ESTABLISHMENT OF SPERMATOGENESIS FOLLOWING TESTICULAR TISSUE ECTOPIC XENOGRAFTING IN ALPACA

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Abstract

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Sperm production depends on development, puberty and germ cell differentiation. Puberty age of alpaca varies and little is known of factors regulating this process. The species is also known for a high incidence of congenital testicular abnormalities and consequently poor fertility. Studies included in this dissertation were designed to investigate male development and testis function in alpaca.

The endocrinological aspects of puberty were investigated through analysis of testosterone and estrogen in blood samples collected before after treatment with human chorionic gonadotropin (hCG, Chorulon®, 3000 IU, IV). Administration of hCG to 60 males aged from 6 to 60 months resulted in a 2 to 4.5 fold increase ($P < 0.05$) in serum testosterone concentration after 2 hours. Testicular sensitivity to hCG resulted in rapid response in males between 9 and 14 months of age and a second increased responsiveness after 21 months of age suggesting biphasic Leydig cell differentiation.

To investigate the cellular aspects of testicular development, 104 male alpacas were castrated and testicular tissues were processed for morphometric and gene expression analysis. The males were divided into five age groups based on months of age: 1.6-10, 2. 11-15, 3. 16-20, 4. 21-24, and 5. over 25. Morphological assessment in these groups was carried out by counting different cell types, seminiferous epithelium development and testosterone concentration. Gene expression analysis suggested that germ cell differentiation is the most variable aspect of alpaca testis development.

Last, pieces of alpaca testis were xenografted onto nude mice and harvested after 24 weeks. Evaluation of germ cell differentiation was achieved by histological examination of grafts and analysis of mouse blood testosterone. Prepubertal animals at 6 to 10 months were selected as donors for testis tissue xenografting. Alpaca testis xenografts produced testosterone and elongated spermatids. Graft survival ranged from 79.1% to 83.3% and grafts with elongating spermatids were 6.8, 4.3, 1.1, 1.1 and 1.7% from donor alpacas aged 6, 7, 8,9,10 months, respectively. These studies suggest that variations of alpaca testis development are associated with Leydig cell maturation and germ cell differentiation. Novel approaches such as testis xenografting can be utilized to investigate factors regulating these processes.

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Dedication

"And your Lord has decreed that you worship none but Him.
And that you be dutiful to your parents"

(Quran, al-Isra' 17: 23)

This dissertation is dedicated to my parents. Without them, I could not be who I am today. Your sacrifices and supplication have allowed me to pursue my lifelong spirits. Together you have both introduced many life-lessons and values. My mother, she will not be happy until she has seen a smile on my face, thank you for your love. My father's words of wisdom were my support and guidelines. Extremely grateful for the manner in which you raised me. I will never forget your efforts.

CHAPTER ONE

1. INTRODUCTION

The long-term goal of this research is to study the spermatogonial stem cell population, spermatogenesis and pubertal development in camelids and define the conditions for successful culture of spermatogonial stem cells. This is the first step in developing technology that can be used for genetic improvement and for the preservation of important genetic stock that may be lost due to reduced genetic diversity maintained in current animal production systems. Reduced fertility in livestock has been linked to increased inbreeding and the reduced numbers of sires used as semen donors as a consequence of a focus on unsustainable production. Thus there is an urgent need to develop techniques for the efficient genetic improvement and genetic preservation for all livestock industries. The research projects described in this dissertation are vital for the study of puberty and the development of molecular and xenografting based technologies. As will be described below, the development of these technologies will lead to rapid and efficient approaches to support the viability of animal production.

At first glance, the testis does not appear to be a suitable tissue for grafting because of the complexity of the seminiferous epithelium and the architecture of its vascular and duct systems. However, transplantation of testicular tissue has been performed since 1920s, and has provided important insights into testicular function. Immunodeficient lines of mice allow xenotransplantation of testicular and other tissues which make it possible to transplant tissue from large animals into mouse hosts (Dobrinski, 2009). Complete cross species spermatogenesis was first reported

in 2002 when pieces of testis tissue from newborn pigs and goats were able to survive and displayed complete development with production of sperm (Honaramooz et al., 2002b). Since then, testis tissue xenografting has been tested in numerous species. The impact of investigation of spermatogenesis goes well beyond the production of few mature spermatozoa because it provides the knowledge of cell biology, animal physiology and technical expertise that will support male fertility.

2. LITERATURE REVIEW

Alpaca breeding has seen tremendous development outside of its countries of origin in recent. The number of alpacas in North America, Australia and Europe has increased substantially in the last 15 years making them a common presenting patients in the veterinary clinics. There is a lack of information on the reproductive process in camelids in general. This literature review aims to give the reader the necessary background to understand the physiological characteristics of the species and to provide a general description of the methodology used throughout this research.

2.1. Animals

2.1.1. Alpaca

The alpaca, V*icugna pacos*, is a domesticated species of the camelidae family that is commonly found in specific areas of South America (primarily Peru). Camelids are pseudoruminants and belong to the mammalian order of Artiodactyla. They are the only living family of the suborder Tylopoda and consist of three genera, *Camelus, Lama and Vicugna.* *Camelus* genus includes the one-humped camel or dromedary (*Camelus dromedaries)* and the two-humped camel or Bactrian (*Camelus bactrianus).* The *Lama* genus includes the llama *(Lama glama)* and the guanaco *(L. ganacoe*). The *Vicugna* genus includes the alpaca (*V. pacos*) and the vicuña (*V. vicugna*) [1]. Alpacas provide means of substances (meat and fiber productions) in a large population of South America countries and particularly in Peru. In the United States alpacas are kept mainly for

shows and fiber production and their number has increased substantially in recent year. Male alpacas possess several reproductive unfavorable characteristics including late puberty and sexual maturity [2], slower testicular growth [3], higher incidence of testes abnormalities [4], low sperm concentration [5], and poorer fertility compared to other domestic species, with pregnancy rate between 40 and 60% [6]. Age at puberty is variable between 1 and 3 years and the factors regulating the pattern of puberty and sexual maturation remains a subject of controversy. The process of spermatogenesis and sperm production in terms of cell type population series that appear in particular seminiferous tubule and regulation of sperm production in terms of genes that are expressed by germ and somatic cells remain poorly studied. The limited information from the literature focuses on changes in circulating testosterone levels and changes in testicular size with age as indicator of sexual maturation pattern. No studies have investigated in details the cellular events occurring within the seminiferous tubules during and around the time of puberty.

Puberty in male alpacas has been traditionally linked to preputial detachment and plasma testosterone concentration. It is well known that the process of preputial detachment in alpaca usually begins at the age of 12–13 months and coincides with an increase in plasma testosterone concentration [2]. Plasma testosterone concentrations in 11-month-old alpaca males have been reported to be similar to those found in adult males [7]. However Bravo et al reported that plasma testosterone levels increase until 20 months of age to reach a concentration of 1.0 ng/mL or more[5, 8]. Our preliminary results showed that there is great variation in testosterone concentration among alpaca in the same age group [9] . In addition, appearance of a lumen in the seminiferous tubules, an indicator of germ cell differentiation leading to sperm production, and presence of spermatozoa were reported at 12 and 15–18 months of age in the alpaca, respectively [10]. Interestingly, a few male alpacas and llamas produce sperm as early as 10–12 months of age [11]. Male alpac-

as are born with descended testes. Testes grow steadily in the first 3.5 years of age to reach a maximum size (3.7 x 2.5 cm) and weight (15g) at about 4 to 5 years of age [8, 12]. Ejaculates collected from mature alpacas by artificial vagina present a wide variation in volume and concentration [13]. Clinical observations showed high incidence of infertility in male alpacas as a result of testicular hypoplasia and degeneration [14].

Traditionally breeders have selected alpacas for breeding based on testicular size and penile-preputial detachment. Male alpaca with normal large testicles size are considered to have higher sperm production as demonstrated in other species. Factors affecting testicular growth have not been yet studied in this species. Based on the available information about camelid reproductive development in males, it is apparent they reach puberty later in life than other large mammals. However, we do not know the time course of germ cell differentiation in male camelids. This represents a gap in our knowledge base and a barrier for our ability to develop reproductive technology in these animals. In addition, dysfunctional reproductive development has been reported in male camelids resulting in infertility. Thus, there is a need to investigate all aspects of male reproductive development in camelids leading to increased knowledge and the development of techniques and therapeutic approaches to treat infertility in camelids. Understanding the molecular and endocrine basis of spermatogenesis is a critical step in male selection for breeding and for the study of causes of infertility.

2.1.2. Immunodeficient mice

Animal models have been used as the front line to test new methods and finding solutions to different disorders, diseases, and pharmacological study before entering the clinic. This has resulted in more interest in many different research animal models. Recently, there has been some signif-

icant progress in the improvement of immunodeficient mouse models. Immunodeficient mouse models have been a central part of preclinical research as well as in advanced significant oncology, transplantation, immunology, and stem cell biology research. Currently a large number of strains and substrains of natural immunodeficiency models and gene deficient transgenic models are available for use in the genetic and immunological studies. Since these mice are athymic (lacking a thymus gland), they do not display normal immunological responds in terms of responding to infections or transplants rejection. This feature makes them extremely valuable as biological hosts for tissue transplants and immunology research. The two most widely used immunodeficient mice models in transplantation research are severe combined immune deficiency (SCID) and Nude mice which are a result of single gene spontaneous or targeted mutations that cause severe immunodeficiency [15]. Nude mice undergo a genetic mutation in homolog 11 gene that is expressed specifically in the skin and the thymus and leads to a nude phenotype with damaged or even completely absent thymus gland and inability to produce T lymphocytes [16]. In 1962, the mouse first appeared at the Virus Laboratory, Ruchill Hospital, Glasgow in Dr. N.R. Grist laboratory. Pantelouris was the first to report in 1968 that nude mice lack a thymus gland{[17]. This chacaracteristic makes them immunodefficient and an excellent model for interspecies tissue grafting (xennografting) because they are less likely to reject grafts A BALB/c Nude (*nu*) mouse is an inbred strain with albino background. They are hairless, pink-skinned, and posses oversized ears and commonly called “nude mice”. Absence of a thymus gland makes the breeding and husbandry of these mice more complicated than in normal mice. Heterozygous parents are generally used to produce a 'nude' mice,. The heterozygotes must be recognized either by the use of dominant chromosome markers or by test matings. There are two markers generally usedfor this: 'rex' *(Re)* and 'trembler' *(tr).* Both mutant mice, ‘rex’ and ‘trembler’, are not

able to produce good mothers and the marker distances are sufficiently large to allow recombination to occur.

Mice that have been used in this study were from *nu/nu* males mated with heterozygous females. Offsprings produced from this matting are fertile and mothers were able to nurse and rear nude litters to weaning age. All weaned males came from dams that were housed in one autoclaved transparent cage with micro filter top cover. Nude mice are used widely in testis tissue grafting and first sperm produce from grafts transplanted onto nude mice was reported by Honaramooz in 2002 [18].

2.2. The testis

The testis contains hundreds of coiled tubules known as seminiferous tubules and surrounded by interstitial tissue. Spermatogonia that reside along the basement membrane of seminiferous tubules produce mature spermatozoa. These germ cells are nursed and structurally supported by somatic cells called Sertoli cells that are present in the tubules and are important for germ cells maturation. Interstitial tissue contains different cell types crucial for spermatogenesis such as myoid cells, macrophages and Leydig cells. Leydig cells are the somatic cells that produce androgens. Therefore, traditionally testis is divided into germ and somatic cells.

2.2.1. Germ cells

Germ cells are the biological path for genetic transmission from one generation to the next. Germ cells undergo several developmental stages called the spermatogenesis process. This process

begin with spermatogonia which differentiate into primary and secondary spermatocytes, round spermatids and eventually ends with spermatozoa. Spermatozoa are the haploid cells that deliver the genetic material to the female side. Germ cells are a result of primordial germ cells (PGCs) differentiation and specifically, gonocytes which are an immediate descendant of primordial germ cells. These cells occupy the center of seminiferous cords during sex differentiation [19]. Gonocytes are morphologically distinguished as large circular cells with a prominent nucleus containing one or two nucleoli [20], and are easily differentiated from the adjacent Sertoli cells. Clermont and Perey [21] were the first to recognize these cells in the seminiferous cords. Gonocytes are the precursor of spermatogonial stem cells (SSCs). They either directly develop to spermatogonia (type A_1) an early differentiation stage or undergo self-renewal and differentiation into daughter cells; undifferentiated spermatogonia type A (A_{undiff}) that are thought to represent the reserve and active SSC pool foundation [22]. Fraction of these cells will support the first wave of spermatogenesis [23]. Before birth, Gonocytes stay mitotically quiescent. After birth these cells are triggered to enter mitotic cell cycles and differentiate into spermatogonia [24]. Spermatogonia undergo multiple cell divisions and differentiate into spermatocytes. They migrate from the basal compartment toward the adluminal compartment for spermatogenesis. The migration of germ cells requires a highly arranged network that involves the autocrine and paracrine signaling, restructuring of the cell adhesion complex and cell-to-cell interaction [25]. Abnormal spermatocytes that failed to repair the DNA are eliminated through apoptosis [26].

2.2.2. Leydig Cells

Leydig cells are located in the interstitial space of the testicular parenchyma between the

seminiferous tubules. They supply testosterone and insulin-like 3 (INSL3) that are essential for normal male sex differentiation and reproductive function. Testosterone is important for male sexual behavior, initiation and maintenance of spermatogenesis. INSL3 controls both testis descent during fetal period and germ cells survival in adults [27]. During the early fetal development, dominant Y-linked *Sry* (sex-determining region of the Y chromosome) gene is expressed by Sertoli cells in XY gonads causes quick changes to the sex indistinguishable gonad to become a testis. A recent study on Leydig cell ontogeny reported that in the fetal testis the coelomic epithelium and vascular-associated cells in the gonad-mesonephric border region are the origin precursor population for mature Leydig cells formation [28]. The process of formation involves progenitor cells proliferation, morphological differentiation, and the ability to produce androgen. Two generations of cells contribute in Leydig cells function. The first generation forms late in gestation through cell proliferation and early phase of differentiation and called fetal Leydig cells which are responsible for the masculinization of the male urogenital system. During puberty, fetal Leydig cells go through further differentiation and a single cell division to form a second generation that is called adult Leydig cells which produces testosterone [29].

Since their description by Franx Leidig in 1850, the endocrine function of Leydig cells was proposed by Pol Bouin and Paul Ancel in 1903. Further studies between 1900's and 1950 confirmed their role in the production of androgens under the control of the pituitary gland. In 1959, Roosen Runge and Anderson [30] identified two populations of Leydig cells (fetal and adult). Later studies utilized histochemical and biochemical techniques to study their endocrine function. Morphologically, Leydig cell precursors (classified as "progenitors by Hardy et al., 1989 [31]) are an elongated spindle-shape mesenchymal-like cells with little smooth endoplasmic reticulum (ER). They differentiate intoround, fetal Leydig cells that contain several lipid inclusions and

expanded smooth ER with increase in the activity of steroidogenic enzymes such as P450scc, P450c17 and 3 β-hudroxysteroid dehydroxylase (3 β-HSD). Adult Leydig cells population is generated by more differentiation and proliferation. These cells are characterized by disappearance of lipid inclusions, increase in size, increased amount of smooth ER, well distinguished nucleolus and an increase in steroidogenic enzyme 17 β-hudroxysteroid dehydroxylase (17β-HSD) activity [32].

Steroidogenesis requires the chronological events that convert cholesterol into different steroid classes. Luteinizing Hormone (LH) is necessary for the biosynthesis of testosterone by normal functionally mature Leydig cells. Therefore, mature Leydig cell population contains high number of membrane-bound receptors (LHR) that belongs to 7-transmembrane G-protein coupled receptor family. This receptor is bound by LH ligand that stimulates the adenylate cyclase and phospholipase C signal transduction systems. Subsequently stimulates synthesis and production of androgens [33]. Binding of LH to its receptor stimulates adenylate cyclase activity which increases cyclic adenosine monophosphate (cAMP) formation. Through a complicated process, cholesterol is carried into the inner mitochondrial membrane where it is instantly converted into pregnenolone. Later, pregnenolone transports to the smooth endoplasmic reticulum where it is metabolized and converted to progesterone. This steroid is converted by 17β-HSD to testosterone [34].

2.2.3. Sertoli cells

Sertoli cells play a fundamental role in development and function of testis. Their location and structure within the seminiferous tubules make them essential in male fertility and spermatogenesis either directly or through secreted factors. The total density of Sertoli cells that occupy the

seminiferous tubules ranges from approximately15% in mice and rabbit to 40% in humans [35]. Sertoli cells are also known as ‘nurse-like’ cells which provide a unique protected microenvironment ‘niche’ that offer a structural and nutritional support to germ cells development and forming blood-testis barrier. Moreover, these cells function as macrophages by phagocytizing degenerating germ cells at all stages of differentiation as well as residual bodies after the release of mature spermatids in order to maintaining the integrity of the seminiferous epithelium [36].

Prior to the onset of morphological sex differentiation, Sertoli cells are the first cells that clearly differentiate in the embryo gonad which initiate the seminiferous cord formation, segregate the germ cells inside the seminiferous tubules and prevent germ cells from differentiation. Furthermore, the Sertoli cells ensure functional arrest of the Leydig cells and also secrete anti-Mullerian hormone which is responsible for Mullerian ducts regression [37]. In 1865, Enrico Sertoli was the first scientist to report Sertoli cells in human testis tissue sections. With very primitive facilities such as microscope, fixative and staining substances, Enrico Sertoli was able to describe and draw these cells. Initially, Sertoli described these cells as a tree-like cell or stringy cell and he used the word “mother cells” in his first publication which means that his observations were positively related to the true function of the Sertoli cells. Since the Anatomy Congress in Pavia, Italy in1900, the term “Sertoli cells” was accepted by scientists to describe the branched “Cells of Sertoli” [38].

The origin of Sertoli cells has been a controversial issue for some time. They either originate from the mesonephros, the coelomic epithelium or both. The mesonephros is a mesenchymal tissue contains the epithelial mesonephric duct that branches into mesonephric tubules. The cells of

these tubules extend toward the gonadal primordium where they dedifferentiate from the epithelial structure at the mesonephric-gonadal junction and contribute to the Sertoli cell population [39]. The other possible source for Sertoli cells is the coelomic epithelium. It is a single layer of cells that cover the entire coelomic cavity including the gonadal primordium. At the beginning of gonadogenesis, the coelomic epithelial cells leave the surface and contribute to the somatic population of the developing gonad [40].

The Sertoli cell plays an important role in sex differentiation. During early embryonic development, about 7–8 wk in humans, or midgestation in the mouse, bipotential gonads are morphologically indistinguishable in XX and XY embryos that contain unorganized combination of germ cells and somatic cells. The first step in the direction of sex differentiation is activation of the *Sry* gene in pre-Sertoli cells of XY gonads which expressed for a short period of time and is consistent with the gene initiation instead of maintenance of testis differentiation [41]. Therefore, the Y chromosome is essential in only Sertoli cells lineage to achieve normal testis development. An ovotestis, a gonad containing a mixture of ovarian and testicular tissues, is a result of low or delayed *Sry* expression [42]. Many other genes have been reported to be involved in sex determination pathway. One of the most candidate genes is *Sox9* that expressed in all Sertoli cells and is upregulated by *Sry*. Both *Sox9* and *Sry* play a critical role in initiation of testis morphogenesis. Since SRY acts on Sox9 for short period of time, it is believed that SOX9 regulates its own expression after the SRY expression has stopped [41]. Cell-fate mapping experiments show that SRY-positive cells exclusively become SOX9-positive Sertoli cells. As soon as SOX9 reaches a significant threshold, SOX9-dependent negative-feedback loop will result in *Sry* suppression [43]. Deletion or overexpression of *Sox9* leads to sex reversal cases due to failure to promote the positive-feedback loops that sustain its own expression [44].

Morphologically, Sertoli cells are irregularly shaped columnar cells that contain long and thin mitochondria and lipid droplets at the base of their cytoplasm. Polar cell extending from the basement membrane to the lumen of the seminiferous tubule, Sertoli cell nucleus are usually oval or pear shape with other multiplicity shapes and contains a large nucleolus. Numerous indentations and cytoplasmic crypt-like processes are noted due to the reshaping of the cell by developing germ cells. At a specific seminiferous epithelium developmental stage, Sertoli cells can be classified into type A or type B Sertoli cells. Type A Sertoli cells are characterized by clear cytoplasmic crypts deeply embedded with mature spermatids that ready to discharge into the tubule lumen, whereas the cytoplasmic crypts in type B Sertoli cells are either difficult to recognize or completely absent. During germ cell development and movement, Sertoli cells transform their shape from type A to type B to adjust to the cellular changes during spermatogenesis [35]. During germ cell development, early preleptotene and late leptotene spermatocytes have to travelfrom the basement membrane of the seminiferous tubules toward the lumen by traversing the blood-testis barrier (BTB).The blood-testis barrier, also called the seminiferous epithelial barrier, is formed by Sertoli-Sertoli and Sertoli-germ cell tight junctions located in the basal third of the seminiferous epithelium. The blood-testis barrier is formed from three different junction types (occluding or tight junctions, anchoring or adhering junctions, and communicating or gap junctions) to facilitate appropriate orientation and migration of germ cells in the seminiferous epithelium. This barrier has a unique architecture and location within the seminiferous epithelium and divides the epithelium into two compartments; a basal compartment in which spermatogonia, preleptotene, and leptotene spermatocytes reside and the other is an adluminal, compartment in which meiotic spermatocytes and spermatids in various stages of spermatogenesis and spermiogenesis exist [45]. Migration process of the developing preleptotene and leptotene spermato-

cytes to the adluminal compartment is a crucial cellular event that requires Sertoli-Sertoli and Sertoli-germ cell junctions be disassembled and reassembled which gives a good example of the remarkable dynamic nature of the blood-testis barrier [46]. There are three main functions for the blood-testis barrier. First, the BTB contributes directly to the complex structural organization of the testis by creating a specialized environment essential for germ cell development, differentiation and movement by producing necessary factors [47]. The second function of the BTB is to regulate the passage of nutrients and wastes molecules from and to seminiferous epithelium [48]. Thirdly, the BTB serves as an immunological barrier to guarantee that the immune system of the living being does not recognize antigens present on the surfaces of germ cells. Therefore, the individual body is immunized against its own spermatozoa. This immunization is a result of antiviral defense system that build by both Sertoli and germ cells by producing special factors such as interferons (IFNs) and cytokines. The BTB also prevents the entrance of Igs and lymphocytes into the adluminal compartment [46].

2.3. Spermatogenesis

Spermatogenesis is a complex process involving a number of cellular events consisting of a proliferative stage, cell migration meiotic stages, and differentiation spermiogenic stage or apoptosis. The process of spermatogenesis was first reported by LeBlond and Clermont [49] in 1952 who described the seminiferous epithelium cycle in mammals. Gonocytes, germ cells that found in seminiferous cords before birth, undergo a transition to Spermatogonial stem cells (SSCs) or spermatogonia. Spermatogonial stem cells (SSCs) are the center for spermatogenesis and are capable of both self-renewal and production of daughter cells that differentiate into spermatozoa.

Germ cell survival and development critically depend on their continuous and close contact with Sertoli cells that provide structural and physiologic support, including paracrine interaction between these two cell types. The end point of spermatogenesis process is producing mature spermatozoa with half number (haploid) of chromosomes [50].

