages (Kaneko *et al.* 2003).

Administration of inhibin antiserum leads to an increase in serum levels of FSH without altering LH concentration in bulls (Kaneko *et al.* 2001). In males, mechanisms leading to sexual maturation may be related to a shift in the sensivity of the FSH-inhibin feedback system (Massicotte *et al*. 1984). It has also been suggested that inhibin may play a role in steroidogenesis through specific control of FSH and LH secretion at the pituitary gland (Massicotte *et al*. 1984; de Kretser and Robertson, 1989). Moreover, immunization against inhibin in bull calves increased FSH levels and subsequent sperm production (Martin *et al.* 1991; Bame *et al.* 1996; Bame *et al.* 1999).

2.3. Testosterone

Testosterone, a steroid derived from cholesterol, is the major male hormone produced by interstitial Leydig cells in the testis in response to LH (Schanbacher and Echternkamp 1978; Amann 1983; Amann and Walker 1983; reviewed in Payne and Youngblood 1995). Biosynthesis of testosterone requires four enzymes: cholesterol side-chain cleavage enzyme (P450scc), 3 β-hydroxysteroid dehydrogenase/delta 5-Δ 4 isomerase (3-β HSD), 17 α- hydroxylase/C17-20 lyase ($P450_{17\text{-}\alpha}$), and 17-ketosteroid reductase (reviewed by Payne and Youngblood 1995; reviewed by Payne and Hales 2004). Promotion and maintenance of spermatogenesis, anabolic-like growth, negative feedback at the hypothalamus and pituitary level on gonadotropins secretion, and stimulatory effects on accessory sex glands have been suggested as the primary actions of testosterone in bulls (Lunstra *et al*. 1978; Rawlings *et al.* 1978; Amann 1983; Amann *et al.* 1986; reviewed by Silva *et al.* 2002; Bagu *et al*. 2006).

Serum concentrations of testosterone are low in the newborn bull and begin to increase to adult levels at approximately 15 to 20 wk of age (Lunstra *et al.* 1978; Rawlings *et al.* 1978; McCarthy *et al.* 1979; Evans *et al.* 1993; Evans *et al.* 1995). Testosterone and dihydrotestosterone (DHT) are required for germ cell development during spermiogenesis through the androgen receptor (AR) expressed on Sertoli cells (Kerr *et al.* 1993; Bremner *et al.* 1994; O'Donnell *et al.* 1994; McLachlan *et al.* 1996). Spermatogenesis depends upon the action of both FSH and testosterone (reviewed by Silva *et al.* 2002). After synthesis by Leydig cells, testosterone is delivered to Sertoli

cells and binds to androgen-binding protein (ABP); thus, maintaining a high concentration inside the seminiferous tubules (reviewed by Silva *et al.* 2002).

2.4. Prolactin

Prolactin (PRL), a protein hormone secreted by the anterior pituitary gland, is considered to be the principal lactogenic hormone (Denamur 1971; reviewed by Ostrom 1990). The actions of PRL are mediated through a single-pass transmembrane receptor (Boutin *et al.* 1988). Prolaction concentrations in serum are correlated with the length of the photoperiod in bull calves (Bourne and Tucker 1975; Mayers and Swanson 1983). PRL regulates the population of testicular LH receptors in rodents, stimulates steroidogenesis in Leydig cells (Takase *et al.* 1990; Chandrashekar *et al.* 1994), and stimulates release of gonadotropins in rodents and rams (reviewed by Bartke 1978; Lincoln *et al.* 1996). Additionally, PRL acts as a pro-gonadal hormone that promotes testicular development and function in conjunction with LH and FSH (Barkey *et al*. 1987; Hair *et al.* 2002; Cavaco *et al.* 2003). Furthermore, PRL-receptors are expressed in Leydig cells, developing germ cells within the testis, and reproductive accessory glands (vas deferens, epididymis, prostate, and seminal vesicle) in rams and humans (Lincoln *et al.* 1996; Jabbour and Lincoln 1999; Hair *et al.* 2002). These findings demonstrate that PRL plays multiple roles on reproductive function of the male through acting on Leydig cells, developing spermatic epithelium, reproductive accessory glands, and as a pro-gonadal gonadotropin like LH and FSH.

2.5. Estradiol

The primary sites of production of estradiol-17β (estradiol) in the testis are Leydig cells (Purvis *et al.* 1981; reviewed by Payne and Youngblood 1995; Sneddon *et al.* 2005) and Sertoli cells (Dorrington and Armstrong 1975; Fujisawa 2001). Testosterone is metabolized to estrogens by aromatase cytochrome $P450_{scc}$ complex (Simpson and Davis 2001) and to dihydrotestosterone (DHT) by 5α-reductase (Viger and Robaire 1995). At the level of the hypothalamus and pituitary, both testosterone and estradiol exert a negative feedback on secretion of GnRH, LH, and FSH (Amann 1983; Amann *et al*. 1986; reviewed Payne and Youngblood 1995). In prepubertal bulls, serum concentrations of estradiol are low; however, after 40 wk of age significant increases in estradiol concentrations were observed in bulls (Evans *et al*. 1993). It was postulated that between 6 and 10 wk of age in bulls, removal of an estradiol-mediated block of GnRH occurs as well as an increase in pituitary GnRH receptors (Amann *et al.* 1986); thus, stimulating early pulses of LH in prepubertal bulls. Additionally, evidence exists that estradiol may modulate Sertoli cell function through its receptor and may support the process of spermatogenesis (reviewed by Hess 2003; Sneddon *et al.* 2005). On the other hand, chronic treatment with estradiol disrupted the spermatogenesis process in bulls (Schanbacher *et al.* 1982).

2.5. Thyroid hormones

The hormones thyroxine (T4) and triiodothroxine (T3) are thyroxine-based hormones produced by the thyroid gland. Thyroid hormones influence steroidogenesis, Leydig cell differentiation, and spermatogenesis in the testis in rodents and small ruminats (Jana and Bhattacharya 1994; Ariyaratne *et al*. 2000; Kim *et al.* 2002; reviewed by Silva *et al.* 2002; reviewed by Todini 2007). Testicular targets for thyroid hormones are Sertoli and Leydig cells (Van Haaster *et al.* 1993), inducing proliferation, differentiation, and lumen formation around puberty (Van Haaster *et al.* 1993; Mendis-Handagama *et al.* 1998; Manna *et al.* 1999; Ariyaratne *et al.* 2000; Silva *et al.* 2002; reviewed Mendis-Handagama and Ariyaratne 2004). In rams, testicular size and secretion of gonadotropins are decreased when the thyroid gland is removed (Parkinson *et al*. 1995). Additionally, thyroid hormones increase glucose transport, production of γ-glutamyl transpeptidase, androgen binding protein (ABP), insulin-like growth factor-I, inhibit aromatase activity, and affect testosterone metabolism in Sertoli cells (Jannini *et al.* 1995; reviewed by Silva *et al*. 2002). In Leydig cells, thyroid hormones directly stimulate expression of LH receptors and steroidogenic enzymes involved in cholesterol transport (Manna *et al.* 2001; reviewed by Mendis-Handagama and Ariyaratne 2004).

2.6. Additional hormones and factors regulating testicular function

Proliferation and differentiation of type A_S spermatogonia are tightly regulated by cellular components at testicular level (see Sertoli and Leydig cells) and endocrine-autocrine mechanisms (see hormones associated to testicular development and function) in vivo. Isolation and culture of male germ stem cells allow characterization of molecular factors associated to proliferation and differentiation of type A_S spermatogonia (reviewed by Parks *et al*. 2003). For instance, Sertoli cells secret stem cell factor, which through c-kit receptor present on type A spermatogonial cells (presumably all type A spermatogonia) support proliferation and differentiation (Dirami *et al*. 1999). It was suggested that the process of self-renewal and differentiation in spermatogonial cells may be regulated by glial cell line-derived neurotrophic factor (GDNF) secreted by Sertoli cells (Meng *et al.* 2000). Additionally, retinoic acid and vitamin A (van Pelt and de Rooij 1990a; van Pelt and de Rooij 1990b), stem cell factor (SCF; de Rooij *et al.* 1999) and cycling D_2 (Beumer *et al.* 2000) have been involved in preventing apoptosis and differentiation of stem cells into A1 spermatogonia in radent. Not only Sertoli cells play an important role in spermatogonial development, but also peritubular cells which secrete leukemia-inhibiting factor (LIF) essential for self-renewal and propagation of type A_S spermatogonial cells in rodent (Piquet-Pellorce *et al*. 2000; reviewed by Smith 2001; reviewed by Parks *et al*. 2003). Additionally, gowth factors added to the culture medium such as transforming growth factor-α and -β (TGF-α and TGF-β, Creemers *et al*. 2002;

reviewed by Parks *et al*. 2003), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), GDNF, and LIF were suggested to support *in vitro* expansion of mice spermatogonia cells (Creemers *et al*. 2002; Kanatsu-Shinohara *et al*. 2003).

In the mouse, adrenocorticotropic hormone (ACTH) can regulate testicular steroidogenesis during fetal development of the Leydig cell (O'Shaughnessy *et al.* 2003). Furthermore, endogenous metabolic hormones such as leptin, insulin, growth hormone (GH), and insulin growth factor-1 (IGF-I) are suggested to contribute directly in testicular development in prepubertal bulls (Brito *et al.* 2007). A recent study suggested that expression of insulin-like peptide 3 (INSL3) is associated with functional maturation of Leydig cells for androgen production (Mendis-Handagama *et al.* 2007).

3. Testicular germ cell transplantation

Male germ cell transplantation is a powerful approach to study spermatogenesis in a host testis or *in vitro*; and alternatively, to improve male fertility in livestock and humans by obtaining donor-derived spermatozoa capable of fertilizing an egg. Testicular germ cell transplantation is a technique where donor spermatogonial stem cells are transplanted into a recipient testis with the ultimate goal to colonize the tubuli, interact with endogenous environment cells, and re-initiate spermatogenesis.

3.1. Male germ cell transplantation in different species

3.1.1. Initial transplantation experiments

More than two decades ago, Brinster and Zimmerman (1994) reported their breakthrough findings in testicular tissue transplantation. In this study, spermatogonial cells isolated from two types of donor male mice (C57BL/6: color coat black and tan mutation and ZFlacZ: presence of the β-galactosidase transgene in donor cells) between postnatal days 4 and 12 colonized recipient seminiferous tubules (sterile mutant W/W and C57BL/6 x SJL mice) and restored spermatogenesis after transfer. Donor cells were directly microinjected immediately after collection into recipient seminiferous tubules using a glass pipette (1 mm outside diameter and 0.75 mm inside diameter with a sharp 40 μm tip) connected to a pressure injector. Since the process of spermatogenesis in mice is about 35 days, transplanted mice were maintained for 48 to 230 days before testes anlaysis. Spermatogenesis was observed, either presence of an active spermatic epithelium or seminifoerous tubules stained blue following treatment with 5-bromo-4-chloro-3-indole-β-D-galactosidase (X-Gal), in recipient mice. This methodology, spermatogonial stem cells transplant, has great potential in clinical applications into biomedical science and biotechnology. These remarkable findings demonstrated that spermatogonial stem cells interacted with the host environment, migrated from the adluminal compartment to the basement of the seminiferous tubule membrane through

Sertoli-Sertoli tight junctions (Griswold 2000), and re-initiated the spermatogenesis cycle (Brinster and Zimmermann 1994).

At the same time, Brinster and Avarbock (1994) reported successful spermatogenesis from mice donor germ cells transplanted into recipient mice. In this study, donor cells were harvested from embryonic day 18 to postnatal day 28 in an attempt to evaluate whether the age of donor affect colonization. Additionally, recipient preparation and transplant technique were similar as described previously (Brinster and Zimmerman 1994). Cells derived from mice at postnatal day 5 and 15 resulted in greater colonization of seminiferous tubules compared to perinatal cells. Donor-derived spermatozoa generated offspring confirmed by haplotyping resulting progeny (Brinster and Avarbock 1994). These finding was confirmed testes of the progeny of male recipient mice that stained blue after incubating testes with X-Gal, indicating that donor cells were able to re-generate spermatogenesis and transmit the transgene (lacZ) to the progeny.

Successful transplantation of mice testicular germ cells after cryopreservation for 5 mo into the donor mice testis was first described by Avarbock *et al.* (1996). In this study, spermatogonial cells from prepubertal or adult donor mice carring a lacZ transgene (as genetic marker) were harvested and frozen. After transplantation of thawed donor cells, different stages of spermatogonia development that stained blue after incubation with X-Gal were noted in recipient testes. Subsequently, transgenic lacZ rat donor germ cells transplanted into mouse testis (xenogeneic) were able to produce differentiated spermatozoa (Clouthier *et al.* 1996). Nagano *et al.* (1998) then successfully cultured transgenic lacZ mice spermatogonial stem cell *in vitro* for up to 4 mo, followed by reconstitution of spermatogenesis (spermatozoa stained blue after incubation with X-Gal)

after transplantation into host mice reciepient. In this experiment, trangenic lacZ donor germ cells from 10 d to 7 wk of age were cultured in 24 well plates (approximately 10^5 to 10^6 cells $well^{-1}$) in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) in presence or absence of a feeder layer (STO) at 32 °C with 5% CO_2 in air. Results from this study indicated that co-culture of donor germ cells, regardless of the age, on a feeder layer resulted in more viable cells and better colonization of seminiferous tubules after transplant. It is clear from this study that the presence of a feeder monolayer such as STO during culture is beneficial for spermatogonia stem cell survival and donor germ cell-derived spermatogenesis in the host testes after transplant.

3.1.2. Cross-species germ cell transfer

Spermatogonial stem cells from donor animals migrate to the basal compartment during the first month after transplantation and colonize the tubuli (Parreira *et al.* 1998; Nagano *et al.* 1999). Spermatogenesis appears to be more conserved and successful between the same species (allogeneic; may be due to species-specific factors such as immunoreaction) and disrupted when using cross-species transplantation (xenogeneic; differentiation and meiosis may not be supported; Hill and Dobrinski 2005). This incompatibility in cross-species could be overcome by co-transplantation of both germ and Sertoli cells from donor to recipient testis (Dobrinski *et al.* 2000; Honaramooz *et al.* 2002b; Izadyar *et al.* 2002b; Shinohara *et al.* 2003). Cross-species transplantation studies

have demonstrated donor spermatogonial survival in host testis (Schlatt *et al.* 1999a; Schlatt *et al.* 1999b; Nagano *et al.* 2001a). Furthermore, transplantation of donor germ cells between mice and rat testes (Clouthier *et al.* 1996; Ogawa *et al.* 1999b; Zhang *et al.* 2003) as well as from hamster into mouse testes (Ogawa *et al.* 1999a) have produced differentiated spermatozoa. Franca *et al.* (1998) suggested that control of the spermatogenesis process, in cross-species germ cell transplant, is primarily through spermatogonial cells rather than Sertoli cells. However, transplantation from non-rodent donors into rodent recipients resulted in seminiferous tubule colonization without complete spermatogenesis (Dobrinski *et al.* 1999; Dobrinski *et al.* 2000; Nagano *et al.* 2001a; Nagano *et al.* 2002).

Successful donor-derived spermatogenesis has been limited to species closely related. Spermatogonial transplatation between species with large phylogenetic distance may create incompatibilities between donor germ cell and the recipient testicular environment that prevent spermatogenesis. Therefore, transplantation from recipient to donor animals within the same species have shown the most promising success in term of donor cell colonization, differentiation, and completed spermatogenesis within the recipient testis environment.

3.1.3. Transplantation experiments in livestock animals

Spermatogonial cell transplantation techniques used in pigs and goats are now used in cattle (Table 3; Honaramooz *et al.* 2002b; Honaramooz *et al.* 2003a; Honaramooz *et al.* 2003b; Izadyar *et al.* 2003b; Jeong *et al.* 2003; Hill *et al.* 2005; Hill and Dobrinski

2005). A schematic summarizing male germ cell transfer approach in cattle is shown in Figure 3 (adapted from Hill and Dobrinski 2005). While allogeneic heterologous germ cell transplantation was successful in pigs and goats, autologous (Holstein-Friesian calves) transplantation of bovine spermatogonial stem cells resulted in a complete regeneration of spermatogenesis (Izadyar *et al.* 2003b).

Table 3. Sequential milestones in the development of mammalian male germ cell transplantation technique (from reviewed by Hill and Dobrinski 2005; reviewed by Sofikitis *et al*. 2005).

Reference	Report	Animal
Brinster and Zimmermann (1994)	First report of successful donor mouse spermatogenesis	Mouse
Brinster and Avarbock (1994)	Germ-line transmission of door mouse haplotype in recipient mice	Mouse and Mice
Jiang and Short (1995)	Rat male germ cell transplantation in recipient rats	Rat
Clouthier *et al.* (1996)	First report of xenogeneic germ cell transplantation from rat into mice	Rat
Avarbock *et al.* (1996)	Transplantation of frozen/thawed mouse spermatogonia into recipient mice	Mouse and Mice
Ogawa *et al.* (1997)	Detailed description of transplantation technique	
Tanaka *et al.* (1997)	Successful colonization and differentiation of hamster germ cell into xenogeneic testis	Hamster
Franca *et al.* (1998)	Control of spermatogenesis cell differentiation process in the rat	Rat
Nagano *et al.* (1998)	Long-term culture of mouse spermatogonial cells	Mouse
Ogawa *et al.* (1998)	Leuprolide enhances recipient tubuli colonization after spermatogonial transplantation	
Ogawa *et al.* (1999b)	Transplantation of donor germ cells from hamster to mouse testes	Hamster and Mouse
Schlatt *et al.* (1999a)	First successful attempt of germ cell transfer into a primate testis	Monkey
Dobrinski *et al.* (1999)	Transplantion of donr germ cells from rabbits and dogs into mice testes	Rabbit, Dog, Mouse
Nagano *et al.* (1999)	Description of colonization process into recipient mice testis	Mouse
Shinohara *et al.* (1999)	New markers for mice spermatogonial cells, β_1- and α_6-integrins	Mouse
Schlatt *et al.* (1999b)	Magnetic cell sorting for enriching viable spermatogonia from rodent and primate	Rodent and Monkey
Ohta *et al.* (2000)	Regulation of proliferation and differentiation of spermatogonial stem cells	
Reis *et al.* (2000)	Attempts to xenogeneic transplantation of human spermatogonia	Human and mouse
Nagano *et al.* (2001a)	Attempts to obtain spermatids after transplantation from a primate to mouse testes	Monkey and mouse
Honaramooz *et al.* (2002*a*)	First spermatogonial stem cell transplantation in a farm animal	Pig
Honaramooz *et al.* (2003*b*)	First report of donor cell-derived sperm production and transmission of the donor haplotype to the next generation	Goat
Izadyar *et al.* (2003*b*)	Successful autologous transplantation of bovine spermatogonial stem cells resulting in a complete regeneration of spermatogenesis	Bovine
Herrid *et al.* (2006)	Successful heterologous transplantation between *Bos taurus* and *Bos indicus* cattle	Bovine
Honaramooz *et al*. (2008)	Transmission of transgene through germ cell line in rodent and non-rodent species	Mice and Goat

Additionally, a recent successful allogeneic heterologous transplantation occurred between *Bos taurus* donor and *Bos indicus* recipient cattle (Herrid *et al.* 2006).

Although preliminary, these finding demonstrated that germ cell transplantation between different cattle breeds is possible. Although they have documented donor spermatzoa production (complete spermatogenesis) into the recipient testis, offspring derived from donor spermatozoa were not always obtained.

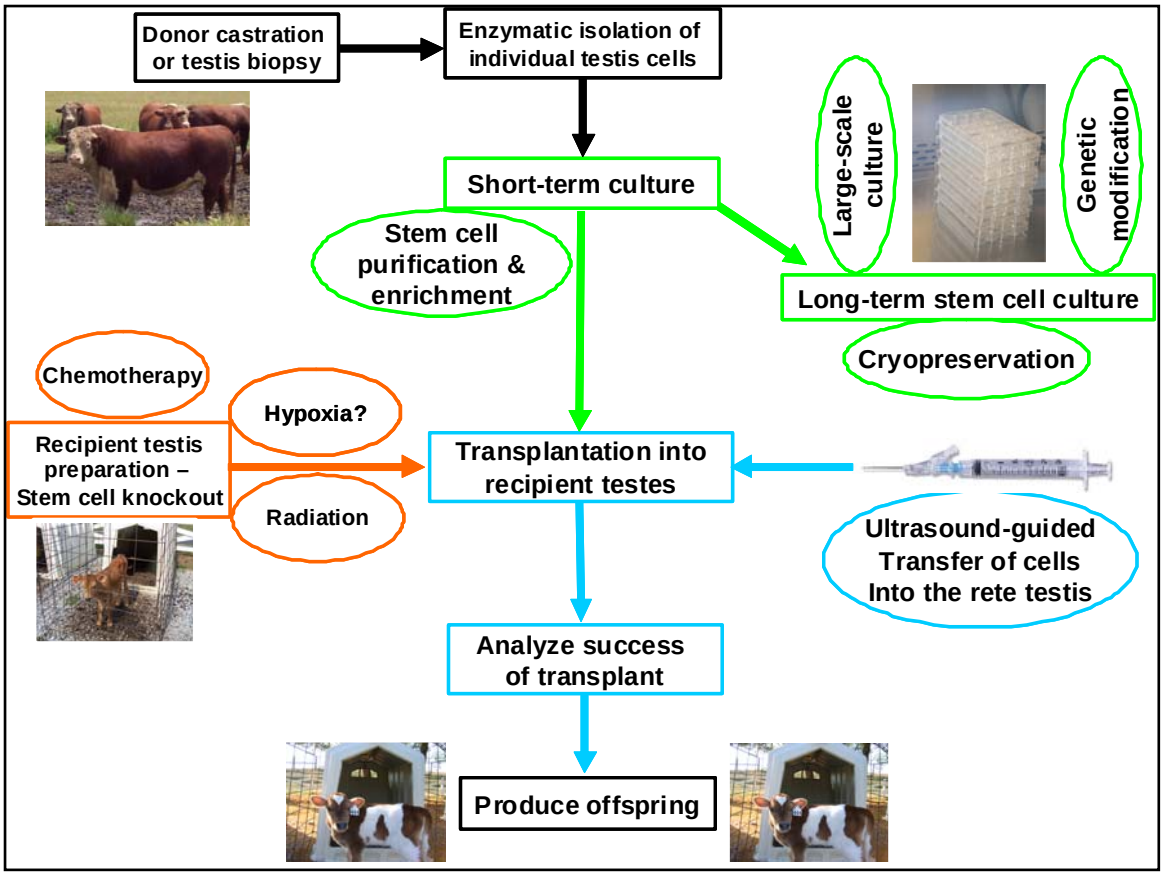

Figure 3. Schematic representation of bovine male germ cell transplantation. Spermatogonial stem cells harvested from donor bulls are isolated by enzymatic digestion. Before transplantation, male germ cells can be maintained and cultured *in vitro* for further cell cryopreservation or genetic modification. Spermatogonial cells are

injected into the rete testis using needle guided-ultrasonography (adapted from Hill and Dobrinski 2005).

3.2. Technique to increase recipient colonization by donor cells

3.2.1. Selection and preparation of donor germ cells

The transplantation process has been modified to improve outcomes since the first experiments by Brinster and Zimmerman (1994) were published. Changes that improve host colonization by donor spermatogonia include 1) enriching the quantity of spermatogonia stem cells in the solution to be transplanted (van Pelt *et al*. 1996; Shinohara *et al.* 200b; van der Wee *et al.* 2001), 2) altering the host environment to deplete endogenous spermatogonia of recipient (Honaramooz *et al.* 2005; Oatley *et al*. 2005), and 3) making technical improvements in the transplantation procedure (Honaramooz *et al*. 2003b; Herrid *et al*. 2006).

Positive identification of bovine type A spermatogonial cells is required to enrich germ stem cells in the solution to be transplanted. If stem cells can be identified and isolated correctly under culture conditions, then spermatogenesis *in vitro* could become a reality, which has major applications in livestock and human male fertility. However, it was reported that out of every 10^3 cells in the mouse testes, only one is a germ stem cell (Tegelenbosch and de Rooij 1993). Selection and preparation of donor spermatogonial

stem cells is a critical step to ensure proper colonization, and eventually, re-initiation of spermatogenesis into recipient testis. The population of spermatogonial stem cells obtained using different methodologies is highly variable; thus, the most effective stem cell-enrichment strategy has to be elucidated. Furthermore, improved culture conditions for male germ line stem cells that allow propagation of undifferentiated cells are still under intense study. Understanding the physical and molecular mechanisms controlling spermatogonial cell development in the testis will help in designing an appropriate microenvironment for *in vitro* culture.

Selection of mouse testicular cells that express α1- and β1-integrin resulted in isolation of enriched germ stem cell population with the ability to colonize the recipient testes (Shinohara *et al.* 1999; Shinohara *et al.* 2000b). Subsequently, immunomagnetic isolation using magnetic beads plus antibodies that recognize the c-kit receptor or the homophilic adhesion molecule (Ep-CAM) demonstrated that type A spermatogonia are able to replicate the testis environment (van der Wee *et al.* 2001). Furthermore, positive c-kit type A spermatogonia from prepubertal bulls proliferated and developed to spermatids-ilike cells in culture (Izadyar *et al*. 2003a). Use of experimental cryptorchid-produced mice provided a relatively high enriched source of spermatogonial stem cell for transplantation (Shinohara *et al.* 2000a; Shinohara and Brinster 2000; Shinohara *et al.* 2000b). Furthermore, the successful use of *in vitro* fluorescence-activated cell sorting (FACS) based on α-6 integrin expression has been reported as a means to enrich spermatogonial stem cells (Shinohara *et al.* 2000b). Type A spermatogonia from 4- to 6-monyh-old calves stained positive for DBA (*Dolbichos bioflorus agglutinin*) were able to form colonies by day 7 of culture and colonize seminiferous tubules of nude mice (nu/nu)

after transplant as confirmed by positive donor type A spermatogonia DBA staining (Aponte *et al*. 2006). Additionally, the use of transgenic mice such as Stra8-enhanced green fluorescence protein (EGFP) has been helpful in isolating pure populations of type A_S spermatogonia (Nayernia *et al*. 2006). Moreover, other markers were expressed on the same EGFP type A spermatogonia in culture such as Rbm, Oct4, c-kit, Tex18, Stra8, Piwil2, Dazl, Hsp90α, β1- and α6- integrins, which were expressed in early stages of type A spermatogonial development (Saitou *et al*. 2002; Nayernia *et al*. 2006). Aditionally, fragilis, stella, mouse vasa homolog (Mvh), and Rnf17 genes were also expressed in transgenic EGFP type A spermatogonia (Saitou *et al*. 2002; Nayernia *et al*. 2006). Furthermore, EGFP type A spermatogonia that proliferated in culture for 2 to 3 wks were able to re-colonize recipient seminiferous tubules after transplant (Nayernia *et al*. 2006).

In a recent study, positive protein gene product 9.5 antigen (PGP 9.5) isolated donor type A spermatogonia from calves and prepubertal rams and were able to colonize seminiferous tubules of recipient animals (Herrid *et al*. 2006; Rodriguez-Sosa *et al*. 2006). Moreover, identification of porcine spermatogonia using expression of PGP 9.5 as a specific marker was reported (Luo *et al*. 2006). These findings suggest that cells staining for PGP 9.5 were likely SSC with the potential to form colonies (Luo *et al*. 2006) or re-colonize foreign seminiferous tubules (Herrid *et al*. 2006; Rodriguez-Sosa *et al*. 2006). Prepubertal pigs type A spermatogonia cells expressed SSEA-1, a specific marker for embryonic stem cells, after 7 days of culture (Goel *et al*. 2007). Alkaline phosphatase (AP) reactivity is highly conserved in undifferentiated embryonic stem cells (Talbot *et al.* 1993) and type A spermatogonia cells (Ginsberg *et al*. 1990). High levels of AP in

spermatogonial cells are related to the undifferentiated stage (Fujino *et al.* 2005). Isolated type A spermatogonia from transgenic mice that proliferate and form colonies after 2 to 3 wks in culture have been stained positive for AP in culture (Jeong *et al.* 2003; Kubota *et al*. 2004; Nayernia *et al*. 2006). Additionally, neonatal testicular cross-section containing peritubular and spermatogonia cells stained both positively for AP (Goel *et al*. 2007).

Seminal alkaline phosphatase (AP) activity has been used in clinical analysis of small and large animals to determine fertility potential of males before breeding. Assays for seminal alkaline phosphatase (AP) can be performed in dogs (reviewed by Johnston 1991) and equine (Pesch *et al.* 2006) to assess semen quality (i.e. azoospermia: collected seminal fluid without spermatozoa) in collected seminal fluid; thus, fertility potential of the male can be determine before mating. In bovine, seminal plasma was negative for AP and sperm acrosome stained positive for AP (Melampy *et al.* 1952). Seminal alkaline phosphatase is produced from epididymal cells (Frenette *et al*. 1986) and is used as a marker of outflow obstruction of upper vas deferens or epididymides. Localization of the cause of azoospermia in dogs could be assessed by physical examination (presence of both testes) and measurement of AP level in seminal fluids (Johnston 2003). Seminal concentrations of AP are greater than 5000 IU L^{-1} in normospermic dogs (reviewed by Johnston 1991). Therefore, lower AP level (100 IU L^{-1}) and absence of spermatozoa are indicative of a potential azoospermia condition. However, incomplete ejaculation containing a pre-sperm fraction (low spermatozoa counting) of semen from prostate gland can also have low levels of AP and dogs should be re-evaluated to discern between incomplete ejaculation and azoospermic problems. Additionally, in stallions, AP was correlated with sperm concentration and volume of collected ejaculate (Pesch *et al.*

2006). It was suggested that levels of AP present in seminal fluid can be used as markers for practitioners to predict the quality of semen samples (Kutzler *et al*. 2003; Pesch *et al.* 2006).

Spermatogonia cells have been cultured in different culture medium containing serum or serum-free in an attempt to study their dynamic of proliferation and differentiation. In general, culture conditions for the studies descrived herein range from 32 °C to 37 °C with 5% CO_2 in air. A potassium simplex optimized medium (KSOM) and minimal essential medium (MEM) without serum were used to culture type A spermatogonial cells isolated from adult mice (Creemers *et al*. 2002) and 3-7 month-old prepubertal bulls (Izadyar *et al.* 2003a). In culture, type A spermatogonia proliferated more in MEM than in KSOM media (data not shown) while more proliferation was observed at 37 °C than 32 °C (Izadyar *et al*. 2003a). Contrarialy, more eight days-old porcine type A spermatogonia cells suvived in KSOM medium than Dolbecco's modified eagle's medium (DMEM; Dirami *et al*. 1999). In these studies, the viability and proliferation of type A spermatogonia cells were decreased in a range of 80 to 90% after one week of culture. Additionally, proliferation of type A spermatogonia cells *in vitro* culture medium without serum or a feeder monolayer resulted in few cells surviving (Dirami *et al*. 1999; Izadyar *et al.* 2003a). However, when 2.5% fetal calf serum (FCS) was added to the medium (KSOM and MEM) proliferation and viability of type A spermatogonia were enhanced after one week of culture. Moreover, in MEM media containing 2.5% of FCS type A spermatogonia proliferated to form two distint types of colonies (round and radial) and some type A spermatogonia differentiated in cells with the structural appearance of spermatocytes and spermatids after 100 days of culture

(Izadyar *et al*. 2003a). Proliferation of type A spermatogonia in culture with colony cells c-kit positive (differentiated) and c-kit negative (presumably stem cells) were reported after a month of culture (Izadyar *et al.* 2003a). It was suggested that the addition of up to 10% of FCS only enhanced somatic cells proliferation over spermatogonia; however, it was not clear how these two populations of cells was evaluated in culture (Izadyar *et al*. 2003a). Isolated type A spermatogonia from prepubertal 2- and 10-weeks-old pigs were maintained and proliferated to form colonies in a simple culture medim (DMEM) plus 5% FBS without addition of growth factors (Marret and Durand 2000; Luo *et al*. 2006) as well as transgenic mice type A spermatogonia in similar midium containing 10% fetal bovine serum (FBS), pyruvate, and lactate (Nagano *et al.* 1998; Goel *et al*. 2007).