Spermatogenesis process can be subdivided into three major phases of germ cell events. The first phase is characterized by proliferation of A_{single} (A_s) spermatogonia, undifferentiated spermatogonia, by the process of mitosis to produce A_{pr} and A_{al} spermatogonia population. A_s spermatogonia but not A_{pr} or A_{al} spermatogonia are capable of colonizing the seminiferous tubules in the testes of recipient mice and subsequently producing sperm [51]. A_{al} spermatogonia can differentiate into type A1 spermatogonia without undergoing mitosis [52]. Daughter germ cells derived from the same stem cell are characterized by the presence of intercellular bridges. This interconnection provides the basis for sharing products between the clonal cells [53]. The second phase or phase of meiotic divisions is recognized by differentiation of type B spermatogonia into the prophase, the first meiotic division. Chromosome number is reduced from diploid to haploid number to form primary spermatocytes. More divisions lead to the formation of secondary spermatocytes and then round spermatids. The third phase, spermiogenesis phase, represents the morphological transformation of the round spermatid into the complex highly structured spermatozoon. Between 25 to75% of the potential number of mature spermatozoa to be produced in adult male testis are lost spontaneously during spermatogenesis by mechanism known as programmed germ cell death or apoptosis [54]. Apoptosis results from overproduction of early germ cells and is also a mechanism that eliminates cells with fragmented DNA [55].

2.4. Hypothalamic-Pituitary-Testicular Axis

During normal male development, first few months of life, the hypothalamic-pituitary-gonadal (HPG) axis is slowly activated and produces gonadotropins and testosterone as an important phase in maturating germ cells in testis [56, 57]. Although endocrine studies of the hypothalamic-pituitary-gonadal axis have been initiated over 40 years ago, details of mechanism and its consequences are not accurately understood. GnRH a neurohormone consisting of 10 amino acids that is released from a distinct population of neurons within the hypothalamus in response to a variety of environmental and endocrine factors. GnRH has to be released in a pulsatile fashion to keep the reproductive axis operative [58, 59]. GnRH stimulates the anterior pituitary gland to synthesis and secretes two gonadotropins by the anterior pituitary, which in turn, acts on the gonads to control gametogenesis. These gonadotropins are luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Luteinizing hormone is also known as interstitial cell stimulating hormone (ICSH) is a heterodimeric glycoprotein that acts on the Leydig cells (interstitial cells) of the testes to stimulate synthesis and secretion of the testosterone. Testosterone can also be secreted by administration of Human chorionic gonadotropin (hCG) which has luteinizing hormone (LH) activity. Human chorionic gonadotropin (hCG) is secreted by the placenta [60]. Higher receptor binding affinity of hCG and a longer circulatory half-life make's it appreciated potent drug of use to stimulate Leydig cells to produce testosterone [61]. Testosterone, the essential hormone for normal male fertility, controlling genital system development and the later initiation and maintenance of spermatogenesis [62, 63], is synthesized from cholesterol that imported into the cell from the circulation [64]. Testosterone binds to androgen receptor (AR) to modulate gene transcription in target cells [65]. Inhibin is also an important hormone in spermatogenesis that produced by Sertoli and Leydig cells Andersson et al., 1998. Inhibin acts as negative feedback to FSH release [66, 67].

2.5. Ectopic Testis Tissue Xenografting

Testicular xenografting is defined as the subcutaneous transplantation of small pieces of testicle tissue from immature donor animal, under the back skin of immunodeficient nude mice (*nu/nu*). These grafts may be able to survive and produce fertilizable sperm as was first demonstrated by Honaramooz [18].

2.5.1. Background

The spermatogenesis process is controlled by the hypothalamic–pituitary–gonadal axis. In castrated mice where no testosterone is released, Leydig cells of testicular grafted tissue respond to the high level of FSH and LH as negative feed-back loop and secrete the testosterone [18]. The castrated mice rely on functional grafts for androgens. This results in a significant increase in seminal vesicles weight compared to the castrated control mice [18]. Testicle grafts from immature animals of several species have been transplanted into castrated mice and were shown to to respond to mice gonadotropins and grow to complete the spermatogenesis process that indicated by producing spermatozoa and androgens [68].

In the past few decades, many efforts have been made to find new alternative techniques to study the testis development and spermatogenesis. Of these procedures germ cell transplantation technique developed by Brinster and Avarbock proved to be valuable for the study of spermatogenesis and the biological activity of spermatogonial stem cell population [69]. Although the protocol has been confirmed to be a helpful tool to study spermatogenesis in species such as pigs [18], goats [70, 71], cattle [72, 73], sheep [74], equine [75] and dogs [76], many limitations in manipulating germ cells from different species makes this technique extremely difficult and expensive.

Incompatibility between the germ cells of large species and the microenviroment of the mouse testis had resulted in incomplete spermatogenesis [77, 78]. Therefore, additional studies are desirable and alternative procedures are needed before this protocol is performed extensively [74]. In 2002, Honaramooz and his college [18] reported that small cubes (0.5-1mm^3, 5-10 mg) of testicle tissue from newborn pigs and goats grafted into multiple sites (2-8 grafts/mouse) under the back skin of immunodeficient mice showed complete development and produced fertile sperm. They concluded that surrounding somatic compartment is necessary for the germ cells to proliferate and differentiate. A In a follow-up of this study in 2007, the same authors showed that transplanted isolated somatic cells and germ cells from neonatal porcine testis onto the back of immunodefficient mice rearrange and form complete functional testicular tissue [79].

2.5.2. Preparation of testicle tissue grafts for xenografting

Testicular tissue is collected from donor animal through standard castration procedure. Testes are immediately placed in Hank's balanced salt solution (HBSS) on ice to prevent RNA degradation. The tunica albuginea and other clear connective tissue are removed within one hour and the testes rinse many times in Dulbecco's phosphate buffered saline (DPBS). The parenchymal tissue is then cut into 3-5mg pieces to expose both Sertoli and Leydig cells to the blood circulation of the mice when transplanted. Fragments are maintained in Dulbecco's modified Eagle's medium (DMEM) on ice until the time of grafting generally within two hours for fresh grafts. To study testis development, samples of testis tissue from each animal are fixed in paraformaldehayde (PFA) 4% or Bouin's solution overnight then kept in 70% ethanol until processed for histology. Grafts can be cryopreserved for long time by adding Dimethylsulfoxide (DMSO) to the DMEM at ratio 1:10. Ten pieces testicle tissue are placed into 2-mL cryovial that contain 1 mL of freez-

ing medium. The cryovial kept on ice until stored in -80ºC freezer. Immediately before transplantation, grafts are thaw by placing the vial in a water bath at 25ºC for one minute then hold on ice [80, 81].

2.5.3. Grafting site

Testicle grafts require suitable environment that mimic the environment in situ. The mammalian testes are located in the scrotum outside the body with temperature that few degrees lower than the standard body temperature with rich blood vasculature which required for normal and continues spermatogenesis. Blood supply and temperature are the main vital factors that secure normal development of the fragments. However, other sites for grafting have been considered and include the anterior chamber of the eye [82], under the kidney capsule [83], into the scrotum and under the skin of the outer ear [84]. Although some grafts exhibited spermatogenesis; most of them showed minor damage immediately after grafting. This might be due to sensitivity to ischemia and the relatively high temperature. For complete spermatogenesis, the perfect site for transplantation is in small pocket under the back skin on either side of the vertebral column of the immunodeficient mice [18, 85, 86]. This area is ideal for grafting because it provides sufficient blood supply to grafts and the absence of hair helps in lowering the temperature to promote spermatogenesis. After placing the grafts of 5-10mg into multiple sites, incisions are closed with suture. Mice are monitored every two weeks for the development of grafts which can be seen as growing protrusion at the site of grafts.

Information regarding germ cell differentiation in alpacas is almost inexistent. This knowledge is required in order to assist in selection of male and understanding o fmale infertility in alpacas. Our objects were to determine the timeline of germ cells development and the key signals that

initiate spermatogenesis in the alpaca and to determine important information regarding factors that negatively impact male development and fertility. Also tracking the appearance of the most advanced cell type at a specific age will increase our knowledge of testis development pattern. We have focused primarily a on prepubertal age which is a crucial period for testicular development. The general hypothesis in the present research was that different alpaca ages will express variable testis development and establishment of spermatogenesis. To test this hypothesis, we started with endocrinology and histology examination and followed by use of the novel testis grafting protocol and gene expression analysis of alpaca testes from different ages.

2.6. Gene expression

Quantitative real-time reverse-transcription polymerase chain reaction (RT-qPCR) represents the current state-of-the-art approach for measuring gene expression. Higuchi, in 1992, and his colleagues first describe this technique and since then it has been extensively used to analyze gene expression [87]. Real-time PCR is a sensitive and reliable procedure to quantify transcript expression levels even in a complex mixture with very low RNA concentration. qRT-PCR is fast and precise to use and offers simultaneous measurement of gene expression in many different samples for a limited number of genes, however, when used in an inappropriate way, it can lead to considerable misinterpretation of results [88-90]. Since the use of qPCR has become very popular, one the other hand, data normalization is complex. Several approaches that can be applied to normalize qPCR results have been used, but the most common one is the use of reference genes as an internal standard. The most conventional reference genes are used regularly are glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or β-actin (ACTB) due to their highly

stability in cells [91, 92]. The most accepted approach to quantification is normalization of the expression level of a gene of interest (target gene) to the expression level of an internal stably expressed gene (reference gene). This allows the direct comparison of normalized transcript expression levels between samples [93, 94].

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CHAPTER TWO

Investigation of Testosterone and Estrogen Response after A Single Treatment of Human Chorionic Gonadotropin in Prepubertal and Pubertal Alpaca*

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***** Theriogenology journal guideline for authors has been used in this manuscript

Abstract

In alpaca, age at puberty is variable and the factors regulating the pattern of puberty and sexual maturation are a subject of controversy. Plasma testosterone concentration is often used as an indicator of sexual maturity. Limited published information is available and suggests that plasma testosterone concentration in 11-month-old alpaca is similar to those found in adults. In order to better investigate sexual maturity in the male alpaca we have tested the response of male alpacas to a single injection of human chorionic gonadotropin (hCG). Our hypothesis is that hCG treatment will cause an increase in testosterone level that is correlated with animal age. The specific aim of the present study was to investigate the testicular tissue response to a single hCG injection by monitoring the serum testosterone concentration. Sixty males ranging in age from 6 to 60 months were used in this project. Alpacas were grouped into seven groups based on their ages. Blood samples were collected from the jugular vein just prior to and 2 hours after intravenous administration of 3000 IU hCG (Chorulon®). The effect of age on basal testosterone level and response to hCG treatment was evaluated by Analysis of Variance. Basal serum testosterone concentrations were very low (≤0.1ng/mL) until 9 months of age. Although basal serum testosterone concentrations increased steadily with age, there was a significant variation amongst males within the same age group. Administration of hCG resulted in an average increase of 2 to 4 fold ($P = 0.05$) in serum testosterone concentration after 2 hours. There was no correlation between the degree of response and age. However, the response to hCG injection presented two modes or biomodal model of testosterone increase depending on the age of animals. The first mode occurred at ages 9 to14 months and the second mode was observed between 22 and 36 months. In conclusion, our results suggest that testicular growth and sensitivity to LH stimulation

may be bimodal in the male alpaca with a rapid increase in growth and sensitivity between 9 and 14 months of age and a second phase of increased responsiveness after 21 months of ages.

1. Introduction

Information available on male alpaca reproduction is scarce. The age animals reach puberty is variable and the factors regulating the pattern of puberty and sexual maturation are a subject of controversy because of the limited research in this area [1, 2]. Early studies showed that serum testosterone concentration increases dramatically early in puberty until 11 months of age then remain stable thereafter [3]. Penile detachment from the prepuce has been used traditionally as a sign of puberty and sexual maturation because it is directly under testosterone influence [4, 5]. Young males display sexual interest and mounting at one year of age but at this age only 8% of males had lost the peno-preputial attachment and can achieve intromission suggesting a dissociation of testosterone regulated reproductive behavior and physiological maturity. Loss of preputial adhesions is achieved in 70% and 100% of the males at two and three years of age, respectively. Male camelids are usually born with descended testes that are relatively small in comparison to other domestic livestock, soft and difficult to palpate [4, 6]. The average size for an adult alpaca male testicle is 3.7 (length) by 2.5 (width) cm [6, 7]. There is a wide variation in testicular size at all ages and body size suggesting that other factors, probably genetic, are also important [8]. Measurement of testosterone and estrogens from a single blood sample is not a reliable indicator of testis function and LH response as testosterone is secreted in a pulsatile fashion [9, 10]. Administration of hormones such as GnRH, LH, or hCG causes an sharp increase in serum testosterone that is proportional to testicular size and Leydig cell function Administration of hCG induces rapid increase in serum testosterone concentration in several species including ram [11] and bull [12], and increase in both testosterone and estrogens in stallion [13]. This technique

provides a diagnostic index of steroidogenic capacity of the testes. Previous studies have reported that hCG (Human Chorionic Gonadotropin) stimulation test is a useful method for examining the function and presence of testicular tissue in stallion [14]. hCG stimulation test has been studied in adult alpacas [15] however there are no studies comparing prepubertal and postpburatal male response to hCG.

The present study investigates the endocrinological regulation and development during puberty in male alpaca to gain better insight on factors affecting spermatogenesis in male alpacas. We hypothesized that hCG treatment will cause an increase in testosterone that is correlated with animal age. The specific aim of this study is to investigate the serum steroids concentration level in pre- and post-pubertal alpaca after a single hCG treatment and its relation to testicle histology.

2. Materials and methods

2.1. Animals and treatment

A total of 60 huacaya alpaca males (*Vicugna pacos*) aged from six months to five years were used and divided into seven age groups: 6 to 8 months (n=12); 9 to 11 months (n=7); 12 to 14 months (n=9); 15 to 17 months (n=14); 18-20 months (n=5); 21 to 24 months (n=3) and 25 to 60 months (n=10). Animals were housed at WSU with ad libitum access to water and Orchard grass hay during the period of study. All procedures were approved by Washington State University Animal Care and Use Committee. Animals were weighed and submitted to an hCG challenge test to determine steroidogenic response before castration for histological analysis.

2.2. hCG stimulation test and blood sampling

All animals received 3000 IU of hCG intravenously (10000 IU/10mL, Chorulon®, Intervet International B.V. Boxmeer-Holland). Blood samples were collected just prior and two hours following hCG administration. Blood was withdrawn by jugular venipuncture into vacutainer tubes without anticoagulant (MONOJECT®, Tyco Healthcare LP, Mansfield, MA, USA). Samples were left to clot at 4ºC overnight then centrifuged at 3000 x *g* for 20 minutes at 4ºC. Sera from the samples were transferred into polypropylene vials and stored at -20ºC until assayed for testosterone and total estrogen concentration.

2.3. Castration and histology

Testes were collected following standard bilateral castration procedures [16]. Animals were anesthetized using an intramuscular treatment combination of xylazine (0.4 mg/kg;AnaSed ® 100 mg/mL, Lloyd, Shenandoah, Iowa), ketamine hydrochloride (4 mg/kg; Ketaset ® 100mg/mL, Fort Dodge Animal Health, Fort Dodge, Iowa), and butorphanol tartrate (0.04 mg/kg IM, Torbugesic® 10 mg/mL, Fort Dodge Animal Health, Fort Dodge, Iowa). Following castration, cubic pieces of testicular parenchyma tissue were collected from each animal and fixed in 4% paraformaldehyde overnight, then stored in 70% ethanol. The tissues were later dehydrated in alcohol, embedded in paraffin, sectioned at 8µm and placed on glass slides. Slides were deparaffinized in histoclear (AGTC Bioproducts LLC, Wilmington, MA 01887 USA**)**, rehydrated in serial ethanol concentrations, and stained with hematoxylin and eosin to evaluate germ and somatic cells using light microscope. The diameters of large ten round seminiferous tubules were measured at 10X magnification using an eyepiece micrometer and averaged. All slides were evaluated by a single operator and in a random order.

2.4. Leydig cells evaluation

Representative cross-sections of testes from each age group were processed for detection of adult Leydig cells. Leydig cells were evaluated based on morphology parameters (shape, size, number, and maturation status). The morphology of stained sections was evaluated using light microscopy at 100X magnification, and digital images were captured. Adult Leydig cells were counted in randomly selected fields through the entire sections without overlap. Mature Leydig cells were identified in the interstitial tissue of prepubertal males (Figure 1) and in adult males (Figure 2) according to the criteria of the cell containing a large rounded nucleus with an obvious nucleolus and little or no cytoplasmic lipid droplets, and a relatively thicker peripheral rim of heterochromatin in their nuclei compared to other interstitial cells.

2.5. Leydig cells apoptosis

Histochemical detection of apoptosis-associated DNA fragmentation in testicular tissue was performed by the TUNEL assay (DeadEnd™ Colorimetric TUNEL System). The TUNEL System end-labels the fragmented DNA of apoptotic cells using a modified TUNEL assays. Biotinylated nucleotide is incorporated at the 3´-OH DNA ends using the Terminal Deoxynucleotidyl Transferase Recombinant (rTdT) enzyme. Horseradish peroxidase-labeled streptavidin (Streptavidin HRP) is then bound to these biotinylated nucleotides, which are detected using the peroxidase substrate, hydrogen peroxide and the stable chromogen, diaminobenzidine (DAB). Using this procedure, apoptotic nuclei are stained dark brown (Promega, Part# TB199, www.promega.com Madison, WI. USA).

The TUNEL assay was carried out on paraffin-embedded testis sections. In brief, testis tissue sections were pretreated by deparaffinized and rehydrated in xylene and decreasing concentra-

tions of ethanol followed by washing in 0.85% NaCl for 5 minutes. Sections were washed twice in PBS for 5 minutes and after each step in this assay. The tissues were permeabilized with 20 µg/mL proteinase K incubation for 25 min at room temperature. Sections on the slides were covered with 100µl of equilibration buffer for 10 minutes followed by addition of 100µl of rTdT TUNEL reaction mixture to the sections for 60 min at 37 C in a humidified chamber. The enzyme reaction was stopped by dipping slides into 20XSSC (liquid buffers Sodium Chloride and Sodium Citrate) for 15 min. Sections on slides were then incubated with 100µl of HRP-conjugated streptavidin (diluted 1:500 in PBS) for 30 min at room temperature, followed by 100µl of DAB until a light brown background appears. The sections were counterstained with hematoxylin and evaluated under a light microscope. To count TUNEL-positive cells in the tissues, five different microscopic fields were scanned at magnification of 40X.

2.6. Hormone assay by Radioimmunoassay (RIA)

The concentration of testosterone and estrogen was determined in serum samples collected pre and post-hCG treatment using a commercial double-antibody RIA kit (Coat-A-Count® total testosterone; Siemens Healthcare Diagnostics Inc. Los Angeles, CA 90045 USA;testosterone Catalog # TKT1, estrogen Catalog # KE2D1). The calibrators contain8, 16, 96, 414, 792 and 1685 nanograms of testosterone per deciliter (ng/dl) in processed human serum. Whereas the estrogen calibrators contain 0, 5,10,20,50,150, and 50 picograms of estrogen per milliliter (pg/mL) in processed human serum. The sensitivity of the assay for testosterone and estrogen was 0.1 ng/mL and 1pg/mL respectively.

2.7. Statistical analysis

All data are presented as mean ± SEM and were analyzed using ANOVA (Tukey test). Analysis was performed using statistical software (minitab15) program. Significance results reported if *P* value is less than 0.05.

3. Results

3.1. Response of testosterone and estrogen to hCG treatment

The basal serum testosterone concentrations were below the detection limit of the assay (<0.1ng/mL) for alpacas from 6 to 11 months of age and then testosterone concentration increased steadily from 12 months old onward from 1.1 ng/mL to 3.06 ng/mL at 25 to 60 months. The standard deviation was high when comparing the data from the animal with the highest testosterone level indicating a wide testosterone concentration variation among individual males within the same age group (Table 1). Administration of 3000 IU of hCG resulted in a significant sharp increase in serum testosterone concentration ($p = 0.05$) two hours after treatment (Figure 3). The intensity of the response to hCG varied according to age of alpacas. A 1.3 fold increase was observed in males aged 6 to 8 months (0.14 to 0.32 ng/mL). The highest responses were observed for males between 12 and 14 months of age (3.4 fold, 1.1 to 3.8 ng/mL) and for males aged 21 to 24 months (4.5 fold, 1.52 to 6.89 ng/mL). The adult group (25 to 60 months) was the only group with a statistically significant difference ($p = 0.05$) to the other groups (Figure 4). Body weight was taken for each animal at the time of bleeding. Mean body weight of 6 to 8 months and 9 to 11months of age groups was 28.1 kg (SD ± 2.9) and 29.9 kg (SD ± 7.6) respectively, whereas the body weight of 12 to 14 months group was 54.4 kg (SD ± 14.7). The weight range between 12 and 24 months was 54.4 kg (SD± 14.7) to 63.5 kg (SD ± 6.4) and the body

weight of 3 to 5 years animal group recorded as 80.1 kg (SD ± 11.9). Body weight in 6-8 and 9-11 months old groups were significant different from all other ages whereas 12-14 and 15-17 months old groups had a significant lower body weight than 25 to 60 months old group (P = 0.05). Testosterone concentrations after the hCG treatment were strongly correlated to body weight (Figure 5).

A total of 19 alpacas produced detectable levels (> 1 pg/mL) of estrogen in response to hCG treatment. The youngest age group that had detectable estrogen was 14-month-old. Post hCG treatment samples with detectable serum estrogen had serum testosterone concentration exceeding 3 ng/mL (Figure 6).

3.2. Histological evaluation of alpaca testes

The morphometric results of the testicle tissues obtained in this study are summarized in Table 2. There is an increase in seminiferous tubule diameter from 77.5 µm in the 9 to 11 month old group to 154.7 µm at 12 to14 months. Statistical analysis showed significant differences between the 9 to 11 months group and the 12 to 14, 18 to 20, 21 to 24, and 25 to 60 month groups. However, these groups were not different from each other. Testosterone concentration in pre- and post hCG treatment samples were correlated to tubule diameter in relation to the age of the animal group (Figure 7).

The proportion of mature Leydig cell increased slowly with advancing age as well as with tubule diameter (Figure 8). However a significant difference in the number of Leydig cells was present between 6 to 8 month of animals and 25 to 60 month old animals. These adult mature Leydig cells were arranged in clusters in the interstitium and around the seminiferous tubules. The number of mature Leydig cellswere 10.5, 37.89, 46.16, 38.04, 43.84, 88.93, and 113.07 in the seven

groups, respectively, with significantly more Leydig cells in 12 to14 and 21 to 24 month groups compared to the other groups (Figure 9).

TUNEL analysis to identify apoptotic cells indicated no fragmented DNA in Leydig cells in all slides from all age groups. However some positive staining cells were present in germ cells within seminiferous tubules among the different ages examined (Figure 10). No significant difference in apoptotic cells was present between ages.

4. Discussion

The large variation in testosterone concentration and Leydig cell number amongst males within each age group suggests that there may be some genetic factors involved in sexual development which are worthy of investigation. The dose of hCG used and the time of second sample collection in the present study was based on previous studies in adult alpacas [15]. As expected, testosterone increased with advancing age, the basal concentrations of the testosterone in prepubertal ages of this study was similar to that described by Bravo [17] at 0.06 to 0.09 ng/mL. There was large variation in androgen secretion among males within the same ages. In samples pooled by age, serum testosterone response to hCG peaked at 12 to14 months. Therefore it appears that the testes in young alpacas (9 to 14 months) are most sensitive to hCG treatment. The largest increase, first peak, of serum testosterone (3.81ng/mL) was seen at 12 to14 months which is in agreement with the observation of sexual activity at this age [5].This endocrine activity may indicate the occurrence of puberty in the alpaca. Response to hCG dropped between 15 to 20 months (2.2 ng/mL) then a second increase in response to hCG was observed at 21 to 24 months of age (6.89 ng/mL). Response to hCG challenge in males ages 24 to 60 months was comparable

to 21 to 24 months group (6.74 ng/mL) which is 4.5 and 2.2 fold higher than the basal level 1.52 and 3.06 ng/mL, respectively. This suggests that the amount of LH secreted naturally by the pituitary gland is insufficient to stimulate a high number of mature Leydig cells. Bravo [17] reported that at approximately 20 months of age, the majority of alpacas have basal testosterone serum concentration that exceeded 1ng/mL as a consequence of males maturation and testes enlargement and these data are in agreement with our results (1.52 ng/mL). Alpacas are considered non-seasonal breeders but recent studies on llamas have shown a negative effect of summer on sperm production and quality [18]. The effect of season of birth on testicular growth and function in alpaca needs further studies.