Alternatively, type A spermatogonia from newborn transgenic mice proliferated for 5 months in culture using StemPro-34 SFM medium (serum-free medium formulated for hematopoetic cells in culture) containing several growth factors (LIF, EGF, FGF, and GDNF) and 1% FCS and spermatogonial cells re-initiated spermatogenesis in infertile mutant mice after transplant (Kanatsu-Shinahara *et al.* 2003). Similarly, isolated type A spermatogonia cultured in DMEM containing 10% FBS and growth factors (LIF, FGF, IGF-I, and platelet-derived growth factor (PDGF)) resulted in more colonies than simple DMEM containing 10% FBS after 4 weeks of culture from transgenic (lacZ) adult mice (Jeong *et al*. 2003) or 6 days-old mice (Anjamrooz *et al*. 2006). All these growth factors mentioned above seemed to enhance, at least in part, spermatogonia cell survival and proliferation; however, it was suggested that GDNF favors type A_S spermatogonia self-renewal when added to serum-free medium in donor mice cells (Kubota *et al*. 2004), to MEM with 2.5% FCS in donor 4- to 6-month-old bovine (Aponte *et al*. 2006), ot to

DMEM with 10% FBS (Oatley *et al*. 2004). Supplementation of culture medium with exogenous glial cell line-derived neutrotrophic factor (GDNF), leukemia inhibitory factor (LIF), and epithermal growth factor (EGF) enhanced survival and proliferation of germ cells (Kanatsu-Shinohara *et al*. 2003; Oatley *et al*. 2004; Meng *et al*. 2000; Nagano *et al*. 2003). It is known from these studies that the addition of serum (FBS, FCS, HS, or Nu serum) to the medium ehanced germ cells survival and proliferation in culture. Additionally, growth factors added to the medium in presence of serum also contributed in more type A spermatogonia proliferating. However, it is difficult to discern what component(s) present in serum are essential for self-renewal of type A_S spermatogonial. Therefore, further studies are needed to discern what component(s) between serum types (i.e. lipidis, proteins, hormones, etc.) support self-renewal of male germ cells.

The role of various types of feeder monolayers has been suggested to enhance maintenance and establishment of donor type A_S spermatogonia in culture (reviewed by Sofikitis *et al*. 2005). Co-culture of type A spermatogonia cells from a 1- to 2-month-old bulls with a feeder cell monolayer such as bovine embryonic fibroblasts harvested from an embryo of 35 days enhanced maintenance and viability of germ cells (BEF, Oatley *et al*. 2004). In this study, added GDNF to the medium of expressed by the BEF cells was suggested to influence maintenance of type A spermatogonia. Moreover, type A spermatogonia from a six-day-old mice survived and proliferated in co-culture with immortalized mice Sertoli cells either SF7 or SG5-1.13 lines for 25 days (van der Wee *et al*. 2001). Furthermore, the addition of 5% Nu serum (low protein serum that allows controlled culture environment) resulted in more surviving spermatogonia than 5% FCS or 5% horse serum (HS) to the medium, suggesting that inhitiory substances were present

in FCS and HS (van der Wee *et al*. 2001). Moreover, type A spermatogonia from 5-7 days of age mice cocultured for 7 days with SIM mouse embryo-derived Thioguanine- and Ouabain-resistant fibroblast (STO), OP9 (bone marrow stroma), or L (fibroblast origin) substantially enhanced germ cell survival by two fold (Nagano *et al*. 2001c; Nagano *et al*. 2003). Recently, a novel three-dimensional soft-agar culture-system that supports spermatogonia proliferation and differentiation (up to post-meiotic level) after 15 days of culture was reported in mice (Stukenborg *et al.* 2008). It was suggested that this three-dimentional culture system mimic structural support of *in vivo* proliferation of type A spermatogonia supporting meiotic differentiation of donor germ cells without addition of any grwth factors (Stukenborg *et al.* 2008).

It seemed reasonable to say that those commun morphological characteristics and no specific markers available to discern between type A (A_S, A_{al}, A_{pr}, and A_1-A_4) spermatogonia cells make difficult to correctly identify type A_S spermatogonia cell (considered stem cell; reviewed by de Rooij 2001). Therefore, an alternative assay is to transfer those cells into recipient seminiferous tubules hoping to restore spermatogenesis as a successful outcome when evaluating presence of donor type A_S spermatogonia in culture or donor-derived progeny (Kanatsu-Shinahara *et al.* 2003). Although this is a powerfull assay to confirm presence of donor-derived spermatogonial cells in recipient animals, the length in days of spermatogenesis or gestation is species-specific making rodents and small ruminants the animal model of election. Moreover, the majority of the data available today about isolation, culture, and transfer of type A spermatogonia are either in rodents or small ruminants, with a few exception to large animals such as bovine as mentioned above. Considerations on how to interprate or apply results from rodents to

large food animals such as bovine should be taken into account due to species-specific characteristics such as length of spermatogenesis. Therefore, field application of spermatogonial stem cell transplant in large food animal such as bovine requires further investigation not only in prepubertal animals but also in adult bulls.

3.2.2. Preparation of recipient animals

Colonization of seminiferous tubules in recipient animals by transplanted spermatogonial stem cells is more efficient when endogenous spermatogonia are depleted from recipient testes. Busulfan, a chemotherapeutic drug, is used widely in rodents to deplete endogenous spermatogonial cells before transplantation (Brinster and Avarvock 1994; Brinster and Zimmerman 1994; Honaramooz *et al*. 2008). However, the dose of busulfan to deplete stem cells is highly toxic and immunosuppression occurs (Ogawa *et al.* 1999b; Brinster *et al.* 2003). Furthermore, the use of busulfan in rams and pigs resulted in a significant depletion of spermatogonial cells; however, animals presented signs of toxicity (Ogawa *et al.* 1999a). In pigs and mice, *in utero* treatment with busulfan was an effective alternative to deplete endogenous germ cells (Brinster *et al.* 2003; Honaramooz *et al.* 2005). Additionally, cold ischemia (Yong *et al*. 1988) and hyperthermic treatment (McLean *et al.* 2002) of testes have been used in preparation of recipient testes.

An alternative method, irradiation, has been used in recipient animals to abolish spermatogenesis in rodents (Creemers *et al*. 2002; Giuili *et al.* 2002), monkey (Schlatt *et*

al. 2002), rams (Oatley *et al.* 2005), and bulls (Izadyar *et al.* 2003b). This method requires costly specialized radiotherapy equipment making it impractical for field application. Another alternative, prolonged administration of the GnRH-agonist Leuprolide, inhibited secretion of LH and FSH followed by reduced testosterone production; thus, disrupting spermatogenesis (Ogawa *et al.* 1989). Additionally, efficiency of colonization recipient testes was markedly higher by donor cells using Leuprolide (Blanchard *et al.* 1998; Dobrinski *et al.* 2001).

These inconsistent results in depletion of recipient germ cells emphasize the necessity to find more practical means of suppressing endogenous spermatogenesis in recipient bulls. Preparation of sound recipient animals will impact efficiency of colonization of seminiferous tubules by transplanted donor germ cells.

3.3. Cyopreservation of male germ cells

The era of successful embryo cryopreservation followed the discovery and development of chemical cryoprotectant compounds (reviewed by William 2007). In the early 1950's, Polge experimented with vitrification and dehydration at low tempartures using speramozoa (Polge *et al*. 1949; reviewed by William 2007). Years later, Wilmut (1972) and Whittingham (1971) had developed independent methods for reliably freezing mouse embryos in dimethylsulfoxide (DMSO). Currently, the most popular mouse and bovine embryo cryoprotectant solutions include DMSO, ethylene glycol, propylene glycol (1-2, propanediol), and glycerol (Leibo 1984; Kaidi *et al.* 2000).

Cryopreservation of germ cells has been demonstrated to produce live offspring after transplantation of frozen-thawed mouse germ cells (Avarbock *et al.* 1996; Izadyar *et al.* 2002a; Kanatsu-Shinohara *et al.* 2003; Nagano *et al.* 2003). These experiments used combinations of fetal calf serum (FCS), DMSO, glycerol, sucrose (sugar), and different cooling rates to evaluate long-term preservation of bovine testicular germ cells. Medium containing 10% FCS, 10% DMSO, and 0.07 M sucrose, using non-controlled rate freezing, showed acceptable viability post-thaw and spermatogonial cells were able to colonized recipient testes (Izadyar *et al.* 2002b; 2003b). The ability to freeze and thaw spermatogonial cells in livestock will facilitate *in vitro* culture and *in vivo* field application of the technique (Izadyar *et al.* 2003a). Therefore, potential donor animals could be selected at convenient times and testicular germ cells stored for long period of time for future *in vitro* and/or *in vivo* studies. Additionally, males from endangered species could be harvested and cryopreserved to protect them from extinction; thus, preserving genetic diversity.

3.4. Methods for germ cell transplantation

Microsurgical puncture directly into the seminiferous tubule was the initial approach to successfully transfer donor germ cells into recipient testes (Brinster and Avarvock 1994; Brinster and Zimmermann 1994). Although this technique requires anesthetized animals, several straight length seminiferous tubules are microinjected under a dissecting microscope with trypan blue dye to monitor the flow of fluid into the tubules.

However, this approach is the most time consuming for large mammal testes such as bull, ram, pig, and goat in terms of infused volume into the testis.

The introduction of donor germ cells into the recipient efferent ducts or rete testis was developed in mice (Ogawa *et al.* 1997). Injecting of donor cells into the efferent ducts or rete testis requires careful surgical dissection, with the rete testis being the less invasive and most rapid method. Rete testis is the simpliest technique for transferring donor cells without the need of a micromanipulator. In other species such as sheep, goat, and bovine, it is considerably more dificult to perform this procedure because the axial rete testis is located deep inside the testis (Ogawa *et al.* 1997).

The ultrasound-guided cannulation (needle) of the rete testis *in vivo* showed to be successful in bull calves and rams (Honaramooz *et al.* 2003a; 2008; Izadyar *et al.* 2003b; Jeong *et al.* 2003; Hill *et al.* 2005). In order to perform this technique, animals are placed under general anaesthesia; thus, the solution can be injected into the rete testis. The spread of fluid into the rete testis can be confirmed by visualization of a characteristic ultrasonographic appearance (Honaramooz *et al.* 2003a; Izadyar *et al.* 2003b). Additionally, a commercially available ultrasound opaque fluid may be used to monitor the solution when injected into the rete testis and donor cell transfer can be assessed immediately (Izadyar *et al.* 2003b).

3.5. Methods to isolate spermatogonia subpopulations

Procedures for isolation of spermatogonial germ cells using velocity sedimentation (Bellve *et al.* 1977; Dirami *et al.* 1999) or elutriation (Bucci *et al.* 1986) have been described. In these methods, a population of up to 90% spermatogonia can be harvested. Spermatogonia were selected base on their size and shape; thus, these methods can only be used in prepubertal animals in which the number of other developing germ cells are minimal (Meachem *et al.* 2001). Using a discontinuous Percoll gradient up to 85% of rat spermatogonia from prepubertal testes (9 days of age) and vitamin A difiecient adult testes can be collected as decribed by Morena *et al.* (1996) and van Pelt *et al.* (1996) respectively. In both studies, cells collected were plated in lectin peanut agglutinin (PNA) pre-coated dishes for 1 hour to remove somatic components, then cells nonadhering to the lectin were subjected to a discontinuous Percoll gradient separation. Isolated population of type A spermatogonia were positive to c-kit marker (Morena *et al*. 1996); however, it was suggested that c-kit may be present in both undifferentiated and differentiated type A spermatogonia. Viable type A spermatogonial cells recovered by this method were used in further *in vitro* experiments.

Magnetic cell sorting can be used to isolate spermatogonia from testicular suspensions of various species (adult hamster, mice, and monkey) using the c-kit antibody to detect the c-kit receptor in the membrane of spermatogonia (Dym *et al.* 1995; von Schonfeldt *et al.* 1999). However, efforts to isolate large numbers of purified type A spermatogonia using the c-kit marker were not accompanied by purity (25-55%) in fully active testes (von Schonfeldt *et al.* 1999). Populations of A_{al} spermatogonia express c-kit; however, these cells are committed to differentiate into A_1 spermatogonia (Schrans-Stassen *et al.* 1999). Additionally, the immunomagnetic bead technique against c-kit to

isolate differentiating A spermatogonial has been used (van der Wee *et al.* 2001). In a similar study, magnetic beads against the β1- and α6-integrins of spermatogonial stem cells resulted in population of cells able to colonize recipient testes after transplant (Shinohara *et al.* 1999). However, the authors did not report viability and proliferative ability under *in vitro* conditions of those sorted spermatogonial stem cells. Cell sorting analysis by fluorescence activation was reported in spermatogonial cells recovered from mouse cryptorchid testes (Shinohara *et al.* 2000a; Shinohara *et al.* 2000b).

Furthermore, the successful use of an *in vitro* fluorescence-activated cell sorting (FACS) based on α-6 integrin expression has been reported as a means to enrich spermatogonial stem cells (Shinohara *et al.* 2000b). Additionally, enriched newborn and adult mouse type A spermatogonial populations were obtained through marker proteins suc as Thy-1, CD9, Egr3, or GFRA-1 expressed on type A (presumably A_S) spermatogonial cells (Kubota *et al.* 2003; Kanatsu-Shinohara *et al.* 2004; Stukenborg *et al.* 2008). Alternatively, differential plating using adhesion molecules (lectin or laminin) to surface plastic and velocity sedimentation are useful to reduce the number of somatic cells harvested from rodents testes (van Pelt *et al.* 1996; Shinohara *et al.* 1999; Lou *et al*. 2006).

Harvesting bovine donor spermatogonia stem cells from animals before puberty, when the seminiferous tubules have higher proportion of undifferentiated germ cells, is the most recommended (Izadyar *et al.* 2002b; Izadyar *et al.* 2003a; Izadyar *et al.* 2003b; Herrid *et al.* 2006). In these studies, type A spermatogonia cells were isolated from 3-7 month-old prepubertal bulls and characterized further by expression of markers such as c-kit and lectin *Dolichos biflorus agglutinin* (DBA; Izadyar *et al.* 2002b; Izadyar *et al.*

2003a; Izadyar *et al.* 2003b) or protein gene product (PGP) 9.5 (Herrid *et al*. 2006). Immediatelly after cell collection, pools of type A spermatogonial and somatic cells were subjected to a Percoll gradient separation to further enrich type A spermatogonia (Izadyar *et al.* 2002b; Izadyar *et al.* 2003a; Izadyar *et al.* 2003b). The most effective way to isolate pure populations of spermatogonial stem cells is to collect all forms of type A spermatogonia (A_s, A_{pr}, A_{al}, and A_1-A_4). Because the markers used in these studies may be expressed in all type A spermatogonia, the age of donors (prepubertal bulls) ensure, at least in part, that spermatogonial stem cells were likely present in the collected solution. On the other hand, isolation of type A spermatogonia from adult testes is more challenging due to the presence of many types of developing germ cells in the spermatic epithelium (reviewed by Meachem *et al.* 2001). In adult testes with similar morphological characteristic, spermatognial stem cells accounts for < 4% of all the spermatogenic cells (reviewed by Meachem *et al.* 2001). However, spermatogonial cells carring a *lac*Z transgene (marker) isolated from adult mice were able to colonize and re-initiate spermatogenesis in recipient mice testes after transplant (Avarbock *et al*. 1996). In this study, the identification of donor-derived spermatogonial stem cell progeny stained blue following incubation with X-Gal. Dobrinski *et al.* (2000) transferred a pool of isolated spermatogonial cells (presumable all types A spermatogonia and spermatocytes) from an adult bull to seminiferous tubules of recipient mice. It was suggested that bovine donor-derived cells colonized seminiferous tubules of recipient mice but do not differentiate into spermatozoa. The species-specific polyclonal antibodies used in this study were generated by immunizing female rabbits with prepubertal testis cells (primarily myoid, germ and Sertoli cells). Therefore, evaluation of colonization after transplant was

performed not only for spermatogonial cells but also for Sertoli cells since antibodies recognized all donor cells inside of the seminifeorus tubules. Although a pool of testicular cells (all types A spermatogonial and spermatocytes) from adult bull have been transferred to mice without further differentiation, information on isolation and culture of adult bovine type A spermatogonia cells is lacking.

3.6. Advances on bovine germ stem cell transplant

The adaptation of the spermatogonial stem cell transplantation in livestock enables the preservation and dissemination of desirable genetics. Significant improvements have been made in donor cell isolation, purification, and recipient preparation in pigs (Luo *et al*. 2006), goats (Honaramooz *et al.* 2003a), and bovine (Izadyar *et al.* 2002a; 2003b; Herrid *et al.* 2006). Long-term and large scale culture systems of bovine germ cells have been reported (Izadyar *et al*. 2003a).

As summary of the advances, the development of an optimal *in vitro* system that allows multiplication of undifferentiated germ cells is a long soughtafter goal. Proliferation of spermatogonial stem cells under *in vitro* culture will allow a continuous supply of cells to be transferred into multiple recipient animals. Additionally, a simple and costly effective method to identify unequivocal viable stem cells for culture is still under investigation. Donor spermatogonial stem cells can colonize the recipient seminiferous tubules more efficiently when the recipient testes present no endogenous spermatogonia. Depletion of donor endogenous germ stem cells has been studied using

irradiation, ischemia, hyperthermia, and chemotherapeutic drug (busulfan). However, a practical method that allows field application to abolish endogenous germ cells while minimizing negative side effects on the recipient animal still remains under investigation.

3.7. Potential applications of germ cell transplantation in livestock

Successful spermatogonial cells transplantation followed by colonization of the recipient seminiferous tubules in cattle and re-initiation of spermatogenesis by trasnplented donor cells requires sound recipient animals within the same specie (Honaramooz *et al.* 2002a; Honaramooz *et al.* 2003b; Izadyar *et al.* 2003a; Honaramooz *et al.* 2005; Herrid *et al.* 2006). Potetial clinical applications and benefits of this technique in livestock are as follow:

a. Enhance dissemination of superior genetic animals: large-scale culture of spermatogonial stem cells will allow a constant supply for transplantation into less-valuable recipient animals.
b. Cross-breeding in environments where artificial insemination is impractical (i.e. heat stress): *Bos indicus* bulls ejaculating *Bos taurus* sperm will enhance the value of the F_1 calves in *Bos indicus* herds.
c. Conservation of endangered species: spermatogonial stem cells can be preserved through transplantation into recipient animals.

d. Introduction of genetic modifications through spermatogonia stem cell line is a promising strategy to obtain transgenic animals used for the efficient production of biopharmaceutical proteins (i.e. insulin, EGF, etc.).

4. Summary and statement of the problem

Spermatogonia stem cells have unique capabilities including self-renewal and production of the end product, spermatozoa. The ability to recover these cells, maintain *in vitro*, and transfer them to another testis would provide a valuable technique to study the process of spermatogenesis. Furthermore, spermatogonial transplantation may be an alternative to transfer economically favorable genes through modification of germ cell lines. The clinical application of culturing SSCs is of great importance in livestock, wildlife, and humans. Animal models may provide adequate knowledge for therapeutic management in patients with oncological and infertility diseases. Preserving SSCs have two advantages (proliferation by mitosis and differentiation to haploid cells by meiosis) as opposed to cryopreservation of spermatozoa (haploid cells cannot undergo mitosis). Therefore, large amounts of the genetic materiel can be preserved through culture of SSCs. Isolation and culture of bovine type A spermatogonia has been performed in prepubertal bulls (Izadyar *et al*. 2002; 2003a) and very little is known on type A spermatogonia cultured from adult bulls. Moreover, male germ cells culture from large food animals such as bovine of known genetic merit is of paramount important for future studies aimed to transfer these valuable genes using recipient animals. The correct

identification and culture of type A spermatogonia will enable dissemination of desired genetic from donor animals through spermatogonial cells transfer.

The present research aims were to: 1) evaluate potential protocols that favor germ stem cell proliferation through hormonal induction in recipient prepubertal bulls, 2) establish an alternative method to abolish endogenous germ stem cell through testicular transiently-induced ischemia in recipient animals, and 3) develop potential protocols for *in vitro* proliferation of undifferentiated spermatogonial stem cells under different culture conditions. Therefore, to accomplish the overall research goal, the following research objectives were pursued:

a. Determine hormone profiles and population of Sertoli, germ, and Leydig cells following immunization against inhibin in Jersey bull calves: *the working hypothesis is that immunization against inhibin will increases germ cell populations and FSH secretion needed during spermatogonia development*.

b. Determine population of Sertoli, germ, and Leydig cells after transiently-induced testicular ischemia (hypoxia) in Jersey bull calves: *the working hypothesis is that transiently-induced testicular ischemia in bull calves will abolish the germ cell epithelium maintaining Sertoli and Leydig cell populations in recipient bulls*.

c. Evaluate SSCs proliferation and differentiation under various culture conditions: *the working hypothesis is that bovine spermatogonial stem cell harvested from prepubertal and adult bulls will proliferate and remain undifferentiated during short-term in vitro culture*.

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Chapter 3 – CHANGES IN THE TESTIS SEMINIFEROUS TUBULES AND INTERSTITIUM IN PREPUBERTAL BULL CALVES IMMUNIZED AGAINST INHIBIN AT THE TIME OF GONADOTROPIN ADMINISTRATION

This chapter is a paper by the same name published in the Journal *Reproduction Fertility and Development* in 2007 by Schuenemann, G.M., Mendis-Handagama, S.M.L.C., Hopkins, F.M., Kania, S.A., and Schrick, F.N. Changes in the testis seminiferous tubules and interstitium in prepubertal bull calves immunised against inhibin at the time of gonadotropin administration. *Reprod. Fertil. Dev*. **19**, 840-849.

My use of "we" in this chapter refers to my co-authors and myself. My primary contributions to this paper include (1) data analysis (2) identification of cell types, (3) collection and interpreting of the literature, (4) writing of this paper.

3.1. Acknowledgments

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3.2. Abstract

The objective of the current study was to evaluate gonadotropin administration at initiation of inhibin passive immunization in Jersey bull calves (age: 27 ± 5 d) on testicular morphology and development. Primary treatments consisted of control (KLH, *n* = 9) or immunization (INH, *n* = 9). Subsets of calves were randomly assigned within primary treatments (TRT) to receive saline (*n* = 3 / TRT), FSH (*n* = 3 / TRT), or GnRH

(n = 3 / TRT). The right testis was removed (age: 118 ± 5 d) to determine volumes of testicular components and cell numbers per testis using stereology. Data were analyzed using MIXED procedure of SAS. Antibody titers against inhibin were increased in INH bulls compared to KLH bulls ($P < 0.05$). In addition, a significant immunization x hormone treatment interaction was noted for number of germ cells. Administration of FSH at the time of initial immunization against inhibin significantly increased number of germ cells (92.2 ± 9 x 10^6 cells) compared to INH-Saline bulls (54.9 ± 10 x 10^6 cells) with INH-GnRH bulls being intermediate (64.5 ± 9 x 10^6 cells; $P < 0.05$). These results suggest that gonadotropin administration at time of inhibin immunization increases number of germ cells in the testis.

Key words: Bull, inhibin, gonadotropin, testis development, germ cells.

3.3. Introduction

Spermatogenesis is a highly organized complex process that involves the coordinated interaction of both germ and somatic cell components. Three primary phases in spermatogenesis occur in the seminiferous tubule (Amann 1983), which include spermatogonial multiplication (mitosis), meiosis, and release of sperm to the lumen of the seminiferous tubule (spermiogenesis). Leydig cells are responsible for the synthesis of testosterone (Abraham 1991; Payne *et al.* 1995) while Sertoli cells are considered the "nurse cell" (Griswold 1995) and are located around the periphery of the seminiferous tubule. Functional Sertoli cells have specific FSH receptors and will absorb testosterone

secreted by Leydig cells (Silva *et al.* 2002). FSH is one of the primary hormones stimulating spermatogenesis (Kerr *et al.* 1992) and early secretion of FSH in bulls is thought to be dependent upon GnRH (Evans *et al.* 1993).

Inhibin is a 31- to 34-kDa heterodimeric glycoprotein (α-β_A is inhibin-A, -α-β_B is inhibin-B) with physiological important roles for regulation of testicular function (Ying 1988; Mather *et al.* 1992). In the male, inhibin is produced by Sertoli cells (de Kretser *et al.* 1989) and the best-established role is an endocrine negative feedback on FSH release at the pituitary gland level (Ying 1988; Bame *et al.* 1999; Kaneko *et al.* 2001). Recent evidence also supports an autocrine or paracrine role for inhibin in regulation of testicular function (Ying 1988; Bardin *et al.* 1989; Mather *et al.* 1992). In support of the overall suppressive effect of inhibin on pituitary and testicular function, immunization against inhibin increases secretion of FSH in rams (Voglmayr *et al.* 1990), and daily sperm production and concentration of FSH secreted in bulls (Martin *et al.* 1991; Kaneko *et al.* 1993; Bame *et al.* 1999; Kaneko *et al.* 2001).

Male germ cell transplantation in livestock has progressed tremendously in the past few years (Honaramooz *et al.* 2003a, 2003b; Izadyar *et al.* 2003; Joerg *et al.* 2003; Hill *et al.* 2005). However, the efficiency of colonization of seminiferous tubules by the transplanted germ cells is low and variable (Dobrinski *et al.* 2000; Nogano, *et al.* 2002; Izadyar *et al.* 2003; Joerg *et al.* 2003; Hill *et al.* 2005). This emphasizes the necessity to find practical means of stimulating transplanted germ cell proliferation in recipient bulls that will result in adequate donor cell colonization without the need for further intervention.

Many reports are available on testicular components in the developing postnatal rat (Roosen-Runge *et al.* 1959; Mendis-Handagama *et al.* 1998), cat (França *et al.* 2003), bull (Curtis and Amann 1981), and other mammals (Russell *et al.* 1990). However, characterization of the testicular components of prepubertal bovine bulls immunized against inhibin is not available. Such data are important for understanding the dynamics of the cell-cell interaction, proliferation, and differentiation in the testis.

Consequently, the primary objective of the current study was to evaluate effects of passive immunization against inhibin in young bull calves and the effectiveness of gonadotropin administration at initiation of inhibin immunization on the testicular cell components. Additionally, the end result would be aimed at developing a protocol that favors germ cells proliferation.

3.4. Materials and Methods

3.4.1. Animals and treatments

Eighteen Jersey bull calves were paired by birth date (± 5 d) at 20 d of age. Bulls were born and raised exclusively at the Dairy Research and Education Center (Lewisburg, TN, USA). During the first month of life, animals were isolated from each other to reduce pathogen transmission. At birth, calves received colostrum in a bottle for the first 48 hours of life (1.2 liters / twice a day). During the initial 8 weeks of life, animals were fed whole waste milk (1.2 liters / twice a day) and offered a starter ration (Tennessee Farmers Cooperative, Lavergne, TN, USA) and a primer ration (Tennessee

Farmers Cooperative, Lavergne, TN, USA) *ad libitum*. Weaning occurred at 8 weeks of age with calves placed in group pens and fed only the primer ration *ad libitum* for the remainder of the experiment.

Calves were randomly assigned into six (6) treatment groups (n = 3 calves / treatment) at 20 d of age. The study was performed using the inhibin peptide (bovine inhibin $\alpha^{1\text{-}26}$) conjugated to keyhole limpet hemocyanin (KLH). Primary treatments (Table 1; initial immunization at 27 ± 5 d of age; d 1 of the experimental period (All tables and figures in this chapter appear in the appendix)) consisted of control (KLH, 250 μg, n = 9) or immunization (INH, 500 μg INH:250 μg KLH, n = 9) with both emulsified in 2 mL of Freund's Complete Adjuvant (FCA). Booster immunizations (identical preparation in Freund's incomplete adjuvant, FICA) occurred every 21 d with the last administration on d 84 of the trial (a week before castration). All immunizations were administered into four sites (two in the neck and two behind the shoulder; subcutaneously; Bame *et al.* 1999). Subsets of calves were randomly assigned within primary treatments (TRT) to receive saline (1 mL, n = 3 / TRT), FSH (20 mg, n = 3 / TRT), or GnRH (50 μg, n = 3 / TRT) every 8 hours (0600, 1400, 2200 h) from D 1-3 of the study (Table 1). Each hormone injection consisted of 1 mL sterile saline (Abbott Laboratories, North Chicago, IL, USA), or 1 mL FSH injection at 20 mg mL^{-1} (Folltropin-V, Bioniche Animal Health Canada Inc., Belleville, Ontario, Canada), or 1 mL GnRH injection at 50 μg mL^{-1} (Cystorelin, Merial Limited, Iselin, NJ, USA). All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee, University of Tennessee, Knoxville, USA. Pain and stress to animals were minimized throughout the experimental period.

3.4.2. Preparation of immunogen

The sequence of bovine inhibin α^{1-26} (his-ala-val-gly-gly-phe-met-arg-arg-gly-ser-glu-pro-glu-asp-gln-asp-val-ser-gln-ala-ile-leu-phe-pro-ala (Bame *et al.* 1999) was synthesized commercially (Bio-Synthesis, Lewisville, TX, USA). In a previous study, (Good *et al.* 1995) showed that antibodies that were generated against inhibin α bound to a carrier cross-reacted with at least eight different molecular variants of bovine inhibin dimmers and inhibin α subunits, but not with activin or the inhibin/activin serum-binding proteins, follistatin and α-macroglobulins. Unlike previous studies in which human alpha globulin (HAG) was utilized (Martin *et al.* 1991; Bame *et al.* 1999), the current experiment was performed using the inhibin peptide conjugated to keyhole limpet hemocyanin (KLH) 2 : 1 (60 mg peptide to 30 mg KLH).

3.4.3. Blood samples, body weight, and scrotal growth

Blood samples were obtained via jugular venipuncture at 1400 h daily from d 0-14 and then weekly until testes collection (Fig. 1; d 91 of the trial). Blood samples were immediately centrifuged at 2000 x g for 15 minutes and serum stored at -20°C until assayed by RIA for FSH, LH, testosterone (T), and determination of inhibin antibody titers. Body weight and scrotal circumference (Good *et al.* 1995) were measured at each

immunization and before testes removal (Fig. 1; approximately 16 weeks of age). The right testis was weighed and used for absolute volume calculation of cell components per testis. The left testis was weighed, fixed in 2.5% glutaraldehyde, and stored.

3.4.4. Collection and processing of testis tissue

At day 91 d of the study, bulls were castrated and both testes were removed and weighed (fresh testicular weight). The right testis was perfused fixed by cannulation of the artery and used for microscopy evaluation and stereology. First, a solution (250 mL) of 0.9% NaCl was flushed through the tissue for approximately 10 minutes to allow blood to clear from testicular vessels. When the testis was clear of blood, a solution (250 mL) containing 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) was administered through the cannula as described by Mendis-Handagama *et al.* (1988).

After perfusion fixation was complete, testes were placed in a plastic container immersed into the same fixative solution for a week. Then testes tunica albuginea were nicked using a 10' blade scalpel and placed back into the container for a week. The testis was weighed with the testicular capsule (tunica) and without the capsule prior to tissue processing for microscopy. From each fixed testicle, 10 tissue samples of approximately 2-3 mm cubes were cut and was post fixed in a 1 : 1 mixture of 2% aqueous osmium tetroxide and 3% potassium ferrocyanide (Russell and Burguet 1977), then dehydrated in a series of graded ethanols and embedded in epon-araldite (Electron Microscopy Sciences, Hatfield, PA, USA) as described by Mendis-Handagama and Ewing (1990).

The polymerization of the tissue blocks took place in an oven at 60ºC for 48 h (Mendis-Handagama *et al.* 1990).

3.4.5. Testis histology and morphometry

From each polymerized tissue block (ten per testis), two 1-μm thick sections were made using a LKB IV ultramicrotome (Pharmacia LKB, Piscataway, NJ, USA) and glass knives. Sections were mounted on pre-cleaned glass slide (Superfrost Plus; Fisher Scientific, Pittsburgh, PA, USA), stained with methylene blue azure II, and cover-slipped under permount (Fisher Scientific, Pittsburgh, PA, USA). The different cell types in the testicular interstitium and inside the seminiferous tubules were identified by their morphological characteristics as previously described (Fig. 2; Curtis and Amann 1983; Berndtson *et al*. 1987*a*; 1987*b*; Mendis-Handagama *et al.* 1987; 1998; Ariyaratne *et al.* 2000). A total of 20, 1-μm thick sections (two per block), per testis were used to evaluate testicular components using the point count method (Weibel 1980).

Volume of components: The volume density (vv%) of testicular components (defined as the volume of a component per unit volume of testis tissue) was obtained by the point-counting method (Weibel 1980) using an ocular grid with 88 test points fitted to a color video monitor at x 400 magnification. To determine the volume density of components of the seminiferous tubules and the testis interstitium, 10 randomly selected ocular fields of each section were scored (10 fields section X 10 blocks testis X 88 points = 8800 points per testis per bull). The absolute volume (mm^3) occupied by each testicular

component was calculated by multiplying the volume density of each component by the testis volume (without the capsule). Because the testis density is nearly 1.0 (range 1.03-4), subsequent morphometric calculations of the testis weight was considered equal to testis volume.

Average volume of a germ cell, and the nucleus of a Sertoli and Leydig cell: The germ cells, and the nuclei of Sertoli and Leydig cells in these tissue sections were reasonably circular to justify that they are spherical or close to spherical in configuration. Therefore, the following methodology was used to determine their average volume. Images of germ cells, Sertoli cells and Leydig cells in the methylene blue-stained testis tissue sections were brought up on a color video monitor using a color video camera (DXC-107A; Sony Corporation, Tokyo, Japan). The diameters of germ cells, and nuclei of Sertoli and Leydig cells were measured using ocular and slide micrometers (n = 50 per animal). The average volume (v) of a germ cell, the nucleus of a Sertoli cell and the nucleus of a Leydig cell was calculated using the formula that determines the volume of a sphere, namely, $v = 4 / 3 \cdot \pi \cdot r^3$, where r is cell diameter / 2 for germ cell or nuclear diameter / 2 for Leydig and Sertoli cells. Results are expressed in μm^3.

Number of germ, Sertoli and Leydig cells per testis: The absolute volumes of germ cells, and the nuclei of Sertoli and Leydig cells were determined similarly to the methodology described above. The number of germ cells per testis was calculated by dividing the absolute volume of germ cells per testis by the average volume of a germ cell. The numbers of Sertoli and Leydig cells per testis were calculated similarly by dividing the absolute volume of nuclei per testis of Sertoli and Leydig cells by the average volume of a nucleus of each cell type. As both Sertoli and Leydig cells contain

one nucleus per cell, number of nuclei per testis of each cell type is the same as the number of cells of each cell type per testis.

Average volume of a Sertoli and Leydig cell: These were calculated by dividing the absolute volume of each cell type per testis by the number of each cell type per testis.

Length of seminiferous tubules: Seminiferous tubules are cylindrical in shape and the formula for the volume of a cylinder (v) is $v = \pi \cdot r^2 \cdot h$, where $\pi \cdot r^2$ is the area of the cross-section of the cylinder, r is radius (diameter / 2), and h is the height and/or length of the cylinder. Average diameter of the seminiferous tubules in each bull calf (n = 50 per animal) was determined by ocular and slide micrometers connected to an Olympus BH-2 light microscope and the radius was calculated. Using the results of absolute volume of seminiferous tubules per testis (STv), the length of the seminiferous tubules per testis (h) was calculated as $h = STv / (\pi \cdot r^2)$. The results were expressed as length (in meters) per testis and per g of testis.

3.4.6. Inhibin antibody titer determination

Peptide ELISAs were performed as described by Kania *et al.* (1997). The volume of each reagent was 100 μL $well^{-1}$. The optimal concentration of the peptide antigens, which was unconjugated inhibin, was determined over the range of two-fold, serial dilutions from 128 μL mL^{-1} to 1 μL mL^{-1} using negative and positive serum. A dilution of 1 μL mL^{-1} was found to be optimal for the peptide. Optimal concentrations of antigen were determined with bull sera diluted 1 : 100. Peptides at a concentration of 1 μg mL^{-1}

in phosphate buffered saline (PBS) were bound to ELISA plates (Immulon 2HB 96 well plates; ThermoLab Systems, Franklin, MA, USA) overnight at 4ºC. The plates were washed 3 times with a plate washer (Elx405 AutoPlate Washer; Bio-Tek Instruments, Inc., Winooski, VT, USA) with PBS containing 0.05% polyoxyethylenesorbitan monolaurate (PBSTW) and soaked for 30 minutes at room temperature with PBSTW. Serum samples diluted 1 : 4,000 in PBSTW were incubated for 60 minutes at 37ºC. This serum concentration was determined to be optimal from a series of serum titrations over the range of 1 : 100 to 1 : 12,800 using negative and positive serum. After 3 washes with PBSTW, peroxidase-conjugated anti-bovine IgG (Bovine IgG no. A10-102P; Bethyl Laboratories Inc., Montgomery, TX, USA) diluted 1 : 10,000 in PBSTW, was added and incubated at 37ºC for 60 minutes. The plates were washed 3 times with PBSTW and then received 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (100 μL $well^{-1}$; TMB substrate no. N301; Pierce Biotechnology, Inc., Rockford, IL, USA). The substrate was allowed to incubate at room temperature for 8 minutes. The oxidation reaction was stopped by adding 100 μL of 0.18 M H_2SO_4 (sulfuric acid) to each well, which in turn changes the blue color to yellow. The optical density of the solution in each well was determined at 450 nm with a plate reader (Elx800 Universal Microplate Reader; Bio-Tek Instruments, Inc., Winooski, VT, USA). ELISA testing was performed on the following experiment days: 0, 7, 10, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 84, and 91 which represents an equal distribution of sampling points over the immunization period.

3.4.7. Gonadotropin and steroid hormone RIA

Serum was removed from storage at -20°C and assays for LH, FSH, and T were performed. Radioimmunoassays (Coat-A-Count; Diagnostic Products, Los Angeles, CA, USA) were performed to determine the concentration of testosterone (Schuenemann *et al.* 2005). Sensitivity of the assay for testosterone was 0.20 ng mL^{-1} with intra- and inter-assay coefficients of variation (CV) of 10% and 2%, respectively. Concentrations of LH and FSH were performed as described by Moura and Erickson (1997). Sensitivity of the assay for LH was 0.031 ng mL^{-1} with an intra- and inter-assay CV of 10% and 15%, respectively. Sensitivity of the assay for FSH was 0.021 ng mL^{-1} with an intra- and inter-assay CV of 14% and 16%, respectively.

3.4.8. Statistical analyses

Variation in BW, SC, LH, FSH, T, anti-inhibin antibody titers, and testicular cell types were evaluated in this study. Data were analyzed by analysis of variance using the MIXED procedure of SAS (SAS 2003). Body weight, SC, anti-inhibin antibody titers, and hormones were arranged in a completely randomized design with factorial and repeated measures. All testicular cell types were analyzed using a completely randomized design with factorial. A mixed model procedure that included vaccination treatment (INH or KLH) and hormonal treatment (Saline, FSH, or GnRH) was used to compare differences among treatments. Age of the calf at castration was used as covariate and calf

(treatment) was included as a random effect. Date of hormone analysis was included as repeated measures. Differences in individual least squares means were evaluated using Tukey-Kramer method. A $P < 0.05$ value was considered statistically significant.

3.5. Results

3.5.1. Hormones, body weight, scrotal growth, testicular weight, and inhibin antibody titers

Body weight (89.8 ± 14.2 kg), SC (14.6 ± 1.3 cm), and single testicular weight (19.2 ± 6.2 g) recorded at the end of the experimental period did not differ among treatments. Serum diluted 1 : 4000 from INH-immunized bulls (1.4 ± 0.1 OD) differed from KLH-immunized bulls (0 ± 0.1 OD; $P < 0.05$) in anti-INH antibody titers in Jersey bull calves (Fig. 3). A significant immunization X date treatment interaction was noted the first 3 weeks of the experimental period in serum anti-inhibin antibody titers in Jersey bull calves ($P < 0.05$; Fig. 3). Additionally, neither serum concentrations of LH (Fig. 4) and T (Fig. 5) differed between any treatment groups ($P < 0.05$). Serum concentrations of FSH increased at the time of FSH administration in INH+FSH and KLH+FSH (0.47 ± 0.04 and 0.48 ± 0.04 ng mL^{-1}) compared to control groups INH+Saline and KLH+Saline (0.25 ± 0.04 and 0.26 ± 0.04 ng mL^{-1}; $P < 0.05$; Fig. 6 and 7). However, concentration of FSH did not differ from the second anti-INH booster to the end of the experimental period for any treatment groups ($P > 0.05$; Fig. 6 and 7).

3.5.2. Testicular components evaluation

Representative light micrographs of testicular components in Jersey bull calves are shown (Fig. 2). Testis volumes and seminiferous tubule length did not differ between treatment groups ($P > 0.05$; Table 2). The volume densities of germ cells, blood vessels, and blood vessel lumen differed between treatment groups ($P < 0.05$; Table 2). Bull calves immunized against INH plus the administration of either FSH (5.7 ± 0.8%) or GnRH (4.8 ± 0.8%) had the higher volume densities of germ cells compared to calves immunized against KLH ($P < 0.05$; Table 2). The remaining volume densities of testicular components were not different for any treatment groups ($P > 0.05$; Table 2). Reduced blood vessel volume densities (0.32 ± 0.1%) and blood vessel lumen diameter (0.08 ± 0.06%) were observed in animals immunized against INH and not administered gonadotropins ($P < 0.05$; Table 2). However, thickening of the walls of blood vessels was unchanged for any treatment groups ($P > 0.05$; Table 2).

Increased absolute volume of germ cells per testis was observed in bull calves immunized against INH plus the administration of FSH (1220 ± 109 mm^3) compared to other treatment groups ($P < 0.05$; Table 3). The remaining absolute volumes of testicular components per testis were unchanged for any treatment groups ($P > 0.05$; Table 3).

A significant immunization x hormone treatment interaction was noted for average volume of germ cells per testis. Increased number of germ cells per testis was found in bull calves immunized against INH plus the administration of FSH (92.2 ± 9 x 10^6 cells) compared to other treatment groups ($P < 0.05$; Table 4). Surprisingly, number of Sertoli cells per testis were decreased in bull calves immunized against INH plus the

administration of FSH (1534 ± 383 x 10^6 cells) compared to other treatment groups ($P <$ 0.05; Table 4). Additionally, animals immunized against INH plus the administration of FSH had the highest average volume of a germ cell (13311 ± 320 μm^3), Sertoli cells nuclei (2066 ± 100 μm^3), and Leydig cells nuclei (1630 ± 60 μm^3; $P < 0.05$; Table 4). Administration of FSH at the time of initial immunization against inhibin significantly increased germ cells number compared to INH-Saline bulls with INH-GnRH bulls being intermediate ($P < 0.05$; Table 4). However, the average volume of a germ cell was increased in bull calves immunized against INH plus the administration of FSH ($P <$ 0.05; Table 4). This indicates that administration of FSH at the time of initial immunization against inhibin significantly increased the average volume of a germ cell and the number of germ cells. However, germ cells number and the average cell volume were not increased following hormone administration in KLH bulls ($P < 0.05$, Table 4).

3.6. Discussion

Early initiation of puberty in the bull calf has been a goal of animal scientists for many years. The main findings of this study demonstrated that 1) there was no direct relationship between plasma anti-inhibin antibody titer and FSH after 3 weeks of the experiment; 2) increased number of germ cells per testis were found exclusively in INH+FSH immunized bulls; and 3) the average volume of germ cells, Sertoli cell nuclei, and Leydig cell nuclei was increased in testes of bull calves immunized against inhibin.

Concentrations of serum FSH are similar to results previously reported (Evans *et al.* 1993; Kaneko *et al.* 2001). Results from previous studies provide clear evidence that

inhibin has a physiological role in the regulation of FSH secretion during the early stages of development in bulls (Bame *et al.* 1999; Kaneko *et al.* 2001). However, the physiological role of FSH during prepubertal development of bulls is unclear. Early secretion of FSH in bulls is thought to be dependent upon GnRH secretion (Evans *et al.* 1993). The FSH response to GnRH administration at 1 month of age is smaller than in other older animals (Schams *et al.* 1981). FSH is necessary to develop the androgen binding protein production by Sertoli cells and to develop the blood-testis barrier (an indirect effect on Sertoli cell function and proliferation). Previous studies on bulls have shown that immunization against inhibin in 3- to 13-mo old bulls increased daily sperm production and the amount of FSH secreted (Martin *et al.* 1991; Kaneko *et al.* 1993; Bame *et al.* 1999; Kaneko *et al.* 2001). In the present study, only administration of FSH (regardless of immunization treatment) resulted in increased FSH concentrations for brief period of time; thereafter, the amount of FSH remained unchanged to the end of the experimental period.

The major production site of inhibin in the testis is Sertoli cells (Kaneko *et al.* 2001). Inhibin secretion may have a direct effect on spermatogenesis by decreasing FSH secretion during early stage of testicular development. Inhibin participates in the regulation of FSH secretion during the early prepubertal stage in bulls (Kaneko *et al.* 2001). Immunization against inhibin in bull calves increased anti-inhibin antibody during titers the first 3 weeks of the experiment and remained high to end of the experimental period. However, the amount of FSH remains unchanged after the second immunization booster among treatment groups for unknown reasons. The present immunization study (utilizing the same bovine inhibin $\alpha^{1\text{-}26}$ as previously reported (Bame *et al.* 1999) with a

different carrier, KLH) clearly indicates that FSH amount remained unchanged after the second immunization booster regardless of treatment combination as reported in previous studies (Evans *et al.* 1993; Bame *et al.* 1999; Kaneko *et al.* 2001). Testosterone concentrations increased at the end of the experimental period for all treatment groups. Leydig cells are responsible for the synthesis of testosterone (Abraham 1991; Payne *et al.* 1995) in response to LH stimulation. In this study, amount of LH remained unchanged among treatments; however, the increased testosterone concentrations at the end of the experiment may be explained by an increase in LH pulse frequency (not determined in the present study).

The most important finding of the present study is the increased germ cell number per testis in 4-mo old bull calves immunized against inhibin at the time of gonadotropin administration. Increased germ cell number per testis is likely responsible for the increase in daily sperm production after puberty as previously reported (Martin *et al.* 1991; Kaneko *et al.* 1993; Bame *et al.* 1999; Kaneko *et al.* 2001). The Sertoli cell is recognized for important contributions to the support of spermatogenesis, and studies with young beef bulls (Berndtson et al., 1987*a*) have revealed large, positive correlations between the total number of Sertoli cells and daily sperm production. Additionally, Berndtson *et al.* (1987*b*) suggested that total Sertoli cell number may be an important determinant of bull's spermatogenic potential. In this study, Sertoli cell number was decreased in bull immunized against inhibin plus FSH administration. Interestingly, immunization against inhibin plus gonadotropin administration favors germ cell development and proliferation. This increased germ cell number per testis could be explained, in part at least, by the increased volume of Sertoli and Leydig cells per testis and the increase in size of each

cell type. Theoretically, increased absolute volume of Sertoli cells per testis is able to support more germ cells; therefore, this parameter may be more important for increased sperm production than the Sertoli cell number. The lower number of Sertoli cells per testis may be due to the possibility that Sertoli cells undergo an early maturation process in bulls immunized against inhibin plus FSH administration; thus losing the ability to proliferate and initiate functions that are essential for support of germ cells during spermatogenesis (Sharpe *et al.* 2003). The onset of puberty in bulls may be also associated with a dramatic increase in mean Sertoli and Leydig cell volume and a peak in the steroid-producing capacity per Leydig cell as shown in boars (Lunstra *et al.* 1986).

In conclusion, these results suggest that gonadotropin administration at the time of inhibin immunization increases germ cell number and the absolute volume of Sertoli, Leydig, and germ cells per testis together with increase in size (average volume of a cell) of all three cell types. Furthermore, the present findings have the potential to develop an alternative therapy to benefit germ cell colonization after stem cell transplantation.

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3.8. Appendix: Figures and Tables

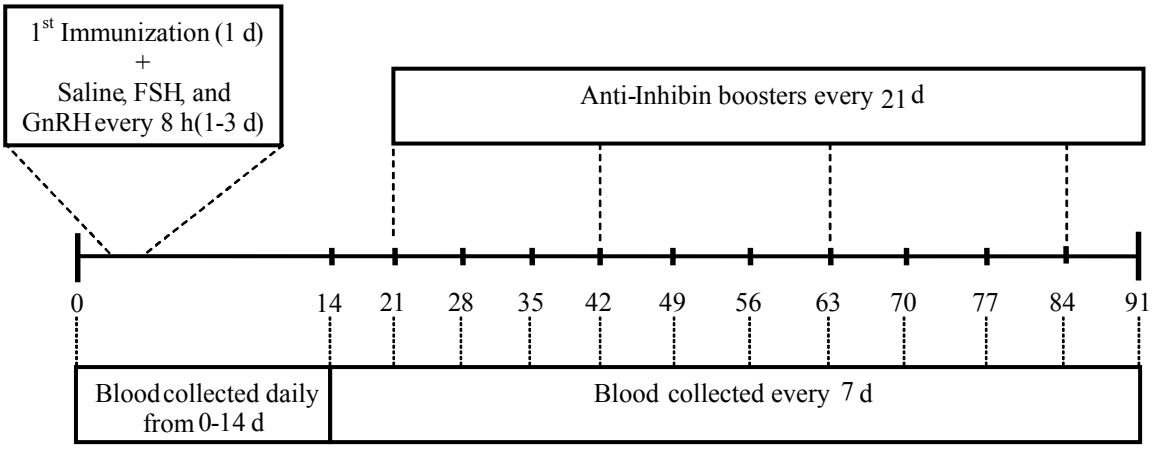

Figure 1. Timeline of primary treatment assignment in Jersey bull calves immunized against INH or KLH plus gonadotropin sub-treatment administration.

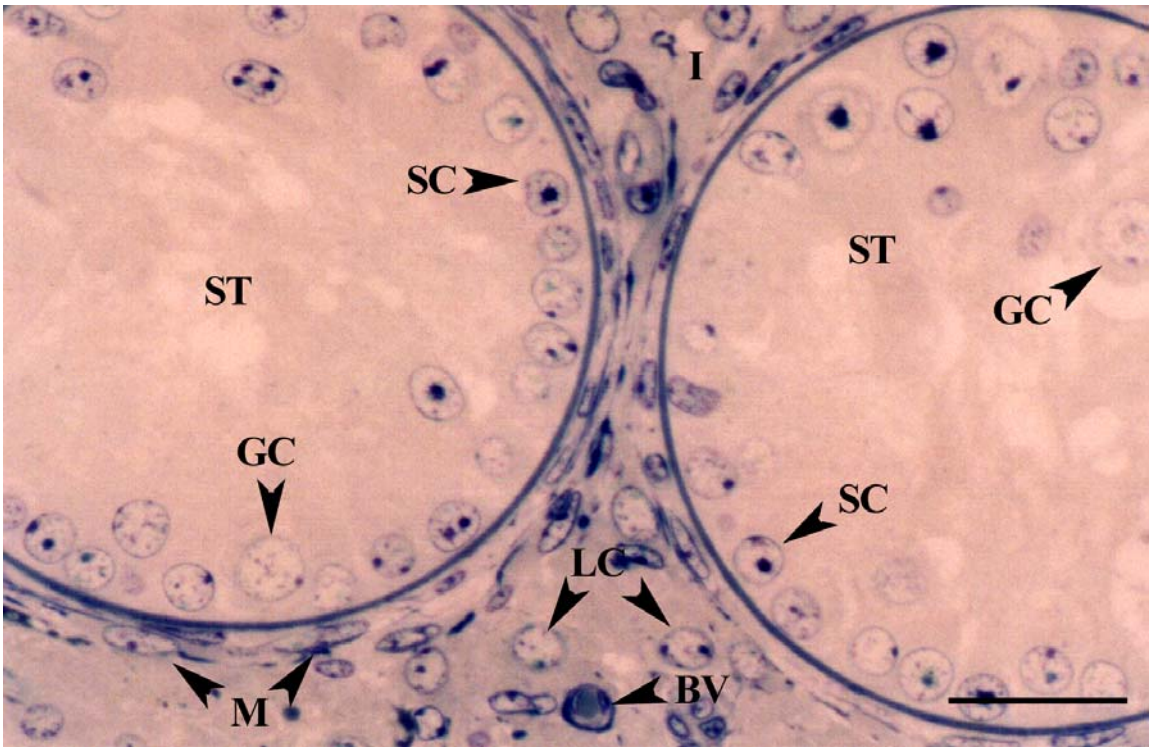

Figure 2. A representative light micrograph of testis tissue of a 4 month old Jersey bull calf showing testicular components. In the seminiferous tubules (ST), germ cells (GC) are fewer in number compared to Sertoli cells (SC). At this stage, lumen is not seen. Leydig cells (LC), myoid cells (M), and blood vessel (BV) are present in the interstitial space (I) of the testis. Bar represent 40 μm.

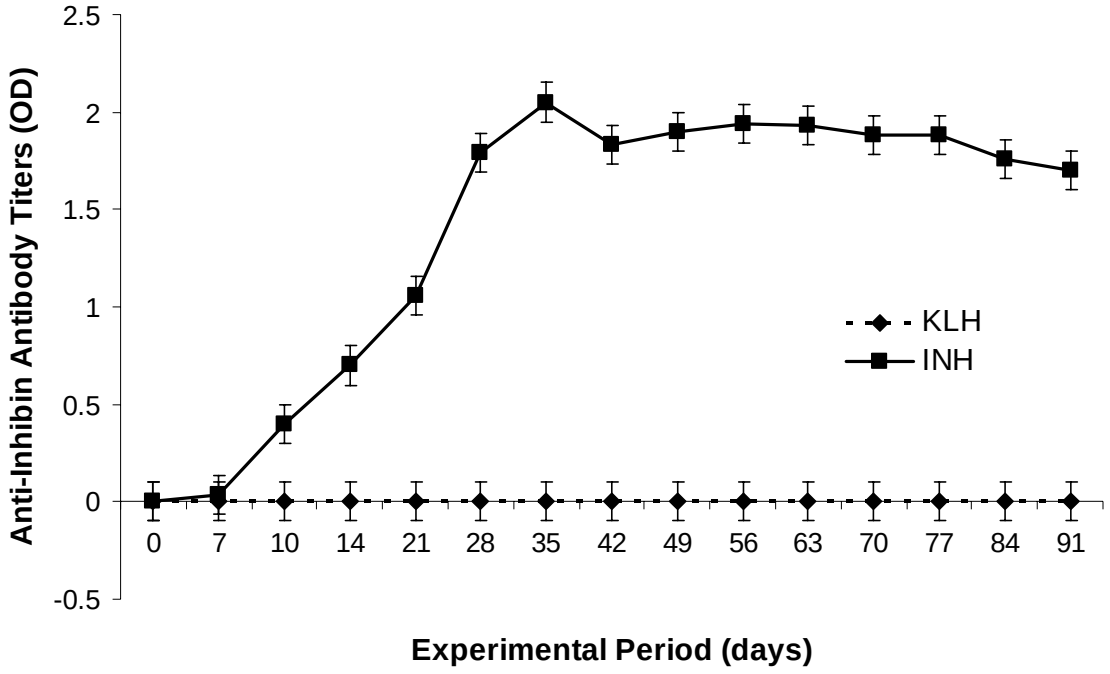

Figure 3. Variation in serum anti-INH antibody titers in Jersey bull calves immunized against INH or KLH treatment groups. Values (mean ± SEM) differ from day 10 throughout the experimental period ($P < 0.05$).

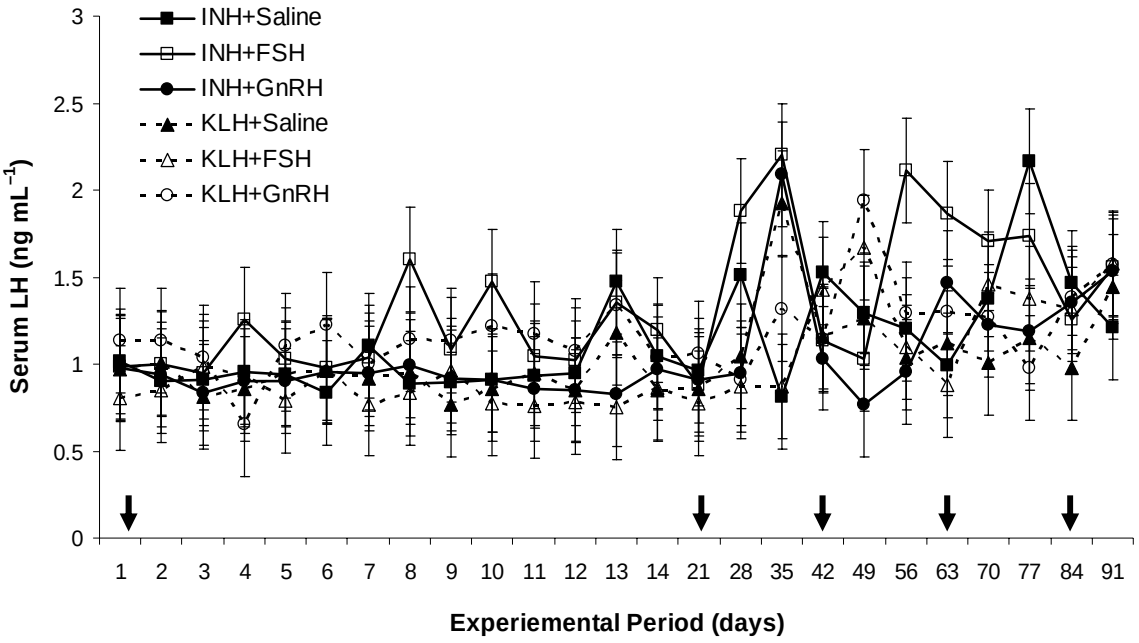

Figure 4. Daily and weekly concentrations of LH (mean ± SEM) in Jersey bull calves immunized against INH or KLH plus gonadotropin administration among treatment groups. Arrows indicate immunization days.

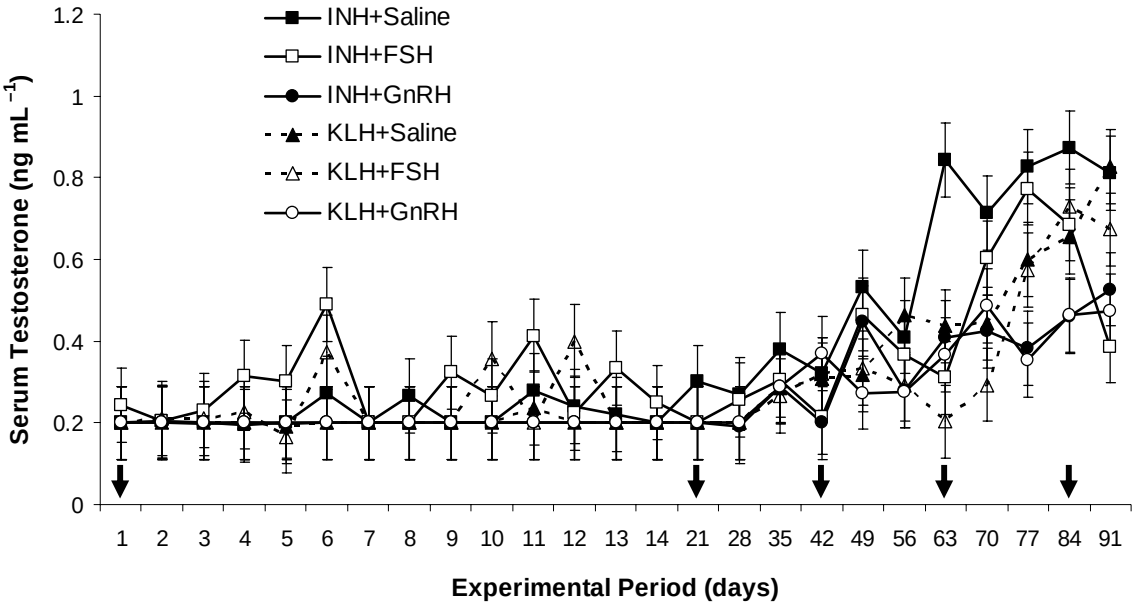

Figure 5. Daily and weekly concentrations of testosterone (T; mean ± SEM) in Jersey bull calves immunized against INH or KLH plus gonadotropin administration among treatment groups. Arrows indicate immunization days.

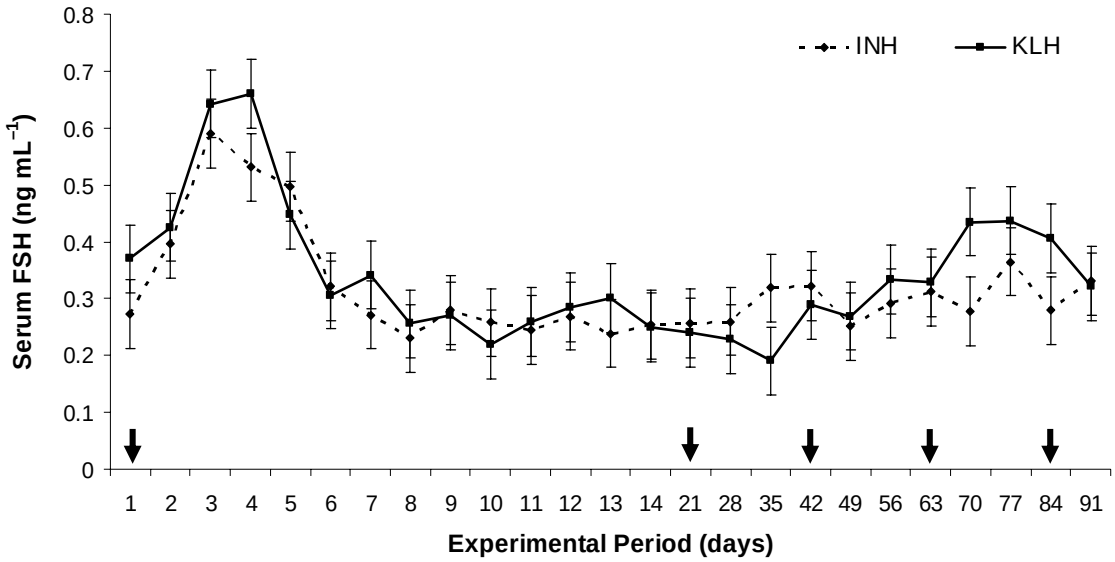

Figure 6. Overall daily and weekly concentrations of FSH (mean ± SEM) in Jersey bull calves immunized against INH or KLH plus gonadotropin administration among treatment groups. Arrows indicate immunization days.

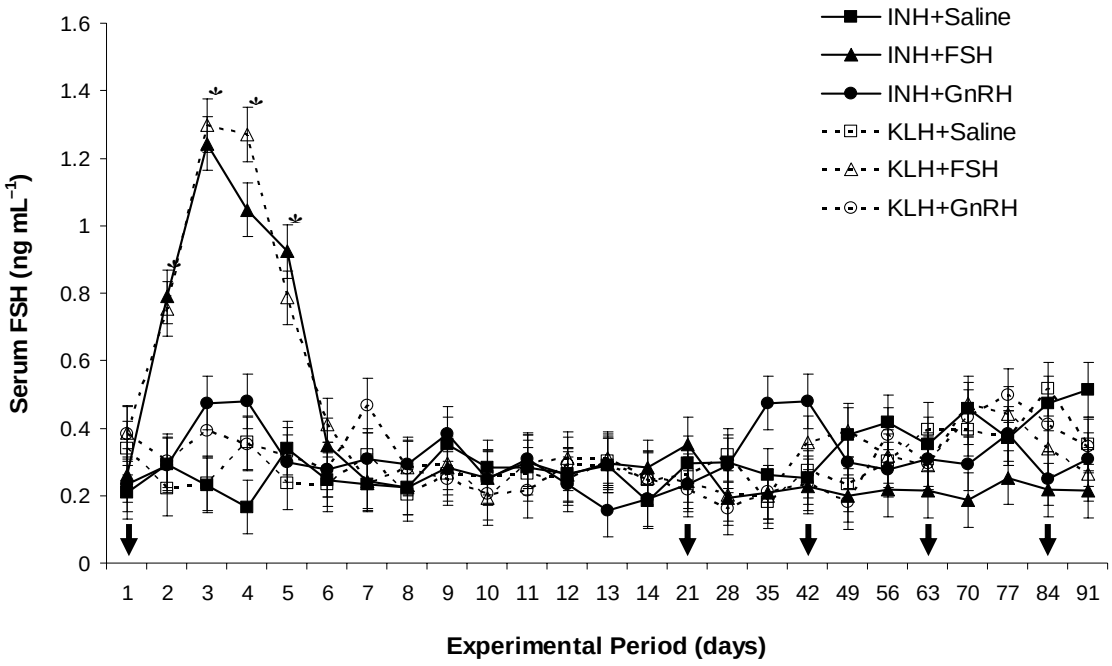

Figure 7. Daily and weekly concentrations of FSH (mean ± SEM) in Jersey bull calves immunized against INH or KLH plus gonadotropin administration among treatment groups. Arrows indicate immunization days. *Asterisks are significantly different ($P < 0.05$).