The bimodal response to hCG has been described in rabbit by Hall and Young [19]. The first response may include two mechanisms, hCG may stimulate the release of testosterone present in the Leydig cells or hCG may stimulate 20α-hydroxylase activity, thereby increasing the conversion of cholesterol to 20α-hydroxycholesterol and finally to testosterone. The second response of hCG is thought to be the result of a general increase in microsomal production of enzymes involved in the synthesis of testosterone. Our experimental design to collect blood samples two hours post hCG treatment was based on previous studies reporting that a peak of testosterone was observed within the first 2 h in humans [20], in ram [21], in stallion [22] and in adult alpacas [15] after hCG treatment followed by a decrease in testosterone between 4 and 24 h after hCG treatment. In this study it appears that each male had variation in serum testosterone levels which was noted by measuring the pre and post hCG treatment for each single male and compared it to the next male in the same age group. We have concluded from the wide variation in the present study a most important finding that testosterone release by the alpaca testes in response to hCG administration is dose-independent, thus the same hCG dose given to all animal resulted in dif-

ferent testosterone concentrations. If there were variations in sensitivity of the testis to hCG between different alpaca ages and among the same age, the response of testosterone release to hCG would not have been of a variable nature that we observed, and either a positive or negative linear relation would have occurred.

Estrogens have an essential role in regulating the hypothalamus–pituitary–testis axis and thus indirectly regulates luteinizing hormone (LH) and testosterone balance through a feedback loop [23]. Estrogen is produced in sizable quantities in the testis and it is also present in very high concentrations in semen of several species. The stallion was the first mammal in which estrogens were discovered to be produced by the testis [24]. Later data have confirmed that the testicular source of estrogens in horse resides quite exclusively in the Leydig cell [25]. In adult stallions, estrogen concentration increases after administration of hCG [13]. The aromatizing ability of the testicular tissue was undetectable in young males until the age of 14 months where the serum estrogen was detected in samples to contain 3ng/mL or higher of testosterone concentration. The only male with detectable estrogen in 13 to 14 months group had four times more Leydig cellsthat the male with the second highest Leydig cells count in the same group. It is possible that the Leydig cell number and aromatase enzyme have an effect on estrogen production which could be mediated by the high testosterone concentration leading to the production of more estrogen.

Differentiation and maturation of adult Leydig cells in alpacas is initiated before 6 months of age. These stages of differentiation and maturation are responsible for increased testosterone production at 12 to 14 and 21 to 24 months of age. The two peaks in testosterone production seen at 12 to 14 and 21 to 24 months confirmed our hypothesis of the correlation between age and testosterone production. Immature and mature Leydig cells resident in the testicular interstitium are

the target cells for hCG action, therefore, histological evaluation of the testes at all ages in this study except 15 to 17 months group, revealed that testosterone production is correlated to Leydig cell numbers. At ages of 6 to 8 months, the average number of Leydig cells per field examined was 7.06 and the testosterone concentration was 0.24 ng/mL, whereas the quantitative distribution and the functional characteristics of these Leydig cell populations at 25 to 60 months was 97.4, and the concentration level of testosterone was 6.74 ng/mL. In 15 to 17 months group where the number of mature Leydig cells was 43.53, the testosterone level was 1.8 ng/mL. the explained can be as the reduction in expression of LH receptor present in Leydig cell plasma membranes. In addition, their ability to bind to hCG and increase testosterone production after hCG stimulation could also lead to lower testosterone in this group. No Leydig cells were apoptotic as determined with the use of the TUNEL assay so changes in testosterone production may be due to the changes in their morphology or steroidogenic ability. Chen [26], reported that the low testosterone concentration in aging male testes is a result of loss of steroidogenic function and not from a reduction of cell numbers. Interestingly, the following group age (18 to 20 months) showed a lower Leydig cells number (31.9) but higher serum testosterone concentration. There is no obvious interpretation for this phenomenon except the morphological features of mature Leydig cells have changed to a way that would not be counted as mature Leydig cells and therefore decrease their ability to produce high concentration of testosterone. This would indicate that there is a strict correlation between the functional properties and the morphological characteristics of these cells.

Previous histological studies on alpacas [27] showed that a diameter of seminiferous tubules varies from 174 to 240 µm, this is in agreement with our observation of an average 206 µm in adult males. Seminiferous tubule diameter is strongly correlated to testosterone level and Leydig cell

numbers. The first dramatic increase in the diameter from 70.7 µm to 155.8 µm occurred between 9 to 11 and 12 to 14 months of age and was accompanied with a strong response to hCG and increased Leydig cell number. The second increase in tubule diameter was seen between 21 to 24 months group and 25 to 60 months group from 147.9 to 206.4 µm when the Leydig cells number increased from 49.9 to 97.4 cells with a high level of testosterone 6.7 ng/mL. This may be explained as an accommodation of the tubules for the release and flow of spermatozoa along the tubules.

The most obvious difference between progenitors and the newly formed adult Leydig cells is the change in cell shape from spindle-shaped to round. The cells which were scattered as singles or in groups in the interstitial tissue each had a characteristic round nucleus with a thick membrane. The nucleus contained coarse chromatin granules arranged at the periphery of the nuclear membrane with one prominent nucleolus in the center (Figures 2). All other cell types were considered as mesenchymal-like cells. These mature adult Leydig cells are larger than the immature adult Leydig cells and have been observed in the central interstitium as well as around its peritubular origin. This finding is contrary of what is described in other species where the mature Leydig cells move from peritubular site toward the center of the interstitial space. The interstitial tissue showed a wide variation from month to month and within the same age in its components, as well as in their morphological association and appearance. The tissue in 6-8 month old alpaca group had few mature Leydig cells (7.06 mature Leydig cells per-field) with predominantly mesenchymal cells and some fibroblasts (82.87%), lymphatic and blood vessels. The mature adult Leydig cells are the dominant cell type of all mesenchymal-like cell lineage between 25 and 60 months. The initial studies which identified fetal and adult Leydig cells as belonging to different cell populations were based on morphological differences between the two cell types. These dif-

ferences are mainly suitable for counting the adult Leydig cells. However, for differentiating between the cells that produce testosterone, the cells must be 3βHSD positive and the morphology needs to be supported by immunohistochemical examination.

In conclusion, we demonstrated that hCG treatment can be used in alpaca to study steroidogenic activity of Leydig cells. The intensity of response to hCG is age dependent. To our knowledge this is the first study to show a bimodal response in this species. A sharp increase in testosterone following hCG treatment is observed in males between 12 and 14 months of age and at 21 months onward. In addition, change in serum estrogen concentration was at detectable level in animals that exceeded 3 ng/mL testosterone concentrations. Response was clearly correlated to number and differentiation of Leydig cells. Studies are now in progress to investigate the factors involved in testis development at the time of germ cells differentiation.

Acknowledgments

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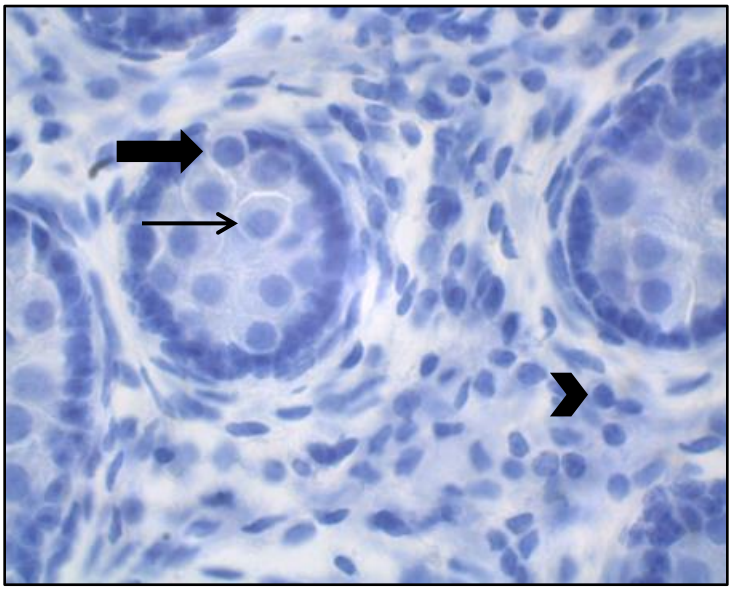

Figure 1: Cross section of alpaca testis at six months of age showing gonocytes in the center of the seminiferous cords (→) and gonocytes that have begun to migrate to the basement membrane (➡). Mature Leydig cells are present in the interstitial space (❯).

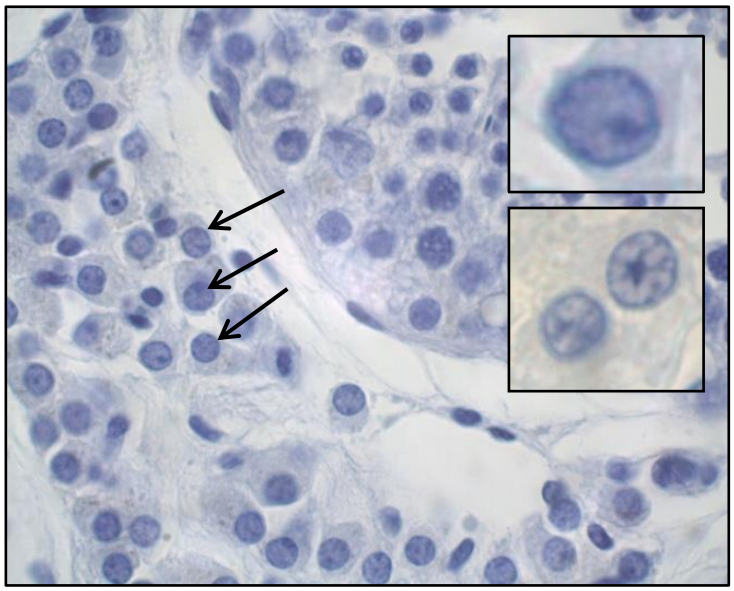

Figure 2: Cross section of an adult alpaca testis showing mature Leydig cells (arrow) in the interstitial space. Higher magnification of Leydig cells ate in inset.

Groups number	1	2	3	4	5	6	7
Age (months)	6-8	9-11	12-14	15-17	18-20	21-24	25-60
Testosterone, Mean *ng*/mL (± SD)							
Pre-hC	0.09 (± 0)	0.85(±1.06)	1.1(±0.74)	0.8 (±0.81)	1.33(±0.58)	1.52(±0.82)	3.06(±2.83)
Post-hCG	0.24(±0.14)	1.62(±1.42)	3.81(±3.08)	1.8(±1.36)	2.72(±1.94)	6.89(±4.72)	6.74(±2.5)

Table 1: Serum testosterone concentrations (ng/mL) pre and post hCG administration in the different age group of alpacas.

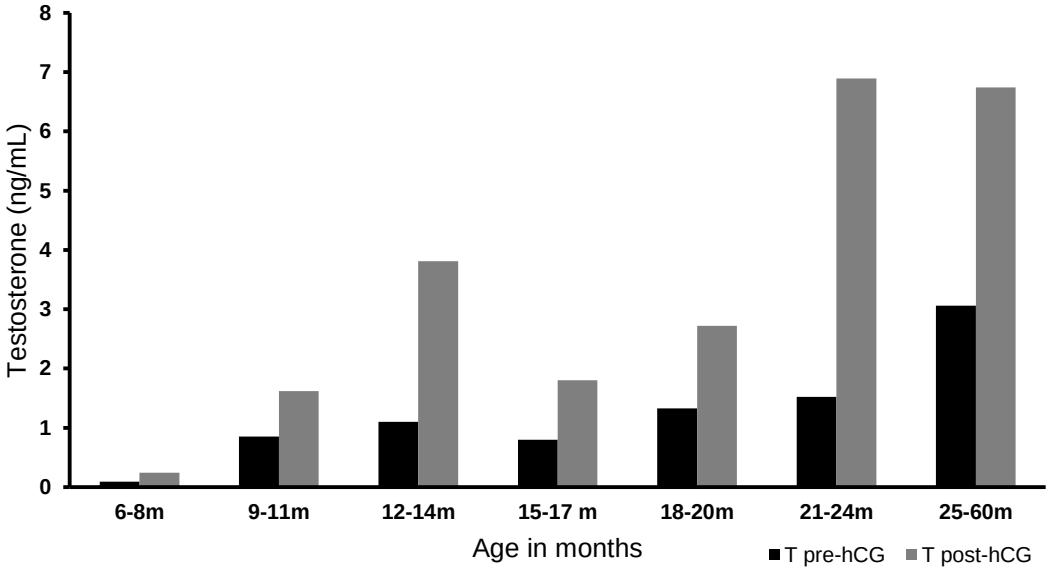

Figure 3: Mean concentrations of testosterone in ng/mL before and after hCG treatment at different ages.

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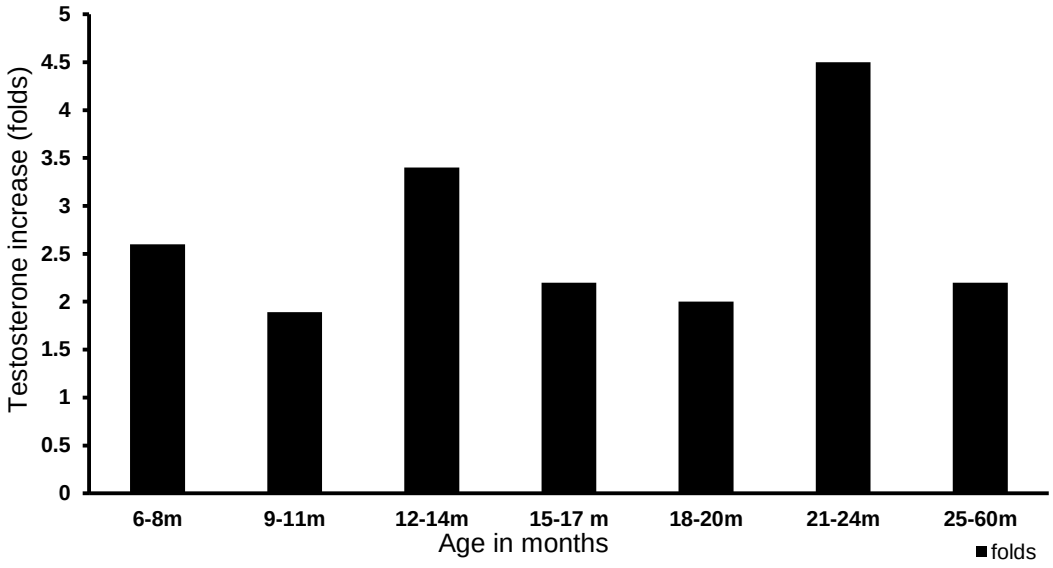

Figure 4: Fold change of testosterone concentration increase after the hCG treatment at different ages.

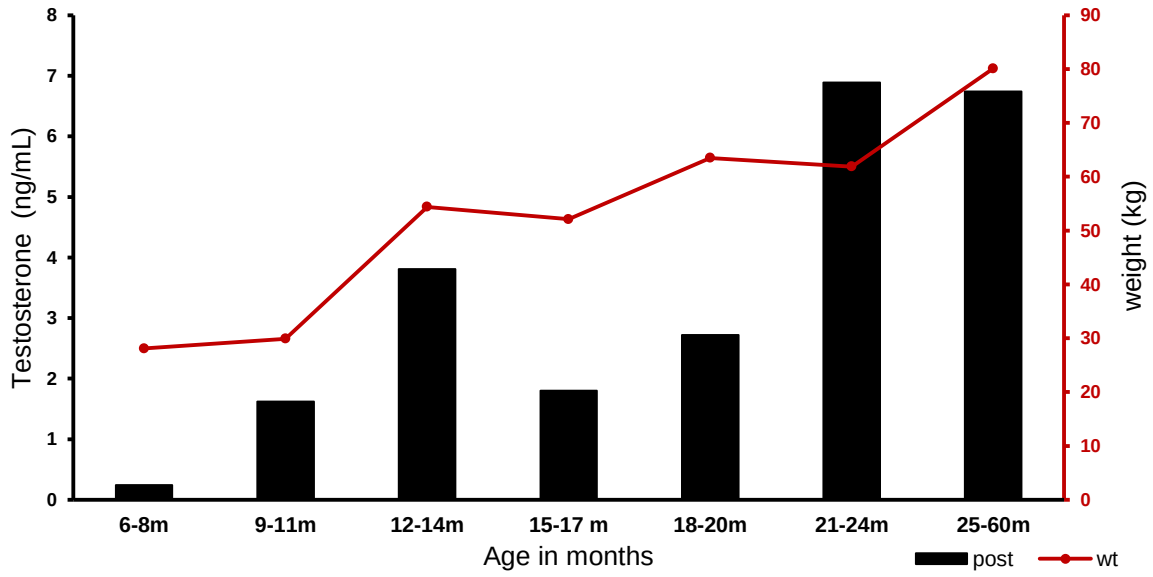

Figure 5: Relationship between post hGC testosterone concentrations and weight of males at different ages.

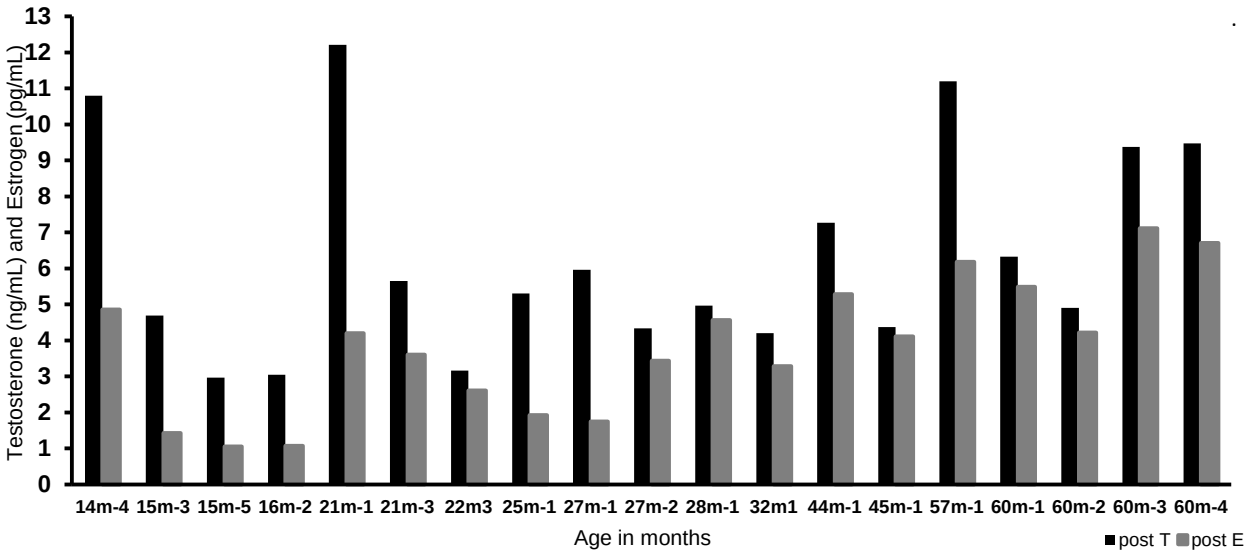

Figure 6: Mean concentration of testosterone (ng/mL) and total estrogen (pg/mL) before and after hCG treatment at different ages.

Age group (in months)	6-8	9-11	12-14	15-17	18-20	21-24	3-5y
Seminiferous Tubules							
Diameter (µm) (±SD)	65.83 (±18.8)	70.78 (±19.7)	155.81 (±50.9)	128.41 (±44.5)	133.19 (±39.4)	147.98 (±32.1)	206.40 (±44.2)
Mature Leydig cells per field (%) (±SD)	28.57 (±32.3)	34.83 (±26.1)	55.32 (±40.3)	50.79 (±34.9)	53.60 (±43.1)	95.00 (±0)	81.56 (±14.9)

Table 2: Seminiferous tubule diameter (µm) and mature Leydig cells percentage per field in the different age group.

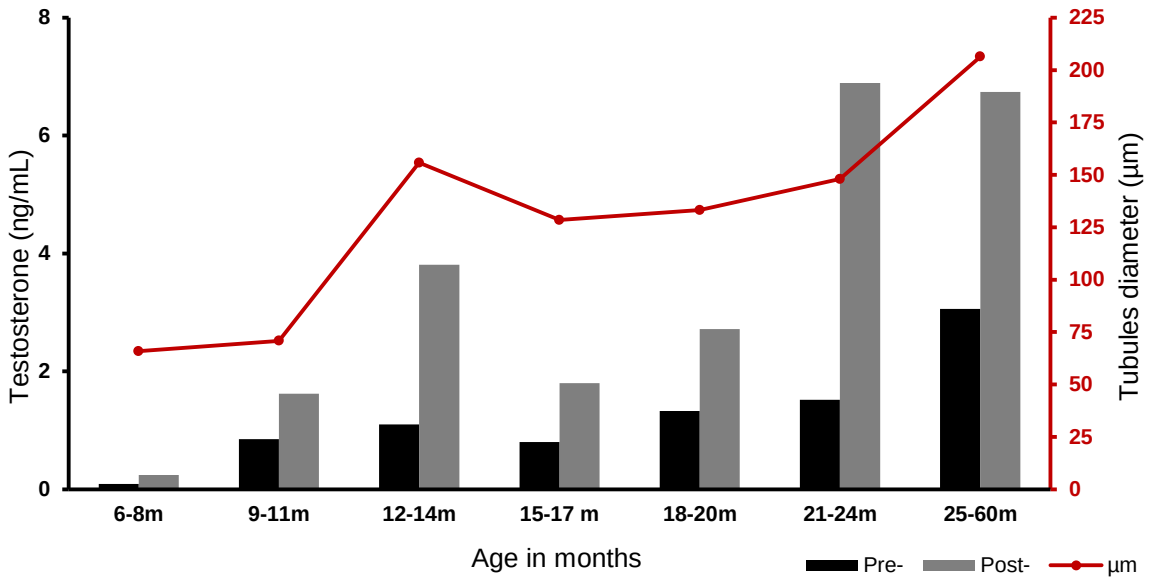

Figure 7: Mean seminiferous tubule diameter (µm) in relation to serum basal testosterone concentration post-hCG treatment at different ages.

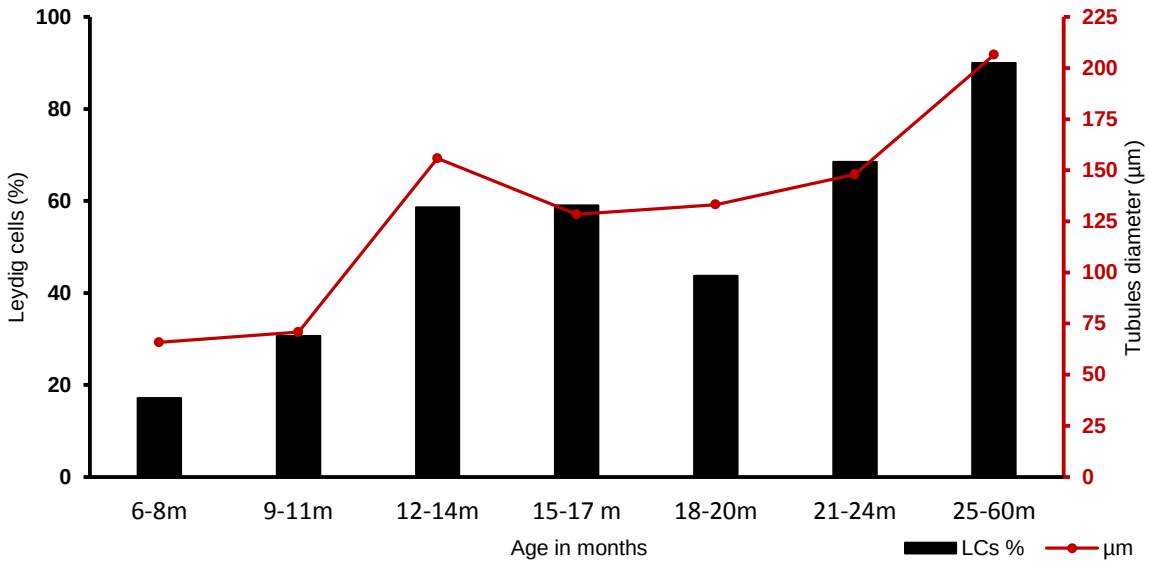

Figure 8: The relationship between mean seminiferous tubule diameter (µm) and Leydig cells number (per microscopic field) at different ages.

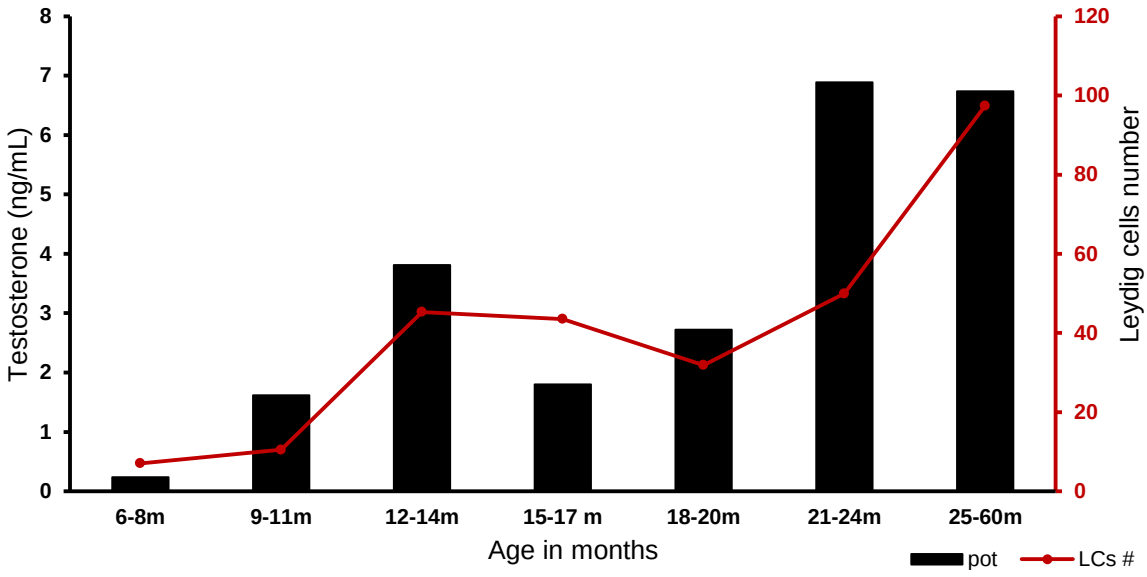

Figure 9: Mean Leydig cell number (per microscopic field) in relation to serum testosterone concentration pre- and post-hCG treatment at different ages.

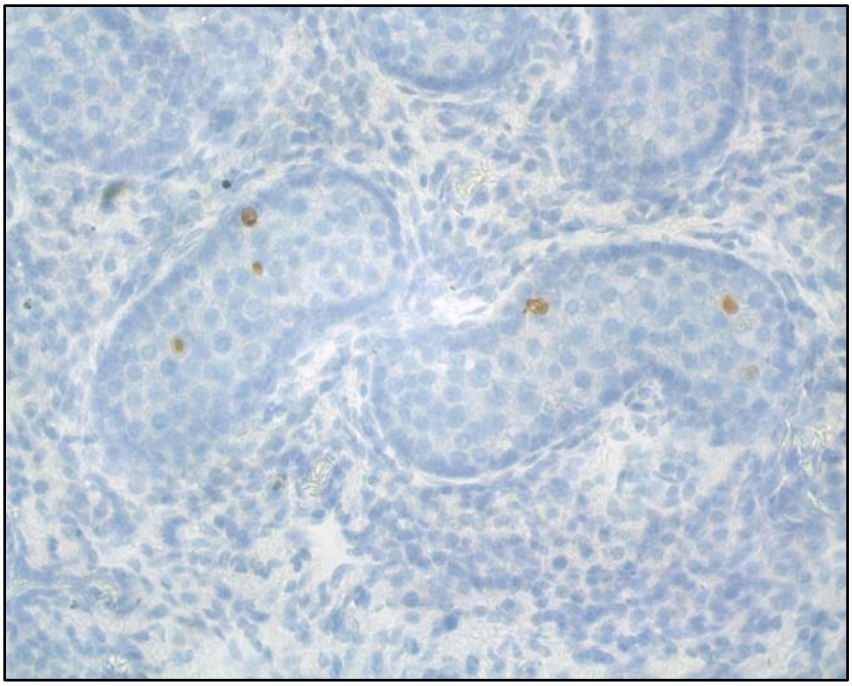

Figure 10: Representative alpaca testis cross-section used for analysis of apoptosis. TUNEL staining for apoptotic cells (Image of 8-month-old male).

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CHAPTER THREE

Testicular morphometric changes and gene expression during sexual maturation in alpaca*

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* Theriogenology journal guideline for authors has been used in this manuscript

Abstract

The present study was performed to investigate the steroidogenic, histological, morphometric, immunohistochemistry, and stereological changes in germ and somatic cells in alpaca from six months of age to adulthood. Male alpacas (n=104), 6 months to 5 years of age, were divided into five groups, 1) 6-10 months, 2) 11-15 months, 3) 16-20 months, 4) 21-24 months, and 5) over 25 months. Body weight, blood samples and testis tissues were collected from each animal. Basal testosterone concentration was measured in serum from all animals to evaluate and correlate with testis activity. The histological and morphometric examination was complemented by evaluating the seminiferous epithelium and interstitial space development, assessment of different cell type morphology and counting cell numbers using hematoxylin counterstaining. Cell markers (DDX-4, GATA-4, and 3BHSD) were used in all ages to analyze germ and somatic cells development at the mRNA and protein levels. The aim here was to improve our understanding of alpaca testis development. Since the testis is a highly dynamic organ, developmental changes for both germ and somatic cell types have been investigated by using variations genes expression primers. RT-PCR of testicular mRNA was applied using GATA-4, c-Kit, AR, FSH-Rα, and GFRα primers and results were compared to histology situation. Basal testosterone was highly correlated (r = 0.99) to age group, however, no significant difference (P=0.05) was noted in testosterone concentration between all groups except group one was lower than all other groups. The ratio of the area occupied by seminiferous tubules to that occupied by interstitial tissue was 25.7: 74.3 in group 1 and 58.0: 42.0 in group 5. Seminiferous tubule, Leydig cell, Sertoli cell numbers were 120.1, 11.4, 26.9 per 1.8 mm^2 and 37.8, 94.2, 9.6 per 1.8mm^2 in group one and five respectively. Tubule diameter in the five groups was 67.5, 124.8, 128.7, 147.9, and 222.2 µm with strong cor-

relation to age (r = 0.94). Percentage of tubules with spermatogonia only and mature spermatozoa were 81.6% and 0.0% in group one and 1.0% and 31.4% in group five. A strong correlation was measured between age and spermatocytes (primary and secondary). DDX-4 and GATA-4 were strongly specific in group one and two, but as the age advanced expression of these markers was not specific until males reach 25 months of age and older. This indicates active change in cells growth. Since accurate investigation was performed in this study, our results have provided a better knowledge of germ cell dynamics from gonocytes migration to mature sperm formation in terms of germ and somatic cells maturation pattern in each group age.

1. Introduction

Spermatogenesis is a complex process that requires the coordinated interaction of somatic cells and differentiating germ cells to produce sperm. Sertoli cells are somatic testicular cells important in supporting germ cell development. Similarly, Leydig cells are essential for testosterone production, the male hormone that sustains the process. Variations in testosterone concentration have been reported among alpaca in the same age group [1]. Reproductive technology depends upon a clear and thorough understanding of all aspects of testicular development in animals. The limited available information on male camelid reproductive development suggests that this animal reaches puberty later in life compared to other domestic large mammals. The time course of germ cell differentiation in male camelids is not completely known. Although testicular structure is similar in mammals, differences can be found even among members of the same species [2]. The spermatogenic cycle length is under the control of germ cells as was demonstrated with the use of germ cell transplantation experiments [3]. The study of the testis morphology and the spermatogenesis process in the alpaca is fundamental for further studies on sperm production, spermatogenesis regulation and factors affecting them. For instance, if complete duration of spermatogenesis is understood, it will be straightforward to estimate sperm production and use the information in male breeding soundness evaluation. In addition, this information will allow better diagnosis of some causes of male infertility. Specific chronological patterning of the seminiferous epithelium is a key element of spermatogenic regulation in animals, and plays an important role in male fertility. In adult alpaca males, the ovoid testes are present in the scrotum and the testicular diameter ranges from 4-5cm (length) and 2.5-3 cm (width)[4]. The diameter of the seminiferous tubules in adult alpaca is between 174 and 240μm. Appearance of a

lumen in the seminiferous tubules, an indicator of germ cell differentiation leading to sperm production, and presence of spermatozoa were reported respectively at 12 and 15–18 months of age in the alpaca [5]. Interestingly, a few male alpacas and llamas produce sperm as early as 10–12 months of age [6] . However, the maturation is variable in animals and reasons for this variation are not known. In addition, an incidence of testicular dysgenesis in alpaca is a concern due to negative impact on fertility. Our clinical observations showed that a high incidence of infertility in male alpaca is a result of testicular hypoplasia and degeneration [7].

Spermatogenesis is a well-organized process that is under the control of highly ordered gene expression culminating with the formation of mature spermatozoa. Each feature of testicular development involves specific genes. The genes that will be evaluated are: GATA-4, FSHR, GDNF, GFRA1, KITLG, NEUROG and AR. Expression, for instance, of GATA-4 in the testis will allow us to evaluate the differentiation and activity of Sertoli cells. Other genes such at KIT and neurogenin3 are specifically expressed by germ cells and provide straightforward determination of germ cell differentiation. Protein GATA-4 (GATA-binding protein 4) is a marker for Sertoli cells. FSHR (follicle stimulating hormone receptor) is a marker for mature Sertoli cells. GDNF (Glial cell line-derived neurotrophic growth factor) and GFRA1 (GDNF family receptor α 1) are both important for the regulation of spermatogonial stem cell self-renewal. KITLG (KIT ligand) produced by the Sertoli cells and its receptor KIT on germ cellsis important for germ cell differentiation. NEUROG has been shown to be a marker for undifferentiated spermatogonia but not spermatogonial stem cells, and AR (Androgen receptor) that is a marker for Sertoli cell maturation.

Lack of establishment of genes regulating the process of spermatogenesis and a high incidence of dysfunctional reproductive development which has been reported in male camelids resulting in

infertility, represents a gap in our knowledge base and a barrier for our ability to develop reproductive technologies in these species. In addition, there is a need to investigate all aspects of male reproductive development in camelids in order to develop diagnostic and therapeutic approaches to male infertility in camelids. This study was designed to address this gap in knowledge and improve our understanding of camelid male reproductive development. The aim of this study was to identify the qualitative and quantitative histological and morphofunctional changes in alpaca testis in different ages and exploring factors contribute to testis development to produce mature spermatozoa. We hypothesized that expression of genes associated with the initiation of spermatogenesis will be at the highest level between 11 and 13 months of age. Our findings here will be helpful in understanding the relationship between these factors and their impact on male fertility.

2. Materials and Methods

2.1. General procedures

A total of 104 male alpacas aged from 6 months to 5 years, were included in this study. Males were grouped by age category into five groups, 1. 6 to 10 months, 2. 11 to 15 months, 3. 16 to 20 months, 4. 21 to 24, and 5. ≥25 months. Testicular histology studies were performed on testicular samples collected from all males whereas gene expression study was conducted in five randomly selected samples from each age group. All animal procedures were approved by Washington State University Animal Care and Use Committee. Immediately before castration, each alpaca's testis was examined by ultrasonography and animal with abnormal testicular ultrasonography were removed from the study.

2.2. Tissue sampling and processing

Testicular tissues were collected following standard bilateral castration procedures[8]. Immediately before castration, each alpaca was weighted, testes were examined by ultrasonography and blood sample was collected from jugular vein. Animals were anesthetized using an intramuscular treatment combination of xylazine (0.4 mg/kg) AnaSed® 100 mg/mL, Lloyd, Shenandoah, Iowa), ketamine hydrochloride (4 mg/kg; Ketaset® 100mg/mL, Fort Dodge Animal Health, Fort Dodge, Iowa),and butorphanol tartrate (0.04 mg/kg IM, Torbugesic® 10 mg/mL, Fort Dodge Animal Health, Fort Dodge, Iowa). Following castration cubic pieces (about 5-7 mm^3) of testicular parenchyma tissues without tunica were collected from testis of each animal. A piece of tissue was fixed in 4% paraformaldehyde overnight then stored in 70% ethanol for histological and immunohistochemical staining. Another piece of testis was placed in TRIzol® reagent (www.invitrogen.com) and later in the lab was homogenized and stored in -20°C for RNA purification and gene expression analysis using RT-PCR. Both tubes were maintained in ice at all times. Blood samples were left to clot at 4ºC overnight then centrifuged at 3000 x *g* for 20 minutes at 4ºC. Sera were transferred into polypropylene vials and stored at -20ºC until assayed for basal testosterone concentration.

2.3. Hormone assay by Radioimmunoassay (RIA)

Concentration of testosterone was determined in serum samples using a commercial double-antibody RIA kit (Coat-A-Count® total testosterone; Siemens Healthcare Diagnostics Inc. Los Angeles, CA 90045 USA Catalog # TKT1).The calibrators contain, respectively, 8, 16, 96, 414, 792 and 1685 nanograms of testosterone per deciliter (ng/dl) in processed human serum. The sensitivity of the assay is 0.1 ng/mL.

2.4. Histological and immunohistochemical staining

2.4.1. Histology

Sections of 8 micrometer-thick were deparaffinized in histoclear, rehydrated in decreased graded ethanol, and washed in tap water. Slides were counterstained with hematoxylin, periodic acid schiff's- hematoxylin (PAS-H), and gemsia. The use of different staining agents helped in evaluation of seminiferous tubules and interstitial cells. At this point, sections were prepared for examination as described above.

2.4.2. Immunohistochemistry

Sections were deparaffinized in histoclear, rehydrated in decreased graded ethanol, and subjected to heat-induced antigen retrieval in sodium citrate 1% + Tween 20 (pH 6) in microwave for 20 minutes. Endogenous peroxidase activity was blocked by incubation in 0.3% H_2O_2 in PBS for 20 min. After washes in PBS (PH 7.4) three times for 5 min each, sections were encircled with hydrophobic slide marking pen to keep reagents localized on the tissue sections. Slides then were incubated for 30 min with 10% normal goat serum in PBS at room temperature to block any non-specific binding followed by blotting the excessive serum from the sections. Primary antibodies (DDX-4, GATA,-4, and 3βHSD) were diluted 1:200 and applied to the sections at 4ºC overnight in a humidified chamber. After the incubation, slides were washed in PBS twice for 5 min each and biotinylated secondary antibody (rabbit anti-goat IgG; Santa Cruz Biotechnology) diluted at 1:200 was added and incubated at room temperature for 60 min. Sections were washed in PBS twice for 5 min each and incubated for 20 min with RTU streptavidin peroxidase complex and

again washed in PBS twice for 5 min each. Sections were developed with impact diaminobenzidine (DAB) substrate for 2-3 min followed by tap water washing. Samples were counterstained with hematoxylin, dehydrated in increased graded ethanol, cleared in histoclear and mounted with a coverslip using Permount. Next day, slides were evaluated by light microscopy and images captured at 100X magnification with a use of Leica DFC 280 digital camera and a Leica DME compound microscope (Leica Microsystems Imaging Solutions Ltd., www.leica-microsystems.com).

2.5. Morphologic study

Assessment of different cell types and thenumber of each cell type were based on previous descriptions [9]. The cells in the seminiferous tubules and intertubular spaces were evaluated according to their appearance in relation to each age. Evaluation of testis function was conducted by determination of Leydig and Sertoli cells efficiency as indicator of spermatogenesis productivity. Interstitial cells, vascular and lymphatic vessels were also examined. We evaluated 300 cross section tubules from two stained sections, 150 from each different section and placed them in one of these groups based on the presence of the most mature germ cell: seminiferous chords with no germ cells; tubules with gonocytes, pre-spermatogonia and spermatogonia; primary and secondary spermatocytes; round spermatids; elongating spermatids and mature sperm cells. Ten seminiferous tubule diameters were measured to nearest 1.0 μm and averaged. The measurement was determined with the use of an ocular micrometer which was calibrated using a stage micrometer by superimposing the two scales. Ten animals from first group and five animals from each other groups were randomly selected and images captured and used for further measurements. All tubules from three different section fields (picture 1.25mm x1.56mm = 1.95mm^2) at

10X magnification were counted and averaged. Area ratio between seminiferous tubules and interstitial tissue area was identified (Figure 1). All these measurements were done with the use of ImageJ (ImageJ 1.46r, Wayne Rasband, National Institutes of Health, USA, Imagej.nih.gov/ij). The purpose of these measurements was to study the development of seminiferous tubules epithelium in relation to age, body weight, and testosterone level of the animals.

2.6. Determination of Cell numbers

2.6.1. Germ cells

Gonocytes were identified by their location in the center of the seminiferous cord and visible cellular surroundings with large round nuclei. Spermatogonium was used as a description of a gonocyte that migrated and attached to the basement membrane. Complete separation of the spermatogonia from basement membrane was a sign of differentiation to spermatocytes. Primary spermatocytes (preleptonene to secondary spermatocytes) were clarified by their clear spherical nuclei that large in size with much of the chromatin deposited on the inner surface of the nuclear membrane ends with formation of secondary spermatocytes. Identified as a small spherical nuclei with a uniformly heterochromatin distribution on the nuclear membrane. Spermiogenesis was recognized by morphological transformation of spherical nuclei to cylindrical nuclei contained a condensed nuclear protein. Mature sperm identified by presence of nuclei and acrosomes, mitochondrial mid-piece, and complete free tails in lumen.

2.6.2. Sertoli cells

Sertoli nuclei cells were also counted in ten different tubules. In group 1, nuclei were identified by the shape of the nucleus and the presence of a distinguishing round nucleolus. Most Sertoli cells at this age are more evenly distributed and their nuclei located near the basal lamina. As age advanced, Sertoli cells were recognized by their distinctive tripartite nucleolus at the basement membrane. In adult males (age ≥ 25months), Sertoli cells were recognized by their irregularly shaped nuclei and difficult observable cell membrane.

2.6.3. Leydig cells

Representative cross-sections of testes from each age group were processed for detection of adult Leydig cells. Leydig cells were evaluated based on morphology parameters (shape, size, number, and maturation status). The morphology of stained sections was evaluated using light microscopy at 100 X magnification and digital images were captured. Adult Leydig cells were counted in randomly selected fields through the entire sections without overlap. Mature Leydig cells were identified in the interstitial tissue according to the criteria of a large rounded nucleus containing an obvious nucleolus and had little or no cytoplasmic lipid droplets, had a relatively thicker peripheral rim of heterochromatin in their nuclei.

2.6.4. Other testicular cells

Interstitial cells, lymphatic and blood vessels in all slides were examined. Unusual distinguishable myoid, mesenchymal, endothelial and macrophages cells number or size was reported and interpreted.

2.7. Real Time-PCR

Small pieces of testis were placed in 2-mL Trizol, on ice to prevent RNA degradation. Later in the lab, testis pieces were homogenized and RNA was isolated according to the following directions: Total RNA was re-suspended in RNase-free water (Ambion), and five μg of total RNA was reverse-transcribed into cDNA using oligo(dT) priming and M-MLV reverse transcriptase (Invitrogen). Complementary DNA synthesis was confirmed using PCR for the *b*-actin gene (ACTB). Complementary DNA (cDNA) samples from all ages were examined for the expression of genes important for testis development and germ cell differentiation (FSHR, GATA4, cKIT, GFRα1, and AR) using real-time RT-PCR. The primers were designed using Primer Express (Applied Biosystems) and obtained from Operon Biotechnologies or Invitrogen (Table 1).
The PCR reaction mixture contained 200 ng cDNA, 20 pmol of each primer, 12.5 μl iQ TM Supermix (Bio-Rad), made up to a volume of 25 μl with water. SYBR Green I (Molecular Probes) was used to detect amplimer production. PCR reactions were conducted in an iCycler iQ TM Real-Time PCR Detection System (Bio-Rad) and consisted of 40 cycles of 95C for 30 sec, 58C for 30 sec, and 72C for 30 sec. Samples were run in duplicate. Relative gene expression was determined based on the expression of ribosomal protein S2 (RPS2) as the baseline using the Q-Gene method and standardized to the beta-actin expression level. Reactions of 25 μL were performed containing 2 mM MgCl2, 0.25 mM deoxynucleotide triphosphates (dNTPs), PCR buffer, 5 pmol of each primer, and 1 U of *Taq* DNA polymerase. Reaction conditions were 94C denaturation for 5 min followed by 35 cycles of 94C for 30 sec, 58C for 30 sec, and 72C for 30 sec, with a final extension of 72C for 10 min.

2.8. Statistical analysis

All data are presented as mean ± SEM and were analyzed using ANOVA (Tukey test). Analysis was performed using statistical software (minitab15) program. Significance results reported if *P* value is less than 0.05.

3. Results

3.1. Age to weight and basal testosterone

Body weight was taken at the time of bleeding. Average body weight was 61.6, 96.8, 113.2, 120.2, and 141.5 lb in groups1, 2, 3, 4, and 5 respectively. Body weight in group 1 was significantly different from all other ages (P = 0.05). Basal serum testosterone concentration was 0.18 ng/mL in group 1, and increased steadily with age with a strong correlation (r = 0.99) from 6 months onward to 2.1 ng/mL at group 5. Standard deviation within groups was high indicating wide variation among individual males within the same age group (Table 2).

3.2. Seminiferous tubule features

The mean number of seminiferous tubules, diameter of tubules and area of distribution in group 1 were 120.1 67.5 µm and 1.95 mm^2, respectively. Presence of a lumen was observed in 10.5% of seminiferous tubule in group 1 (6-10 months of age). Subsequently, numbers of tubules within a similar area decreased as the tubule diameter expanded (r = - 0.84) to reach 222.2 µm in adult ages (Figure 2). Presence of a lumen in the seminiferous tubules increased from 10.5% in group 1 to reach 95% in groups 4 and 5 (Table 3). The seminiferous tubules occupied 25.7% of an area

of 1.95 mm^2 in group 1 while the highest percent area occupied by seminiferous tubules (58.0%) was observed in group 5 (Figure 4).

3.3. Histology of the seminiferous and interstitial compartments

Testicular development in alpaca was evaluated at the morphometric level by assessing interstitial cell (Table 4) and seminiferous epithelium (Table 5) development. Perfect correlation has been detected between age and cellular changes (Figure 5).