Tables

Table 1. Assignment of primary treatments in Jersey bull calves immunised against inhibin or keyhole limpet haemocyanin plus gonadotropin subtreatment administration.

Primary TRT	Sub-TRT	Day 1	Day 1–3 (Every 8 h)	Day 21	Day 42	Day 63	Day 84	Day 91
INH (n = 9)	1. Saline (n = 3)	Immunisation (INH)	Saline (1 mL)	Booster immunisations FICA	Booster immunisations FICA	Booster immunisations FICA	Booster immunisations FICA	Castration
	2. FSH (n = 3)		FSH (20 mg)					
	3. GnRH (n = 3)		GnRH (50 μg)					
KLH (n = 9)	4. Saline (n = 3)	Immunisation (KLH)	Saline (1 mL)					
	5. FSH (n = 3)		FSH (20 mg)					
	6. GnRH (n = 3)		GnRH (50 μg)					

Primary treatment (TRT): Jersey bull calves were either immunised against inhibin (INH) hormone or immunised against the keyhole limpet haemocyanin (KLH)-conjugated adjuvant. Sub-treatment (TRT): Subsets of calves were randomly assigned within primary treatments (TRT) to receive saline, follicle-stimulating hormone (FSH) or gonadotrophin-releasing hormone (GnRH) every 8 h (0600, 1400 and 2200 hours) from Day 1 to Day 3 of the study. Day 1: Primary treatments administered to Jersey bull calves (initial immunisation at 27 ± 5 days of age; Day 1 of the experimental period) consisted of control (KLH; 250 μg; n = 9) or immunisation (INH; 500 μg INH : 250 μg KLH; n = 9), with both emulsified in 2 mL Freund's complete adjuvant (FCA). Days 21–84: Booster immunisations with Freund's incomplete adjuvant (FICA) occurred every 21 days, with the last administration on Day 84 of the trial. Day 91: Castration and testes collection

Table 2. Mean testis volume, volume density of testicular components, and seminiferous tubule length. In each row, values (mean±s.e.m.) with different superscript letters are significantly different ($P<0.05$).

Parameter	Treatment groups					
	INH+Saline	INH+FSH	INH+GnRH	KLH+Saline	KLH+FSH	KLH+GnRH
Testis volume (mm^3)	21810.5±4827[a]	23094.1±7482[a]	17274±2191[a]	21065±3925[a]	16448.7±6031[a]	15977.3±1720[a]
Seminiferous tubules (%)	62.7±2.2[a]	61.8±2.2[a]	63.2±2.2[a]	62±2.2[a]	61.6±2.2[a]	57.6±2.2[a]
Seminiferous tubule cells (%)	17.4±1.2[a]	19.4±1.2[a]	20.3±1.2[a]	17.9±1.2[a]	16.8±1.2[a]	16.6±1.2[a]
Sertoli cells (%)	14.5±0.9[a]	13.6±0.9[a]	15.5±0.9[a]	13.9±0.9[a]	14.3±0.9[a]	14±0.9[a]
Germ cells (%)	3.2±0.8[b]	5.7±0.8[a]	4.8±0.8[a]	3.8±0.8[b]	2.5±0.8[b]	2.7±0.8[b]
Seminiferous tubule length per gram of testis (m)	448±91[a]	398±91[a]	689±91[a]	422±91[a]	512±91[a]	423±91[a]
Seminiferous tubule length per testis (m)	10117±3730[a]	10515±3728[a]	12386±3729[a]	9013±3729[a]	11020±3729[a]	7252±3729[a]

INH+FSH, immunisation against inhibin (INH) plus follicle-stimulating hormone (FSH) administration; INH+GnRH, immunisation against INH plus gonadotrophin-releasing hormone (GnRH) administration; INH+saline, immunisation against INH plus saline administration; KLH+FSH, control conjugated to keyhole limpet haemocyanin (KLH) plus FSH administration; KLH+GnRH, control conjugated to KLH plus GnRH administration; KLH+saline, control conjugated to KLH plus saline administration.

Table 2, continued

Parameter	Treatment groups					
	INH+Saline	INH+FSH	INH+GnRH	KLH+Saline	KLH+FSH	KLH+GnRH
Interstitial cells (%) (all cell types)	35.9±1.7[a]	36.3±1.5[a]	34.9±1.6[a]	36.7±1.6[a]	36.3±1.6[a]	38.6±1.6[a]
Blood vessels (%)	0.32±0.1[c]	0.52±0.1[bc]	0.77±0.1[ab]	0.43±0.1[bc]	0.76±0.1[abc]	1.1±0.1[a]
Blood vessels lumen (%)	0.08±0.06[c]	0.2±0.05[abc]	0.3±0.05[a]	0.1±0.05[bc]	0.3±0.06[ab]	0.3±0.05[a]
Blood vessel wall (%)	0.2±0.1[a]	0.2±0.1[a]	0.3±0.1[a]	0.2±0.1[a]	0.4±0.1[a]	0.6±0.1[a]
Leydig cells (%)	32.8±1.5[a]	32.8±1.3[a]	31.5±1.4[a]	33.4±1.4[a]	33.6±1.4[a]	35.5±1.4[a]
Macrophages (%)	0.9±0.2[a]	1.2±0.2[a]	1±0.2[a]	1±0.2[a]	0.8±0.2[a]	0.8±0.2[a]
Mesenchimal cells (%)	2.1±0.2[a]	2.2±0.2[a]	2.3±0.2[a]	2.2±0.2[a]	1.9±0.2[a]	2.3±0.2[a]

INH+FSH, immunisation against inhibin (INH) plus follicle-stimulating hormone (FSH) administration; INH+GnRH, immunisation against INH plus gonadotrophin-releasing hormone (GnRH) administration; INH+saline, immunisation against INH plus saline administration; KLH+FSH, control conjugated to keyhole limpet haemocyanin (KLH) plus FSH administration; KLH+GnRH, control conjugated to KLH plus GnRH administration; KLH+saline, control conjugated to KLH plus saline administration.

Table 3. Mean absolute volume of testicular components. In each row, values (mean±s.e.m.) with different superscript letters are significantly different ($P<0.05$).

Parameter	Treatment groups					
	INH+Saline	INH+FSH	INH+GnRH	KLH+Saline	KLH+FSH	KLH+GnRH
Seminiferous tubules (mm^3)	13149±1749^a	14788±1744^a	11187±1744^a	12806±1744^a	11857±1744^a	9780±1744^a
Seminiferous tubule cells (mm^3)	3584±435^a	4390±430^a	3600±432^a	3648±431^a	3079±431^a	2818±433^a
Sertoli cells (mm^3)	2999±434^a	3171±431^a	2769±432^a	2895±432^a	2632±432^a	2403±433^a
Germ cells (mm^3)	641.3±117^{bc}	1220±109^a	839.6±112^b	738.9±111^{bc}	448.9±114^c	454.5±113^c
Interstitial space (mm^3)	7835±1191^a	8937±1194^a	6510±1187^a	7962±1186^a	6761±1188^a	7016±1188^a
Interstitial cells (mm^3)	7621±1141^a	8488±1134^a	6182±1133^a	7622±1135^a	6501±1138^a	6433±1138^a
Blood vessels (mm^3)	68.2±34^a	126±32^a	136±33^a	88.2±32^a	135±33^a	193.5±33^a
Blood vessels lumen	21.4±18^a	65±17^a	72.6±17^a	39.5±17^a	52.8±18^a	69±17^a
Blood vessel wall	46.9±25^a	59.8±24^a	65.6±24^a	48.2±24^a	83.2±24^a	124.3±24^a
Leydig cells	7003±1055^a	7652±1046^a	5588±1049^a	6936±1049^a	6031±1051^a	5928±1051^a
Macrophages (mm^3)	187.3±46^a	284.6±44^a	178.5±45^a	221.2±44^a	137.4±45^a	130.4±45^a
Mesenchimal cells (mm^3)	442.9±71^a	541±69^a	413.4±70^a	465.8±69^a	332.5±70^a	375.9±70^a

INH+FSH, immunisation against inhibin (INH) plus follicle-stimulating hormone (FSH) administration; INH+GnRH, immunisation against INH plus gonadotrophin-releasing hormone (GnRH) administration; INH+saline, immunisation against INH plus saline administration; KLH+FSH, control conjugated to keyhole limpet haemocyanin (KLH) plus FSH administration; KLH+GnRH, control conjugated to KLH plus GnRH administration; KLH+saline, control conjugated to KLH plus saline administration.

Table 4. Mean cell number per testis and average volume of Leydig, Sertoli, and germ cells.

Parameter	Treatment groups					
	INH+Saline	INH+FSH	INH+GnRH	KLH+Saline	KLH+FSH	KLH+GnRH
Leydig cell number (x 10^6)	5316±815^{a}	4700±805^{a}	5334±810^{a}	5755±810^{a}	5837±812^{a}	5395±812^{a}
Sertoli cell number (x 10^6)	2432±386^{ab}	1534±383^{b}	3249±384^{a}	2789±384^{a}	2909±385^{a}	2229±385^{ab}
Germ cell number (x 10^6)	54.9±10^{b}	92.2±9^{a}	64.5±9^{ab}	66.5±9^{ab}	50.3±9^{b}	45±9^{b}
Average volume of a Leydig cell nuclei (μm^3)	1318±60^{ab}	1630±60^{a}	1048±60^{b}	1204±60^{ab}	1033±60^{b}	1098±60^{b}
Average volume of a Sertoli cell nuclei (μm^3)	1238±120^{b}	2066±100^{a}	852±60^{c}	1038±100^{bc}	905±60^{c}	1078±100^{bc}
Average volume of a germ cell (μm^3)	11999±440^{c}	13311±320^{a}	13042±370^{b}	11013±340^{d}	8995±380^{f}	10083±370^{e}

In each row, values (mean±s.e.m.) with different superscript letters are significantly different (P<0.05). INH+FSH, immunisation against inhibin (INH) plus follicle-stimulating hormone (FSH) administration; INH+GnRH, immunisation against INH plus gonadotrophin-releasing hormone (GnRH) administration; INH+saline, immunisation against INH plus saline administration; KLH+FSH, control conjugated to keyhole limpet haemocyanin (KLH) plus FSH administration; KLH+GnRH, control conjugated to KLH plus GnRH administration; KLH+saline, control conjugated to KLH plus saline administration.

Chapter 4 – RECIPIENT PREPARATION FOR SPERMATOGONIAL STEM CELL TRANSPLANTATION: ALTERATION IN TESTICULAR CELL COMPONENTS FOLLOWING TRANSIENTLY INDUCED ISCHEMIA IN BULLS

This chapter is a revised version of a paper by the same name submitted for publication in the Journal *Reproduction, Fertility, and Development* in 2008 by Gustavo Schuenemann, Charmindrami Mendis-Handagama, Tulio Prado, and Neal Schrick.

My use of "we" in this chapter refers to my co-authors and myself. My primary contributions to this paper include (1) aided in design of the experiment and data analysis (2) sample and tissue collection, (3) identification of cell types, (4) collection and interpreting of the literature, (5) writing of this paper.

4.1. Acknowledgments

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4.2. Abstract

The aim of the present study was to evaluate testicular transiently induced ischemia (using elastrator bands) in Jersey calves on testicular morphology and development. Treatments (at 27 ± 5 days of age) consisted of control (0, $n = 4$), banding for 2 h (2, $n = 4$), 4 h (4, $n = 4$), and 8 h (8, $n = 4$) periods. After castration (age: 60 ± 5 days), the right testis was used for calculation of cell components per testis according to the point counting method. Data were analyzed using MIXED procedure of SAS

program. Bodyweight (59.8 ± 6.2 kg) and SC at banding (9.1 ± 0.2 cm) did not differ between treatments. Fresh testis weight (TW), scrotal temperature immediately before band removal (ST), and daily scrotal circumference growth (SC) were decreased (4 and 8 h) in ischemic testes compared to controls ($P < 0.05$). In addition, Sertoli and Leydig cells were severely reduced in the 8 h ischemic treatment ($P < 0.05$). Transiently induced ischemia significantly decreased number of germ cells in 8 h (12.6 ± 5 x 10^6 cells) compared to 0 (38.1 ± 6 x 10^6 cells), 2 (31.9 ± 6 x 10^6 cells), and 4 h (33.4 ± 5 x 10^6 cells; $P < 0.05$). These results suggest that transiently induced ischemia significantly decreases number of germ, Sertoli and Leydig cells in the testis.

Key words: germ cell, testis development, recipient animals, spermatogonial transplantation.

4.3. Introduction

Spermatogenesis is a highly organized complex process that involves the production of spermatozoa through a continual supply of A-spermatogonia. There are three primary phases in spermatogenesis that occur in the seminiferous tubule (Amann 1983) that include spermatogonial multiplication (mitosis), meiosis, and release of the sperms to the lumen of the seminiferous tubule (spermiogenesis). Spermatogenesis is supported by both spermatogonial stem cells and the somatic components of the testis (Amann 1983).

Male germ cells transplantation in livestock has progressed tremendously in the past few years (Honaramooz *et al.* 2003a, 2003b; Izadyar *et al.* 2003; Joerg *et al.* 2003; Hill and Dobrinski 2006). However, the efficiency of colonization of seminiferous tubules by the transplanted germ cells is low and variable (Dobrinski *et al.* 2000; Nogano, *et al.* 2002; Izadyar *et al.* 2003; Joerg *et al.* 2003; Hill *et al.* 2005). Depletion of endogenous spermatogonial stem cells using busulfan (Brinster *et al*. 2003) or irradiation (Izadyar *et al*. 2003) have both been used in preparation of recipient animals prior to transplantation; however, both techniques are not without problems (severe bone morrow depression or specialized radiotherapy equipment required). This emphasizes the necessity to find practical means for depleting endogenous germ cells in recipient animals that will result in adequate donor cell colonization environment without the need for further intervention (Hill and Dobrinski 2006).

Many reports are available on testicular components in the developing postnatal rat (Roosen-Runge *et al.* 1959; Mendis-Handagama *et al.* 1998), cat (França and Godinho 2003), bulls (Curtis and Amann 1981), and other mammals (Russell *et al.* 1990). However, characterization of the testicular components of prepubertal bovine bulls following transiently induced ischemia is not available. Induced testicular ischemia in rams altered spermatic epithelium with germ cell-depleted seminiferous tubules (Markey *et al*. 1994). These data are important for understanding the dynamics of the cell-cell interaction, proliferation, and differentiation in the testis.

Consequently, the objective of the current study was to evaluate the effects of transiently induced ischemia on testicular components and depleting endogenous germ cells in the testes of prepubertal bull calves.

4.4. Materials and methods

4.4.1. Animals and treatments

Sixteen Jersey bull calves were paired by birth date at 27 ± 5 d of age. The bulls were born and raised exclusively at the Dairy Research and Education Center (Lewisburg, TN, USA). During the first month of life, animals were isolated from each other to reduce pathogen transmission. At birth, calves received colostrum in a bottle for the first 48 hours of life (1.2 liters / twice a day). During the initial 8 weeks of life, animals were fed whole waste milk (1.2 liters / twice a day) and offered a starter ration (Tennessee Farmers Cooperative, Lavergne, TN, USA) and a primer ration (Tennessee Farmers Cooperative, Lavergne, TN, USA) *ad libitum*. Weaning occurred at 8 weeks of age with calves placed in group pens and fed only the primer ration *ad libitum* for the remainder of the experiment.

Calves were randomly assigned into four (4) treatment groups (n = 4 calves / treatment) at 27 days of age. The study was performed using the elastrator method to induce different periods of transiently induced ischemia by decreasing blood supply to the testes with a heavy green rubber band. Treatments (Fig. 1; initial banding at 27 ± 5 days of age; Day 1 of the experimental period (All tables and figures in this chapter appear in the appendix)) consisted of control group (0, no band application, n = 4), banding for a period of 2 h (2, n = 4), 4 h (4, n = 4), and 8 h (8, n = 4). Bulls were

castrated at 30 days after banding and testes collected for further histological analysis. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee, University of Tennessee, Knoxville, USA (UT-IACUC no. 1324).

4.4.2. Bodyweight, scrotal growth, and scrotal temperature

Bodyweight (BW) and scrotal circumference (SC; Hahn *et al*., 1996) were measured at banding (approximately 1 month of age) and before testes removal (Table 1; approximately 2 month of age). The testes were weighed and used for absolute volume calculation of cell components per testis. Scrotal temperatures (ST; Schuenemann *et al*. 2005) were determined immediately before banding, immediately before band removal, and 1 hour after band removal using an infrared thermography camera (eMerge Vision DTIS 500, eMerge International Inc., Sebastian, FL, USA). The camera had an opaque chopper and internal calibration that allowed determination of absolute temperatures. Recorded images (Fig. 2) were analyzed by EResearch software. In brief, images were imported into the software and a region of interest (ROI) was drawn on each testis. The same region was taken on each testis and each ROI contained the same number of pixels. This information allowed the program to calculate absolute temperature for each ROI.

4.4.3. Collection and processing of testis tissue

At Day 30 of the study (approximately 2 month of age), bulls were castrated and both testes were removed and weighed (fresh testicular weight; TW). Immediately after castration, testicular cords were visually inspected at the banding site for abnormalities in the vascular plexus. The testes were perfusion fixed by cannulation of the artery. First, a solution (250 mL) of 0.9% NaCl was flushed through the tissue for about 10 minutes to allow blood to clear from testicular vessels. When the testis was clear of blood, a solution (250 mL) containing 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) was administered through the cannula as described by Mendis-Handagama *et al.* (1988) and Schuenemann *et al*. (2007).

After perfusion fixation was completed, testes were placed in a plastic container immersed into the same fixative solution for 1 week. Then, the tunica albuginea was nicked using a #10 blade scalpel and placed back into the container for 1 week. Testes were weighed with and without the testicular capsule (tunica) before tissue processing for microscopy. From each fixed testicle, 10 tissue samples (approximately 2-3 mm cubes) were cut and post-fixed in a 1:1 mixture of 2% aqueous osmium tetroxide and 3% potassium ferrocyanide (Russell and Burguet 1977). Tissue samples were then dehydrated in a series of graded ethanols and embedded in epon-araldite (Electron Microscopy Sciences, Hatfield, PA, USA) as described by Mendis-Handagama and Ewing (1990). The polymerization of the tissue blocks took place in an oven at 60ºC for 48 h (Mendis-Handagama and Ewing 1990).

4.4.4. Testis histology and morphometry

From each polymerized tissue block (10 per testis), two 1-μm sections were cut using a LKB IV ultramicrotome (Pharmacia LKB, Piscataway, NJ, USA) and glass knives. Sections were mounted on pre-cleaned glass slide (Superfrost Plus; Fisher Scientific, Pittsburgh, PA, USA), stained with methylene Blue-Azure II and cover-slipped under permount (Fisher Scientific, Pittsburgh, PA, USA). The different cell types in the testicular interstitium and inside the seminiferous tubules were identified by their morphological characteristics as described previously (Mendis-Handagama *et al.* 1987, 1998; Ariyaratne *et al.* 2000). In total, 20 1-μm sections (two per block) per testis were used to evaluate testicular components using the point-counting method (Weibel 1980).

Volume of components: The volume density of testicular components (defined as the volume of a component per unit volume of testis tissue) was obtained by the point-counting method (Weibel 1980) using an ocular grid with 88 test points fitted to a color video monitor at x400 magnification. To determine the volume density of components of the seminiferous tubules and the testis interstitium, 10 randomly selected ocular fields of each section were scored (10 fields section X 10 blocks testis X 88 points = 8800 points per testis per bull). The absolute volume (mm^3) occupied by each testicular component was calculated by multiplying the volume density of each component by the testis volume (without the capsule). Because the testis density is nearly 1.0 (range 1.03-4), subsequent morphometric calculations of the testis weight were considered equal to testis volume.

Average volume of a germ cell, and the nucleus of a Sertoli and Leydig cell: The germ cells, and the nuclei of Sertoli and Leydig cells in these tissue sections were reasonably circular to justify that they are spherical or close to spherical in configuration. Therefore, the following methodology was used to determine their average volume. Images of germ cells, Sertoli cells and Leydig cells in the methylene blue-stained tissue sections were displayed on a color video monitor using a color video camera (DXC-107A; Sony Corporation, Tokyo, Japan). The diameters of germ cells, and nuclei of Sertoli and Leydig cells were measured using ocular and slide micrometers (n = 50 per animal). The average volume (v) of a germ cell, the nucleus of a Sertoli cell and the nucleus of a Leydig cell was calculated using the formula that determines the volume of a sphere, $v = 4 / 3 . \pi . r^3$, where r is cell diameter/2 for germ cell or nuclear cell diameter/2 for Leydig and Sertoli cells. Results are expressed in μm^3.

Number of germ, Sertoli and Leydig cells per testis: The absolute volumes of germ cells, and the nuclei of Sertoli and Leydig cells were determined similar to the methodology described above. The number of germ cells per testis was calculated by dividing the absolute volume of germ cells per testis by the average volume of a germ cell. The numbers of Sertoli and Leydig cells per testis were calculated by dividing the absolute volume of nuclei per testis of Sertoli and Leydig cells by the average volume of a nucleus of each cell type. As both Sertoli and Leydig cells contain one nucleus per cell, number of nuclei per testis of each cell type is the same as the number of cells of each cell type per testis.

Average volume of a Sertoli and Leydig cell: These were calculated by dividing the absolute volume of each cell type per testis by the number of each cell type per testis.

Length of seminiferous tubules: Seminiferous tubules are cylindrical in shape and the formula for the volume of a cylinder (v) is $v = \pi \cdot r^2 \cdot h$, where $\pi \cdot r^2$ is the area of the cross-section of the cylinder, r is radius (diameter/2), and h is the height and/or length of the cylinder. Average diameter of the seminiferous tubules in each bull calf (n = 50 per animal) was determined by ocular and slide micrometers connected to an Olympus BH-2 light microscope and the radius was calculated. Using the results of absolute volume of seminiferous tubules per testis (STv), the length of the seminiferous tubules per testis (h) was calculated as $h = STv / (\pi \cdot r^2)$. The results were expressed as length (m) per testis and per g of testis.

4.4.5. Statistical analyses

Variation in BW, SC, ST, TW, regressed spermatic epithelium, and testicular cell types were evaluated in this study. Data were analyzed by ANOVA using the MIXED procedure of SAS program (SAS 2003). Bodyweight, SC, ST, TW, regressed spermatic epithelium, and testicular cell types were analyzed using a randomized block design with factorial. A mixed model procedure that included ischemia treatments (0, 2, 4, or 8 h) was used to compare differences among treatments. Age of the calf at castration was used as covariate and date of castration was included as a random effect. Differences in individual least squares means were evaluated using Tukey-Kramer method. A value of $P < 0.05$ was considered statistically significant.

4.5. Results

4.5.1. Effects of treatments on testicular blood flow, scrotal growth, and scrotal temperature

Body weight (59.8 ± 6.2 kg), SC at banding (9.1 ± 0.2 cm), and ST immediately before banding did not differ between treatments. However, single fresh testicular weight (6.6 ± 0.4 g; Fig. 3), SC growth (0.01 ± 0.007 cm d^{-1}), and SC at the time of castration (9.5 ± 0.2 cm) were decreased in bull calves subjected to 8 h of transiently induced ischemia ($P < 0.05$; Table 1). Scrotal temperature immediately before band removal decreased in bull calves exposed to 4 h (28.9 ± 0.6^{o}C) and 8 h (28.5 ± 0.6^{o}C) of transiently induced ischemia treatments ($P < 0.05$; Table 1), suggesting a reduction of blood supply to the testes (Fig. 2). Conversely, increased temperatures were observed in bull calves subjected to 4 h (33.7 ± 0.4^{o}C) and 8 h (34.2 ± 0.4^{o}C) of transiently induced ischemia 1 h after band removal ($P < 0.05$; Table 1).

4.5.2. Evaluation of testicular components

Representative light micrographs of testicular tissue cross section in 2-month of age Jersey bull calves are shown in Fig. 5. Reduced testis volumes and seminiferous tubule cells and diameter were observed in prolonged (8 h) ischemic group ($P < 0.05$; Table 2). Seminiferous tubules, seminiferous tubule length, and macrophages did not

differ between treatment ($P > 0.05$; Table 2). However, the volume densities of germ cells, Sertoli cells, Leydig cells, and mesenchimal cells differed between treatment groups ($P < 0.05$; Table 2). Bull subjected to 8 h (0.7 ± 0.6%) of ischemia had the lowest volume densities of germ cells compared to control (2.7 ± 0.6%) bulls ($P < 0.05$; Table 2). The same trend was observed for Leydig and Sertoli cells ($P < 0.05$; Table 2 and Fig. 3). Increased blood vessel volume densities (1.4 ± 0.1%), blood vessel lumen diameter (0.78 ± 0.1%), and the thickening of the walls of blood vessels (0.61 ± 0.08%) were observed in 8 h ischemic group compared to control testes ($P < 0.05$; Table 2).

Decreased absolute volume of germ (48.2 ± 45 mm^3), Sertoli (617±77 mm^3), and Leydig (1224 ± 167 mm^3) cells per testis were observed in 8 h bull calves compared with the other treatment groups ($P < 0.05$; Table 3). The same trend was observed for the remaining absolute volumes of testicular components per testis in 8 h ischemic group ($P < 0.05$; Table 3). Increased absolute volumes of blood vessel and blood vessel lumen were observed in bull subjected to prolonged ischemic period ($P < 0.05$; Table 3).

Bull calves exposed to 8 h of transiently induced ischemia had the lowest number of germ, Sertoli, and Leydig cells per testis ($P < 0.05$; Fig. 4). Interestingly, the number of germ cells was reduced by approximately 80% in the 8 h ischemic group while Sertoli and Leydig cells were reduced approximately 50% ($P < 0.05$; Fig. 4). Furthermore, the 8 h treatment resulted in the lowest average volume of Sertoli (915 ± 112 μm^3) and Leydig (758 ± 50 μm^3) cells nuclei while the average volume of germ cells remain unchanged in the same treatment group (4228 ± 975 μm^3; $P < 0.05$; Fig. 4). This indicates that 8 h transiently induced ischemia at one month of age significantly reduced the number of germ cells per testis in Jersey bulls ($P < 0.05$; Table 4 and Fig. 4). However, similar

trends were observed in the number of Sertoli and Leydig cells and the average cell volume in 8 h group ($P < 0.05$; Table 4 and Fig. 4).

The spermatic epithelium is composed primarily of Sertoli cells, germ cells, and an array of committed spermatocytes and spermatids. At two months of age (day of castration), the spermatic epithelium inside the seminiferous tubules (130 μm, average diameter) is formed by a single layer of rounded Sertoli cells nuclei (12 μm, average nuclei diameter) on the basement of the tubules and rounded scattered germ cells (19 μm, average cell diameter; Fig. 5). Bull calves subjected to 8 h of transiently induced ischemia had more than 80% of the spermatic epithelium (Sertoli plus germ cells combined) regressed compared to controls ($P < 0.05$; Fig. 4 and Fig. 5). This indicates that Sertoli and germ cells were the most sensitive cell types to the reduction in blood supply following the 8 h of ischemia compared to other treatment groups (Fig. 5).

4.6. Discussion

The necessity to find practical means for depleting endogenous germ cells in recipient animals that will result in sound recipient animals and adequate donor cell colonization environment without the need for further intervention is needed. The primary finding of the present study demonstrate that: (1) testicular transiently induced ischemia was confirmed by scrotal thermography temperatures; (2) germ cells population were the most sensitive cell type following prolonged ischemic treatment in bull calves; and (3) although testicular weight was significantly reduced, seminiferous tubules structure and interstitial cells were present after prolonged ischemia.

Transient occlusion of the blood supply to the testes exhibited focal damage of the spermatic epithelium with germ cell-depleted seminiferous tubules. Similar patterns of focal damage were observed in partial occlusion of the internal artery in rams (Markey *et al*. 1994). As this oxygen-dependent spermatic epithelium is in a state of near transient anoxia, such a decrease in blood flow may have profound effects on tissue morphology. The testis, specifically the seminiferous tubule cells, may be susceptible to oxidative damage, as the blood vessels supplying these tissues are interrupted. Macrophages and lymphocytes have been identified in the interstitial region of the testis (Miller *et al*. 1983; Schuenemann *et al*. 2007). Markey *et al*. (1994) reported that macrophages were laden with lipofuscin pigment, suggesting a role in phagocytosis of cellular debris that accumulated as a result of testis ischemic damage in rams. Testicular macrophages are known to generate high concentration of reactive oxygen species (Wei *et al*. 1988). Free radicals are powerful oxidizing agents and the combination of these with high concentrations of polyunsaturated fatty acids in ischemic testes provides an optimum environment for lipid peroxidation and formation of lipofuscin (Markey *et al*. 1994).

Prepubertal bulls subjected to 8 h of transiently induced ischemia had a significant decrease in testis weight at castration compared to controls. This observation and partially depleted cells in seminiferous tubules observed in tissue cross sections suggest that testis cells are particularly sensitive to such oxidative processes; therefore, confirming the reduction of tubules cells. Blood flow to the testes correlates with scrotum temperature in bulls (Purohit *et al*. 1985; Schuenemann *et al*. 2005). Indeed, quantitation of scrotal thermography temperature in this study confirmed that testicular banding impaired blood supply to the testes, creating a transiently induced ischemia for each

banding periods. However, blood supply to the testes was restored as illustrated by scrotal temperatures following band removal.

Histological cross sections of the seminiferous tubules revealed a significant reduction of spermatic epithelium (Sertoli plus germ cells) and interstitial cells in bulls subjected to prolonged ischemia as opposed to controls. Moreover, germ cells were the most sensitive cell type to vascular disturbance (approximately 70% depletion of germ cells), indicating that spermatogonial stem cells were destroyed inside of the tubules by transiently induced ischemia. The most significant finding of the prolonged 8 h ischemic testis was the partial depletion of the seminiferous tubule cells (Sertoli and germ cells) and decreased average volume of Sertoli and Leydig cells. This finding suggests that transient disturbance (8 h) of the blood supply to the testis is an aggressive insult with morphological consequences to the seminiferous tubules. Interestingly, while transiently ischemic treatments voided germ cell development and proliferation, other testicular components remain within the testes.

Detrimental effects on testicular somatic cell viability and function must be avoided when preparing recipient animals before donor germ cell transplantation. In this study, the functionality in term of testosterone (Leydig) and inhibin or FSH (Sertoli) production in prepubertal bull calves at 1- and 2-mo of age was not evaluated. However, testicular cell components were present at castration in testes cross sections following ischemic treatments. This finding may indicate that testicular cells survive and were viable at castration; however, functionality of Sertoli and Leydig cells need to be elucidated.

In conclusion, these results suggest that transiently induced ischemia decreased the spermatic epithelium and germ cell populations while maintaining a number of Sertoli and Leydig cells per testis. Further studies are needed to evaluate the functionality of Sertoli and Leydig cells following testicular ischemia.

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4.8. Appendix: Figures and Tables

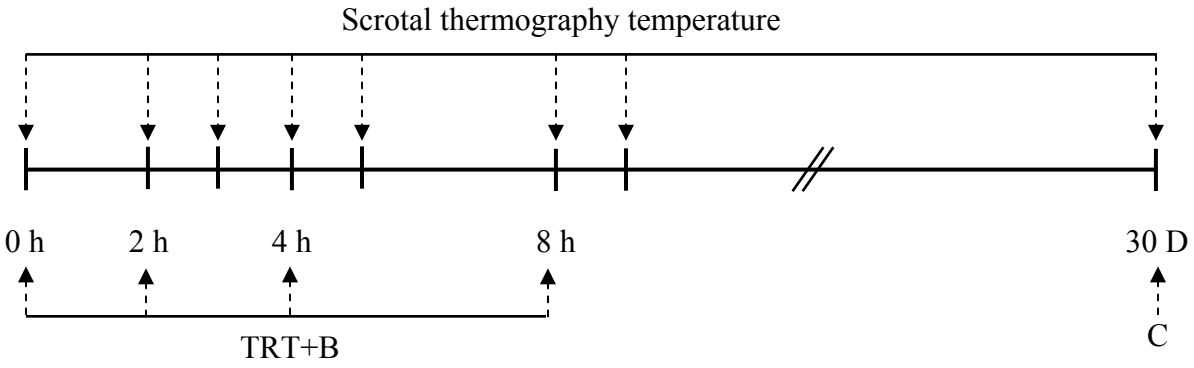

Figure 1. Assignment of transiently induced ischemia treatment groups in Jersey bull calves. *Treatment groups (TRT)*: intact Jersey bull calves (n = 16) at 27 ± 5 d of age (Day 1 of the experiment) were randomly assigned to four transiently induced ischemia treatments, group 1 (control, n = 4), group 2 (2 h ischemia, n = 4), group 3 (4 h ischemia, n = 4), and group 4 (8 h ischemia, n = 4) by banding method. *Scrotal thermography temperature (TT)*: testicular temperature in intact Jersey bull calves was recorded immediately before banding, 1 h after banding, and immediately before castration using a thermography camera. *Banding (B)*: four transiently induced ischemia periods were induced by placing a rubber band on the neck of the scrotum in intact Jersey bull calves. *Castration (C)*: testes were collected 1 mo after banding (approximately at 2 mo of age) by castration procedure for further histological analysis.