3.4. Group 1 (6 to10 months old)

Most males of this group (88.3%) had only gonocytes as germ cells present in seminiferous cords (Figure 6). They were large, round cells with a pale cytoplasm and contained between 1 to 5 visible nucleoli. Some of these cells had migrated and relocated at basement membrane and some remained in the central area of the tubule. The percent of gonocytes that had made contact with the basal lamina in this group varied from 95% of tubules to 0.0% regardless of the age of the animals within the group. Gonocytes were strongly positive after using anti DDX-4 antibody and found to be positive in 19.3 cells per seminiferous cord (Figure 7). Spermatogonia were recognized by an oval nucleus at the basal lamina in this group. A few (6 out of 51) males in 8, 9, 10 months old had germ cell differentiation and thus had started spermatogenesis. Immature Sertoli cell nuclei had a variable shape and size between columnar to oval with a regular border; these cells were located at basement membrane of the cords and arranged at different heights. Sertoli cell nuclei were easily distinguished from gonocytes. Using anti-GATA-4 antibody for IHC has confirmed this finding (Figure 7). The total number of Sertoli cells per tubule in this group was 26.9 (SD= 5.68). The basal lamina was very thick and surrounded the cell types (gonocytes and

Sertoli cells) present at that stage. The interstitial tissue (space between the seminiferous tubules) was characterized by large areas of very loose mesenchymal-like-cell tissue. Seminiferous cords and tubules occupied only 25.7% of this area. Scattered Leydig cells in the interstitium have been counted. Their number of Leydig cells per section varied between 0.4 in 6-month-old to 64.8 in 10-mont-old (11.4 SD±14.37). Leydig cells clusters of three to five cells first appeared between 9 and ten months of age. The myoid cells were elongated and located along the basal membrane of the tubules and were distributed uniformly around the tubules. These cells were present in all examined samples. Small blood vessels, mostly capillaries and lymphatic ducts were also found. Resident macrophages were not recognized in this group because they were either are not present or did not stain with hematoxylin. Other interstitial cells such as elongated spindle-shaped cells like fibroblasts, endothelial cells, pericytes and peritubular myoid cells were present in all examined ages in this group. These cells were collectively categorized as mesenchymal cells and were distributed irregularly between the developing tubules.

3.5. Group 2 (11 to 15 months old)

At 11 months, the basal lamina of the seminiferous tubules was thinner than 12 months of age and older. The lumen was formed on average in 39.4% of the tubules (range 2-100%) and 80.8% of gonocytes completed their relocation to the basil lamina of the seminiferous tubule. Seminiferous tubules with post-meiotic cells were found in 17.2% of samples and only 0.08% of samples had elongating spermatids out of all tubule counted (Figure 8).

Sertoli cells in this group were more consistently distributed and their nuclei located near the basal lamina. The number of Sertoli cells per cross section decreased, but not significantly, from 26.9 in group 1 to 23 in group 2. The interstitial compartment in this group was generally disor-

ganized. Different cell types can be recognized in most slides. The Leydig cells were generally polygonal in shape and arranged in large clusters (184.1 in 15-month-old) except for some that appeared in small groups (11.8 in 11-month-old). Mature Leydig cells were 52.7% of all cells counted and immature Leydig cells were also present. Macrophages were clearly observed with their faint stain and characteristic kidney shaped nucleus. Immunohistochemistry examination has revealed that markers, DDX-4 and GATA-4 can be used to identify germ and Sertoli cells as described previously (Figure 9).

3.6. Group 3 (16 to 20 months old)

Group three had little difference from group 2 in terms of most parameters measured (Table 5). Post-meiotic germ cells were represented in 25.6% of all tubules examined (Figure 10). Interestinglyin this group was the highest (but not significant different) percentage of 5.57% tubules with one layer of cells in all five groups (Figure 11A). Testicular tissues from this group had few but clear identified interstitial cells. Mature Leydig cells number and percentage to immature cells were lower than that found in group 2 even with higher testosterone level in this group. Macrophages were smaller in size than that seen in group 2 but comparable in number to Leydig cells.

3.7. Group 4 (21 to 24 months old)

Examination of testis tissues in this group revealed a dramatic increase in testicular development compared to group three. For instance, formation of lumen was presented in 95% of seminiferous tubules and 68.5% of Leydig cells were mature. Seminiferous epithelium assessment showed a complete disappearance of tubules containing only gonocytes. The percentage of tubules with

elongating spermatids increased from 8.4% in group 3 to 45.7% in this group(Figure 12). Tubules occupied 46.0% of the total are of the testis cross-section. Overall, 79.8% of seminiferous tubules evaluated in this group contained post-meiotic germ cells. In addition to Leydig cells (mature and immature), interstitial space contained active macrophages, approximately half the Leydig cell number and few mesenchymal cells (Figure 13A).

3.8. Group 5 (25 months and older)

Histology of the adult group was characterized by the presence of the most advanced cell types in seminiferous tubules. The lowest total number of seminiferous tubules (37.8) and highest tubule diameter (222.3 μm) was measured in this group. The tubules occupied 58% of the total section area. Less than 5% of tubules had non-differentiated germ cells (Sertoli cell only) and 90.0% contained post-meiotic germ cells with 31.4% of the seminiferous tubules containing mature spermatozoa ready to be released in the lumen (Figure 14). The number of Sertoli cell nuclei was 9.6 per tubule (Figure 15A). Mature Leydig cells number was the highest (94.2 per field examined) in this group among all five groups. This number is significantly correlated to basal testosterone concentration (2.14 ng/mL) and more strongly correlated (R-Sq (adj) = 77.7%) to Sertoli cells. Mature Leydig cells and active macrophages clearly share the interstitial space with few other mesenchymal cells (Figure 15B). Immunohistochemistry was utilized to confirm the presence of cells in this group (Figure 16A&B).

3.9. Gene expression

Subsequent to qRT-PCR analysis and data collection, raw expression levels of CT values for each gene in different five age groups (figure1) and the expression stability for β-actin (figure 2)

were transformed to relative quantities using $\Delta\Delta C_T = \Delta C_{T(test)} - \Delta C_{T(calibrator)}$ equation. Generally, the five target genes exhibited a wide expression range with CT values from 16.6 to 39.5 in the different age groups. The gene expression results confirmed the results determined from histological examination. GATA-4 expression was higher in the 11-15 month old and 21-24 month of age group compared to the other ages. Androgen receptor (AR) expression was low until 21-24 months of age when it increased. The decrease after this age is likely due to the increase of differentiating germ cells. FSHR receptor was low throughout development compared to other genes. FSHR expression was higher at 11-15 months of age and 21-24 months of age consistent with the biphasic testicular development associated with testosterone production in alpaca. GATA-4, AR and FSH are all expressed in Leydig and Sertoli cells so the similar expression patterns suggest these cells develop in synchronous manner.

The germ cell specific expressed genes GFRa1 and c-kit were higher in the 21-24 month group compared to other age groups. These data support the conclusion that post-20 month of age represents that time with testicular development has been achieved in most male alpaca and germ cell differentiation is progressing toward spermatid production.

4. Discussion

To our knowledge this study is the most comprehensive investigation to date on the seminiferous epithelium development and investigation of testis function in alpacas. Testis tissue development consists of a series of events occurring in the seminiferous epithelium and changes in interstitial cells. Spermatogenic activity starts with the migration of gonocytes from the center of seminiferous cords to relocate at the basement membrane and differentiation to spermatogonia. Germ cell

differentiation ends with the formation of mature sperm that is released into the lumen. The interstitial space of the testis begins with compact mesenchymal-like-cells to recognizable functional cells such as mature Leydig cells. It is well known that spermatogenesis and sperm production is a function of age. Histological and morphometrical investigation was achieved with careful evaluation related to male age. Males in this work were divided into five groups with based on maturity and based on information from the literature. Moreover, to obtain additional confirmatory and accurate results, RT-PCR for genes that are hallmarks of germ, Sertoli and Leydig cell differentiation was performed on selected samples and was compared to histological findings.

Group 1 which contained ages 6 to 10 months was an important group because we hypothesized that these ages are the transitional period from prepubertal age entering puberty changes. Fifty one healthy males included in this group were examined. As was expected, males of this group had many common features, for instance all these animals had gonocytes that were starting the migration to basement membrane. Testosterone concentration was low in all males in this group except three males that exceeded 0.5 ng/mL which were 10 month old and the heaviest in weight. Tubules diameter was 67.5 μm and a lumen was observed in 10.4% of total seminiferous tubules examined which was earlier than that reported by Montalvo [5]. Overall no significant differences were observed between the ages of animals included in this group indicating that in general this is the pre-pubertal stage of alpaca development.

Changes in seminiferous epithelium and cells in interstitial area continued slowly in groups 2, 3, and 4 with no significant difference between values from each group. This might be attributed to slow development of the testis and the delay of puberty in alpaca. Dramatic increase in the seminiferous tubules epithelium was observed in group 4. Animals in this age group show the largest

change in all aspects of differentiation including the increase in area occupied by developing seminiferous tubules from 25.7% to 79.8% , cell differentiation from 8.5 to 45.7% (tubules with elongating spermatids) and from an increase of seminiferous tubules with spermatozoa from1.6 to 9.8% from group 3 to 4. These changes indicated that these group ages were in puberty and preparing for maturity.

In adult ages (group5), full testis function was observed in all animals. It was interesting to know that basal testosterone concentration (2.1 ng/mL) was significantly correlated to all parameters examined especially with Sertoli cell number and seminiferous tubules with mature spermatozoa. This finding provides strong evidence of the effect of testosterone in testis development in the alpaca and how delays in certain animals are likely due to delays in the development of the hypothalamic-pituitary-gonadal axis or Leydig cell differentiation. Leydig cell maturation is stimulated by a variety of factors so delayed Leydig maturation could be due to low production of factors such as Anti-Mullerian Hormone (AMH). The nature of the delay in puberty requires further investigation but our data suggest that the Leydig cell maturation is a key component.

To support our histological results, most important testis development genes (GATA-4, AR, FSHR, c-Kit, and GFRa1) were evaluated. The gene expressions pattern was consistent with the morphological findings. As GATA-4 is expressed by somatic cells, including Sertoli and Leydig cells, higher expression demonstrates that Sertoli cells are differentiating during pre-pubertal (11-15 months of age) and pubertal (21-24 months of age) time periods. Highest Sertoli cells counted in group 1 with lowest GATA-4 mRNA expression means that the functional Sertoli cells at that age are low. The highest significant level of AR that was seen in group 4 could be a reason of high functional activity of Leydig cells. FSHR was expressed at low levels in all five groups which is consistent with other species. However, the higher expression at 11-15 months

and 21-24 months of age compared to the other ages is consistent with the biphasic pattern of testicular development based on testosterone production and morphological analysis. This pattern is consistent with pigs and humans.

The expression pattern of GFRa1 and c-Kit were consistent among all ages with highest expression of both genes in group 4 which the age that require high self-renewal and differentiation of spermatogonial stem cells to produce sperm production with a strong influence on the initiation of continual sperm production.

In summary, alpaca testis development has been studied in this work with the use of histological examination and supported by testosterone concentration determination and gene expression analysis. Pre-puberty ages examined in group 1 (6 to 10 months) had almost the same features between animals with no significant difference among these males. This group is characterized by formation of the lumen (10% of samples) and relocation of gonocytes to the basement membrane in 61.4% of tubules. Testis development progresses slowly from this group onward. The notable difference was observed in fifth group that included adult males. Full post-meiotic tubular activity (90.0%) and high testosterone level along with high Leydig cells per field examined (94.2). Testis growth in alpaca is very slow and differences are not very obvious between close ages. This study provides background for further studies on factors affecting age at puberty and testicular development in alpacas.

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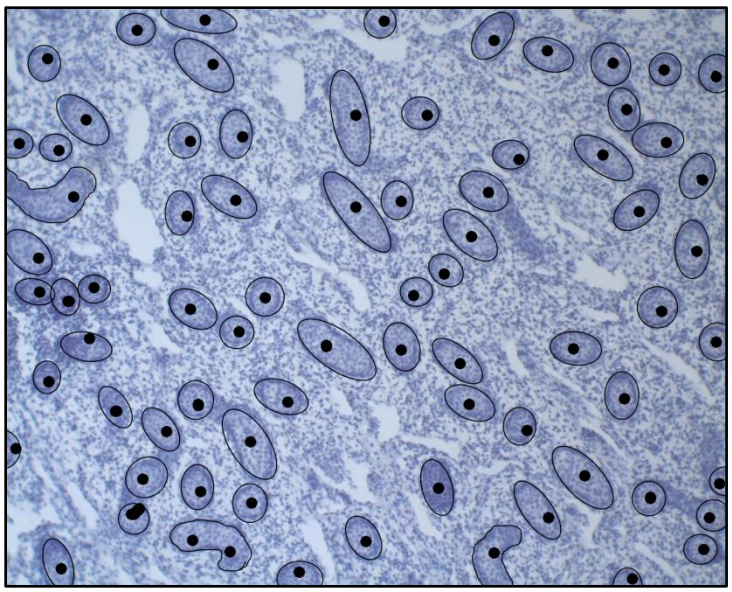
A.



B.

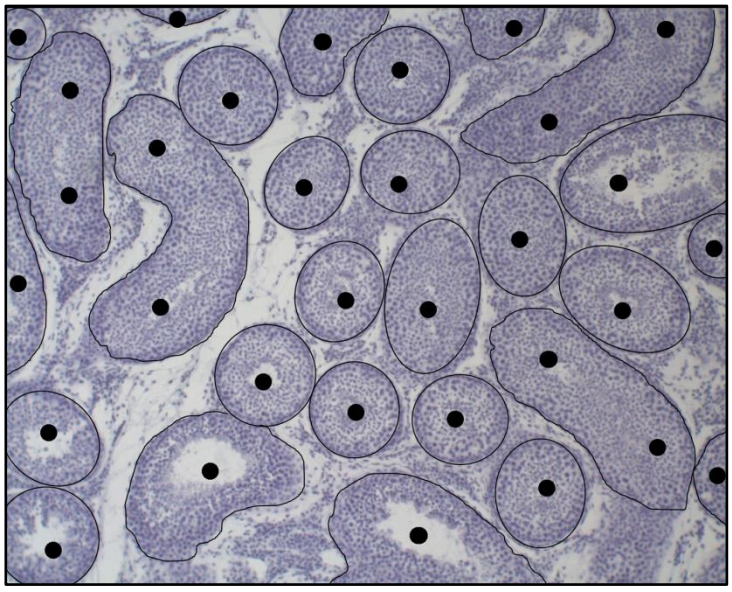


Figure 1: Representative image of alpaca testis and how seminiferous tubules were counted and area was determined A. 6-month-old (area = 19.2% lowest in the study), B. adult male (area = 61.0% highest in the study).

Antibody	5’ Forward primer	3’ Reverse primer
GATA-4	gtg tag ggc cag tgt tac cag at	ttt gag ccg cgg aag ga
GFRa1	cca cca gca tgt cca atg ac	gag cat ccc ata gct gtg ctt
AR	aga ggc cca agg aca tga aa	aga gag gga aaa aac agg gtt ga
FSHR	ctg ctg ctc ata gcc tca gtt g	aaa aag ccg gca gca tca c
c-Kit	tgg agt gca ggg ctt ata acg	gaa cag ggt gtg agc atg ga

Table 1: Sequence of Primers used in this study.

Groups number	1	2	3	4	5
Ages (months)	6-10	11-15	16-20	21-24	25-60
Males/group	51	18	20	8	7
B.W., kg (± SEM)	27.95 (±9.63)	43.92 (±35.25)	51.36 (±25.91)	54.54 (±31.58)	64.21 (±19.23)
Testosterone, µm (± SEM)	18.9 (±37.68)	68.4 (±70.56)	100.3 (±91.06)	155.4 (±152.15)	214.4 (±189.18)

Table 2: Alpaca body weight and basal testosterone level for each group

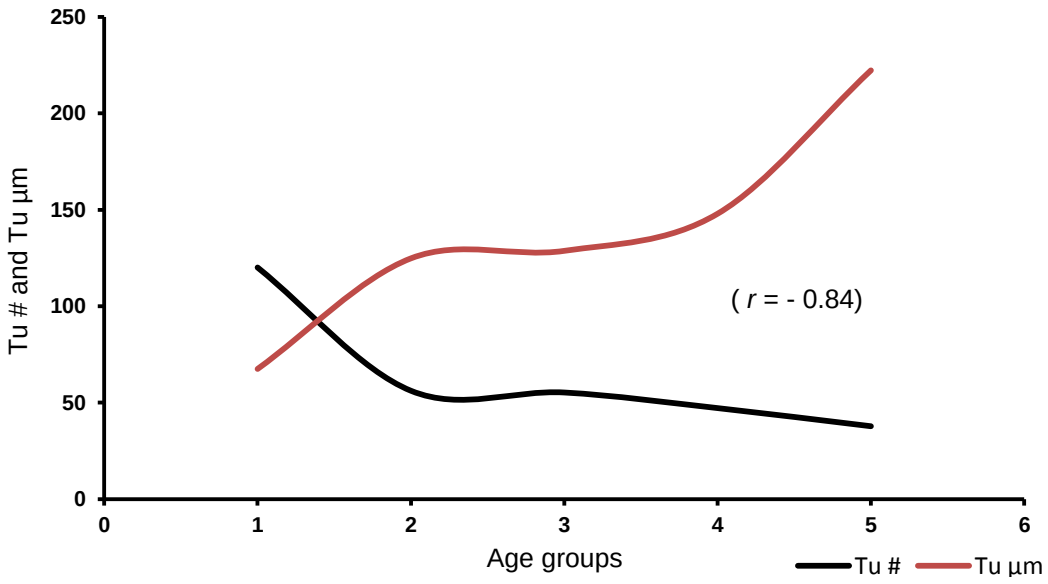


Figure 2: Graphical representation of the relationship of the number of seminiferous tubules (Tu #) and the seminiferous tubule diameter (Tu µm) during alpaca testis development.

Groups number	1	2	3	4	5
Tubules number	120.1 (±31.17)	56.2 (±16.41)	62.6 (±20.46)	47.2 (±13.09)	37.8 (±11.3)
Tubules diameter, µm	67.5 (±20.51)	124.8 (±50.70)	128.7 (±38.17)	147.9 (±41.16)	222.3 (±41.33)
Presence of lumen, %	10.5 (±22.58)	39.4 (±38.34)	41.9 (±39.01)	95.6 (±4.17)	95.4 (±1.81)
Tubular area to interstitial area %	25.7:74.3	46.3:53.7	41.7:58.3	46.0:54.0	58.0:42.0

Mean (±SD)

Table 3: Seminiferous tubule measurements in different ages and percentage of seminiferous tubules occupied the testis area.

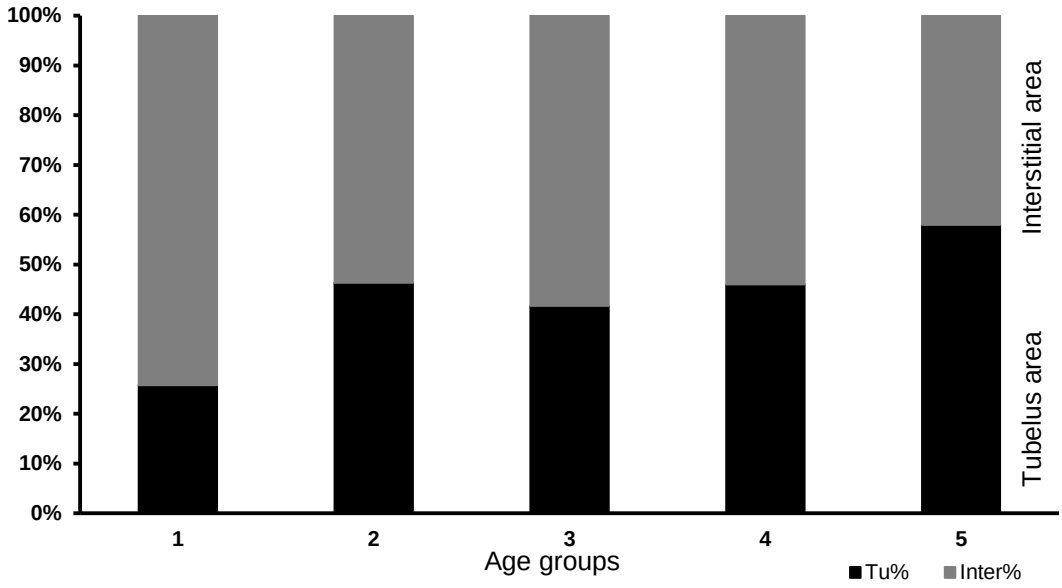


Figure 3: Graphical representation of the ratio between seminiferous tubule area and interstitial space area (%) in alpaca testes during development.

Groups number	1	2	3	4	5
Mature Leydig cells %	22.4 (±24.18)	52.7 (±32.68)	48.3 (±35.72)	68.5 (±40.71)	90 (±5.45)
Mature Leydig cells number Per field (1.95 mm^2)	11.4 (±14.37)	40.4 (±38.45)	36.7 (±24.65)	49.9 (±39.20)	94.2 (±35.31)

Table 4: Assessment of Leydig cell in five groups of alpacas.

Groups number	1	2	3	4	5
Gonocytes per cord	19.3 (±8.3)	22.2 (±7.6)	*	*	*
Gonocytes	0	34.3 (±22.4)	*	*	*
Primary spermatocytes	8.33 (±5.6)	66.9 (±26.1)	47.5 (±26.3)	17.8 (±18.7)	8.1 (±9.2)
Round spermatocytes	0	11.4 (±10.5)	15.6 (±15.4)	25.7 (±11.2)	32.3 (±7.2)
Elongating spermatids	0	5.7 (±11.8)	8.4 (±12.6)	45.7 (±24.3)	26.3 (±5.1)
Mature spermatocytes	0	0.08 (±0.2)	1.6 (±4.5)	8.9 (±7.2)	31.4 (±6.9)
Tubules with one layer	0	1.16 (±1.7)	5.6 (±9.1)	2.3 (±3.5)	0.86 (±1.2)
Sertoli cells	26.9 (±5.6)	23.0 (±9.2)	*	*	9.6 (±2.2)
Tubules with gono-cytes at basement membrane %	61.5 (±29.46)	80.8 (±26.02)	74.6 (±32.61)	84.6 (±29.34)	95 (±0.00)

Mean (±SD)
* identified not possible

Table 5: Determination of cellular components of the seminiferous epithelium in five groups of alpacas.

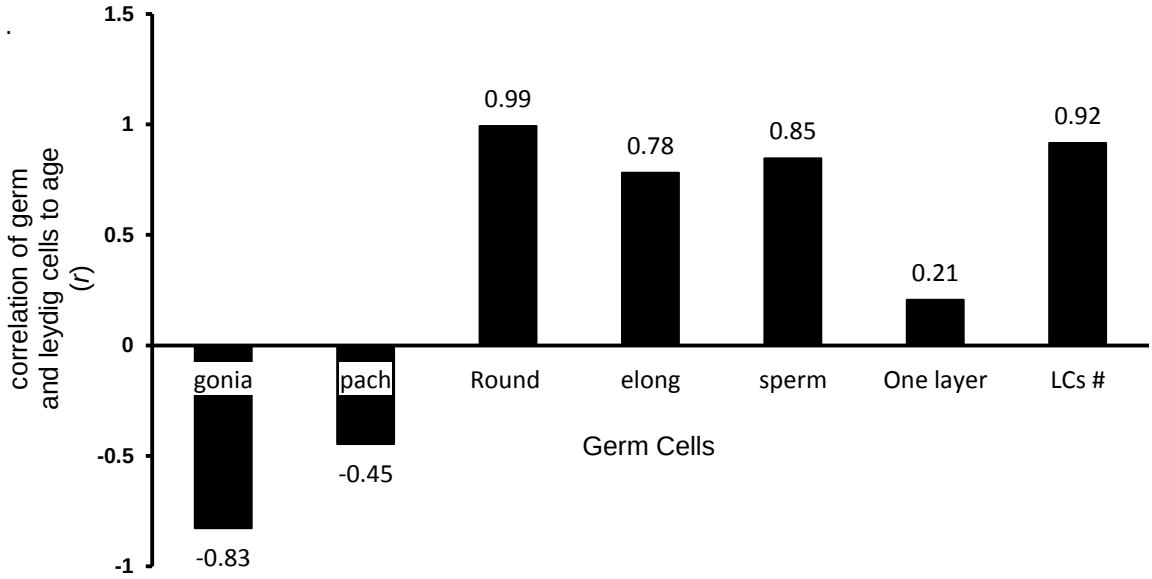


Figure 4: Correlation between alpaca age and germ cells and testis cellular compartment. Gonia(Gonocytes), pach (pachytene), round(round spermatocytes), elong(elongated spermatocytes), spem(spermatocytes), one layer(one layer cells), LC#(Leydig cells).

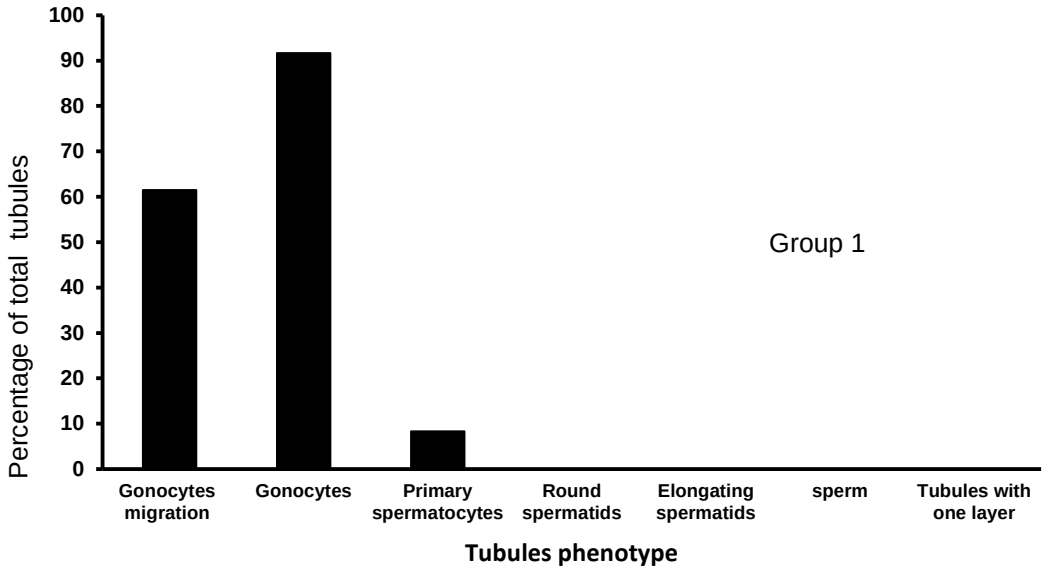
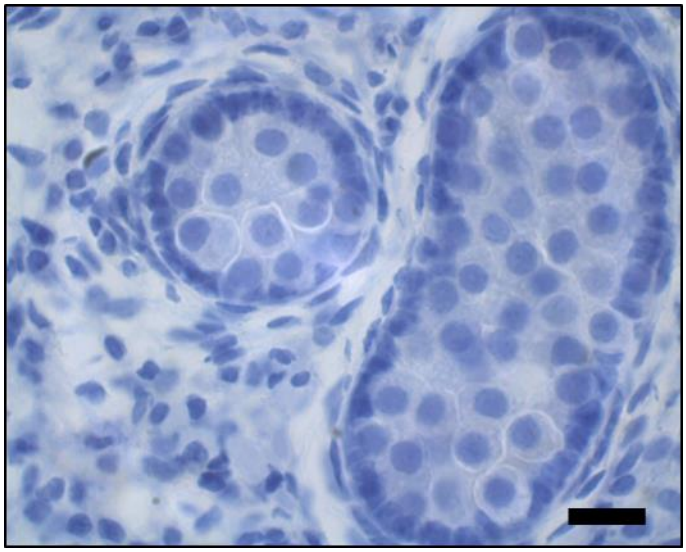
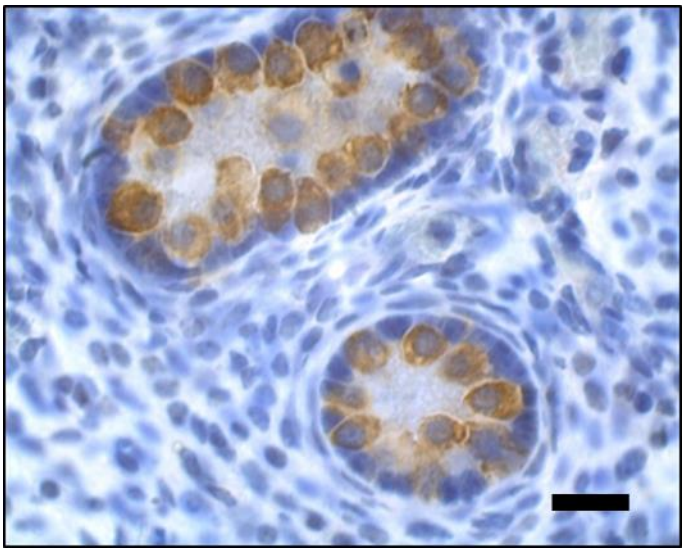


Figure 5: Percentage of total seminiferous tubules containing different germ cells in alpaca group 1 (6 to 10 months of age).

A.



B.



C.

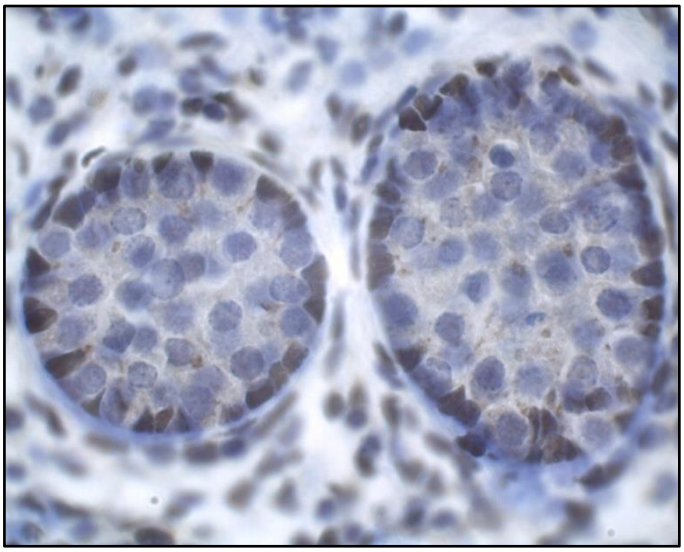


Figure 6: Representative cross-section of alpaca testis tissue from group 1, A. Gonocytes appear in the center and at the basement membrane of seminiferous cords (hematoxylin), B. Gonocytes stained with DDX-4 (brown), C. Sertoli cells stained with GATA-4 (brown)

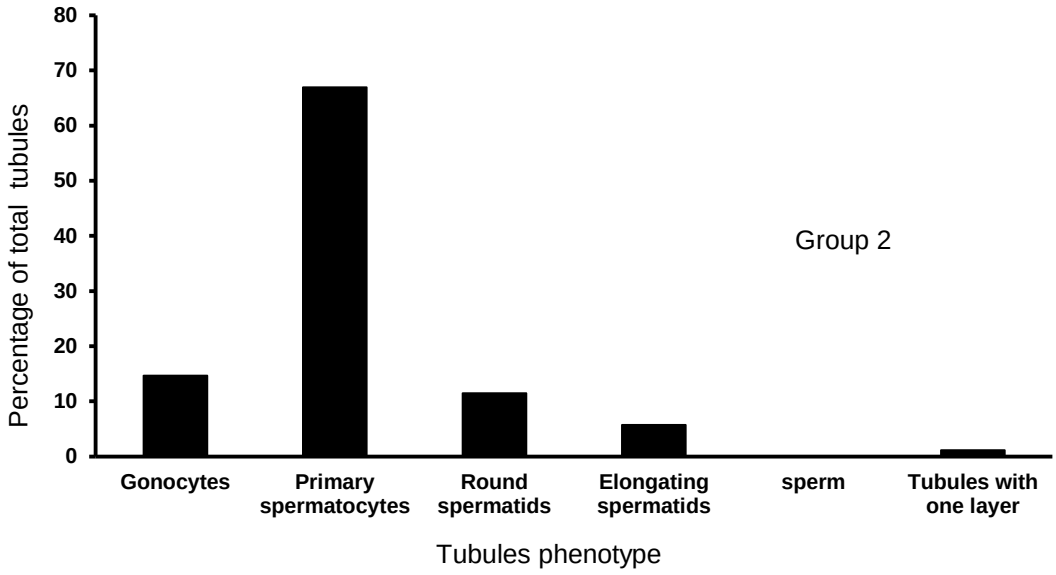
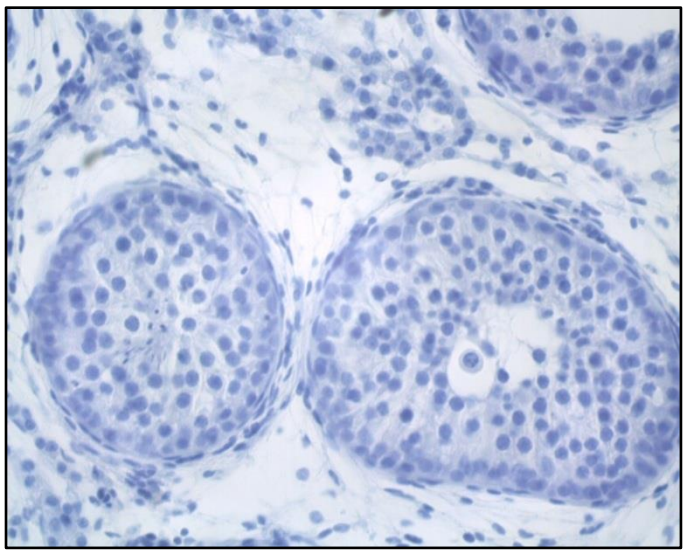
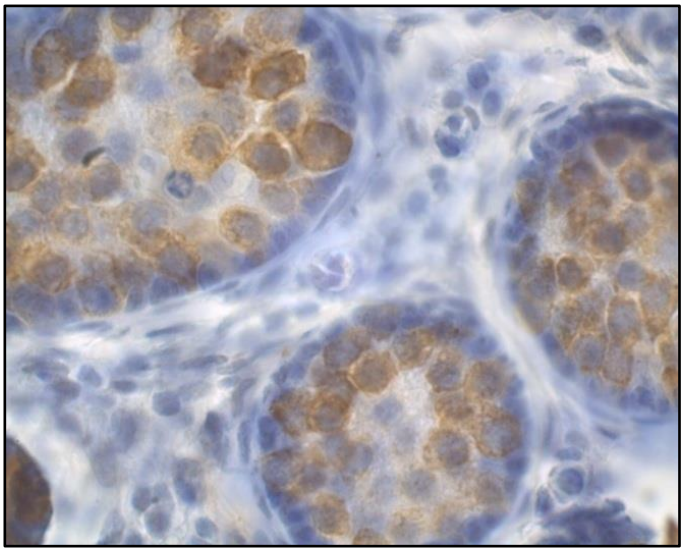


Figure 7: Percentage of total seminiferous tubules containing different phenotypes and germ cell types in group 2 (11 to 15 months).

A.



B.



C.

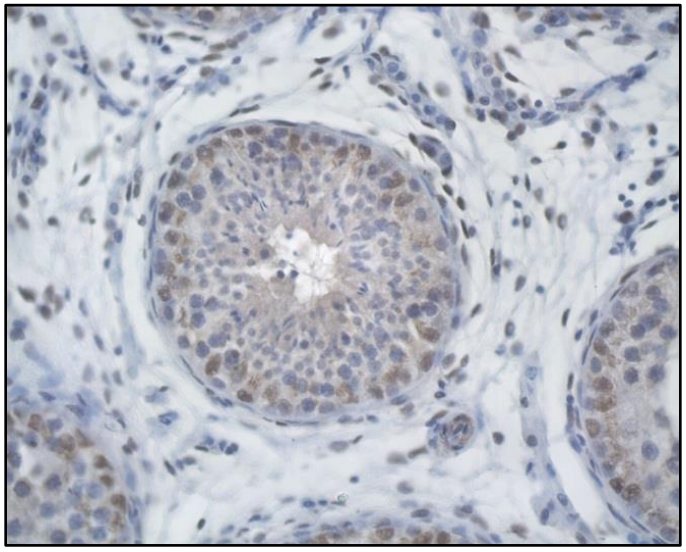


Figure 8: Cross-section of testis tissue from group 2, A. Differentiated germ cells in seminiferous tubules and mature Leydig cells in interstitial space (hematoxylin), B. Germ cells stained with DDX-4, C. Sertoli cells stained with GATA-4.

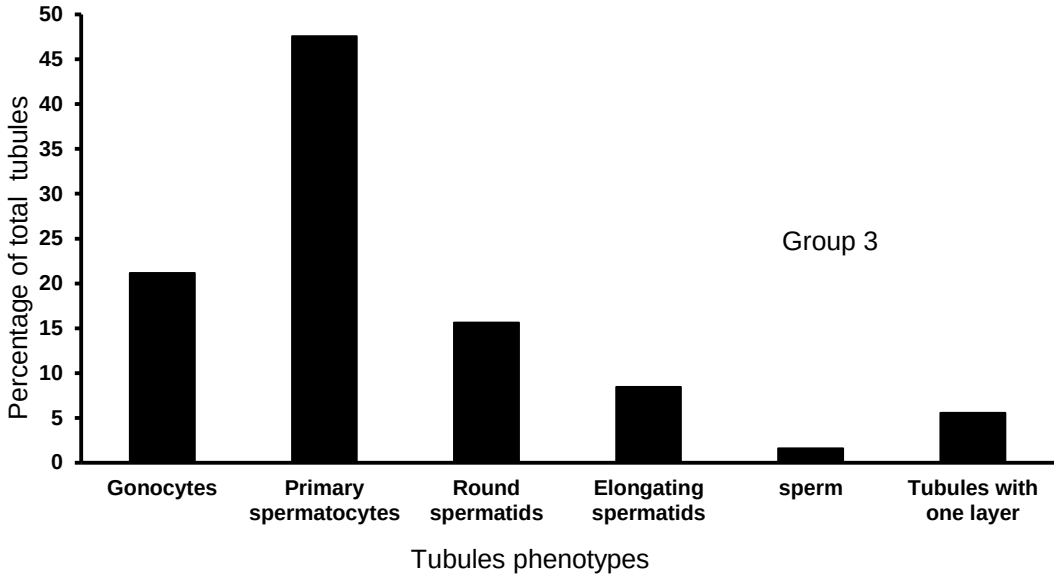
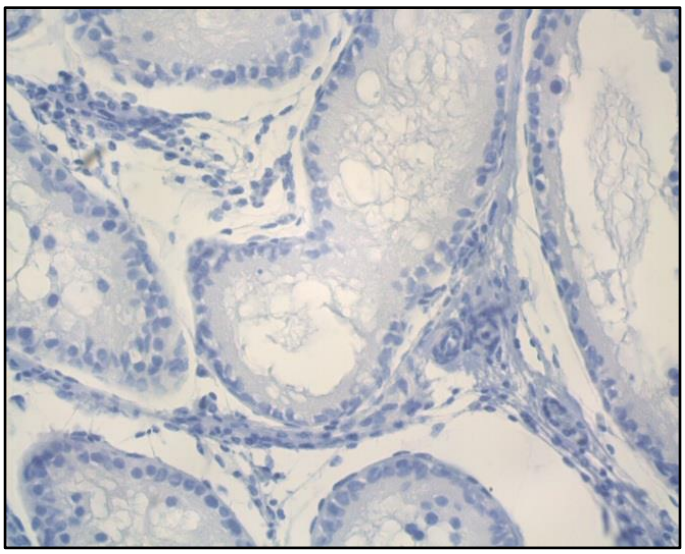
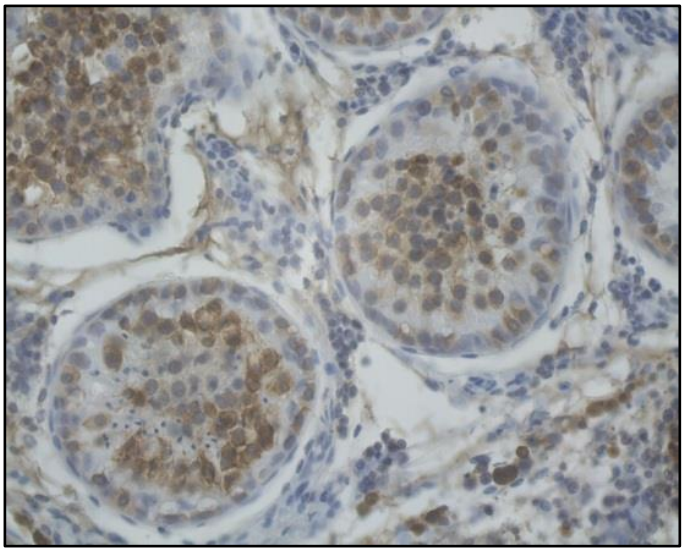


Figure 9: Percentage of total seminiferous tubules containing different phenotypes and germ cell types in group 3 (16 to 20 months).

A.



B.



C.

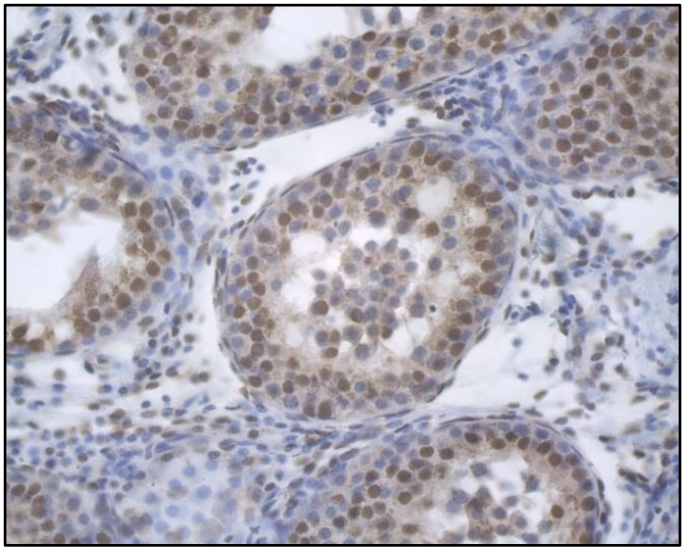


Figure 10: Cross-section of testis tissue, A. one-layer-cell tubules (hematoxylin), B. Germ cells stained with DDX-4, C. Sertoli cells stained with GATA-4.

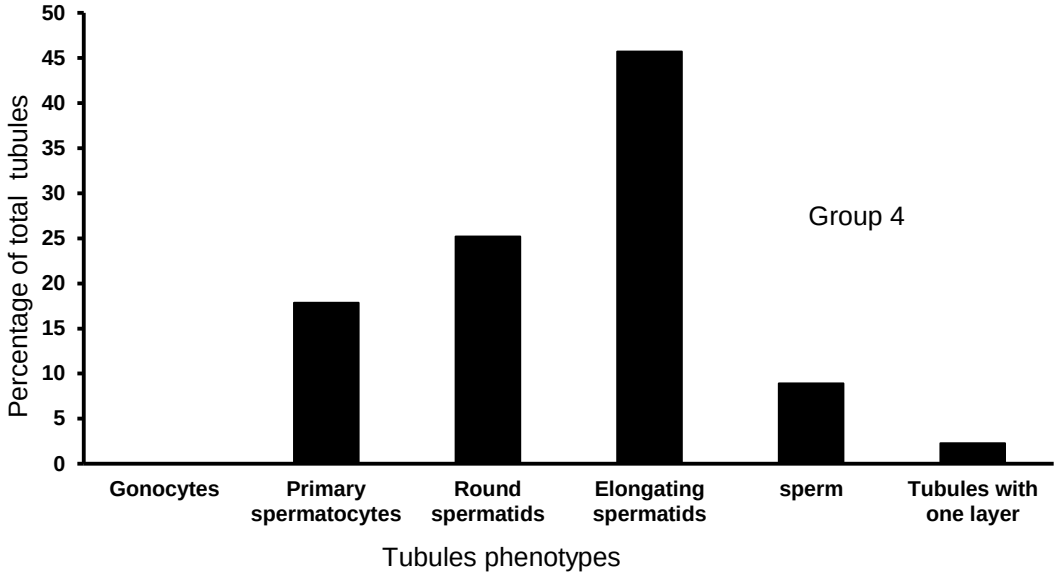
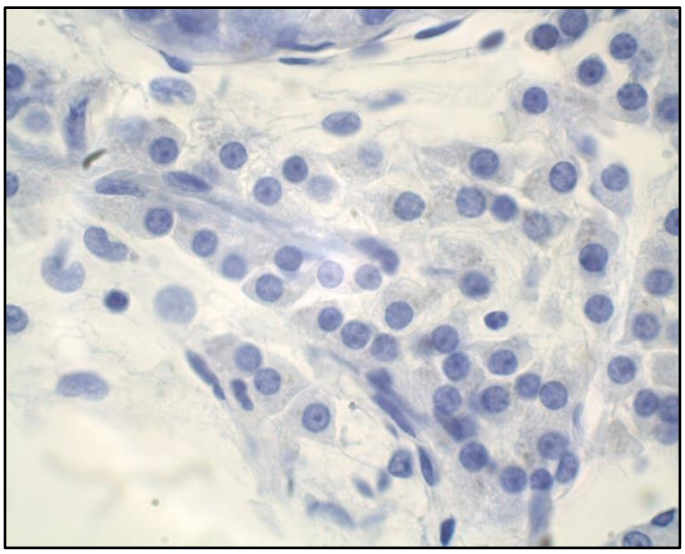
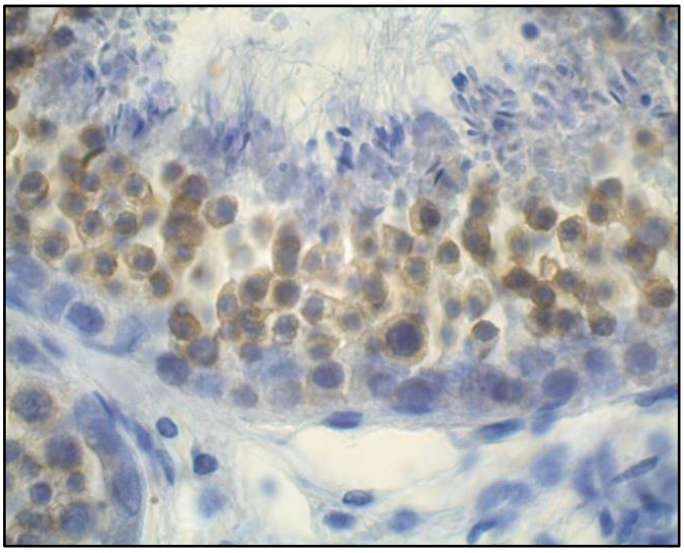


Figure 11: Percentage of total seminiferous tubules containing different phenotypes and germ cell types in group 4 (21 to 24 months).

A.



B.



C.