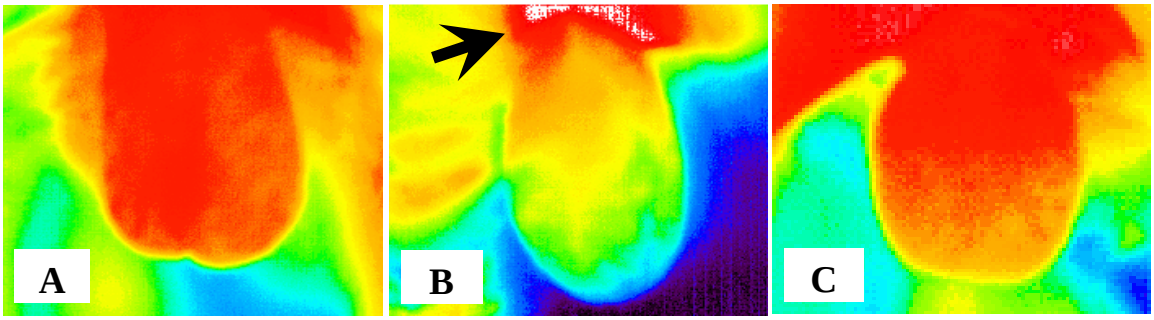

Figure 2. Transient changes on testicular temperature in prepubertal Jersey bull calves immediately before, during, and 1 h after transiently induced ischemia treatments. Panel A = thermography temperature before transiently induced ischemia in testes. Panel B = thermography temperature during transiently induced ischemia showing depleted blood flow into the testes (arrow indicates site of band on). Panel C = thermography temperature 1 h after transiently induced ischemia showing regained blood flow into the testes. Color pattern temperature range from warm (red) to cool (blue).

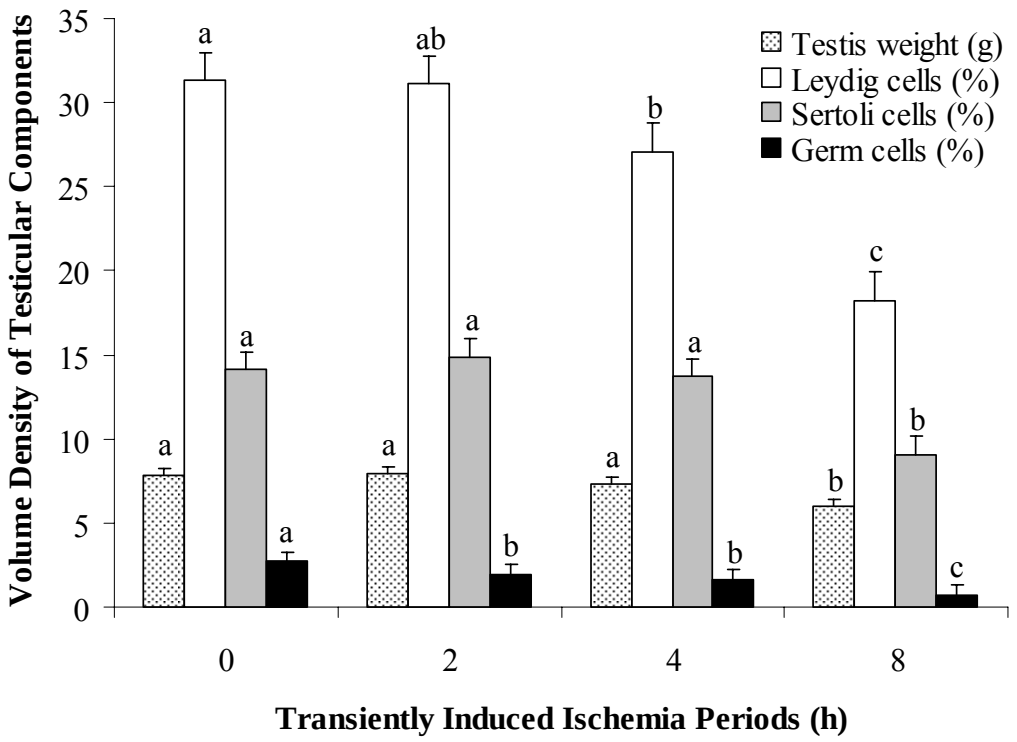

Figure 3. Mean testis weight and percentage volume density of testicular Leygig, Sertoli, and germ cells in bulls subjected to different transiently induced ischemia treatments for depletion of endogenous germ cells. 0 = untreated animals, 2 = animals exposed to 2 h of testicular transiently induced ischemia, 4 = animals exposed to 4 h of testicular transiently induced ischemia, and 8 = animals exposed to 8 h of testicular transiently induced ischemia. Same color bars with different letters differ, $P < 0.05$. Data are presented as the least squares means ± SEM.

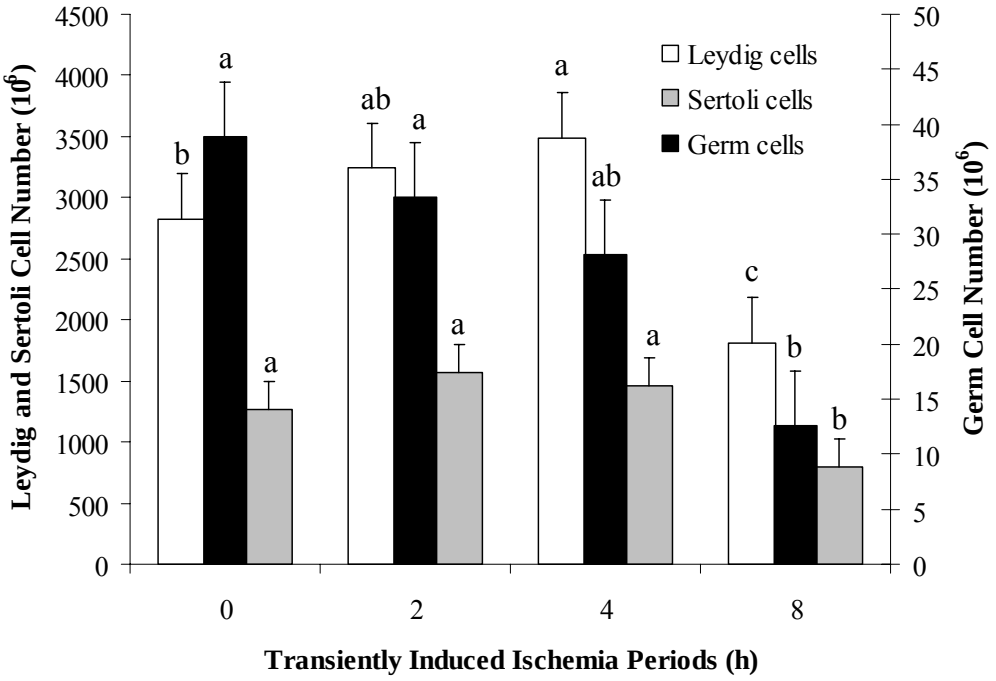

Figure 4. Mean cell number per testis in cross sections of bulls subjected to different transiently induced ischemia periods. 0 = untreated animals, 2 = animals exposed to 2 h of testicular transiently induced ischemia, 4 = animals exposed to 4 h of testicular transiently induced ischemia, and 8 = animals exposed to 8 h of testicular transiently induced ischemia. Same color bars with different letters differ, $P < 0.05$. Data are presented as the least squares means ± SEM.

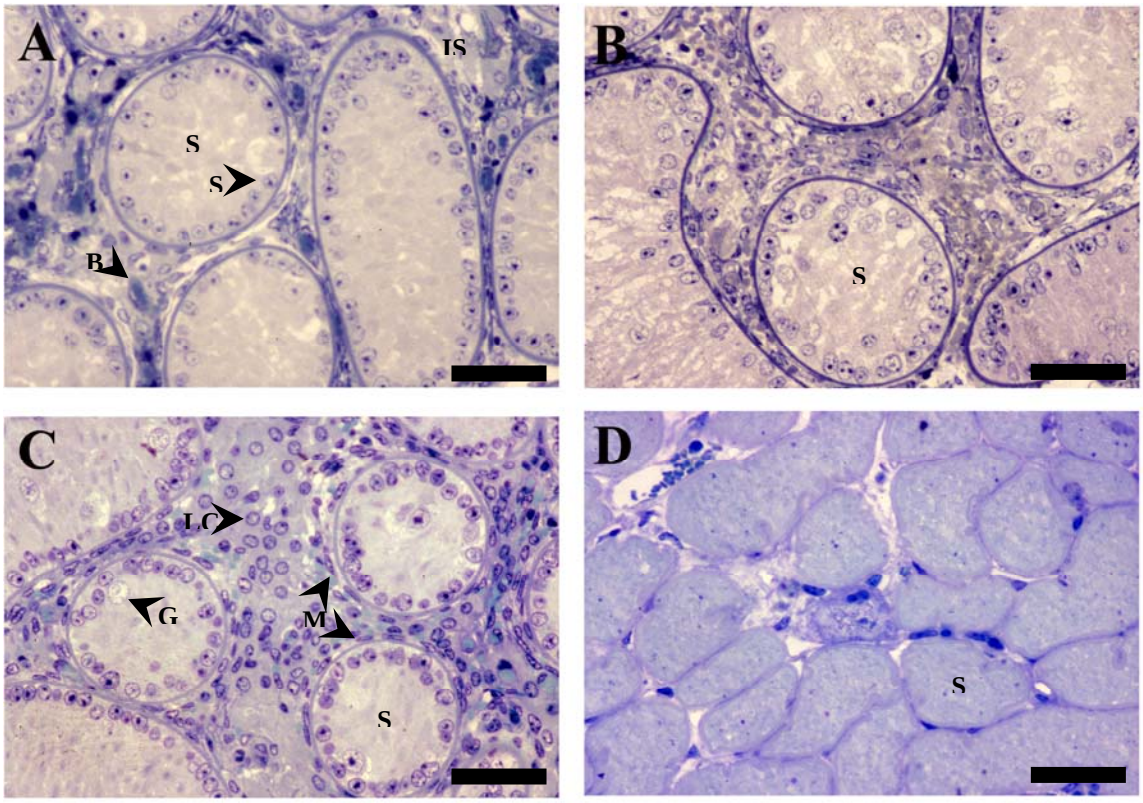

Figure 5. Representative light micrographs of testicular tissue cross section in 2-mo of age Jersey bull calves subjected to different transiently induced ischemia periods showing testicular components. Panel A= control bull testicular components. Panel B = testicular components in bulls exposed to 2 h of testicular transiently induced ischemia with regressed tubules (arrow head). Panel C = animals exposed to 4 h of testicular transiently induced ischemia containing germ cell depleted siminiferous tubules (arrow head). Panel D = animals exposed to 8 h of testicular transiently induced ischemia containing germ cell depleted siminiferous tubules (arrow head). In the seminiferous tubules (ST), germ cells (GC) are fewer in number compared with Sertoli cells (SC). At this stage the lumen is not seen. Leydig cells (LC), Myoid cells (MC), and blood vessel (BV) are present in the interstitial space (IS) of the testis. Scale bar = 100 µm.

Tables

Table 1. Mean scrotal circumference (SC), SC growth, scrotal temperature, and single fresh testicular weight (TW) at castration.

Parameter	Treatment groups			
	0 h	2 h	4 h	8 h
SC at banding (cm)	8.98±0.2[a]	9.18±0.2[a]	9.1±0.2[a]	9±0.2[a]
SC at castration (cm)	10.2±0.2[a]	10±0.2[a]	10.3±0.2[a]	9.5±0.2[b]
SC growth (cm d^{-1})	0.04±0.007[a]	0.03±0.007[a]	0.04±0.007[a]	0.01±0.007[b]
TW at castration (g)	7.8±0.4[a]	7.9±0.4[a]	7.3±0.4[a]	6.6±0.4[b]
ST immediately before banding (^{o}C)	31.7±0.7[a]	31.8±0.7[a]	30.3±0.7[a]	31.5±0.7[a]
ST immediately before band removal (^{o}C)	30.7±0.6[a]	30.6±0.6[a]	28.9±0.6[b]	28.5±0.6[b]
ST 1 h after band removal (^{o}C)	30.5±0.4[b]	30.5±0.5[b]	33.7±0.4[a]	34.2±0.4[a]

In each row, values (mean ± s.e.m.) with different superscript letters are significantly different ($P < 0.05$). 0 h, untreated animals; 2 h, animals subjected to 2 h of testicular transiently induced ischemia; 4 h, animals subjected to 4 h of testicular transiently induced ischemia; 8 h, animals subjected to 8 h of testicular transiently induced ischemia.

Table 2. Mean seminiferous tubule diameter, length and volume density of testicular components.

Parameter	Treatment groups			
	0 h	2 h	4 h	8 h
Seminiferous tubules (%)	54.3±2[a]	54.6±2.1[a]	49.7±1.9[a]	51.7±2.1[a]
Seminiferous tubule cells (%)	15.8±1.6[a]	17.5±1.7[a]	15.6±1.6[a]	10±1.7[b]
Seminiferous tubule diameter (μm)	138.6±6.3[a]	129±6.4[ab]	122.3±5.6[b]	115.8±6.4[b]
Seminiferous tubule length per g of testis (m)	325 ± 52[a]	424 ± 46[a]	438 ± 45[a]	389 ± 52[a]
Seminiferous tubule length per testis (m)	2469 ± 733[a]	3628 ± 639[a]	4258 ± 633[a]	2588 ± 732[a]
Interstitial space (%)	36.05±1.2[a]	38.4±1.3[a]	31.7±1.1[b]	36.1±1.3[ab]
Interstitial cells (all cell types; %)	33.4±1.7[a]	33.1±1.7[ab]	28.8±1.6[b]	19.3±1.7[c]
Blood vessels (%)	0.54±0.1[b]	0.97±0.1[ab]	0.84±0.1[b]	1.4±0.1[a]
Blood vessels lumen (%)	0.19±0.1[c]	0.42±0.1[bc]	0.52±0.09[ab]	0.78±0.1[a]
Blood vessel wall (%)	0.28±0.07[b]	0.4±0.08[ab]	0.36±0.06[ab]	0.61±0.08[a]
Macrophages (%)	0.4±0.08[a]	0.4±0.08[a]	0.4±0.07[a]	0.3±0.08[a]
Mesenchimal cells (%)	1.6±0.1[a]	1.6±0.1[a]	1.2±0.1[ab]	0.8±0.1[b]

In each row, values (mean ± s.e.m.) with different superscripts are significantly different ($P < 0.05$). 0 h, untreated animals; 2 h, animals subjected to 2 h of testicular transiently induced ischemia; 4 h, animals subjected to 4 h of testicular transiently induced ischemia; 8 h, animals subjected to 8 h of testicular transiently induced ischemia.

Table 3. Mean absolute volume of testicular components. In each row, values (mean ± s.e.m.) with different superscripts are significantly different ($P < 0.05$).

Parameter	Treatment groups			
	0 h	2 h	4 h	8 h
Seminiferous tubules (mm^3)	4321±181[a]	4404±193[a]	4609±154[a]	3440±199[b]
Seminiferous tubule cells (mm^3)	1254±91[a]	1497±97[a]	1430±81[a]	680±97[b]
Sertoli cells nuclei (mm^3)	1138±70[a]	1228±75[a]	1282±60[a]	617±77[b]
Germ cells (mm^3)	243.4±45[a]	191.2±44[ab]	140.7±45[b]	48.2±45[c]
Interstitial space (mm^3)	2838±186[a]	3021±190[a]	2881±178[a]	2389±191[b]
Interstitial cells (all cell types; mm^3)	2663±159[a]	2638±165[a]	2659±147[a]	1297±166[b]
Blood vessels (mm^3)	42.9±13[b]	77.5±14[ab]	72.3±11[ab]	95.9±14[a]
Blood vessels lumen (mm^3)	17.7±8.3[b]	35.6±8.7[ab]	41.2±7.6[a]	52.4±8.4[a]
Blood vessel wall (mm^3)	23.9±6.3[a]	35.1±6.7[a]	29.2±5.4[a]	41.2±6.9[a]
Leydig cells nuclei (mm^3)	2494±161[a]	2461±167[a]	2499±150[a]	1224±167[b]
Macrophages (mm^3)	32.2±6.2[ab]	31.9±6.5[ab]	41.7±5.3[a]	18.1±6.5[b]
Mesenchimal cells (mm^3)	132.5±10[a]	131.3±11[a]	114.5±9[a]	55.5±11[b]

0 h, untreated animals; 2 h, animals subjected to 2 h of testicular transiently induced ischemia; 4 h, animals subjected to 4 h of testicular transiently induced ischemia; 8 h, animals subjected to 8 h of testicular transiently induced ischemia.

Table 4. Average volume of a Leydig, Sertoli, and germ cells. In each row, values (mean ± s.e.m.) with different superscripts are significantly different ($P < 0.05$).

Parameter	Treatment groups			
	0 h	2 h	4 h	8 h
Average volume of a Leydig cell nucleus (μm^3)	973 ± 48^{a}	825 ± 50^{bc}	922 ± 44^{ab}	758 ± 50^{c}
Average volume of a Sertoli cell nucleus (μm^3)	1045 ± 111^{ab}	891 ± 112^{b}	1139 ± 108^{a}	915 ± 112^{b}
Average volume of a germ cell (μm^3)	6493 ± 973^{a}	5759 ± 886^{a}	5388 ± 944^{a}	4228 ± 975^{a}

0 h, untreated animals; 2 h, animals subjected to 2 h of testicular transiently induced ischemia; 4 h, animals subjected to 4 h of testicular transiently induced ischemia; 8 h, animals subjected to 8 h of testicular transiently induced ischemia.

Chapter 5 – EFFECTS OF CULTURE ENVIRONMENT ON PROLIFERATION OF SPERMATOGONIA STEM CELLS FROM PREPUBERTAL AND ADULT BULLS

This chapter is a manuscript by the same name which will be submitted for publication in the journal of *Reproduction, Fertility, and Development* in 2008 by Gustavo Schuenemann, Lannett Edwards, Arnold Saxton, Louisa Rispoli, and Neal Schrick.

My use of "we" in this chapter refers to my co-authors and myself. My primary contributions to this paper include (1) aided in design of the experiment and data analysis, (2) tissue and sample collection, (3) cell isolations, (4) cell culture, (4) identification of cell and colony types, (5) collection and interpreting of the literature, and (6) writing of this paper.

5.1. Acknowledgments

Authors wish to thank Henry Dowlen, Hugh Moorehead, and Phillip Lunn at the Dairy Research and Education Center (Lewisburg, TN) and Charlie Young at the East Tennessee Research and Education Center (Knoxville, TN) for providing prepubertal bovine testes. Appreciation is also extended to Russell Harris at Brown Packing Co., (Gaffney, SC) for providing testes from mature bulls, and Dr. John Dunlap at the Microscope Core Facility, Division of Biology, The University of Tennessee for providing assistance with phase contrast microscopy.

5.2. Abstract

The objective of the current study was to evaluate type A spermatogonial cell (SSC) proliferation and differentiation under various culture conditions. SSC were harvested from 3-4 mo-old prepubertal bulls (PB; Dairy) and adult bulls (AB; Beef). Spermatogonia according to origin (prepubertal or adult) were cultured on the presence or absence of a feeder monolayer (FL or NF) with either stem cell media (ELSC) or regular media (RSC) supplemented with either regular fetal bovine serum (FBS-S) or charcoal stripped (FBS-SF). Immediately after digestion and Percoll purification, cells from prepubertal bulls had higher viability than adult bulls ($P < 0.0001$). In both PB and AB, positive staining for protein gene product (PGP) 9.5 was scattered across seminiferous tubules and distributed randomly within somatic components of tubules. Following Percoll purification, the change in spermatogonial cells staining positive to PGP 9.5 improved slightly in PB (1.3 ± 0.84%) and was reduced in AB (-2.2 ± 0.09%; $P < 0.0001$). The number of colonies increased gradually from day 4 to 15 of culture and was not affected by bull type. Additionally, three types of colonies (round, radial, and irregular) were present in culture. Overall, radial colonies were the most predominant type of colony in culture ($P < 0.0001$). Furthermore, co-culture of spermatogonial cells with a defined FL yielded more colonies than without FL ($P < 0.0001$). In both PB and AB, co-culture of spermatogonial cells in the presence of FL, RSC media plus FBS-S or FBS-SF treatment combination resulted in more colonies at day 7 and 15 compared to NF monolayer ($P < 0.0001$). Furthermore, the maximum number and size of colonies were obtained in a FL with RSC media containing FBS-S in prepubertal and adult bulls ($P <$

0.0001). Furthermore, colonies stained positive for alkaline phosphatase (AP). These findings suggest that SSC were present in culture and capable of proliferating and forming colonies. These results, although preliminary, will provide the basis for potential production of offspring through *in vitro* manipulation or following SSC transfer.

5.3. Introduction

Spermatogenesis is a highly organized process that is maintained by continuous proliferation of stem cells (reviewed by de Rooij 2001). Over three decades ago, both Huckins (1971) and Oakberg (1971) proposed an accepted model of spermatogonial self-renewal and differentiation. To begin, the As (A single) spermatogonia are the stem cells of spermatogenesis (Huckins 1971; de Rooij 2001). Divisions of type A spermatogonia give rise to intermediate (In) and B spermatogonia that become spermatids after the process of meiosis, and finally differentiate into mature spermatozoa (reviewed by de Rooij 2001). Currently, the molecular or cellular mechanisms controlling progression of spermatogonia to become specialized and differentiated remain unknown.

Previous studies in spermatogonial stem cell transplantation in mice demonstrated that spermatogonial stem cells could be harvested from donor animals, maintained *in vitro* for a few hours, and re-initiate spermatogenesis in the host testis after transplantation with spermatozoa capable of fertilizing an oocyte resulting in offspring (Brinster and Avarbock 1994; Brinster and Zimmerman 1994). Subsequent studies have successfully adopted these procedures in other species such as rat (Clouthier *et al*. 1996), dog (Dobrisnki *et al*. 1999), pig (Honaramooz *et al*. 2002), ram (Rodriguez-Sosa *et al*.

2006), goat (Honaramooz *et al*. 2003), and bull (Izadyar *et al*. 2003b, Herrid *et al*. 2006). However, offspring were not always obtained which may be related to issues in the culture of type A spermatogonia cells following collection from donor testes.

In vitro culture of type A spermatogonia cells (SSC; which include A_S, A_{al}, A_{pr}, and A_1-A_4) has the potential to provide a powerful tool to investigate spermatogonial proliferation and differentiation (reviewed by Sofikitis *et al*. 2005). The ideal *in vitro* system, one that supports self-renewal of type A_S spermatogonia (presumably the stem cell; reviewed by de Rooij 2001) or complete the process of spermatogenesis from a population of SSC, would result in generation of germ cells capable of colonizing tubuli in recipient animals and producing spermatozoa (van der Wee *et al*. 2001; Izadyar *et al*. 2003a; reviewed by Sofikitis *et al*. 2005). Culture systems for type A spermatogonia cells facilitate propagation of spermatogonia (Izadyar *et al*. 2003a; 2003b), which can then be transferred into recipient testes (Izadyar *et al*. 2003b; Herrid *et al*. 2006), genetically modified (Nagano *et al*. 2001), or cryopreserved for future interventions (Oatley *et al*. 2004).

Primarily, isolation and culture of bovine type A spermatogonia have been performed from prepubertal bulls (Izadyar *et al*. 2002; 2003a). However, very little is known on type A spermatogonia culture from adult bulls. Moreover, male germ cells cultured from large food animals such as bovine of known genetic merit is of paramount importance for future studies aimed at transferring this genetic potential using recipient animals. The correct identification and culture of type A spermatogonia will enable dissemination of desired genetic from donor animals through spermatogonial cells transfer. However, isolation and purification of type A (A_S, A_{al}, A_{pr}, and A_1-A_4)

spermatogonial cells are more difficult in adult than prepubertal mammals due to common morphological characteristics of developing germ cells (Dobrinski *et al*. 2000; Izadyar *et al*. 2002). Therefore, field application of spermatogonial stem cell transplant in large food animal such as bovine requires further investigation not only in prepubertal animals but also in adult bulls.

Furthermore, co-culture of germ cells (2-month-old bulls and 6-day-old mice) with a feeder cell monolayer such as bovine embryonic fibroblast (BEF; Oatley *et al*. 2004), immortalized mice Sertoli cells (van der Wee *et al*. 2001) or SIM mouse embryo-derived Thioguanine- and Ouabain-resistant fibroblast (STO; Nagano *et al*. 2001) substantially increased viability and proliferation (van der Wee *et al*. 2001). Proliferation of type A spermatogonial cells from 2-month-old porcine (Dirami *et al*. 1999) or 3-7-month-old bovine (Izadyar *et al.* 2003a) in *in vitro* culture medium without serum or a feeder monolayer resulted in fewer cells surviving. Again, the focus of these studies investigating culture systems has been primarily aimed at prepubertal animals and not adult animals.

Type A spermatogonial cells from a 5-month-old bull cultured in minimal essential medium supplemented with serum yielded more viable cells and proliferation compared to absence of serum (Izadyar *et al*. 2003a). Using the same media in long-term culture of bovine germ cells resulted in differentiated spermatogonia resembling spermatocytes and spermatids (Izadyar *et al*. 2003a). It was suggested that different levels of serum (ranging from 1 to 10%) such as Nu-serum (serum replacement with low proteins) or horse serum (HS; van der Wee *et al*. 2001), fetal calf serum (FCS; Aponte *et al*. 2006), or fetal bovine serum (FBS; Izadyar *et al*. 2003a) added to the medium

enhanced type A spermatogonia survival and proliferation. However, it is difficult to discern what component(s) present in serum (i.e. lipids, proteins, hormones, etc.) are essential for self-renewal of type A_S spermatogonial.

Difficulties arise when attempting to obtain a large number and pure population of viable SSC from donor testes. In part, methods of isolation, purification, and identification of SSC may contribute to the success in establishing lines of male germ cells (reviewed by Sofikitis *et al*. 2005). In a recent study in calves, isolated donor spermatogonia identified using protein gene product 9.5 antigen (PGP 9.5) were able to colonize seminiferous tubules of recipient animals (Herrid *et al*. 2006). Moreover, identification of porcine spermatogonia using expression of PGP 9.5 as a specific marker was reported (Luo *et al*. 2006). These findings suggest that cells staining for PGP 9.5 were likely SSC with the potential to form colonies (Luo *et al*. 2006) or re-colonize foreign seminiferous tubules (Herrid *et al*. 2006).

The objective of the current study was to evaluate spermatogonial stem cells, isolated from prepubertal and adult bulls, during short term *in vitro* culture under various conditions.

5.4. Materials and methods

All chemical were purchased from Sigma Chemical Company (Sigma, St. Louis, MO, USA) unless otherwise noted.

5.4.1. Media preparation

Minimum essential medium (MEM; Sigma, St. Louis, MO, USA) was supplemented to contain 14 mM L^{-1} $NaHCO_3$, 4 mM L^{-1} L-glutamine, 100 mg mL^{-1} MEM non-essential amino acids, 15 mM L^{-1} Hepes (all from Sigma, St. Louis, MO, USA), 100 IU mL^{-1}-100 mg mL^{-1} penicillin-streptomycin (Chemicon International Inc., Temecula, CA, USA), and 50 µg mL^{-1} gentamycin (Chemicon International Inc., Temecula, CA, USA).

Enzymatic MEM as described above were supplemented with 1 mg mL^{-1} hyaluronidase type II, 2 µg mL^{-1} DNase I (2000 units mg^{-1}; Unit: 1 µg of plasmid DNA is digested to oligonucleotides in 10 min at 37 °C), 1 mg mL^{-1} collagenase type IA (all from Sigma, St. Louis, MO, USA), 0.5 mg mL^{-1} trypsin : 0.53 mM EDTA without Ca^{2+} and Mg^{2+} (Chemicon International Inc., Temecula, CA, USA), 100 IU mL^{-1}-100 mg mL^{-1} penicillin-streptomycin and 50 µg mL^{-1} gentamycin immediately before testes digestion.

Embryonic-like stem cell culture medium (ELSC; Robertson 1987 with modifications*)* consisting of Dulbecco's minimal essential medium (DMEM) containing low glucose (1000 mg L^{-1}) but without phenol red was supplemented with 3500 mg L^{-1} L-glucose, 14 mM L^{-1} NaHCO3, 4 mM L^{-1} L-glutamine, 100 mg mL^{-1} MEM non-essential amino acids (all from Sigma, St. Louis, MO, USA), 100 IU mL^{-1}-100 mg mL^{-1} penicillin-streptomycin, 50 µg mL^{-1} gentamicin, 1x nucleosides, 0.1 mM 2-mercaptoethanol (Sigma, St. Louis, MO, USA), and either 10% fetal bovine serum with steroids (FBS-S; BioWhittaker, MD, USA) or 10% FBS steroids free (FBS-SF; Hyclone, Logan, UT, USA).

Regular stem cell culture medium (RSC): DMEM low glucose (1000 mg L^{-1}) without phenol red containing 14 mM L^{-1} NaHCO3 (Sigma, St. Louis, MO, USA), 100 IU mL^{-1}-100 mg mL^{-1} penicillin-streptomycin, 50 µg mL^{-1} gentamicin, and either 10% FBS-S or 10% FBS-SF.

5.4.2. Collection and processing of testes

Testes from 3 to 4-mo-old prepubertal Jersey (*n* = 2) and Holstein bull calves (*n* = 1) and adult beef bulls (*n* = 3; Hereford, Charolais, and Angus) were collected by opportunistic retrieval of testes following castration (prepubertal; East Tennessee Research and Education Center, Knoxville, TN, USA and Dairy Research and Education Center, Lewisburg, TN, USA) or harvesting (adult; Brown Packing Inc., Gaffney, SC, USA). Testes were placed immediately on ice and transported to the laboratory for processing.

Prepubertal: Testes from prepubertal bulls were placed in a glass container with approximately 100 mL MEM as described earlier supplemented with 100 IU mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, and 0.25 µg mL^{-1} amphotericin B (ABAM; Sigma, St. Louis, MO, USA) and transported to the laboratory on ice within 3 to 4 h. In the laboratory, testes were rinsed with water to remove blood and debris. Using a #10' blade scalpel, removal of the tunica dartos, epidydimis, excess connective tissue, and testis cord was performed. A single testis was placed in a square 100 mm petri dish (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) and weight recorded (with tunica

albuginea). Afterwards, a longitudinal incision to the testis was made in the tunica using a #10' blade scalpel. Approximately 20 g of testicular tissue was removed using scissors and hemostats, then rinsed in sterile Dulbecco's phosphate buffer saline without Ca^{2+} and Mg^{2+} (DPBS; Sigma, St. Louis, MO, USA) for cell isolation. An aliquot of testis tissue was pulled apart to visualize fragments of seminiferous tubules (without digestion), minced (using forceps and hemostats), and then resuspended in MEM to collect tubules using a mouth glass pipette (FisherBrand, Fisher Scientific, Pittsburgh, PA, USA) under stereo microscope (Nikon, Japan). Aspirated seminiferous tubules were fixed into a 2 mL dolphin nose cap tube with 3.7% formaldehyde (Sigma, St. Louis, MO, USA) for subsequent immunological and microscopic evaluation. The remaining of testicular tissue was used for isolation of type A spermatogonial cells by enzymatic digestion.

Adult: Similar procedures were performed as described above with minor modifications. Testes from adult bulls were harvested at the abattoir and placed directly into a ziplock bag and transported to the laboratory on ice within 3 to 4 h after collection. Approximately 20 g of testis tissue were removed and dispersed in small fragments using a scalpel and scissors before digestion.