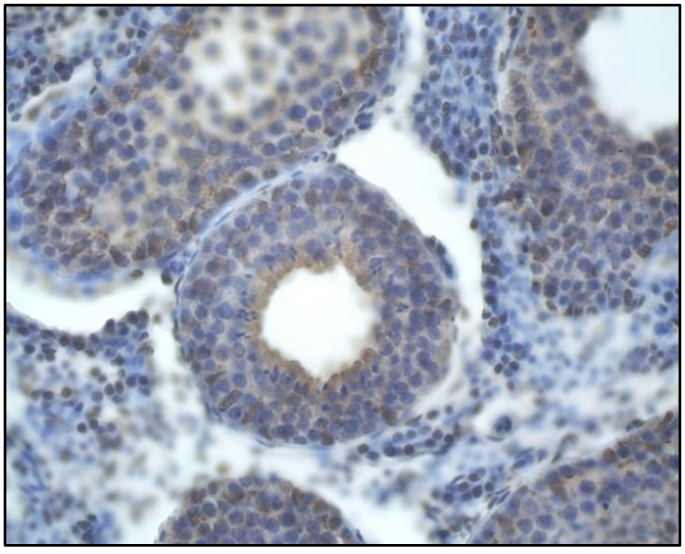


Figure 12: Cross-section of testis tissue, A. interstitial space shows mature Leydig cells and macrophages (hematoxylin), B. Germ cells stained with DDX-4, C. Sertoli cells stained with GATA-4.

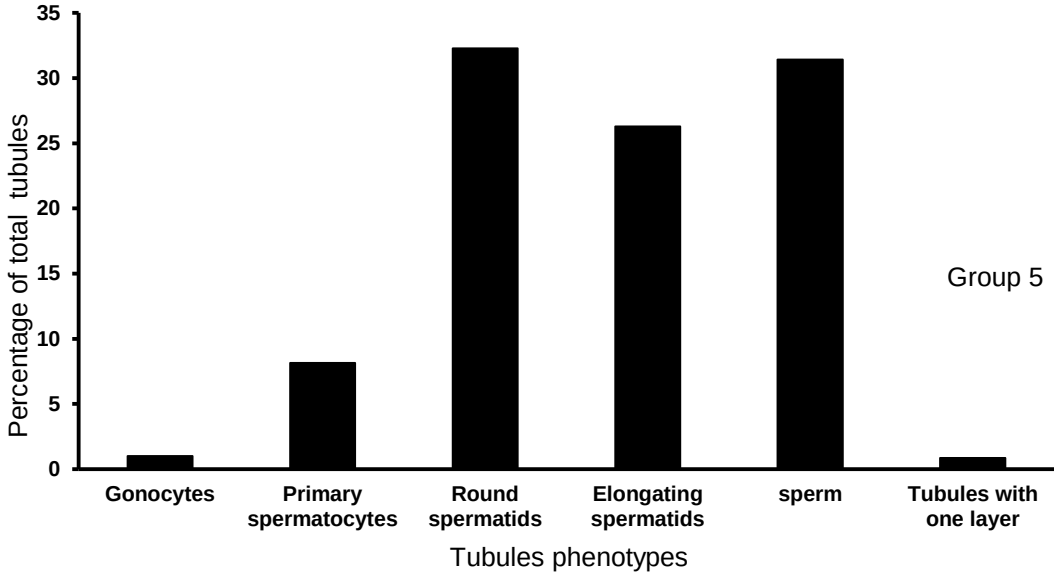
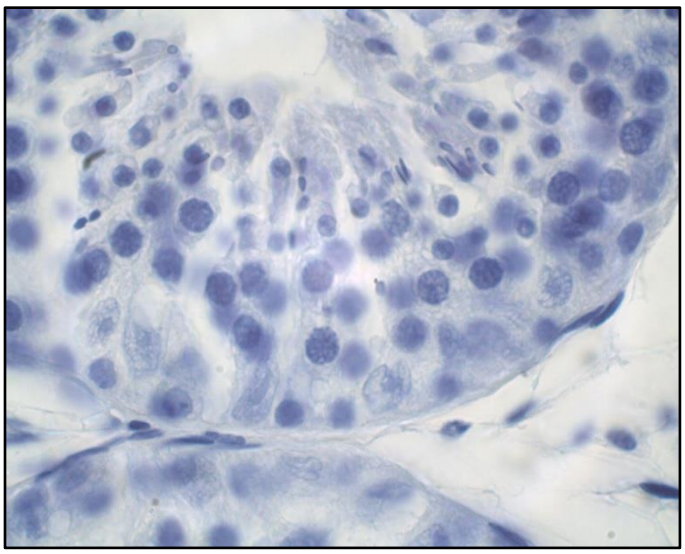


Figure 13: Percentage of total seminiferous tubules containing different phenotypes and germ cell types in group 5 (25 months and older).

A.



B.

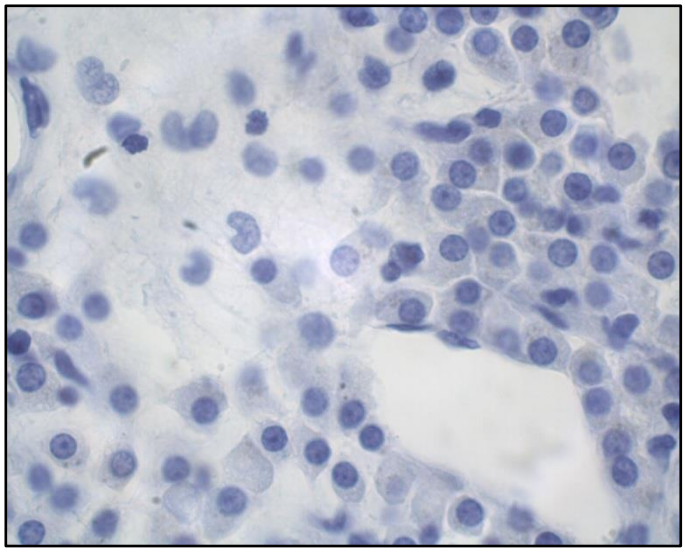
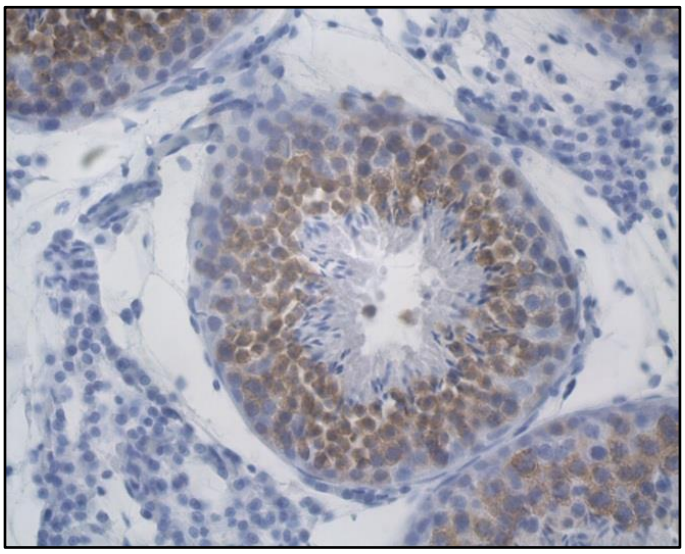


Figure 14: Cross-section of testis tissue, A. Tubules with differentiated germ cells and Sertoli cells, B. interstitial space shows mature Leydig cells and macrophages (hematoxylin).

A. 

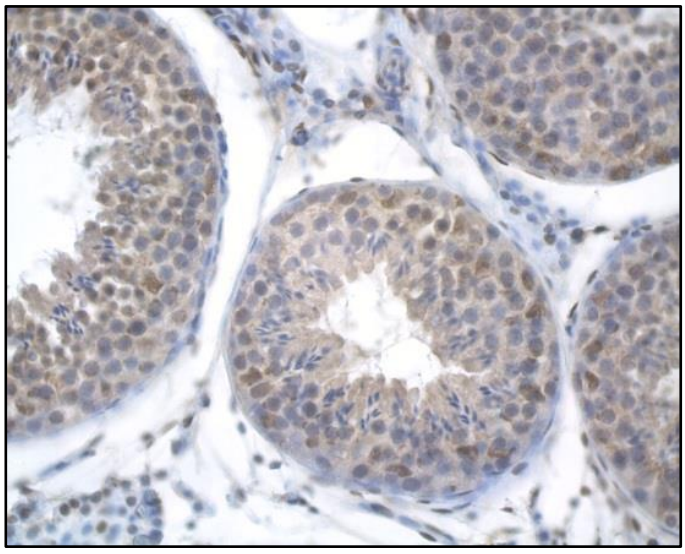
B. 

Figure 15: Cross-section of testis tissue, A. Germ cells stained with DDX-4, B. Sertoli cells stained with GATA-4.

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CHAPTER FOUR

Establishment of Spermatogenesis in Alpaca Testis Tissue Xenografted onto Nude Mice is Age Dependent*

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Keywords: Alpaca, Testis, Xenografting, Nude mice

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* Theriogenology journal guideline for authors has been used in this manuscript

Abstract

Testicular development in camelids is remarkably variable between individuals. Multiple factors can contribute to testicular dysgenesis including improper germ cell differentiation, disrupted rete testis and genetic factors. Ectopic testis tissue xenografting could prove to be an effective model to investigate testis development in alpacas. The objective of this study was to evaluate development of testicular tissue from pre-pubertal alpacas xenografted onto castrated nude mice. We hypothesized that testicular tissue from 8-month-old alpaca would result in the most effective establishment of spermatogenesis follow grafting due to the presence of only undifferentiated spermatogonia and mature Leydig cells at this age. Testis tissue from 17 alpacas at 6, 7, 8, 9, and 10 months were xenografted onto nude mice. Each recipient mouse had four testis explants (5-10 mg) grafted onto the back and grafts were harvested 24 weeks later. Analysis consisted of evaluation of recipient mouse serum testosterone, testis graft weights and histological examination of grafts. Serum testosterone concentration was highest (1.1 ng/mL, SD±1.4) ($P = 0.05$) in recipient mice grafted with 8-month-old donor tissue and the lowest (0.2ng/mL, SD±0.1) in recipients with 6-month-old donor tissue. The vesicular gland weights were largest (126.5 mg, SD±185.1) in mice grafted with 7-month-old donor tissue. Graft survival and collection ranged from 79.1% to 83.3% and functional tubules that contained active differentiated germ cells ranged from 75.8% to 90.5%. The only significant difference ($P = 0.05$) was detected in graft weights in mice grafted with 8 month old and 10 month old testis tissue. The percentage of seminiferous tubules in grafts with elongating spermatids was 6.8, 4.3, 1.1, 1.1 and 1.7% from donor alpacas aged 6, 7, 8, 9 and10 months respectively. Additionally, 6-month-old donor grafts showed the greatest overall percentage (100%) of grafts that produced elongated spermatids. In

contrast, grafts from 9-month-old donors contained highest percentage (19.4%) of degenerated seminiferous tubules. Survival rate and the percentage of seminiferous tubules with differentiating germ cells was highest in 8-month-old donor grafts. Xenografting of alpaca testes produced testosterone and successfully supported maturation of pre-pubertal testes to produce elongated spermatids. Thus, this approach can be used as a model to study disrupted spermatogenesis in camelidae.

1. Introduction

The alpaca, *vicugna pacos*, is a domesticated species of camelidae family that originate in South America. Unfortunately, male alpaca possess several reproductive unfavorable characteristics including late puberty and sexual maturity [1], slower testicular growth [2], higher incidence of testes abnormalities [3], low sperm concentration [4], and poor fertility with low pregnancy rate between 40 and 60% [5]. Since the puberty age is variable and the factors regulating the pattern of puberty and sexual maturation are controversial issues in this species, investigation of endocrine and cellular maturation has increased. Thus the process of sperm production in terms of the cells that appear in particular seminiferous tubules and regulation of sperm production in terms of genes that are expressed by germ and somatic cells during spermatogenesis in general remains poorly studied. The limited information from the literature on alpaca puberty focuses on changes in circulating testosterone levels and changes in testicular size with age as indicator of sexual maturation pattern. No studies have investigated in detail the cellular events occurring in the seminiferous tubules during and around the time of puberty. Based on the available information about camelid reproductive development in males, it is apparent they reach puberty later in life than other large mammals. However, we do not know the time course of germ cell differentiation in male camelids. This represents a gap in our knowledge base and a barrier for our ability to develop reproductive technology in these animals. Thus, there is a need to investigate all aspects of male reproductive development in camelids leading to increased knowledge and the development of techniques and therapeutic approaches to treat infertility in camelids.

In the last few years several alternative procedures have been used to study testes development and spermatogenesis. In 1994, Brinster and Avarbock used a germ cell transplantation technolo-

gy in rodents to study spermatogonial stem cell biology [6]. Other approaches and technique to study germ cell development focus on maintaining the germ-somatic cell association wwithout removing them from their surrounding tissue. Testis grafting is a useful approach to study effects of drugs and toxins on the developmental stages of spermatogenesis without the need of using the expensive experimentation. In this approach, small pieces of testicular tissue (0.5-1mm) from various species can be transplanted onto the back of castrated immunodeficient nude mice. Testis grafts survive and produce sperm capable of fertilization at the time of collection and evaluation [7]. Investigation of testis development with the use of grafting demonstrated that testicular tissue from calves at different immature ages grew, differentiated, and produced testosterone and elongating spermatids regardless of grafting period [8]. In castrated mice where no testosterone is released, Leydig cells of testicular grafted tissue respond to elevated FSH and LH serum concentrations to regulate testicular somatic cell maturation and secrete testosterone [7]. The castrated mice rely on functional grafts for the androgens that can be biologically detected by the maintenance of seminal vesicles weight compared to regression that occurs in castrated mice [7]. For many species including pigs [7] and bulls [9], testicle grafts from immature animals transplanted into castrated mice are able to respond to mice gonadotropins and grow to support spermatogenesis indicated by producing spermatozoa and androgens [10]. In addition, it simulates the endocrinology of pre-pubertal animals providing the optimal environment for testis development. Since first reported, testis tissue xenografting has been used in numerous species to investigate germ cell differentiation and the formation of the spermatogonial stem cell population. Xenografting of testicular tissue from mature males of several species such as pigs and goats containing post-meiotic germ cells results in degeneration of these grafts due to either ischemia or inability to induce angiogenesis [11, 12].

Spermatogenesis is a complex process requiring highly dynamic interaction between germ cells and supporting somatic cells [13]. Testis tissue grafting provides many options: preserving genetic material from males when sperm collection is not possible; production of fertile sperm from sexually immature males for instance for pig and goat [7]; and studying the effect of toxins and drugs on testicle tissue without the need for extensive experimentation in the donor species [14]. Transplantation of alpaca testicular tissue onto nude mice was one of the objectives to be investigated at the level of cross-species compatibility. Rodriguez-Sosa and Dobrinski, [10] reported that spermatogenesis process will arrest in some stage with no differentiation beyond the spermatogonial stage when only germ cells from donors of larger species are transplanted into mouse testes. This is due to an incompatibility between the donor germ cells and the microenvironment of the mouse testis. They concluded that transplantation of germ cells with the surrounding somatic compartment onto the back of mice, either by transplanting a piece of testicular tissue or a combination of isolated testicular somatic cells and germ cells will result in production of elongated spermatids. Therefore surrounding somatic compartment seems to be necessary for germ cells to proliferate and differentiate. The second objective of this study was to determine the age of donor that is suitable to ectopic xenografting and which age can be supported by the mice to potentially accelerate the testicular tissue development. Studies have reported that xenografting of testicular tissue from mature males of several species such as pig and goat contained post-meiotic germ cells resulted in degeneration of these grafts due to either the ischemia or inability to induce angiogenesis [11, 15]. It is well known that cryoprotectant agents can cause some damage to testis tissue during the freezing and thawing process. The revascularization of the frozen-thawed testis tissue after grafting has reported to take longer time than with non-frozen tissues which means there will be a delay in spermatogenesis process or tissue death Jahnukainen and

colleagues [16] reported that the cellular testis development is delayed in grafted testis tissue after cryopreservation in rhesus monkeys. We expect to see more cell stages delay and tissue degeneration in frozen grafts than in fresh ones. Grafted tissue is very sensitive to the new environment under the skin of the nude mice, therefore tissues from immature animal might have a greater likelihood to survive and build vasculature than the mature tissues. Based on our preliminary histological data, we hypothesized that the grafted testis tissues will show normal developmental spermatogenesis and produce elongating spermatids and the critical age of testis development in alpaca is between 6 and 7 months. In addition we hypothesized that grafting tissue from animals at these ages will result in complete spermatogenesis after the transplant onto the back of nude mice.

2. Materials and Methods

2.1. Donor alpacas and recipient nude mice

Seventeen male alpacas were included in this study and divided into five groups according to age: Group1 (n=3, 6 months), group 2 (n=4, 7 months), group 3 (n=3, 8 months), group 4 (n=3, 9 months), and group 5 (n=4, 10 months). Adult immunodeficient intact male mice (NCR nu/nu CrTacNCR-Foxn1) were used (8 mice/age group) as recipients. In addition, five mice were grafted with testes from 14-day old pigs as a reference for this study. Mice were housed in filter-cover cages and provided food and water ad libitum. All animal procedures were approved by Washington State University Animal Care and Use Committee.

2.2. Castration, control and xenografts testis tissues preparation

Testicular tissues were collected following standard bilateral castration procedures[17]. Immediately before castration, all males were examined ultrasonography for any testicular abnormalities. Animals were anesthetized using an intramuscular treatment combination of xylazine (0.4 mg/kg) AnaSed ® 100 mg/mL, 0.4 mg/kg IM,Lloyd, Shenandoah, Iowa), ketamine hydrochloride (4 mg/kg; Ketaset ® 100mg/mL, Fort Dodge Animal Health, Fort Dodge, Iowa),and butorphanol tartrate (0.04 mg/kg IM, Torbugesic® 10 mg/mL, Fort Dodge Animal Health, Fort Dodge, Iowa). Males were placed in Lateral recumbency with the right side up. Following castration, pieces of testicular parenchyma tissues were collected from the first testis of each animal, as a reference for pre-grafting control, and were fixed in 4% paraformaldehyde overnightand stored in 70% ethanol. The second testis from each animal was immediately placed in Hanks balanced salt solution (HBSS) on ice and processed by removing the so theparenchymal tissue could be cut into small pieces (5-10 mg). The tissue was washed twice in Dulbecco's Modified Eagle's Medium (DMEM) for five minutes and maintained in fresh DMEM on ice for fresh transplantation. In addition to fresh grafts, frozen graft pieces were also prepared to assess the development of cryopreserved tissue. About 15 pieces of testis tissue were transferred into freezing medium, DMEM, fetal bovine serum (FBS), and dimethyl sulfoxide (DMSO) at ratio of 8:1:1. Tissues in1.0 mL freezing medium were held on ice for 20 min until moved to -20ºC for one hour. Afterward, vials were transferred and maintained at -80ºC until the time of transplanting. The tissues were thawed by placement in a 37°C water bath for 90 sec. Two milliliters of DMEM was added to each vial for 5 min and then testis tissue grafts were washed twice in 2-mL fresh DMEM for 5 min each to remove the cryoprotectant [18].

2.3. Testis tissue xenografting procedures

Recipient immunodeficient mice were anesthetized by intraperitoneal injection with a mixture of 0.1 mg/kg body weight ketamine (Ketaset®, ketamine HCL inj. USP. 100 mg/mL. Fort Dodge Animal Health. Fort Dodge, Iowa. USA) and 0.05 mg/kg body weight xylazine (Tranquived®, xylazine sterile solution. 20 mg/mL. vedco inc. st. Joseph, MO. USA) in sterile physiological saline. Mice were castrated at the same time just before grafting using an abdominal midline approach. On the back of the animal, four small incisions, two on either side, were made along the vertebral column according to the technique described by Honaramooz and co-workers [7]. The muscle was scored in individual areas; four pieces of previous prepared grafts were transplanted individually into subcutaneous pouches on each recipient mouse. The grafts remained onto the recipients for 24 weeks.

2.4. Recipient mouse parameters

At 24 weeks post-grafting, the mice were humanely scarified by CO_2 inhalation. Blood was collected by cardiac puncture and subsequently serum was obtained by centrifugation to evaluate testosterone concentration (ng/mL) in response to graft development. Concentration of testosterone was determined in all samples collected; using a commercial double-antibody Radioimmunoassay (RIA) kit for this analysis (Coat-A-Count® total testosterone; Siemens Healthcare Diagnostics Inc. Los Angeles, CA 90045 USA).The sensitivity of the assay for testosterone was 0.1 ng/mL. Vesicular glands were also removed and weighed (mg) to assess the effect of testosterone on these glands as an indicator of the level of bioactive androgens released by grafts.

2.5. Graft harvesting and preparation

The visible xenografts were localized and evaluated in their site for their size and appearance, removed, weighed, fixed in 4% paraformaldehyde overnight, and stored in 70% ethanol. Thereafter, the tissues were dehydrated in alcohol, embedded in paraffin, sectioned at 8μm and placed on glass slides. Slides were deparaffinized in histoclear, rehydrated in serial ethanol concentrations, and stained with hematoxylin to evaluate germ and somatic cells using light microscope and digital images captured with a Leica digital camera. Analysis of cross-sections included assessment of the average seminiferous tubule diameter, the diameters (μm) of large ten round seminiferous tubules were measured at 10X magnification using an eyepiece micrometer and averaged. Sections were also were evaluated to determine the percentage of seminiferous tubule cross sections with spermatogonia, meiotic germ cells, elongating spermatids, or no germ cells. Spermatogonia were identified by the presence of condensed nuclei and cellular basal location within the seminiferous tubule. Meiotic germ cells were identified by the presence of diffuse nuclear staining. Elongating spermatids were identified by the presence of highly condensed elongating nuclei. Interstitial space was evaluated for the immature and mature Leydig cells according to their morphology. All slides were evaluated by a single operator and in a random order.

3. Statistical analysis

Two analyses were conducted; the first analysis determined the difference between testis tissue grafts from different donor ages removed from recipient mice at 24 weeks and the same tissue from the donor males. The second analysis, data were pooled for donor age to determine if any overall difference existed between means. Student's t-test and ANOVA for significance were used to determine the differences between means for testis graft weight, the percentage of semi-

niferous tubule cross sections with germ cell types per testis graft per mouse, mouse vesicular gland weight, and mouse serum testosterone concentration.

4. Results

This study was conducted to histologically examine the grafted tissue to investigate if donor age had any effect on the ability of germ cells to differentiate and establish the spermatogenesis.

4.1. Donor testis tissue pre-xenografting histological analysis

Histological examination of donor testicular tissue at the time of grafting was conducted to evaluate differences in germ cell populations between donor ages. The ability of germ cell differentiation to commence and the extent of differentiation that occurs following grafting may be influenced by the stage of germ cell development at the time of grafting. However, the percentage of tubules with gonocyte migration to the basement membrane was non-significantly higher (80.0%) in 9 month testicular tissue compared with to testis tissue from 10 month old animals (70.0%). The majority of cords at 10 months of age had gonocytes still present in the center of the cords. Transition of gonocytes to spermatogonia was not evident for all ages and seminiferous tubules with lumen ranged from 0.67% to 31.6%. Histological results of pre-grafting testicular tissue are summarized in Table 1.

At 6 months of age the mean size of seminiferous cords was the smallest (60.0 μm) of all groups with 27.7 gonocytes in the center and only 0.67% of these cords had a defined lumen. Sertoli cells were also counted to assess the potential support and development of gonocytes. Cords that contained gonocytes in the center as primary cell type was 76.6, 73.7, 75.0, 80.0, and 70%, in 6,

7, 8, 9, and 10 month old males respectively. Few gonocytes (pre-spermatogonia) had migrated to the basement membrane as determined as one gonocyte touching the membrane. The beginning of the migration phase of gonocytes to the basement membrane is one indication of the cellular initiation of germ cell differentiation. The basal lamina was very thick and surrounding cell types present at this stage were immature Sertoli cells and gonocytes. The interstitial space contained few adult Leydig cells, 1.97 cells average/ field in 6-month-old animals. Examination of testicular tissue from 10 months of age showed a slight increase in seminiferous tubule diameter (63.5 μm) and the beginning of formation of a lumen in some samples. Statistically there was no significant difference in tubule cross section diameter between donor ages. Most of the gonocytes had relocated to the periphery of the seminiferous cords. Sertoli cells at this age are more evenly distributed and their nuclei located near the basal lamina (Figure 1).