5.4.3. Spermatogonia stem cell isolation and purification

Prepubertal: After removing the tunica, the entire testes were minced (as described above) into small pieces and suspended in enzymatic MEM as described earlier. Testis tissue containing spermatogonia cells was dispersed by a two-step

enzymatic digestion described by van Pelt *et al.* (1996) and Izadyar *et al.* (2002) with minor modifications. In brief, minced testis pieces were suspended in enzymatic MEM and incubated at 37^{o}C for approximately 45 min in a shaking water bath operated at 130 cycles min^{-1}. After the first digestion, debris and large fragments of undigested testis tissue were eliminated by filtration through a 55- to 77-µm nylon filter (Em-Con, Immuno Systems Inc., Spring Valley, WI, USA). Undigested fragments of seminiferous tubules that passed through the mesh filter were collected in a 50 mL conical blue cap tube (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) and pelleted by centrifugation at 30 x *g* for 2 to 5 min at 9 °C. After centrifugation, the pellet composed primarily of fragments of seminiferous tubules was resuspended and incubated in enzymatic MEM for 45 min as described above. Free cells were separated from the remaining undigested tubule fragments by centrifugation at 30 x *g* for 2 to 5 min (if needed). The pool of cells in the supernatant was collected and transferred to a 15 mL conical blue cap tube (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) for centrifugation at 3000 x *g* for 1 min at 9°C and then washed twice in sterile DPBS supplemented with 10% FBS to remove enzymes. Washing steps were performed without the addition of antibiotics (potential risk of contamination).

Harvested pools of cells were incubated overnight as described by van Pelt *et al.* (1996) with minor modifications. Briefly, a 100 mm Falcon dish was pre-coated with 100 µg mL^{-1} peanut agglutinin (PNA, Sigma, St. Louis, MO, USA) in 1 mL of DPBS for at least 1 h at 37.5^{o}C, and then aspirated before plating pool of cells. Pools of cells in RSC with the addition of 10% FBS-S were then incubated overnight in pre-coated PNA dishes

in a humidified atmosphere at 37.5°C with 5% CO_2 balance air in an attempt to eliminate somatic cell components (myoid, peritubular, and Sertoli cells; van Pelt *et al*. 1996).

After attempted removal of somatic components, presumptive spermatogonia in suspension were collected and loaded onto a discontinuous Percoll density gradient for further cell purification as described by van Pelt *et al.* (1996) with minor modifications. Briefly, an iso-osmotic Percoll (Sigma, St. Louis, MO, USA) suspension containing 82.2% Percoll in MEM supplemented with 6 mg mL^{-1} bovine serum albumin (BSA) and 2 μg mL^{-1} DNase (all from Sigma, St. Louis, MO, USA) was prepared. This method selects type A spermatogonia cells on the basis of size, shape, and centrifugation forces (van Pelt *et al*. 1996). Later, a discontinuous density gradient was built by diluting the iso-osmotic Percoll suspension in MEM as described earlier supplemented with 7 mg mL^{-1} BSA and 2 μg mL^{-1} DNase. Percentages of each 1 mL column were as follows: 20, 30, 40, 50, and 65% of Percoll densities and later the interface between fractions referred to as fractions 1, 2, 3, and 4 respectively. The gradient was constructed in a sterile polypropylene tube (round bottom, 17 x 100 mm long and 15 mL; Evergreen Scientific, Los Angeles, CA, USA) using an automatic pipette (Accu-Jet, Sigma, St. Louis, MO, USA) to load 1 mL of each Percoll suspension. Each one mL of the Percoll suspension was layered slowly into the tube by starting with the most concentrated (65%) column on the bottom until the five fractions were placed into the tube and a visible interface line between fractions was confirmed.

Finally, cell suspensions were layered on top of the gradient column in one mL RSC plus 10% FBS-S. The gradient was then centrifuged at 800 x *g* for 30 min at 9°C. Immediately after centrifugation, cells found in fraction 2 were collected in prepubertal

bulls (Izadyar *et al*. 2002) and fraction 1 for adult bulls (visible fraction; Figure 1 (All tables and figures in this chapter appear in the appendix)). Cell suspensions containing presumptive type A spermatogonia were washed 3 times in DPBS plus 10% FBS. Cells in collected fractions were counted using a hemacytometer (Fisher Scientific, Pittsburgh, PA, USA), viability determined by trypan blue (0.4%, Diagnostic Systems, Inc., Raritan, NJ, USA) exclusion method (membrane integrity) under microscopy at 200x (TE300, Nikon, Japan), and then plated in 24 well plates for culture. Aliquots of presumptive spermatogonia and somatic cells were placed in a 2 mL dolphin nose cap tube with 3.7% formaldehyde. Additionally, glass slide smears from freshly isolated cells were obtained and air dried at room temperature overnight. Fixed cells were used to determine proportion of type A spermatogonial cells present in fractions.

5.4.4. Determination of germ cell populations

To identify the presence of germ cells, enriched populations of type A spermatogonia cells and fragments of seminiferous tubules were fixed in 3.7% formaldehyde after tissue digestion and stained as described by Luo *et al.* (2006) with modifications. Briefly, cells and fragments of seminiferous tubules contained in 1.5 mL vials were permeabilized with 0.3% Triton-X100 in Tris Buffer Saline (TBS) overnight (~ 16-18 h) at 4°C and then incubated for 1 h at room temperature in 5% mouse normal serum (MNS)/TBS (Immuno Pure, Rockford, IL, USA) followed by 15 min of Avidin and 15 min of Biotin blocking (Vector Laboratories, Burlingame, CA, USA). For

localization of PGP 9.5 in type A spermatogonia cells, rabbit anti-human PGP 9.5 (1° AB, Biogenesis Inc., Kingston, NH, USA) was diluted 1:200 in 5% MNS/TBS and incubated overnight at 4°C (~ 16-18 h). Biotinylated goat anti-rabbit IgG (H+L) (Vector Laboratories, Burlingame, CA, USA) was diluted 1:100 in 5% MNS/TBS and incubated for 1 h at room temperature as the corresponding secondary antibody. Secondary biotynilated antibody was visualized by using conjugated steptavidin Cy3 diluted 1:200 in TBS (Jackson ImmunoResearch Laboratory, Inc., West Grove, PA, USA) for 10 min. Cells and seminiferous tubules incubated without primary antibody were used as controls. Finally, cells and tubules were spread on glass slides and mounted in mounting medium containing 0.5 μg mL^{-1} Hoechst 33342 (Sigma, St. Louis, MO, USA) for nuclei staining. Somatic components were determined based on nuclei staining (blue) and spermatogonial cells staining positive to PGP 9.5 (red).

Confirmation of PGP 9.5 staining was performed by identifying cells with morphological characteristics of germ cells (large round cells compared to somatic cells; Schuenemann *et al*. 2007) in prepubertal bulls (Figure 2). Furthermore, staining of seminiferous tubules was performed to identify the presence of SSC and provide confirmation of the staining procedure (Figure 3). To calculate percentage of spermatogonia in the initial pool of cells and after cell separation by a discontinuous Percoll density gradient (fraction II for prepubertal bulls and fraction I for adult bulls), four images were recorded (200x) for each fraction and pool of cells (Figure 4 and 5; Luo *et al.* 2006). Cells positive to PGP 9.5 were counted using computer software (Metamorph 6.3r2, Molecular Devices, Sunnyvale, CA, USA). The percentage of PGP 9.5 cells (spermatogonia stem cells) and somatic components (Sertoli cells, peritubular

cells, etc.) was calculated in the pool of cells before Percoll and after Percoll in each fraction (I and II) used for culture. To assess the efficiency of spermatogonial purification by a discontinuous Percoll density gradient, the difference between spermatogonia cells (stained positive to PGP 9.5) in the pool of cells and fractions (I and II) was calculated as mentioned above.

The discontinuous density gradient was validated using a Percoll density bead kit (Sigma, St. Louis, MO, USA). Prior to validation, one mL of DPBS was added to each vial of beads and allowed to equilibrate overnight. Density marker beads were used as a standard in a separate centrifuge tube in parallel to tubes containing experimental samples. Both standard and experimental tubes were loaded using the same Percoll suspensions as described above. The standard tube received 10 µL of each colored bead type and the experimental tube received the pool of cells (10-37 $x10^6$ cells mL^{-1}). After centrifugation, colored beads were visualized easily within the standard tube in each Percoll suspension (Figure 1). Distribution of density marker beads was comparable to those described by van Pelt *et al.* (1996). This method selects spermatogonia on the basis of size, shape, and centrifugation forces using a Percoll density gradient. In a study using prepubertal bulls (5 to 7 month of age), the percentage of isolated type A spermatogonia after Percoll (van Pelt *et al*. 1996) was greater (Izadyar *et al*. 2002) in the same fraction used in this study. Although in this study only cells collected from fraction 1 (adult bulls) or 2 (prepubertal bulls) were evaluated, density marker beads illustrated that Percoll columns in this study were similar to the studies described above.

Adult: Processing of testes was similar to methods described above with minor modifications. Tissue samples were removed from the equatorial region of testes and

minced into small pieces, digested once for 45 min in a shaking water bath, and filtered to remove debris and undigested large fragments of testis tissue. Pools of cells were collected into a 50 mL conical blue cap tube, pelleted at 3000 x *g* for 1 min at 9°C, and washed twice in DPBS plus 10% FBS to deactivate enzymes from media.

5.4.5. Cell culture and treatments

A monolayer of mitomycin C-treated (Sigma, St. Louis, MO, USA) male bovine fetal fibroblasts (BFF-8; kindly provided to Dr. Lannett Edwards by Ann Powell, USDA, Beltsville, MD, USA) was evaluated for use in culture of SSC. As described by Robertson (1987) and Skibinski *et al*. (2007) with minor modifications, BFF-8 cells (from passages 9 to 16) were seeded on a 24-well plate (alternating columns, one column with and one without feeder monolayer; Figure 6) at a concentration of 100 x 10^3 cells cm-^{2} (190 x 10^3 cells well^{-1}) for 2 to 3 days in ELSC medium. When BFF-8 cells reached 60-90% confluency, cells were inactivated mitotically using 10 µg mL^{-1} of mitomycin-C for 2-3 h, and then washed twice in DPBS plus 10% FBS-S before replacing with ELSC medium containing 10% FBS-S.

Prior to initiation of the experimental procedures, effect of cell density on viability and concentration of type A spermatogonia cells to be used was determined. Three concentrations of cells (20, 50, and 75 x 10^3 cells well^{-1}) were cultured in 24-well plates prior to initiation of the experiment. Cell confluency was evaluated at 2, 4 and 7 d of culture. Cells reached approximately 40% confluency after 2 d of culture and a complete cell monolayer was obtained after 4 to 7 d of culture using approximately 50

$x10^3$ cells per well. Isolated cells from fraction II (prepubertal bulls) and fraction I (adult bulls) were cultured in 24-well plates (Costar, Corning Inc., Corning, NY, USA) during the experiment at a concentration of 100 x 10^3 viable cells mL^{-1} (0.5 mL $well^{-1}$, approximately 50 x 10^3 cells $well^{-1}$). Cells were incubated at 37.5°C in a humidified atmosphere with 5% CO_2 balance air.

At the onset of culture, type A spermatogonia cells isolated from prepubertal and adult bulls were seeded onto 24-well plates and remain in culture under various conditions for 15 days. Media treatments consisted of two media types (ESCL or RSC) supplemented with either steroids (FBS-S) or steroids low (FBS-SF) serum to evaluate proliferation of type A spermatogonia (Figure 6). Medium was changed every 2-3 d. Type A spermatogonia cells were cultured for 4, 7, and 15 d. At each time period, one 24-well plate was removed from the incubator, fixed in 3.7% formaldehyde, and stored at 4°C to evaluate spermatogonia proliferation. All culture experiments were replicated three times and each experiment comprised at least triplicate cultures. Type A spermatogonia proliferation and differentiation from two bull types (prepubertal and adult) were evaluated under presence or absence of a feeder monolayer with media types (ELSC or RSC) supplemented with 10% of serum (FBS-S or FBS-SF).

5.4.6. In vitro proliferation of type A spermatogonia

After fixation at 4, 7, and 15 d of culture, colonies in each treatment were counted, typed by shape (round, radial, and irregular; Figure 7; Izadyar *et al*. 2003a), and

measured for area (μm^2) using a computer digital caliper (Metamorph 6.3r2, Molecular Devices, Sunnyvale, CA, USA) connected to an inverted light microscope (TE300; Nikon, Tokyo, Japan) equipped with Hoffmann modulation contrast at constant magnification (100 x). Additionally, cell morphology within and surrounding colonies was evaluated under inverted light microscope (TE300; Nikon, Tokyo, Japan) equipped with phase contrast and a digital camera connected to computer software (Qcapture, Qimaging, Surrey, BC, Canada) for capture of images. However, multilayer colonies prevented morphological characterization of cell type present within and surrounding colonies.

5.4.7. Alkaline phosphatase activity

After culture, colonies present in a subset of 8 wells (2 rows of 4 wells each from the outer part of the 24-well plates; Figure 6) containing all treatment combination were evaluated for expression of alkaline phosphatase (AP) as described by Talbot *et al.* (1993). Briefly, cells were fixed *in situ* with 3.7% formaldehyde and stored at 4°C as described previously. Fixative solution was removed from wells and then washed twice with ultrapure water (Milli-Q, Millipore, Bellerica, MA, USA). Fixed cultures were incubated for 15 min at room temperature in fresh distilled water containing 1 mg mL^{-1} fast red TR salt and 40 µg mL^{-1} naphthol AS-MX phosphate (both from Sigma, St. Louis, MO, USA) at pH 8.4. After incubation, fixed cells were washed twice with ultrapure water to end the reaction and observed under an inverted light microscope (TE300;

Nikon, Tokyo, Japan) equipped with an epifluorescence mercury lamp with the rhodamine filter as described by Ziomek *et al*. (1990). Because colonies presented autofluorescence, data from rhodamine filter were not reported in this study. Therefore, classification of positive AP colonies was based on colorimetric evaluation under an inverted light microscope (TE300; Nikon, Tokyo, Japan) as described by Edwards *et al*. (2003). Based on the intensity of red color in each colony, a subjective four-increment intensity score (from 0 = no color, 1 = slight, 2 = mild, 3 = moderate; Figure 8) was assigned by an independent evaluator unaware of treatments to each individual colony.

5.4.8. Evaluation of bovine spermatogonia in culture

After each time period of culture (4, 7, and 15 d), type A spermatogonia cells present in colonies were evaluated using a dual immunoflourescence labeling for localization of PGP 9.5 in undifferentiated spermatogonial cells and c-kit in differentiated cells. Tissue samples contained in 24-well plates were fixed in 3.7% formaldehyde and stained for PGP 9.5 as described previously for immunolocalization in pools and cell fractions. Immediately after PGP 9.5 immunolabeling, the presence of c-kit, goat polyclonal antibody raised against the mouse c-kit receptor (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was evaluated on same cell tissue samples in wells following the same steps as described previously for PGP 9.5. Secondary rabbit biotinylated antibody (Vector Laboratories, Burlingame, CA, USA) was visualized by using conjugated Fluorescein Avidin DN diluted to 15 µg mL^{-1} in TBS (Vector Laboratories,

Burlingame, CA, USA) for 10 min. Finally, mounting medium (solution containing 50% Glycerol v/v final concentration in DPBS) containing 0.5 μg mL^{-1} Hoechst 33342 was added to cell tissue samples for nuclei staining. Somatic components were determined based on nuclei staining (blue). Additionally, type A spermatogonia cells staining positive to PGP 9.5 (red) or positive to c-kit (green) was determined. Due to autofluorescence in control colonies present in 24-well plate, results were not obtained.

5.4.9. Statistical analyses

Variables measured on bulls, fresh testis weight (FTW), spermatogonial cell viability (at collection, before and after Percoll), percentage of germ cells, and percentage of somatic cells present in fractions (I and II) and pool of cells (at collection and seeding) were tested in a completely randomized design. Fixed treatment was bull type (PB or AB).

Laboratory data collected from plates were analyzed using a randomized block design with factorial treatment arrangement (Proc Mixed, SAS 2003). Quantitative end points were total number of colonies, size of colonies (μm^2), total colonies positive to AP and size of AP reactivity on colonies (μm^2), and percentage of PGP 9.5 and c-kit assigned to colonies. Fixed treatment effects were presence of a feeder monolayer (FL = presence or NF = absence), fetal bovine serum type (FBS-S or FBS-SF), media type (ELSC or RSC), bull type (PB or AB), day of culture (4, 7, and 15 d) and all interactions.

Percentage of cell viability at seeding and percentage of germ and somatic cells at seeding were used as covariates. Plate (bull type*day) were random blocking factors.

Categorical data such as colony type (1 = round, 2 = radial, and 3 = irregular), colonies reactive to AP (presence or absence), intensity (0-3) of AP staining, colony type (1-3) reactive to AP, colonies positive to PGP 9.5 or c-kit markers (presence or absence) were analyzed by the GLIMMIX procedure (SAS 2006). Presence of a feeder monolayer (FL = presence or NF = absence), bull type (PB or AB), fetal bovine serum (FBS-S or FBS-SF), media type (ELSC or RSC), and interactions were used as fixed effects. Because irregular colonies were very few in number (n = 32) compared to round (n = 852) or radial (n = 1182) colonies, the statistical model failed to provide information on irregular colonies. Therefore, only statistical information for radial and round colonies is presented.

Differences were considered significant when the P value was < 0.05. For all analyses, least squares means were estimated and compared using Tukey mean separation, complex interactions were "sliced" (Littell *et al*. 2006) by day of culture to focus interpretation and comparisons of interest.

5.5. Results

Testicular components characteristics at collection and prior to culture

Single fresh testes weight after harvesting differed between prepubertal (24.09 ± 0.1 g) and adult bulls (399.03 ± 0.5 g; $P < 0.0001$). After testicular enzymatic digestion,

seminiferous tubules, fragments of seminiferous tubules, and pools of spermatogonial cells were obtained from prepubertal and adult bovine bulls (Figure 2). Immediately after digestion, cells from prepubertal bulls (92.0 ± 0.13%) had higher viability than adult bulls (84.9 ± 0.14%; $P < 0.0001$). Following culture overnight and immediately before Percoll separation, cell viability in adult bulls (61.8 ± 0.33%) was lower compared to prepubertal bull (91.0 ± 0.38%; $P < 0.0001$). Moreover, immediately after Percoll separation, cell viability in fraction II of prepubertal bulls (78.8 ± 0.20%) was higher compared to fraction I of adult bulls (44.9 ± 0.22%; $P < 0.0001$).

To evaluate the susceptibility of these two pools of cells (from prepubertal and adult bulls) to handling and manipulation, the change (difference) in viability at collection and immediately before seeding (approximately 18 hours later) was assessed. After overnight culture and Percoll separation, the decline in cell viability was more pronounced in adult bulls (39.9 ± 0.21%) compared to prepubertal bulls (12.4 ± 0.20%; $P < 0.0001$). To further narrow possible causes affecting cell viability in pools of cells immediately before and in cell fractions immediately after Percoll, cell susceptibility to handling and manipulation was evaluated. Cells from adult bulls (17.2 ± 0.12%) were more susceptible (percent reduction in cell viability) to Percoll separation than prepubertal bulls (11.5 ± 0.11%; $P < 0.0001$). Based on these results, approximately 50% of reduced viability in adult bulls occurs during Percoll separation while almost the entire decrease in cell viability in prepubertal bulls was due to Percoll separation.

Enrichment of spermatogonial cells by a discontinuous Percoll gradient

Type A spermatogonia within seminiferous tubules isolated from both prepubertal and adult bulls stained positive for PGP 9.5 (Figure 3). In prepubertal 3- to -4 month old bulls, positive staining to PGP 9.5 was scattered across the tubules (Figure 3) and was randomly distributed within the somatic components of the seminiferous tubules. In adult bulls, spermatogonia cells positive to PGP 9.5 were also randomly distributed within the active spermatic epithelium (Figure 3). Since immunolocalization for PGP 9.5 was performed on whole fragments of seminiferous tubules, the anatomical localization (lumen or basement) of positive PGP 9.5 type A spermatogonial cells within the seminiferous tubules could not be confirmed. In both types of bulls, cells in suspension and fragments of seminiferous tubules stained strongly for PGP 9.5, confirming the presence of spermatogonial cells (Figure 4).

After cell collection, the percentage of spermatogonial cells positive to PGP 9.5 in prepubertal bulls (10.4 ± 0.14%) was lower than adult bulls (17.4 ± 0.15%; Figure 4; $P < 0.0001$). Following cell purification by a discontinuous Percoll density gradient, percentage of spermatogonial cells positive to PGP 9.5 present in fraction II of prepubertal bulls (11.5 ± 0.17%) differed from fraction I adult bulls (15.5 ± 0.19%; Figure 4; $P < 0.0001$). The efficiency of a discontinuous Percoll density gradient as a means to enrich spermatogonial cell populations was assessed by calculating the difference between spermatogonial cells in suspension before (pool of cells) and after Percoll separation (fraction II of PB and fraction I of AB). The change in spermatogonial cells staining positive to PGP 9.5 following Percoll separation improved slightly in prepubertal bulls (1.3 ± 0.84%) and was reduced in adult bulls (-2.2 ± 0.09%; $P <$

0.0001); however, the overall spermatogonial cell enrichment in both cases was low (prepubertal bulls) or detrimental (adult bulls).

On the other hand, testicular somatic components present in the pool of cells (90.0 ± 0.15%) and fraction II (88.4 ± 0.17%) in prepubertal bulls was higher than in the pool of cells of adult bulls (82.3 ± 0.16%) and fraction I (84.5 ±0.19%; $P < 0.0001$). Moreover, somatic components after cell enrichment by Percoll separation was reduced in prepubertal bulls (-1.3 ± 0.07%) or low in adult bulls (2.5 ± 0.08%; $P < 0.0001$).

Effects of bull type and culture treatment on type A spermatogonia cells

In general, low number of colonies was observed on day 4 of culture, however, number and size of colonies increased by day 15 of culture. Furthermore, the presence of a FL improved the establishment of colonies. The mixture of type A spermatogonial cells used in culture yielded three types of colonies (round, radial, and irregular) in this experiment. Overall, radial colonies were the most predominant type present in culture.

Colony number: The number of colonies gradually increased from day 4 to 15 of culture (Tables 1-3). Low number of colonies per well were observed on day 4 compared to day 7 or 15 (Table 1-3) of culture. However, the number of colonies per well was not affected by bull type (PB or AB; $P = 0.88$), media type (ELC or RSC; $P = 0.13$), or serum type (FBS-S or FBS-SF; $P = 0.16$). The maximum number of colonies were observed at day 7 in prepubertal bulls and on day 15 in adult bulls (Table 1-3). Overall, the presence of a feeder monolayer yielded more colonies compared to absence of a feeder monolayer (Table 1-3). Numerically, co-culture type A spermatogonia with a feeder monolayer

resulted in more colonies per well at day 7 (prepubertal bulls; Table 2) and day 15 (adult bulls; Table 3) of culture; however, no significant differences were observed between bull type (Table 2 and 3). Moreover, this lower number of colonies on day 15 in prepubertal bulls was coincident with a large number of wells presenting a detached feeder monolayer at the time of colony evaluation. Regardless of bull type, presence of a FL and RSC media yielded more colonies on day 15 of culture (Table 3). Additionally, co-culture of type A spermatogonia in presence of a FL, RSC plus FBS-S resulted in maximum number of colonies at day 15 of culture from prepubertal and adult bulls (Table 3).

Colony area: As expected, colonies formed by day 4 and 7 of culture were smaller in size compared to colonies at day 15 (Table 1-3). Colony size was not affected by bull type (PB or AB; $P = 0.24$) or media type (ELSC or RSC; $P = 0.80$). There was a bull type (PB or AB) x day of culture (4, 7, or 15) x feeder monolayer (FL or NF) x media type (ELSC or RSC) x FBS type (FBS-S or FBS-NF) interaction (five way interaction; Tables 1-3). The presence of a FL resulted in larger colonies regardless of bull type or media type (Table 1-3). Moreover, both media type supplemented with FBS-S yielded larger colonies (Table 1-3). Furthermore, the maximum size of colonies was obtained in presence of a FL, RSC plus FBS-S (Table 3).

Colony type: The type of colony observed in this study included round, radial, and irregular. Because most of the colony evaluation was performed using Hoffman contrast, it was difficult to discern between cell type within and surrounding colonies. Colonies were evaluated under phase contrast in an attempt to better describe the morphology of cells within colonies; however, due to the small diameter of the well (24-well plate), a

distortion of the phase contrast rings was produced. Only colonies present in the center of the well could be evaluated, and often times no colonies were present. Morphologically, round colonies had a well-defined rim between the edge of the colony and the feeder monolayer interaction (Figure 7). Round colonies were composed mostly of a homogeneous type of cells tightly interconnected. This type of colony appeared to have a cone-like pattern of cellular growth and was composed of several layers of cells. In contrast, radial colonies were formed by cells regularly seen to be interconnected (Figure 7). Between the edge of the colony and the feeder monolayer, a transitional type of cell was characterized morphologically with finger-like cytoplasm protrusions present (Figure 7). Cells within the colony were organized in a loose pattern as opposed to round colonies and single cells were observed often. Finally, irregular colonies (Figure 7) were composed of cells with similar morphology as round colonies but presented in an irregular shape, resembling different patterns of cellular growth.

Because irregular colonies were very few in number (n = 32) compared to round (n = 852) or radial (n = 1182) colonies, the statistical model failed to provide information on irregular colonies. Therefore, only statistical information for radial and round colonies is reported. Overall, radial colonies were the most predominant type of colony in prepubertal and adult bulls (Table 1-3). However, type of colony was not affected by day of culture (4, 7, and 15; P = 0.99), bull type (PB or AB; P = 0.99), feeder monolayer (FL or NF; P = 0.99), media type (ELSC or RSC; P = 0.99), or serum type (FBS-S or FBS-SF; P = 0.99). The establishment of type A spermatogonia colonies (round or radial) in presence of a NF was minimum in adult bulls (Table 1-3) compared to prepubertal bulls (Table 1-3) across the experimental period. Furthermore, co-culture of type A

spermatogonia in a FL, ELSC plus FBS-SF resulted in more round colonies in prepubertal (73 ± 5%) than adult bulls (39 ± 5%; Table 3) at day 15 of culture. On the other hand, the same treatment combination resulted in more radial colonies in adult (61 ± 5%) than prepubertal bulls (27 ± 5%; Table 3) at day 15 of culture.

Alkaline Phosphatase reactivity

Alkaline phosphatase (AP) staining was performed in an attempt to better describe cells present on colonies. The percentage of positive AP colonies was not affected by day of culture (4, 7, and 15; $P = 0.21$), bull type (PB or AB; $P = 0.99$), or feeder monolayer (FL or NF; $P = 0.24$). Additionally, there was a bull type (PB or AB) x day of culture (4, 7, or 15) x feeder monolayer (FL or NF) x media type (ELSC or RSC) x FBS type (FBS-S or FBS-NF) interaction (Tables 4-6). The percentage of the total colonies present at day 4 that stained positive for AP was greater compared to day 7 and 15 of culture (Table 4-6). Furthermore, colonies on a FL were more reactive to AP than colonies without a feeder monolayer across the experimental period (Table 4-6). Additionally, co-culture of type A spermatogonia in a FL, ELSC or RSC plus the addition of FBS-S resulted in more positive AP colonies at day 15 of culture in prepubertal bulls (Table 6).

The type of colony (round or radial) that stained for AP was affected by day of culture (4, 7, and 15; $P < 0.016$). The percentage of positive AP round (48 ± 10%) and radial (82 ± 6%) colonies was greater at 15 days of culture compared to round (18 ± 6%) and radial (52± 10%; $P < 0.016$) at day 7 of culture. The color intensity (0 = none, 1 =

slight, 2 = mild, and 3 = moderate) of positive AP colonies was evaluated. However, the intensity of positive AP colonies was not affected by day of culture (4, 7, and 15; P = 0.23), media type (ELSC or RSC; P = 0.96), serum type (FBS-S or FBS-SF; P = 0.97), and interactions. For those colonies that stained positive, the proportion between the colony area and the area that stained red was determined. The total area of colonies that stained for AP was not affected by day of culture (4, 7, and 15; P = 0.95), bull type (PB or AB; P = 0.73), and feeder monolayer (FL or NF; P = 0.93), media type (ESLC or RSC; P = 0.37), or FBS type (FBS-S or FBS-SF; P = 0.14).

Expression of PGP 9.5 and c-kit in proliferating spermatogonial cells in culture

To evaluate differentiation of type A spermatogonial cells present in culture, immunolabeling with PGP 9.5 and c-kit positive cells were assessed at three time periods (4, 7, and 15 days of culture) under various conditions. Because autoflourescence was observed in control samples, it was difficult to discern between positive or false positive staining signal. Therefore, information regarding PGP 9.5 and c-kit was not presented. A colorimetric immunolabeling using PGP 9.5 and c-kit may be an alternative to avoid this problem.

Discussion

Type A_S spermatogonial cells (SSC) have unique capabilities including self-renewal and production of the end product, spermatozoa. The ability to recover these

cells from donor animals and perform *in vitro* culture may provide a valuable tool to study SSC proliferation and differentiation *in vitro*. Primary findings of this study demonstrate that: 1) viable type A spermatogonial cells can be harvested from prepubertal and adult bulls, 2) spermatogonia cells survived *in vitro* and were able to proliferate and form different types of colonies, 3) co-culture of spermatogonial cells with a defined feeder monolayer plus FBS-S enhanced colony number (may be due to increasing cell viability), and 4) different types of colonies stained positive for AP.

Spermatogonial cells from prepubertal bulls used in this study (3.5 mo with scrotal circumference of 15 cm and single fresh testis weight of 24 g) were morphologically similar to previous reports in cattle (Izadyar *et al*. 2002; 2003a; Herrid *et al.* 2006). However, spermatogonia cells collected from adult bulls (testis weight of ~400 g) were morphologically different, with the entire array of developing germ cells plus spermatozoa obtained in the mixture (Figure 2). Although spermatogonial cell viability at collection differed between prepubertal (~92%) and adult bulls (~85%), they were in accordance with prior studies using calves (Izadyar *et al.* 2002; 2003a), goats (Honaramooz *et al*. 2003) and pigs (Honaramooz *et al*. 2002; Luo *et al.* 2006). Furthermore, it has been reported previously that spermatogonial cell viability was reduced after overnight culture (Luo *et al*. 2006) as seen in adult bulls in the current study.

In this experiment, a discontinuous Percoll density gradient was examined as a procedure to enrich spermatogonial cells based on previous reports (van Pelt *et al*. 1996; Izadyar *et al*. 2002). This method selects spermatogonia on the basis of size, shape, and centrifugation forces. In an attempt to determine the susceptibility of spermatogonial cells

to a discontinuous Percoll gradient separation, cell viability was evaluated from collection to seeding. After overnight culture, the decline in cell viability was more pronounced in adult bulls (~40%). However, cell viability in both bull types was affected by Percoll separation method, giving better viability in fraction II of prepubertal (~79%) compared to fraction I of adult bulls (~45%). Although spermatogonial cells were slightly higher after an enrichment method in prepubertal bulls, the efficiency was low or detrimental in terms of cell viability. While enrichment of cell populations seemed to work best in prepubertal bulls, the degree of enrichment was not comparable to those reported by Izadyar *et al*. (2002) using similar methodology and animals. In adult bulls, the combination of developing germ cells and spermatozoa appears to be more susceptible to handling and more challenging to purify. Despite low cell viability in adult bulls, spermatogonial cells were able to establish colonies *in vitro*. It has been suggested that the ability of germ cells to form colonies indicates that overall cellular health is not compromised (Izadyar *et al*. 2003a) or at least supported by the culture system.

PGP 9.5 is a specific marker for type A spermatogonia cells in cattle (Wrobel 2000; Herrid *et al.* 2006) and pigs (Luo *et al.* 2006). In this study, positive PGP 9.5 spermatogonia cells followed the distribution pattern of gonocyte localization within seminiferous tubules as reported by Luo *et al.* (2006) in neonatal pigs and Herrid *et al*. (2006) in prepubertal bulls. In adult bulls, positive PGP 9.5 cells were also present within seminiferous tubules (Figure 3). Seminiferous tubules were 3 to 4 times larger in diameter than prepubertal bulls (Figure 3) and developing germ cells such as spermatids were visualized (Figure 3). For both types of bulls, PGP 9.5 was a reliable and strong marker for identification of spermatogonial cells in suspension; thus, making it an

excellent tool to identify spermatogonia during *in vitro* manipulation. To be able to maintain unequivocally type A_S spermatogonia (considered stem cell; reviewed by de Rooij 2001) in culture for several weeks is a difficult task, but it is of paramount important for future studies aimed at manipulation of germ cell lines.

The first step when culturing SSC is to confirm that spermatogonia are present in the culture system. Spermatogonial cells have the ability to self-replicate (Wrobel 2000; reviewed by Brinster 2002; Kubota *et al.* 2004) and to re-colonize recipient testis after transplantation (Dobrinski *et al*. 2000) or to form colonies *in vitro* (Izadyar *et al*. 2003a). In the current experiment, both prepubertal and adult bovine SSC were able to form colonies *in vitro* from day 4 of culture onward. Morphological characteristics of colonies present were in accordance with the pattern of SSC proliferation of *in vitro* produced colonies reported previously in prepubertal bulls (Izadyar *et al.* 2003a), neonatal and prepubertal pigs (Luo *et al*. 2006; Goel *et al.* 2007), and mice (Anjamrooz *et al.* 2006; Koruji *et al*. 2007). Additionally, SSC from adult bulls proliferated into pairs, chains of spermatogonia, and ultimately formed colonies in culture with similar morphological characteristics as prepubertal bulls (Figure 7). Although SSC from adult bulls have been isolated previously and proliferated for a few weeks in recipient mice testes after transplantation (Dobrinski *et al.* 2000), information regarding *in vitro* performance is lacking.

In the current study, SSC from adult bulls were able to establish colonies in culture; thus, confirming that SSC retained stem cell capacity. In this study, type A spermatogonia from prepubertal and adult bulls were cultured along with testicular somatic components (i.e. Sertoli cells). It is known that Sertoli cells from prepubertal

bulls (3-7 month of age) are in the proliferative phase of development. Also, it was suggested that somatic component (myoid, Sertoli cells, etc.) can form a feeder monolayer in culture which eventually support spermatogonial proliferation (Izadyar *et al*. 2003a). On the other hand, adult somatic components (myoid, Sertoli cells, etc.) do not proliferate; therefore, no structural support exists for spermatogonial cells proliferation. In this study, type A spermatogonial colonies were observed in presence of a feeder monolayer (BFF-8) or without it in prepubertal bulls. However, most of the colonies from adult bulls were restricted to a presence of feeder monolayer (BFF-8). This finding may suggest that in fact type A spermatogonia cell were present in culture and formed colonies. While type A spermatogonia from prepubertal bulls formed colonies on either FL or NF, cells from adult bulls formed colonies on a FL. This finding suggested that type A spermatogonia in the current experiment proliferate and form colonies; thus, SSC were present in the cell suspension collected and in culture. Therefore, we suggest that SSC were present in the culture system and were able to proliferate and to form colonies (Figure 7) from prepubertal and adult bulls.

After 15 days of *in vitro* culture, three types of colonies of spermatogonial cells were observed: round, radial and irregular. Izadyar *et al.* (2003a) reported in a similar study that *in vitro* culture of bovine SSC yielded more radial than round colonies while the latter were few in numbers. In the current experiment, radial colonies were morphologically similar to those reported by Izadyar *et al.* (2003a); however, *in vitro* culture in the present study yielded more round colonies of smaller size than previously reported (Izadyar *et al.* 2003a). Numerically, radial colonies were the most predominant type of colony followed by the appearance of round colonies. Morphologically, round

colonies were tightly organized and intercellular bridges between cells could not be confirmed. It appears that this type of colony grew following a cone-like pattern of cellular growth. However, radial colonies were formed from cells regularly seen to be interconnected for intercellular bridges. Between the edge of the colony and the feeder monolayer, a transitional type of cell morphologically characterized by finger-like cytoplasm protrusions was present. Cells within the radial colony were organized in a loose pattern as opposed to round colonies.

During the differentiation process of single type A spermatogonia, formation of intercellular bridges occur (Wrobel 2000; Izadyar *et al*. 2003a). Although type A_P and A_C spermatogonia are considered differentiated cells, agreement exists that type A_S has stem cell capacity (Wrobel 2000). Although PGP 9.5 and c-kit were used in attempts to confirm the presence of type A spermatogonia in culture, control and test colonies of spermatogonial cells stained positive to PGP 9.5 and c-kit. Immunolocalization of spermatogonial cells in culture was performed by immunofluorescence; however, both the sample to be tested (primary and secondary antibody) and negative control (without primary antibody) looked similar. At first, it appears that autofluorescence caused by either the 24-well plate (shape of the well) or plastic (reflecting light) may have interfered with the signal. In previous studies, detection of SSC in culture (against PGP 9.5 and c-kit proteins) has been performed by immunlocalization either using fluorescence or colorimetric reagents (Izadyar *et al*. 2003a; Luo *et al*. 2006). The latter may be an alternative for future studies.

Alkaline phosphatase (AP) reactivity is highly conserved in undifferentiated embryonic stem cells (Talbot *et al.* 1993) and type A spermatogonia cells (Ginsberg *et al*.

1990). High levels of AP in spermatogonial cells are related to the undifferentiated stage (Fujino *et al.* 2005). Therefore, AP activity was evaluated on SSC in culture as a means to detect presence of type A spermatogonia. Although the majority of the colonies stained negative for AP, colonies formed on FL showed more AP intensity (Figure 8). Therefore, this may indicates that FL supports proliferation and maintenance of type A spermatogonia in culture as previously reported (van de Wee *et al.* 2001).

In the present study, several culture conditions were tested. Co-culture of SSC on a feeder monolayer resulted in more and larger colonies. Similar results were observed in mice spermatogonia co-cultured with a defined feeder monolayer of adult Sertoli cells, resulting in proliferation of undifferentiated SSC for up to 25 days (van de Wee *et al.* 2001). In the current experiment, FL supported survival and proliferation of SSC in both type of animals as opposed to the absence of a feeder monolayer. Additionally, the presence of colonies on NF in prepubertal bulls could be explained, at least in part, since SSC were obtained from prepubertal calves, and somatic components (i.e. Sertoli cells) actively proliferate at this age, which in turn form a monolayer where SSC can proliferate and form colonies. Furthermore, approximately 85 to 89% of the cells present in fraction II (prepubertal bull) and I (adult bulls) at seeding were somatic components. On the other hand, colonies from adult bulls were fewer in number with NF, possibly due to adult Sertoli cells not proliferating as in prepubertal bulls; thus, less structural support exists for colony formation. The ability of type A spermatogonia from adult bulls to form colonies may be indicative, in part, that spermatogonia were present in our culture system as mentioned above. Adult Sertoli cells do not proliferate as prepubertal Sertoli cells,

indicating that isolated type A spermatogonia cells from adult bulls have the potential (stem cell) to form colonies in culture.

The presence of a FL favors SSC proliferation in prepubertal and adult bulls; however, it seemed that SSC from adult bulls are more sensitive to the microenvironment (cell-cell interaction) since most of the colonies were observed in the presence of a FL. This finding highlights the importance of close contact between SSC and their cellular environment (intercellular communication) for spermatogonial proliferation as reported previously (van der Wee *et al*. 2001). In this study, the importance of structural support for spermatogonial survival and colony formation was evident primarily in adult bulls. The overall success of culture system could be attributed to the presence of a FL (prepubertal and adult bulls) and, at least in part, to actively proliferating Sertoli cells (present in the mixture) collected from prepubertal calves, which eventually formed a monolayer that supported SSC survival and proliferation. This is in agreement with results reported by Izadyar *et al*. (2003a), where SSC collected from prepubertal bulls (similar to the present study) formed colonies *in vitro* without using a defined FL.

To be able to maintain undifferentiated SSC in culture is a valuable tool to understand proliferation mechanisms of this specialized cell. It was suggested that proliferation and differentiation of SSC are regulated by intrinsic factors associated to SSC and factors associated with their environment (Nagano *et al*. 2003). Maintenance of spermatogonial cells from prepubertal and adult mice was enhanced by the presence of a feeder monolayer and addition of GNDF factor (Nagano *et al.* 2003). In the current culture system, the presence of a FL definitely enhanced SSC proliferation, possibly

through close cell-cell interactions or some factor(s) secreted by cells forming the FL as reported above.

Spermatogonial cells can be maintained and proliferated in culture; however, how hormones affect protein synthesis and proliferation of SSC in culture remains to be elucidated. In this study, two serum types were evaluated in order to discern whether the presence (FBS-S) or low absence (FBS-SF) of steroids may affect SSC survival and proliferation. Additionally, culture media (ELSC or RSC) consisted of free-phenol red (a pH indicator), a chemical known to have significant estrogenic like-activity on cells in culture (Berthois *et al.* 1986). It is known from these studies that the addition of serum such as FBS, FCS, HS, or Nu serum (low protein replacement of serum) to the medium enhanced type A spermatogonia cells survival and proliferation in culture (van der Wee *et al*. 2001; Izadyar *et al*. 2003a; Anjamrooz *et al*. 2006; Goel *et al*. 2007). Additionally, growth factors added to the medium in presence of serum also contributed in more type A spermatogonia proliferating. However, it is difficult to discern what component(s) present in serum are essential for self-renewal of type A_S spermatogonial. Therefore, further studies are needed to discern what component(s) between serum types (i.e. lipids, proteins, hormones, etc.) support self-renewal of type A_S spermatogonial. Furthermore, testosterone and dihydrotestosterone are required for germ cell development during spermiogenesis (Kerr *et al.* 1993; O'Donnell *et al.* 1994; McLachlan *et al.* 1996). Estrogens are known to stimulate diverse biosynthetic pathways in different target tissues, including Sertoli cells (reviewed by Hess 2003; Sneddon *et al.* 2005). In the present experiment, SSC co-cultured with a FL and a combination of RSC media plus

FBS-S resulted in more radial and round colonies at 15 days of culture regardless of bull type.

Based on biochemical analysis of FBS-SF used in this experiment (a charcoal/dextran treated FBS) provided by the manufacturer, concentrations of the major steroid sex hormones were low (17 β-estradiol 1 pg mL^{-1}; testosterone <3 ng mL^{-1}; and progesterone <10 ng mL^{-1}). This may explain, in part, that addition of FBS-S to the media (presumably with higher level of sex steroid hormones than FBS-SF) favors survival and proliferation of SSC in culture. Since phenol red was not present in the media, sex steroids hormones present in FBS-S may be responsible for the successful outcome in this experiment. In a recent study, the ability to form colonies in co-cultured mouse SSC exposed to different combinations of EGF, FSH, and testosterone was evaluated (Anjamrooz *et al*. 2006). It was concluded that addition of EGF to the media improved *in vitro* SSC proliferation. Moreover, media type (DMEM) and level of FBS (10%) in the study by Anjamrooz *et al*. (2006) was comparable to our *in vitro* media conditions. Therefore, we can support this finding by adding that FL and steroid hormones present in culture conditions improved SSC proliferation and colony formation. It appears reasonable to suggest that growth factor(s) present in media or secreted by FL aided in SSC proliferation. Furthermore, culture media used in the current experiment consisted of a widely used synthetic culture medium (DMEM) either low in glucose (RSC) or containing high glucose plus non-essential amino acids and nucleosides as described earlier (ELSC). Our results demonstrate that RSC media resulted in a greater number of colonies regardless of bull type. It seemed that addition of glucose, non-essential amino acids and nucleosides to the media did not improve proliferation of SSC

in culture while low levels of glucose present in RSC media did. In recent studies, the use of comparable media (DMEM or MEM) plus serum resulted in successful spermatogonia survival and proliferation in bulls and pigs (Izadyar *et al*. 2003a; Luo *et al*. 2006). One reasonable explanation of the low success in ELSC media containing high level of glucose at 15 day of culture in prepubertal bulls may be related to large number of wells presenting detached feeder monolayer before day 15 of culture. Izadyar *et al*. (2003) reported that the addition of up to 10% of serum to a similar media (DMEM) favors only somatic components.

In conclusion, viable bovine SSC from two types of bulls (PB and AB) were isolated successfully, maintained *in vitro*, and proliferated to form large colonies in a short-term culture system. Expression of PGP 9.5 was found to be a strong and reliable marker for bovine SSC. Positive AP colonies were present, suggesting that type A spermatogonia proliferated and was supported by culture conditions. Results provide the basis for future studies aimed at improving culture condition and ultimately modification of SSC before transplant and/or production of offspring by intracytoplasmatic sperm injection (ICSI). If stem cells can be identified and isolated correctly under culture conditions, then spermatogenesis *in vitro* could became a reality, which has major applications in livestock and human male fertility.

5.7. References

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5.8. Appendix: Figures and Tables

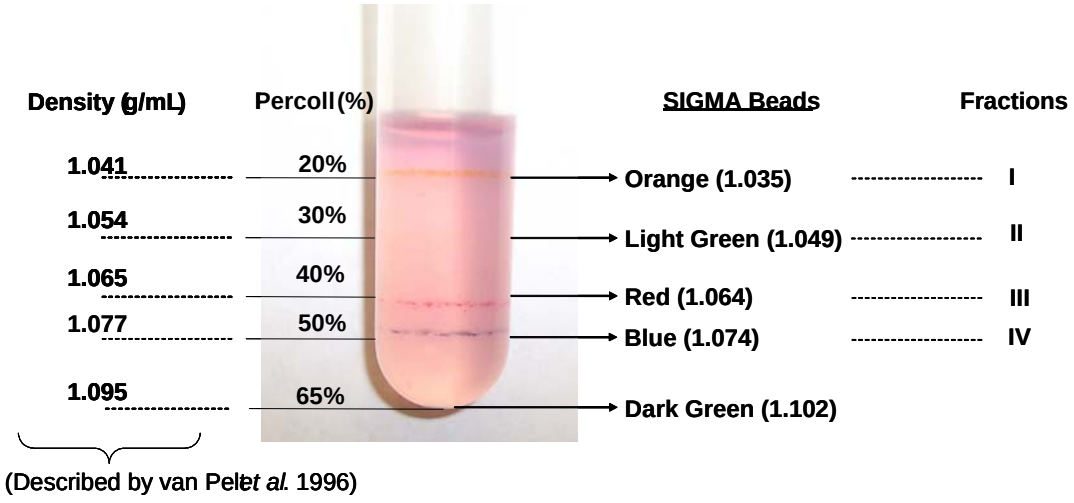

Figure 1: Representation of a discontinuous Percoll density gradient illustrating the position of each cell fraction and corresponding density marker beads after centrifugation.

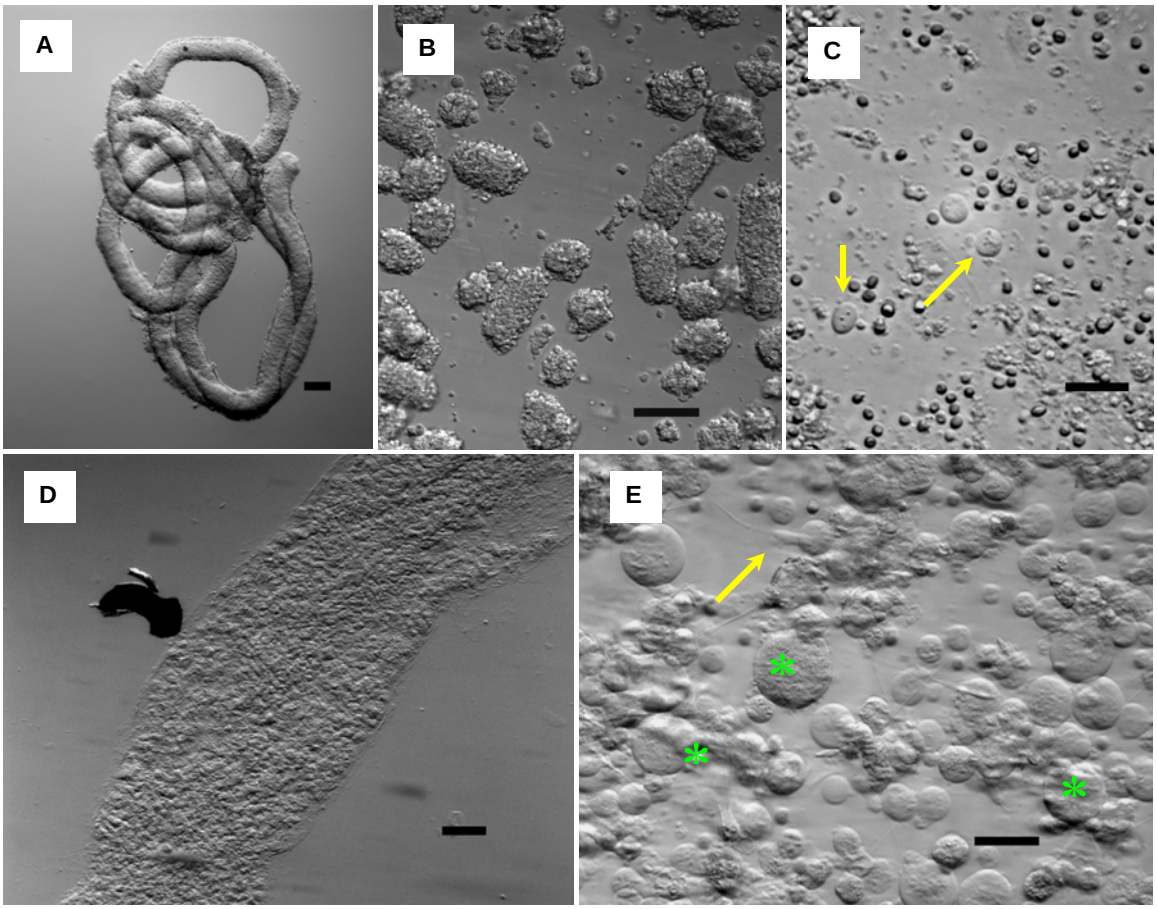

Figure 2: Representative images of bovine testicular components obtained before enzymatic digestion of seminiferous tubules. **A)** Fragment of seminiferous tubule from a prepubertal bull (100 x). Note that seminiferous tubule has little interstitial components associated with it. **B)** Smaller fragments of seminiferous tubules obtained after the first enzymatic digestion (200 x). Spermatogonia cells from these seminiferous tubules fragments were used in culture. **C)** Corresponding spermatogonial cells after the second enzymatic digestion of seminiferous tubule fragments (400 x). Note the homogeneous cells with one or two nuclei (arrows), morphologic characteristic of gonocytes at this stage of development in prepubertal bulls (~ 3 mo of age). These cells represent the pool of cells used for Percoll separation. **D)** Representative Hoffman view of seminiferous tubules from adult bulls (200 x). Note the size of seminiferous tubules compared to prepubertal bulls. **E)** Corresponding spermatogonial cells after enzymatic digestion (400 x). The entire array of developing cells, different in size (asterisks), was recovered from active spermatic epithelium. This pool of cells was obtained after the first enzymatic digestion and no fragments of seminiferous tubules were observed as in prepubertal bulls. Sperm present in the mixture of cells after digestion (arrows). **A, B, and C)** Bars = 100 µm. **C and E)** Bars = 25 µm.

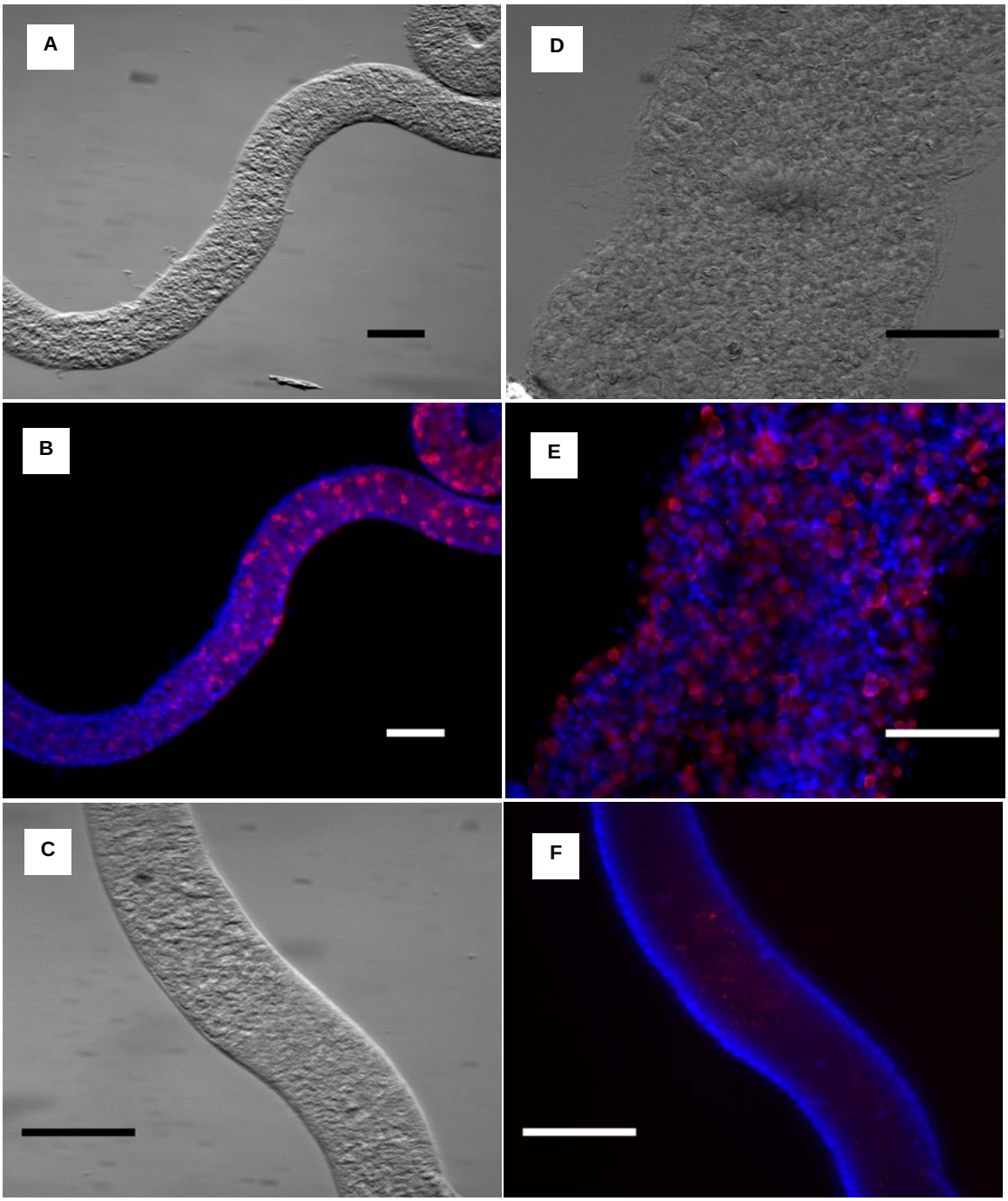
A
D
B
E
C
F

Figure 3: Immunolocalization of spermatogonial cells positive to PGP 9.5 in seminiferous tubules from prepubertal and adult bulls. **A** and **D)** Representative images from repubertal bull at 3 months of age (**A**, 100 x) and adult bull (**D**, 200 x). **B** and E**)** Immunolocalization of positive PGP 9.5 cells within the seminiferous tubules in prepubertal (**B**, 100 x) and adult bulls (**E**, 200 x). **C)** Representative images from prepubertal (200 x) and **F)** same image from prepubertal stained negatively for PGP 9.5 (control; 200 x). Note that positive PGP 9.5 cells (red) are distributed randomly within the seminiferous tubules. Also note the somatic components (blue) in prepubertal and adult bull. Recognize that some of positive PGP 9.5 cells are single or interconnected by intercellular bridges, characteristic of developing A-pair spermatogonia. Bars = 100 μm.

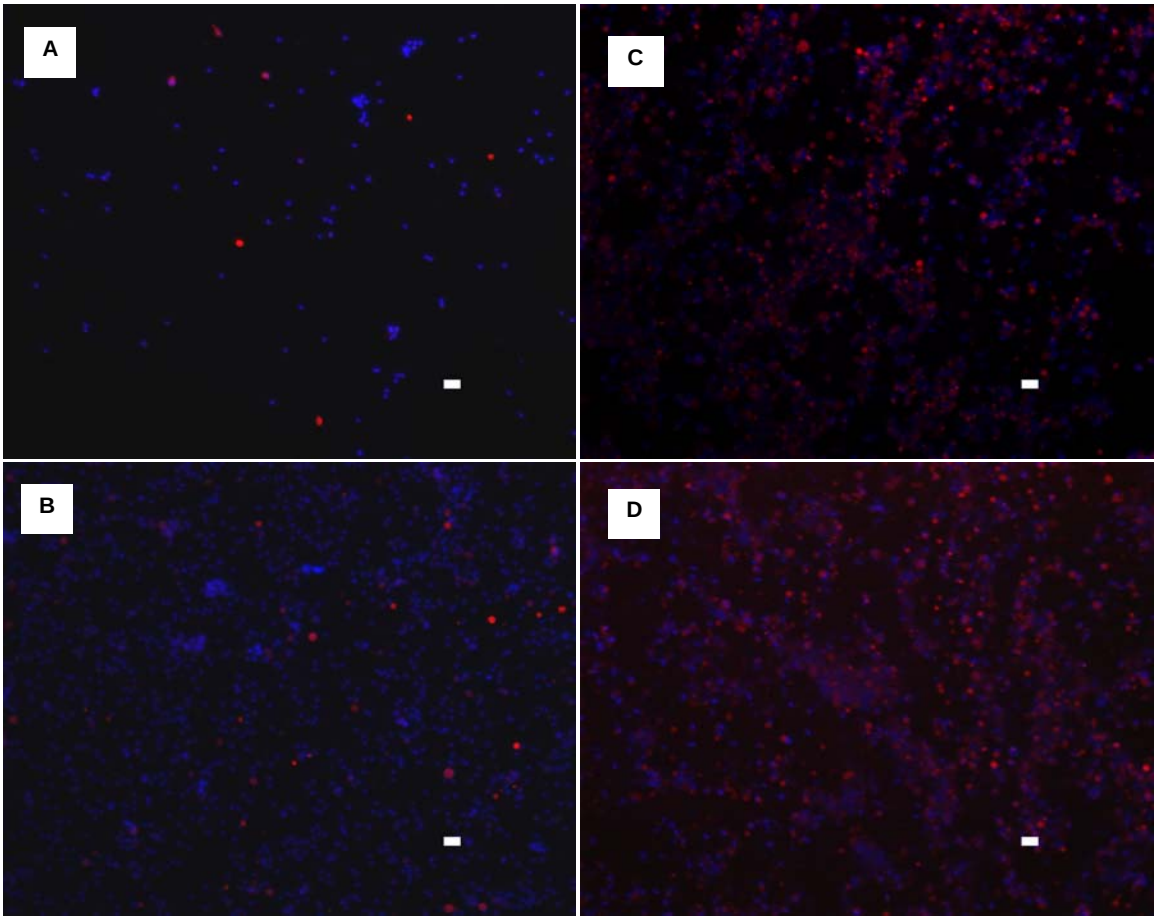

Figure 4: Spermatogonial cell characterization in pools of cells and fractions (I and II) from prepubertal and adult bulls. **A** and **C)** Immunolocalization of positive PGP 9.5 cells in prepubertal and adult bulls (100 x). Type A spermatogonia are positively stained (red) for PGP 9.5 and somatic components stain blue. Note the strong and dense staining pattern of positive PGP 9.5 cells (**A**, prepubertal bull, and **C**, adult bull). **B** and **D**) Cells obtained following Percoll density gradient as fraction II in prepubertal (**B**, 100 x) and adult bull (**D**, 100 x). Bars = 25 μm.

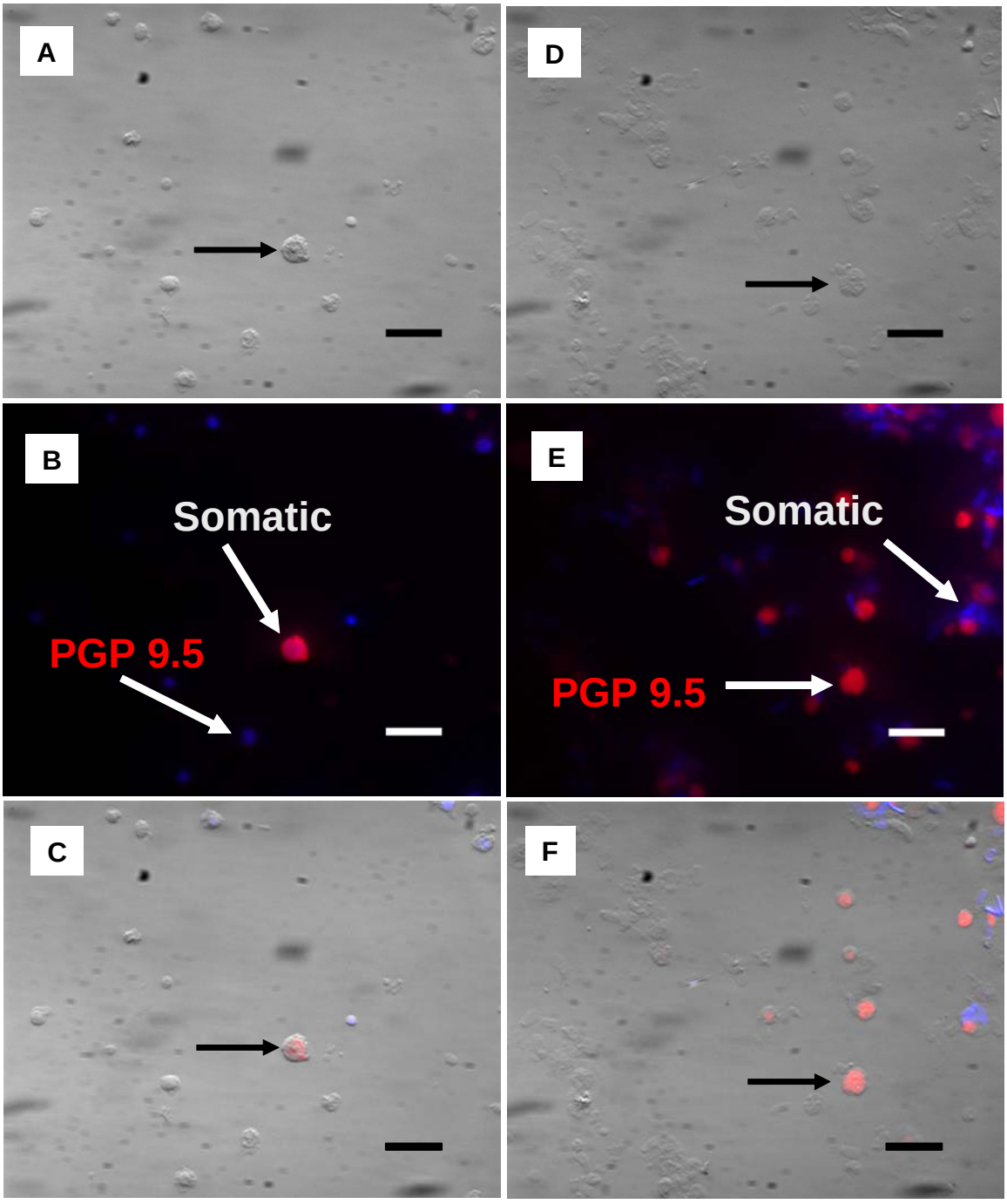
A
D
B
Somatic
PGP 9.5
E
Somatic
PGP 9.5
C
F

Figure 5: Morphological characteristics of spermatogonial cells before and after culture. Representative micrographs showing positive PGP 9.5 cells from prepubertal and adult bulls. **A-C** and **D-F**) Micrographs correspond to spermatogonial and somatic components from prepubertal and bulls. **A** and **D**) A Hoffman view of nuclei, morphological characteristic of spermatogonial cells, present in prepubertal and adult bulls (arrows, 400 x). Spermatogonia cells are rounded and large in size (arrows) compared to testicular somatic components (asterisk). **B** and **E**) Spermatogonia cells positive to PGP 9.5 stained intensively (red) and somatic components (blue, 400 x). **C** and **F**) Merged micrographs showing the exact location of positive PGP 9.5 spermatogonial cells (400 x). **G** and **H**) Immunolocalization of single PGP 9.5 cells after 15 days of culture (400 x). Microphotographs showing positive PGP 9.5 cells (**G**) and a Hoffman view of the same cells in culture layered on top of a feeder monolayer (**H**). Note that single cells are rounded and large in size (arrow) and present the nuclei, characteristic of spermatogonia cells. Bars = 25 μm.

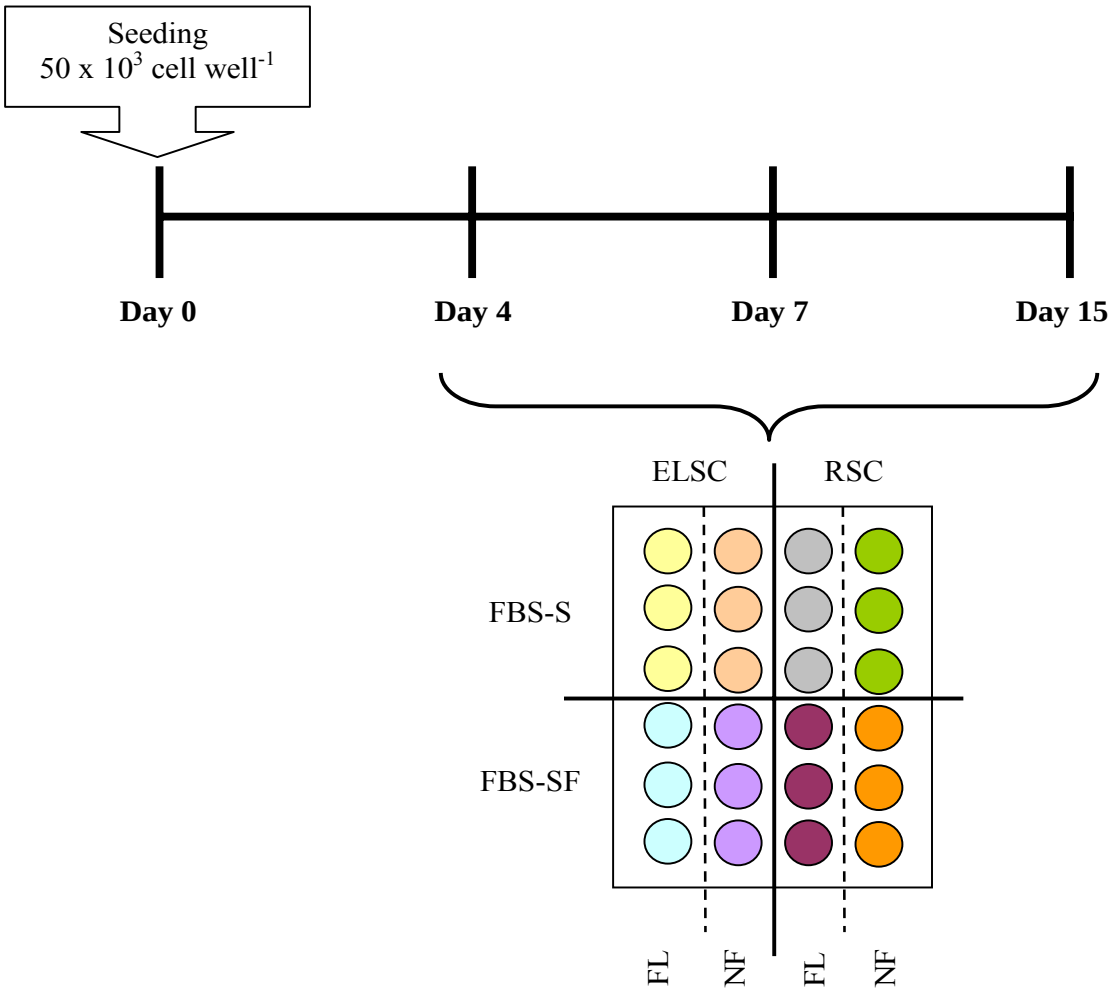

Figure 6: Schematic representation of experimental design. At the onset of culture (day 0), presumptive type A spermatogonia from two bull types (prepubertal and adult) were seeded into 24-well plates and remained in culture for 4, 7 and 15 d. Spermatogonia were cultured under the presence (FL) or absence (NF) of a feeder monolayer with two types of media (ESCL or RSC) supplemented with (FBS-S) or without (FBS-SF) steroids. At each time period, one 24-well plate was removed from the incubator and tissue was fixed in 3.7% formaldehyde.

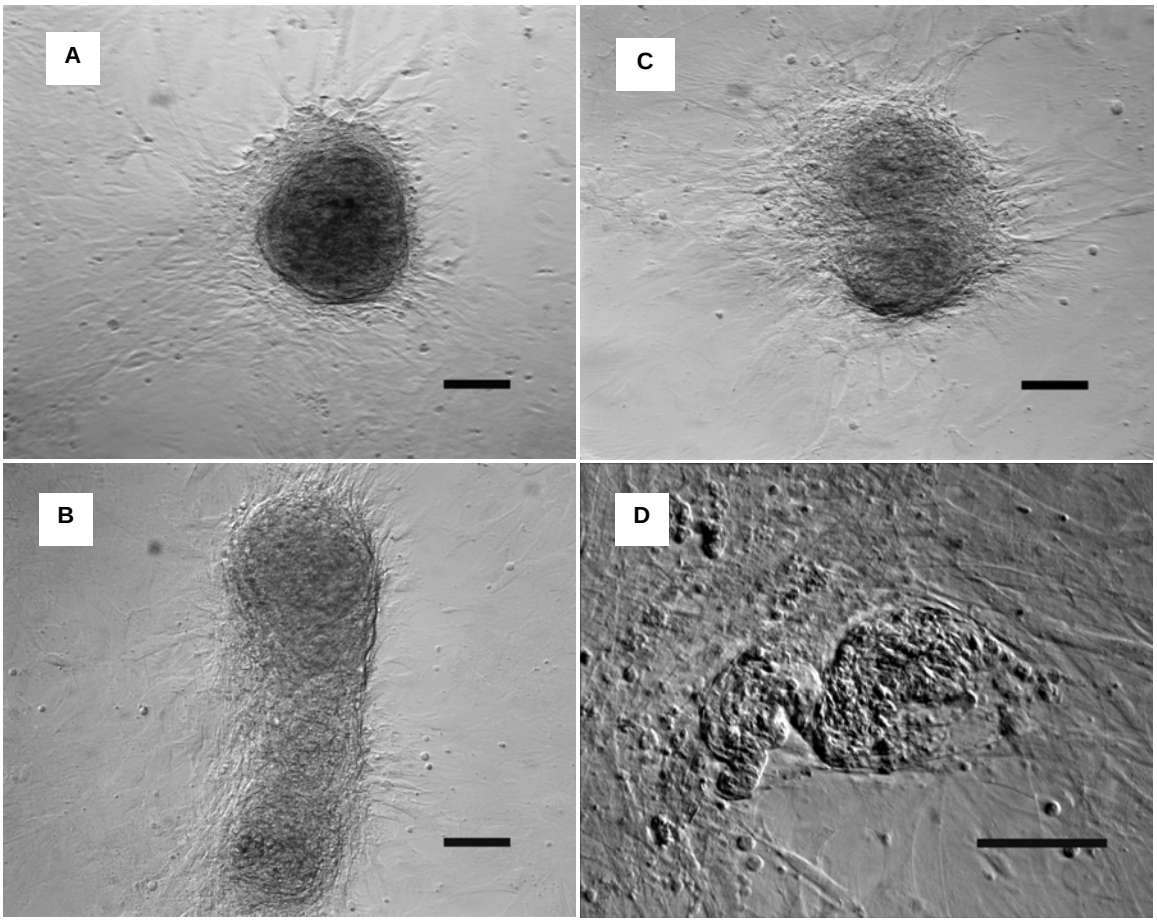

Figure 7: Representative Hoffman contrast images of colonies from prepubertal and adult bulls on day 15 of culture. **A**) Round colony from prepubertal bull (100 x). **B** and **C**) Radial colonies from prepubertal bull (100 x). **D**) Irregular colonies from adult bull (200 x). Bars = 100 μm.

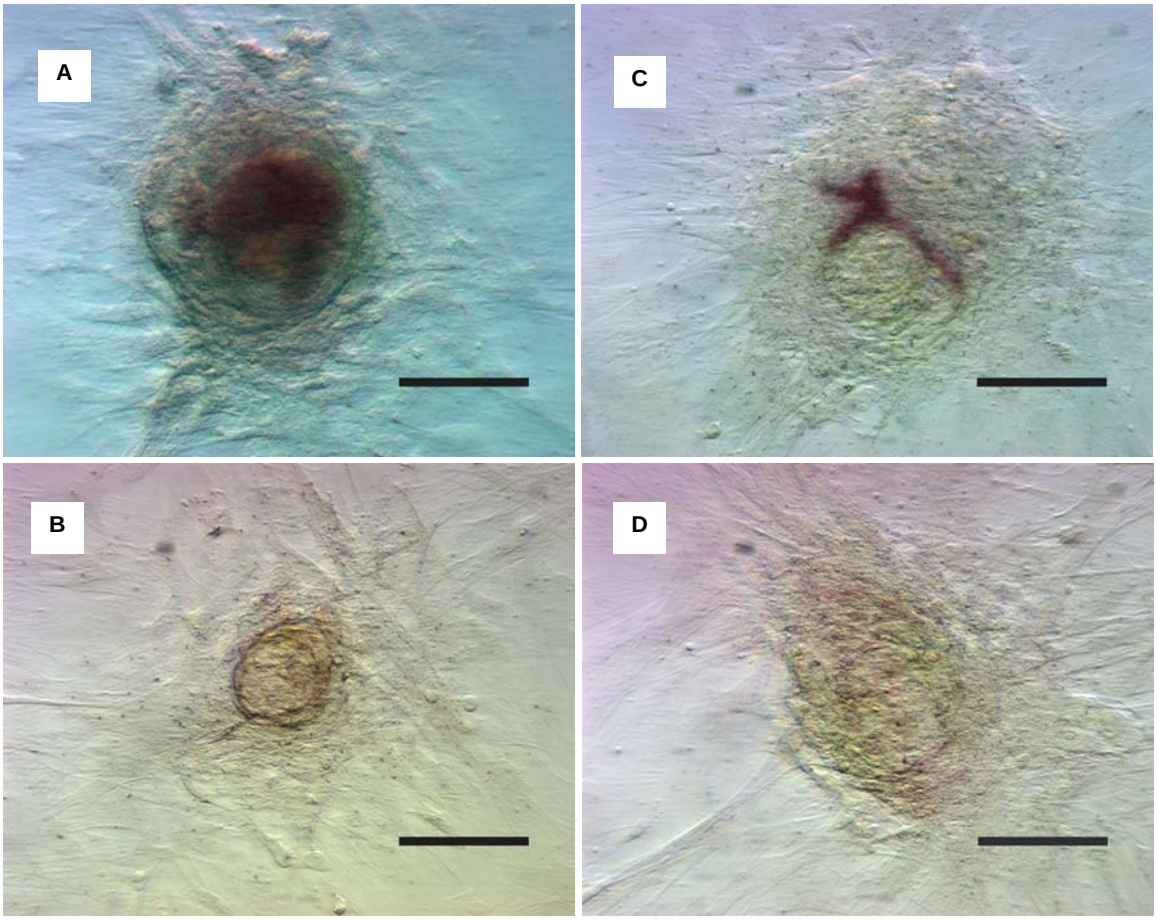

Figure 8: Representative microphotographs showing different alkaline phosphatase (AP) reactivity on different types of spermatogonial colonies. **A**) A round colony that stained moderately for AP activity in cells from prepubertal bull (200 x). **B)** A bright field microphotograph showing a round colony with no staining for AP in cells from prepubertal bull (200 x). **C)** A radial colony stained mild for AP in cells from prepubertal bull (200 x). **D**) Radial colonies slightly stained for AP reactivity in cells from prepubertal bull (200 x). Bars = 100 µm.

Table 1: Colony characteristics of type A spermatogonia cells on day 4 of culture. Data are presented as least square means.

Bull Type	Day of Culture	Feeder Monolayer	Media Type	FBS Type	Total Colonies	Colonies per well	Colony Area (μm^2)	Type of Colony (%)	
								Round	Radial
AB	4	FL	ELSC	S	3	0.33[a]	33341 ± 14354[a]	66 ± 2[a]	33 ± 2[a]
AB	4	FL	ELSC	SF	3	0.33[a]	7008 ± 14354[a]	0.0 ± 0.0[a]	100 ± 0.0[a]
AB	4	FL	RSC	S	9	1[a]	25016 ± 8287[a]	0.0 ± 0.0[a]	100 ± 0.0[a]
AB	4	FL	RSC	SF	5	0.55[a]	6841 ± 11119[a]	0.0 ± 0.0[a]	100 ± 0.0[a]
AB	4	NF	ELSC	S	0	-	NA	NA	NA
AB	4	NF	ELSC	SF	0	-	NA	NA	NA
AB	4	NF	RSC	S	0	-	NA	NA	NA
AB	4	NF	RSC	SF	0	-	NA	NA	NA
Pooled SE						3.21			
PB	4	FL	ELSC	S	24	1.60[a]	13636 ± 4875[a]	53 ± 8[a]	47 ± 8[a]
PB	4	FL	ELSC	SF	13	0.86[a]	10741 ± 6895[a]	7.6 ± 7[a]	92.4 ± 7[a]
PB	4	FL	RSC	S	9	0.60[a]	11662 ± 8287[a]	22 ± 13[a]	78 ± 13[a]
PB	4	FL	RSC	SF	46	3.30[a]	12790 ± 3626[a]	39 ± 7[a]	61 ± 7[a]
PB	4	NF	ELSC	S	0	-	NA	NA	NA
PB	4	NF	ELSC	SF	0	-	NA	NA	NA
PB	4	NF	RSC	S	0	-	NA	NA	NA
PB	4	NF	RSC	SF	0	-	NA	NA	NA
Pooled SE						2.49			

[a] Least square means with different letters differ ($P < 0.0001$). **NA** = not applicable.
AB = adult bull; **PB** = prepubertal bull; **NF** = non feeder monolayer; **FL** = feeder monolayer; **ESCL** = embryonic-like stem cell medium; **RSC** = regular stem cell medium; **FBS-S** = fetal bovine serum with steroids; **FBS-SF** = fetal bovine serum steroids reduced. **PB** = 15 wells total for treatment combination. **AB** = 9 wells total for treatment combination.

Table 2: Colony characteristics of type A spermatogonia cells on day 7 of culture. Data are presented as least square means.

Bull Type	Day of Culture	Feeder Monolayer	Media Type	FBS Type	Total Colonies	Colonies per well	Colony Area (μm^2)	Type of Colony (%)	
								Round	Radial
AB	7	FL	ELSC	S	32	3.55^{abc}	19415 ± 4539^{ab}	20 ± 7^{a}	80 ± 7^{a}
AB	7	FL	ELSC	SF	24	2.66^{abc}	14953 ± 5075^{ab}	41 ± 10^{a}	59 ± 10^{a}
AB	7	FL	RSC	S	38	4.22^{abc}	14869 ± 4033^{ab}	15 ± 5.9^{a}	85 ± 5.9^{a}
AB	7	FL	RSC	SF	31	3.44^{abc}	11127 ± 4465^{ab}	19 ± 7^{a}	81 ± 7^{a}
AB	7	NF	ELSC	S	1	0.11^{abc}	4741 ± 24862^{ab}	100 ± 0.0^{a}	0.0 ± 0.0^{a}
AB	7	NF	ELSC	SF	0	-	NA	NA	NA
AB	7	NF	RSC	S	0	-	NA	NA	NA
AB	7	NF	RSC	SF	0	-	NA	NA	NA
Pooled SE						3.21			
PB	7	FL	ELSC	S	132	8.8^{a}	22101 ± 2214^{a}	26 ± 4^{a}	74 ± 4^{a}
PB	7	FL	ELSC	SF	111	7.40^{ab}	21172 ± 2437^{a}	31 ± 5^{a}	69 ± 5^{a}
PB	7	FL	RSC	S	150	10^{a}	23201 ± 2079^{ab}	36 ± 4^{a}	64 ± 4^{a}
PB	7	FL	RSC	SF	145	9.66^{a}	18973 ± 2086^{ab}	32 ± 5^{a}	68 ± 5^{a}
PB	7	NF	ELSC	S	13	0.86^{c}	2683 ± 7177^{b}	8 ± 7^{a}	92 ± 7^{a}
PB	7	NF	ELSC	SF	62	4.36^{abc}	4208 ± 8790^{b}	87 ± 11^{a}	13 ± 11^{a}
PB	7	NF	RSC	S	20	1.33^{bc}	5009 ± 6030^{b}	17 ± 9^{a}	83 ± 7^{a}
PB	7	NF	RSC	SF	8	0.53^{c}	5767 ± 8790^{ab}	75 ± 15^{a}	25 ± 15^{a}
Pooled SE						2.49			

[a,b] Least square means with different letters differ ($P < 0.0001$). **NA** = not applicable.
AB = adult bull; **PB** = prepubertal bull; **NF** = non feeder monolayer; **FL** = feeder monolayer; **ESCL** = embryonic-like stem cell medium; **RSC** = regular stem cell medium; **FBS-S** = fetal bovine serum with steroids; **FBS-SF** = fetal bovine serum steroids reduced. **PB** = 15 wells total for treatment combination. **AB** = 9 wells total for treatment combination.

Table 3: Colony characteristics of type A spermatogonia cells on day 15 of culture. Data are presented as least square means.

Bull Type	Day of Culture	Feeder Monolayer	Media Type	FBS Type	Total Colonies	Colonies per well	Colony Area (μm^2)	Type of Colony (%)	
								Round	Radial
AB	15	FL	ELSC	S	206	22.88^{abc}	16012 ± 1736^{abc}	40 ± 4^{bd}	60 ± 4^{bd}
AB	15	FL	ELSC	SF	126	14^{bcde}	10808 ± 2250^{bd}	39 ± 5^{bd}	61 ± 5^{bd}
AB	15	FL	RSC	S	278	30.88^{a}	44179 ± 1491^{ac}	53 ± 4^{abd}	47 ± 4^{abd}
AB	15	FL	RSC	SF	232	25.77^{ab}	14615 ± 1632^{bd}	37 ± 4^{d}	63 ± 4^{d}
AB	15	NF	ELSC	S	1	0.11^{fg}	2330 ± 24862^{abc}	0.0 ± 0.0^{abd}	100 ± 0.0^{abd}
AB	15	NF	ELSC	SF	0	-	NA	NA	NA
AB	15	NF	RSC	S	3	0.33^{fg}	4975 ± 14354^{abc}	33 ± 27^{abd}	67 ± 27^{abd}
AB	15	NF	RSC	SF	0	-	NA	NA	NA
Pooled SE						3.21			
PB	15	FL	ELSC	S	54	3.60^{cdef}	10530 ± 3383^{abc}	42 ± 7^{abd}	58 ± 7^{abd}
PB	15	FL	ELSC	SF	64	4.26^{bcdef}	16618 ± 3132^{abc}	73 ± 5^{a}	27 ± 5^{a}
PB	15	FL	RSC	S	152	10.13^{abcdf}	33630 ± 2016^{ab}	67 ± 4^{ac}	33 ± 4^{ac}
PB	15	FL	RSC	SF	72	4.80^{bcdef}	20152 ± 2930^{cd}	68 ± 5^{ab}	32 ± 5^{ab}
PB	15	NF	ELSC	S	6	0.40^{eg}	16930 ± 10150^{abc}	33 ± 19^{abd}	67 ± 19^{abd}
PB	15	NF	ELSC	SF	7	0.46^{eg}	19013 ± 9397^{abc}	28 ± 10^{abd}	72 ± 10^{abd}
PB	15	NF	RSC	S	22	1.46^{eg}	39583 ± 5425^{abc}	38 ± 10^{abd}	62 ± 10^{abd}
PB	15	NF	RSC	SF	20	1.33^{def}	35924 ± 5559^{abc}	15 ± 8^{abd}	75 ± 8^{abd}
Pooled SE						2.49			

^{a,b,c,d,e,f,g} Least square means with different letters differ ($P < 0.0001$). **NA** = not applicable.
AB = adult bull; **PB** = prepubertal bull; **NF** = non feeder monolayer; **FL** = feeder monolayer; **ESCL** = embryonic-like stem cell medium; **RSC** = regular stem cell medium; **FBS-S** = fetal bovine serum with steroids; **FBS-SF** = fetal bovine serum steroids reduced. **PB** = 15 wells total for treatment combination. **AB** = 9 wells total for treatment combination.

Table 4: Alkaline phosphatase characteristics on day 4 of culture of colonies derived from type A spermatogonia cells. Data are presented as least square means ± SEM.

Bull Type	Day of Culture	Feeder Monolayer	Media Type	FBS Type	Total Colonies	Percentage of Colonies	Intensity (#)		
							1	2	3
AB	4	FL	ELSC	S	2	0.0 ± 0.17^{bde}	-	-	-
AB	4	FL	ELSC	SF	1	0.0 ± 0.25^{bde}	-	-	-
AB	4	FL	RSC	S	3	33.3 ± 25^{ac}	-	-	1
AB	4	FL	RSC	SF	0	NA	-	-	-
AB	4	NF	ELSC	S	0	NA	-	-	-
AB	4	NF	ELSC	SF	0	NA	-	-	-
AB	4	NF	RSC	S	0	NA	-	-	-
AB	4	NF	RSC	SF	0	NA	-	-	-
PB	4	FL	ELSC	S	12	50 ± 14^{ab}	4	2	-
PB	4	FL	ELSC	SF	4	0.0 ± 0.1^{abc}	-	-	-
PB	4	FL	RSC	S	4	0.0 ± 0.1^{e}	-	-	-
PB	4	FL	RSC	SF	16	50 ± 12^{cd}	6	2	-
PB	4	NF	ELSC	S	0	NA	-	-	-
PB	4	NF	ELSC	SF	0	NA	-	-	-
PB	4	NF	RSC	S	0	NA	-	-	-
PB	4	NF	RSC	SF	0	NA	-	-	-

[a,b,c,d,e] Least square means with different letters differ ($P < 0.0001$). **NA** = not applicable. **AB** = adult bull; **PB** = prepubertal bull; **NF** = non feeder monolayer; **FL** = feeder monolayer; **ESCL** = embryonic-like stem cell medium; **RSC** = regular stem cell medium; **FBS-S** = fetal bovine serum with steroids; **FBS-SF** = fetal bovine serum steroids reduced. **PB** = 5 wells total for treatment combination. **AB** = 3 wells total for treatment combination. AP intensity staining (0 = no AP, 1 = slight, 2 = mild, 3 = moderate).

Table 5: Alkaline phosphatase characteristics on day 7 of culture of colonies derived from type A spermatogonia cells. Data are presented as least square means ± SEM.

Bull Type	Day of Culture	Feeder Monolayer	Media Type	FBS Type	Total Colonies	Percentage of Colonies	Intensity (#)		
							1	2	3
AB	7	FL	ELSC	S	21	4.7 ± 4.5^{abcde}	1	-	-
AB	7	FL	ELSC	SF	10	20 ± 12^{abcde}	1	1	-
AB	7	FL	RSC	S	18	0.0 ± 0.0^{abcde}	-	-	-
AB	7	FL	RSC	SF	14	7.1 ± 6.8^{abcde}	1	-	-
AB	7	NF	ELSC	S	0	NA	-	-	-
AB	7	NF	ELSC	SF	0	NA	-	-	-
AB	7	NF	RSC	S	0	NA	-	-	-
AB	7	NF	RSC	SF	0	NA	-	-	-
PB	7	FL	ELSC	S	52	15.4 ± 4.5^{ab}	7	1	-
PB	7	FL	ELSC	SF	41	17 ± 5.8^{cd}	7	-	-
PB	7	FL	RSC	S	46	10.9 ± 4.5^{b}	3	1	1
PB	7	FL	RSC	SF	55	32.7 ± 6.2^{a}	14	4	-
PB	7	NF	ELSC	S	5	NA	-	-	-
PB	7	NF	ELSC	SF	21	NA	-	-	-
PB	7	NF	RSC	S	7	NA	-	-	-
PB	7	NF	RSC	SF	3	NA	-	-	-

^{a,b,c,d,e} Least square means with different letters differ ($P < 0.0001$). **NA** = not applicable. **AB** = adult bull; **PB** = prepubertal bull; **NF** = non feeder monolayer; **FL** = feeder monolayer; **ESCL** = embryonic-like stem cell medium; **RSC** = regular stem cell medium; **FBS-S** = fetal bovine serum with steroids; **FBS-SF** = fetal bovine serum steroids reduced. **PB** = 5 wells total for treatment combination. **AB** = 3 wells total for treatment combination. AP intensity staining (0 = no AP, 1 = slight, 2 = mild, 3 = moderate).

Table 6: Alkaline phosphatase characteristics on day 15 of culture of colonies derived from type A spermatogonia cells. Data are presented as least square means ± SEM.

Bull Type	Day of Culture	Feeder Monolayer	Media Type	FBS Type	Total Colonies	Percentage of Colonies	Intensity (#)		
							1	2	3
AB	15	FL	ELSC	S	69	0.0 ± 0.0^{abc}	-	-	-
AB	15	FL	ELSC	SF	56	1.7 ± 1.7^{abc}	1	-	-
AB	15	FL	RSC	S	117	2.5 ± 1.5^{abc}	2	-	1
AB	15	FL	RSC	SF	86	0.0 ± 0.0^{abc}	-	-	-
AB	15	NF	ELSC	S	1	0.0 ± 0.0^{abc}	-	-	-
AB	15	NF	ELSC	SF	0	NA	-	-	-
AB	15	NF	RSC	S	2	0.0 ± 0.0^{abc}	-	-	-
AB	15	NF	RSC	SF	0	NA	-	-	-
PB	15	FL	ELSC	S	27	22.2 ± 8.1^{ab}	4	2	-
PB	15	FL	ELSC	SF	22	9 ± 6.1^{bc}	-	2	-
PB	15	FL	RSC	S	92	16.3 ± 3.9^{a}	7	6	2
PB	15	FL	RSC	SF	17	0.0 ± 0.0^{c}	-	-	-
PB	15	NF	ELSC	S	3	0.0 ± 0.0^{abc}	-	-	-
PB	15	NF	ELSC	SF	3	0.0 ± 0.0^{abc}	-	-	-
PB	15	NF	RSC	S	3	0.0 ± 0.0^{abc}	-	-	-
PB	15	NF	RSC	SF	9	0.0 ± 0.0^{abc}	-	-	-

[a,b,c,d,e] Least square means with different letters differ ($P < 0.0001$). **NA** = not applicable. **AB** = adult bull; **PB** = prepubertal bull; **NF** = non feeder monolayer; **FL** = feeder monolayer; **ESCL** = embryonic-like stem cell medium; **RSC** = regular stem cell medium; **FBS-S** = fetal bovine serum with steroids; **FBS-SF** = fetal bovine serum steroids reduced. **PB** = 5 wells total for treatment combination. **AB** = 3 wells total for treatment combination. AP intensity staining (0 = no AP, 1 = slight, 2 = mild, 3 = moderate).

Chapter 6 - SUMMARY AND CONCLUSIONS

Spermatogenesis is a highly organized process that is maintained by continuous proliferation of stem cells. Spermatogonia stem cells (SSC) have unique capabilities including self-renewal and production of the end product, spermatozoa. The ability to recover these cells from donor animals, perform *in vitro* culture and introduce genetic modification, and transfer back into a host testis would provide a valuable tool for transfering economically favorable genes in livestock. To this end spermatogonial stem cell isolation, culture, and transplantation procedure in livestock has progressed tremendously in the past few years (Honaramooz *et al.* 2003; Izadyar *et al.* 2003a). However, efficiency of cultured transplanted germ cells to colonize within seminiferous tubules is highly variable (Dobrinski *et al.* 2000; Nogano, *et al.* 2002; Izadyar *et al.* 2003a). Depletion of endogenous SSC in recipient animals using busulfan, cold treatment, or irradiation has been used in preparation of recipient animals prior to transplantation. However, these techniques are not without compromise such as severe bone marrow depression and specialized radiotherapy equipment required.

The clinical application of culturing SSC is of great importance in livestock, wildlife, and humans. Animal models may provide adequate knowledge for therapeutic management in patients with oncological and infertility diseases. Furthermore, the ability to maintain and proliferate undifferentiated SSC in culture will enable SSC transplantation, cryopreservation, and *in vitro* manipulation of these specialized cells.

Characterization of testicular components of prepubertal bovine bulls is important for understanding the dynamics of early stages of cell-cell communication (primarily type A spermatogonia) and proliferation within the testis. As a first step, we evaluated gonadotropin administration (GnRH and FSH) at the time of inhibin immunization in

prepubertal bulls in an attempt to increase number of germ cells. Therefore, a successful protocol could be used as a mean to favor proliferation of transplanted spermatogonial cells into recipient testes. Our results confirmed that concentrations of serum FSH were similar to those reported previously (Evans *et al.* 1993; Kaneko *et al.* 2001). Furthermore, it is clear that inhibin played a crucial role in spermatogonial development before puberty. This is in accordance with results from previous studies that provide clear evidence that inhibin has a physiological role in the regulation of FSH secretion (negative feedback) during the early stages of development in bulls (Bame *et al.* 1999; Kaneko *et al.* 2001). The significance of these results suggested that gonadotropin administration at the time of inhibin immunization increases germ cell number and the absolute volume of Sertoli, Leydig, and germ cells per testis together with increase in size (average volume of a cell) of all these cell types. Furthermore, the present findings could be used to develop an alternative therapy that benefit type A spermatogonia cell colonization after germ cell transplant.

In order to elucidate alternative procedures to deplete endogenous spermatogonial cells in recipient animals, we explored the effects caused by transiently induced ischemia in prepubertal bulls in our second experiment. It is known that partial occlusion of the blood supply to the testes reduces the spermatic epithelium with germ cell-depleted seminiferous tubules in rams (Markey *et al*. 1994). In this experiment, we explored a simple method of inducing ischemia at the testes level, through elastrator bands, to induce different periods of transiently induced ischemia by decreasing blood supply to the testes. It was hypothesized that few cells would survive such insult to the testis. Although our results suggested that transiently induced ischemia decreased the

spermatic epithelium and germ cell populations, a number of Sertoli and Leydig cells per testis survived. One limitation from this experiment is that functionality in term of testosterone (Leydig), inhibin or FSH (Sertoli) production in prepubertal bull calves was not evaluated. However, it can be suggested that testicular cell components were present at castration in testes cross sections following prolonged (8 h) ischemic treatments, indicating that cells survived and some degree of cellular functionality still remained. The significance of the study revealed that transiently induced ischemia significantly decreased the number of germ, Sertoli and Leydig cells in the testis while maintaining structural components of the testis.

In our final experiment, we explored the feasibility of isolating viable SSC from prepubertal and adult bulls and further propagate these cells in culture. Large numbers of viable SSC were isolated from prepubertal and adult bulls. Type A spermatogonia were present in solutions collected and stained positive to PGP 9.5 which showed to be a reliable and strong marker for spermatogonia cells in both bull type. Under *in vitro* conditions, SSC survived and were able to proliferate and form of colonies. Not only spermatogonial cells from prepubertal bulls but also from adult bulls formed colonies, showing that both populations of cells can be harvested and cultured. In this study, three types of colonies (round, radial, and irregular) were present. Overall, radial colonies were the most predominat type of colony in culture. Furthermore, co-culture of type A spermatogonia with a feeder monolayer resulted in more and larger colonies. Moreover, co-culture of spermatogonial cells with a feeder monolayer in a media containing FBS-S (with steroids) enhanced colony number. Lastly, colonies from both types of bulls stained positive for AP across the experimental period.

In conclusion, this research provides evidence that spermatogonial cell dynamics can be altered through *in vivo* and/or *in vitro* models. First, we were able to increase spermatogonial population in an attempt to favor *in vivo* germ cell proliferation important to develop alternative protocols that support survival of spermatogonia after transplant. Second, using similar animals (breed and age) spermatogonial cells were reduced in number through disturbance of the blood supply to the testis in prepubertal bulls while structural components of the testis remain. Lastly, viable SSC were isolated and different types of colonies were formed in culture from prepubertal and adult bulls. If stem cells can be identified and isolated correctly under culture conditions, then spermatogenesis *in vitro* could became a reality, which has major applications in livestock and human male fertility. Our results provide the basis for futures studies aimed at improving culture condition and ultimately modification of SSC before transplant and/or production of offspring by intracytoplasmatic sperm injection (ICSI).

VITA

Gustavo M. Schuenemann was born November 25, 1972 in Claraz, Argentina. He attended primary and secondary school in Claraz, Buenos Aires, Argentina. Gustavo graduated from Edgardo Giachino Argricultural High School in 1991. From there, he went to the National University of the Centre, Tandil, Buenos Aires where he received a Doctor in Veterinary Medicine degree in 1998. In 2004, Gustavo received a Master of Science degree in Animal Science, The University of Tennesse under the guidance of Dr. F. Neal Schrick. In the summer of 2008, he graduated with a Doctor of Philosophy degree in Animal Science, The University of Tennessee.