4.2. Recipient mouse response to grafts (seminal vesicle weight and testosterone analysis)

Recipient mouse seminal vesicular gland weights were measured to evaluate testosterone produced by Leydig cells (Figure 2). The gland weights were greatest (126.5 mg) in mice grafted with 7-month-old donor tissue which indicates that the testosterone produced by grafts was correlated with seminal vesicle size. The lowest weight (18.3 mg) was reported in glands collected from mice grafted with 6-month-old testis tissue. Growth was noticed in vesicles from mice that had well developed grafts compared with vesicles from mice that had degenerated grafts. This result means that the Leydig cells in donor grafts are functional and produced testosterone. Testosterone production was confirmed by RIA analysis of the recipient mice blood. In control groups of nude mice, the average mass of the seminal vesicles in intact mice was 280-314mg (n = 3) in intact mice and in castrated mice the seminal vesicles were regressed, and difficult to lo-

cate and measure (n = 5). Additionally grafts removed from recipient mice of 7 month testis tissue had larger vesicular glands than mice with 6 month testis grafts. Overall, analysis of recipient seminal vesicle weights indicated that testosterone production by grafted alpacas testis tissue strongly correlated (r = 0.92) with seminal vesicle size (Figure 3).

No significant difference (P =0.05) was observed in vesicular gland weights in mice that were grafted with different aged donor testis tissue during the specific grafting periods even though 8-month-old donors had largest weight (126.5 mg) compared to 6-month-old donors (18.3 mg). Castrated recipient mouse serum testosterone concentration was measured to evaluate Leydig cells differentiation and function in grafted alpaca testicular tissues and their response to recipient mouse LH. This observation revealed that Leydig cell maturation pattern was based on their status before grafting. This observation revealed that mature Leydig cell number before grafting was correlated with testosterone concentration in recipient mouse serum (r = 0.52). Recipient mouse serum testosterone concentration was highest for 8 month's testis tissue (1.1 ng/mL) grafts but was not significantly different from any other ages. These findings indicate that all alpaca testis xenografts produced functional bioactive testosterone. Serum from control male mice 2 days after castration had a testosterone concentration below the low standard of the assay (0.92 ng/mL).

4.3. Mouse bodyweight, graft growth and gross morphology, graft recovery rate, and graft weight analysis

The response of alpaca testis tissue hormones produced by recipient mice was investigated following xenografting by assessing the survival status of alpaca grafted testis tissue.

Mice body weights were measured to determine if recipient mouse weight had an effect on alpaca testis tissue graft growth and final weight. Testis graft weights were between 25 to 36 mg with no significant difference based on recipient mouse size. Growth of donor testicular tissue under the skin of the recipient mice was visible and could be visually identified in most sites on recipients during the grafting period. The testicular tissues created a vascular, capsulated area under the skin andalthough different in sizes tissue were straightforward collect at the time of dissection (Figure 4A.).

Graft recovery ranged between 79.1% in 7-month-old donor and 85.0% in 8-month-old donor. There was no significant difference in the percentage of grafts recovered or functional status between the donor ages. The weights of all grafts were measured individually at time of harvest to compare the relationship of growth rate to the age of testis tissue donor. At the time of grafting, the weight of the testis tissue fragments was approximately 5 mg. Although some recovered tissue grafts appeared slightly smaller than when they were implanted, these grafts were also collected and processed for histological evaluation (Figure 4B).

Analysis of testis graft weights at removal indicated that growth potential variation did not depend on donor age. The weight of most of the grafts was 2 to 4 fold greater than the weight at time of grafting. Of the grafts recovered from the 31 recipient mice, 85.2% were functional. Average weight of grafts from 8 month-old donor alpaca was significantly larger than 10 month donors with an adjusted R-square of 31.1%, but not significantly different to other age donors (Figure 5).

4.4. Grafts histology

Grafts were prepared for histological examination and stained by hematoxylin. seminiferous tubules contain meiotic germ cells have been seen in all ages, 13.5, 9.2, 2.6, 2.5, and 5.8% in 6, 7, 8, 9, and 10 month old donor respectively.

4.4.1. Tubular diameter and density

Average seminiferous tubule diameter was significantly larger in 6 and 7 month donor testis tissue compared to 10 month old donors (P = 0.05). There was no significant difference in seminiferous tubule cross section diameter between other donor ages. Ten month donor grafts had the smallest (130.9 µm) seminiferous tubule cross section diameters among other donor ages (*P* = 0.05). In contrast, overall, the average diameter of seminiferous tubules in recovered grafts had no relationship (*r* = -0.08) with testosterone level (Figure 6).

4.4.2. Tubules morphology

Seminiferous tubule cross sections were evaluated to determine the germ cell differentiation stage. Advanced stages of spermatogenesis containing meiotic germ cells and elongated spermatids were present within the grafts collected from all donor ages. The stages of germ cell differentiation in donor testis tissue following grafting varied even within the same age group. The average percentage of tubules containing meiotic cells in 8 month donor grafts was 87.9%, whereas, meiotic cells were present in an average range of 43.5% of the tubules in grafts from 6 month donor testes. Grafts from 8 month old donor contained the highest percentage, of tubules with meiotic germ cells than grafts from 6 month donors that contained lowest percentage but difference were not statistically significant. Germ cell differentiation had progressed through meiosis as evidenced by the presence of elongating spermatids in grafts. The average percentage of sem-

iniferous tubules in grafts with elongating spermatids was 6.7, 4.2, 1.0, 1.1 and 1.6% from donor alpacas aged 6,7,8,9, and 10 months, respectively (Figure 7). Maturation of germ cells to meiosis status and elongating spermatids were seen in grafts from all donor ages (Figure 8). Seminiferous tubules with abnormalities were also observed including one layer of cells only (spermatogonia and/or Sertoli) have been obseved in grafts from all different donor ages (Figure 10). The grafts recovered from 6 month old donors had the highest percentage of one layer cells only tubules (12.8%) but this value was not significantly different from other ages.

Tubules from 8-month-old donors contained the highest percentage (90.5%) of functional tubules that contain germ cells that could be morphologically identified. Germ cell developmental stage at the time of grafting has been discussed to influence the differentiation of germ cells following grafting. In this study, 6 month donor tissue grafted for 24weeks had the poorest germ cell differentiation. These grafts had the lowest percentages (57.0 %) of seminiferous tubule cross sections containing meiotic germ cells. In all ages except 6 month, recovered grafts had 1.6, 4.5, 19.5, 2.9 % degenerated tubules mainly in the center of the grafts (Figure 11).

Defect in factors contributing to proliferation or maturation of both Sertoli and germ cells may have caused this phenotype. Analysis of testis tissue grafts from these donors in this study indicated that germ cell differentiation did not accelerate in tissue from donors at these ages because elongating spermatids were present in testis grafts of 6 month after 6 month grafting period as well as in 10 months testis grafts. This correlates to a first normal appearance of elongating spermatids observed in alpaca testis at 12-13 months of age. These results revealed that alpaca's testis tissue survived on the back of nude mice and are responsive to its gonadotropin hormones.

5. Discussion

Ectopic testicular xenografting is a method that has the potential for use in investigating spermatogenesis in non-rodent mammalian species when experimentation in the target species is difficult or expensive. Since the onset of puberty and testicular development in the alpaca takes several months, evaluation of spermatogenesis establishment in ectopically xenografted testicular tissue from prepubertal donor alpacas in the current experiment was designed to include a range of ages before the expected age of puberty. During the prepubertal phase of testicular development, somatic, Sertoli and Leydig cells undergo proliferation and differentiation. However, the developmental stage of both the somatic and germ cells during the prepubertal period could have an impact on the establishment of spermatogenesis in grafted testicular tissue. To increase the efficiency of sperm production in grafted testicular tissue, it is important to gain an understanding of changes in testis cell differentiation throughout the early postnatal period of testicular development. Evaluation of recipient mouse serum testosterone level, vesicular gland weights, and histological examination of grafts after harvest will provide a clear understanding of differences in growth of different donor alpaca ages testicular tissue after 24 weeks grafting period.
Leydig cell proliferation and function can be evaluated by measuring testosterone concentration in mouse blood to confirm that recipient mouse gonadotropins stimulate Leydig cells proliferation and steroidogenic activity. In addition, seminal vesicle mass recovered from the host mice can be used as indicator of testosterone concentration that produced by grafts. Considerably low concentrations of testosterone (0.2 to 1.1 ng/mL) were observed in serum samples collected from all nude recipient mice included in this study compared to control uncastrated mice and in other donor species. However, all grafts collected had the ability to produce testosterone that was

greater than 0.1 ng/mL. The low concentration of circulating testosterone could be attributed to an inadequate release of testosterone by alpaca xenografts as a consequence of low number of mature Leydig cells or could be due to low sensitivity of Leydig cells in these grafts to mice gonadotropins since testosterone is produced by Leydig cells in response to LH. Interestingly, no significant difference has been noticed in the ability of Leydig cells to produce testosterone between all donor ages in current experiment. This data suggests the number of both immature and mature Leydig cell populations in pre-grafting testis tissue in different ages had no effect on the testosterone production after 24 weeks grafting period. Serum testosterone concentration in the recipient mice carrying 8 month alpaca testis tissues was found to produce the highest level of testosterone (1.1 ng/mL) among the ages. Eight month donors had the highest mature Leydig cells percentage 27.3 % which suggests that these cells were respsonsive to the high LH concentration in recipient mice and caused initiation of Leydig cells function. The lowest recipient testosterone concentration (0.2 ng/mL) was found in 6 month old alpaca testis xenografts. One possible reason can be the low (3.0%) mature Leydig cells population in donor testis tissue and inability of the immature Leydig cells to initially respond to LH. In spite of low testosterone concentration in general, presence of post-meiotic germ cells in the grafts would be a result of an adequate intratesticular physiological testosterone concentration to maintain germ cells differentiation. Our data shows that serum testosterone concentrations are variable in recipient mice which could be explained by the pulsatile nature of testosterone secretion. Testosterone production from xenografts was also indirectly evaluated by seminal vesicle weight since they are highly dependent on testosterone for their development [19]. The weight of seminal vesicles from transplanted mice was in general lower than those from control non-castrated mice. The highest seminal vesicle weights in recipient mice were observed in animals with 8 month old alpaca tes-

tis tissue. This confirms the steroidogenic activity of Leydig cell from transplants from this age group. In contrast, seminal vesicles were atrophied in mice with no graft development. No significant differences were found between vesicle weights in different donor ages.

Testis tissue grafts survival and growth was principally examined by measuring the weight of all collected tissues after transplantation. Graft difference in weight could be attributed initially to the ability of Sertoli cells to respond to recipient mouse pituitary-released gonadotropins. In order for these testicular tissues to respond and grow, vascularization must occur around and in the newly grafted tissues to provide nutrients for survival and function. Castrated rodents have FSH level five times higher than controls [19]. Therefore, Sertoli cell proliferation and differentiation following grafting could be a result of FSH receptor expression, blood flow enriched with FSH to these cell or other factors. Grafts survival and collection ranged from 79.1% to 85%. The largest weight (16.6 mg, a threefold increase from the weight of the starting tissue) was observed in 9-month donor and second largest weight (15.8 mg) was in 8-moth donor tissue may be due to high number of immature Sertoli cells that were responsive to high recipient FSH. However, the grafts weights of 10-month-old donor were the lowest (6.58 mg). These data suggest Sertoli cells in alpaca testes complete maturation during 9-10 months of age since the growth of grafts declines with these donors. Although graft weights were numerically different between groups, , no statistically significant differences were determined.

In spite of using nude recipient mice that at the same age, atrophied testis tissue grafts were, in few cases, observed on the same single recipient mouse along with the large functional grafts which ruled out the effect of recipient mice endocrine environments on grafts difference development.

All collected grafts were processed for histological analysis and each one was compared to its testicular tissue before grafting. During testis development, gonocytes have to migrate from the center of seminiferous cords to the basement membrane to establish the niche preparing for differentiation to spermatogonia and proceeding spermatogenesis [20]. Pre-grafting donor testis tissues had over 70% of tubules with gonocyte migration with highest (80%) in 9-month old donor tissue. Since begin and end of gonocyte migration in alpaca is not clear, degeneration of these cords after grafting could be one potential reason for the inability of niche assembly. Assessment of Leydig cell development in donor testis tissue showed that 8 month old donors had highest mature Leydig cells percentage 27.3% whereas only 3.0% of mature Leydig cells observed in 6 month old donor alpaca. Low mature Leydig cells means low cells sensitive to recipient LH leads to low seminal gland and tubule epithelium proliferation.

Proliferation and differentiation of germ and Sertoli cells after grafting and changes in morphology were determined by seminiferous tubule diameter. Tubule diameter and number were 193.8 µm and 72.7 in 6 months and were 134.9 µm and 120.5 in 9 month old grafts respectively. Interestingly, no significant difference was observed between all examined grafts. Large tubules were found at the periphery area of the graft in most of the grafts with more organized advanced germ cell differentiation which is understandable because this area exposed to blood supply early. Inadequate blood vessel distribution in the graft resulted in differences in tubules diameterSmall tubules with no lumen or even degenerated tubules in the center of the graft were present in most grafts Although small or degenerated tubules were present in grafts, progression of germ cells to meiotic stages was obvious in all grafts from all donor ages. In the alpaca, the time frame of germ cell differentiation is not well established. However, no significant differences were detected between higher 87.9% and lower 43.5% average percentages of total tubules containing mei-

otic germ cells found in 8 and 6 month old donors respectively. Possible cause for this high percentage could be related to stage of Sertoli and germ cells development before grafting. In 8 month old donors the maximal FSH concentrations provided by castrated recipient gonadotropin stimulated the growth of these grafts or could be due to the meiotic arrest since the percentage of tubules containing stages of post-meiotic germ cells (round and elongating spermatids) was the lowest (2.6%) among other ages included in this study. In contrast, seminiferous tubules with post-meiotic germ cells were the highest (12.5%) in 6 month old donor with 6.76% for round and 6.7% for elongating spermatids. This finding suggests that germ cells were able to progress to more advanced stages than pachytene spermatocytes in this age.

Honaramooz and coworkers [7], reported that porcine testis xenografts can reach efficiency to produce fertilizable sperm similar to that produced by intact males relative to per gram tissue basis. In bull for instance, only 10.9% of the seminiferous tubules of grafts show elongated spermatids in bull [9], suggesting inefficient spermatogenesis. Since the outcome of porcine testis tissue xenografting is well established, piglet testes grafts were used as a control in the present experiment. Testis tissues from pigs have been grafted during the experiment and with the same protocol to ensure the efficiency of the protocol. Pig grafts that were collected and examined after 20 months had tubules with mature spermatozoa (Figure 12). Average testosterone concentration was 0.21 ng/mL.

Interestingly, production of elongating spermatids in the 6 month alpaca testis tissue grafts was higher (6.76%) than that of 10 month donors (1.65%) despite the fact of the same grafting period (24 weeks). In the intact alpaca, appearance of mature sperm in the seminiferous tubules lumen has been reported to occur as early as 10-12 months old [21] and in our histology experiment around 13 months (data not shown). Absence of observed mature sperm in 10-month-old donor

alpaca grafts after 24weeks grafting period may suggest that a comparable delay or lack of factors responsible for sperm morphogenesis occurred after the formation of elongated spermatids. Allowing 6 month donor testicular grafts more time on recipient mice could provide the opportunity to study a production of mature sperm in grafts. Overall, the appearance of elongating spermatids clearly existed for all grafts regardless of donor ages.

Differentiation of germ cells seems to be sensitive to the hypoxic damage associated with testicular tissue collection, preparation, and grafting procedure. However, neither pre-grafting stages of germ cell development nor recipient host endocrine environment has any effect on tubule developmental pattern. Degeneration and unclassified tubules phenomenon was 19.5%, 4.3% in 9 months old and 0.0%, 30.1% in 6 months respectively. This phenomenon is probably due to ischemia around and in grafting vesicular tissue [15] that reduced the ability of germ cells to survive. Some alpaca testis xenografts fail to survive despite of presence grafts supporting germ cell differentiation on the same individual nude mouse. The appearance of seminiferous tubules containing one cell layer, either Sertoli or Sertoli and germ cell phenotype was evident in the present study for all testicular grafts, with slightly higher variation between different donor ages. The highest (12.8%) and lowest (0.77%) percentages of these tubules in 6 and 8 months, respectively could be attributed to few gonocytes with the ability to migrate and attach to the basement membrane resulting in Sertoli cells only tubules. On other hand inability of Sertoli cell proliferation and maturation could led to disruption in supporting germ cell normal differential function resulting in tubules with few Sertoli and no germ cells. In intact testis tissue from various mammalian species, the percentage of tubules with Sertoli cell only phenotype decreases rapidly when post-meiotic germ cells appear, and the percentage of tubules with round and elongated spermatids increases. These changes were less definite in the grafts. Abnormally large tubules

were reported in bovine [22] and in mouse [12] xenografts in normal growing grafts. Schlatt in 2003 [12] explained this phenomenon by an accumulation of fluid produced by the Sertoli cells in absence of efferent duct tissue. In this study, tubules diameter ranged between 130.9 to 193.8 µm and evident diameter in age-matched control testes was 124 to 152 µm which can be a result of fluid accumulation as explained earlier.

In conclusion, the benefit of using testicular tissue from prepubertal donors was because of the presence of only pre-spermatogonia population in seminiferous cords which is enriched with spermatogonial stem cells and grafting this tissue will be a credible method to understand the establishment of spermatogenesis in different species. For a superior knowledge of this technique in alpaca, testicular tissue from five different pre-puberty ages were ectopically xenografted under the skin on the backs of castrated adult nude mice for 24 months period time to take full advantage of and to mimic the appearance of seminiferous tubules with elongating spermatids in intact males. The percentage of seminiferous tubule cross sections with elongating spermatids were observed in all grafts. Statistical analysis indicates that no significant difference in grafts from all five age groups, 6 month old origin was the best for grafting based on the most advanced germ cell stages that differentiated into round and elongated spermatids in 24-weeks period time. Remarkably, no significant differences have been observed in grafts from 6 month age group in term of the onset of meiosis compared with the intact age-matched males. Ectopic alpaca testis xenografting is a promising technique for the study of testicular physiology and pathophysiology in alpaca.

Acknowledgments

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Age (months)	Tubules diameter, µm (±SD)	Tubules with lumen % (±SD)	Mature Leydig cells % (±SD)	Gonocytes Migration % (±SD)	Mature Leydig cells num-ber (±SD)	Gonocytes per cord (±SD)	Sertoli cells per cord (±SD)
6	60.00 (±13.2)	0.67 (±1.2)	3.00 (±1.0)	76.67 (±7.6)	1.97 (±0.9)	27.70 (±10.4)	31.37 (±4.9)
7	65.00 (±5.8)	26.75 (±42.5)	23.00 (±22.6)	73.75 (±7.5)	9.63 (±7.8)	24.40 (±9.6)	27.80 (±5.8)
8	77.17 (±12.1)	31.67 (±54.8)	27.33 (±21.6)	75.00 (±26.0)	7.87 (±4.7)	32.57 (±3.8)	32.47 (±2.4)
9	63.00 (±11.1)	1.33 (±2.3)	5.33 (±4.2)	80.00 (±5.0)	7.07 (±5.3)	21.67 (±11.2)	31.40 (±2.4)
10	63.50 (±10.3)	2.75 (±3.0)	18.75 (±27.5)	70.00 (±25.5)	11.33 (±4.4)	20.95 (±9.2)	28.33 (±5.4)

Table 1: Histological results of pre-grafting (donor) testicular tissue in five different ages.

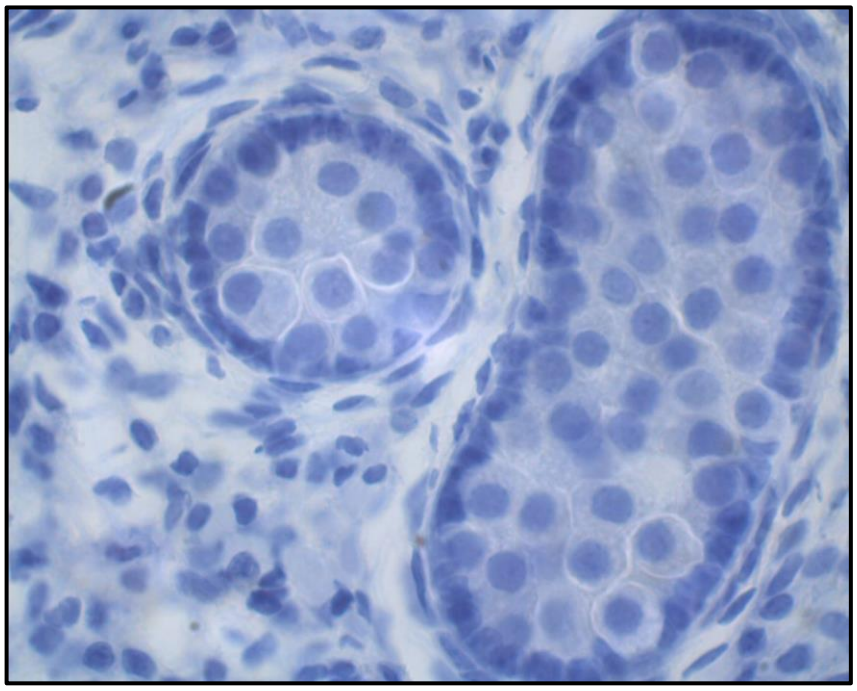
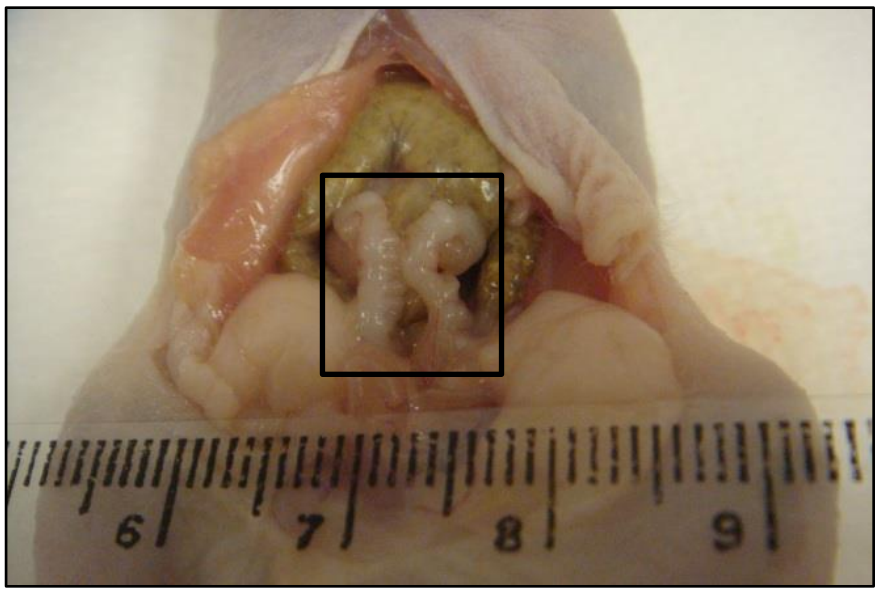


Figure 1: Cross-section of 6-month-old male alpaca shows the gonocytes in the center of the tubules as well as at the basement membrane, Sertoli cells lined at the membrane (hematoxylin).

A.



B.

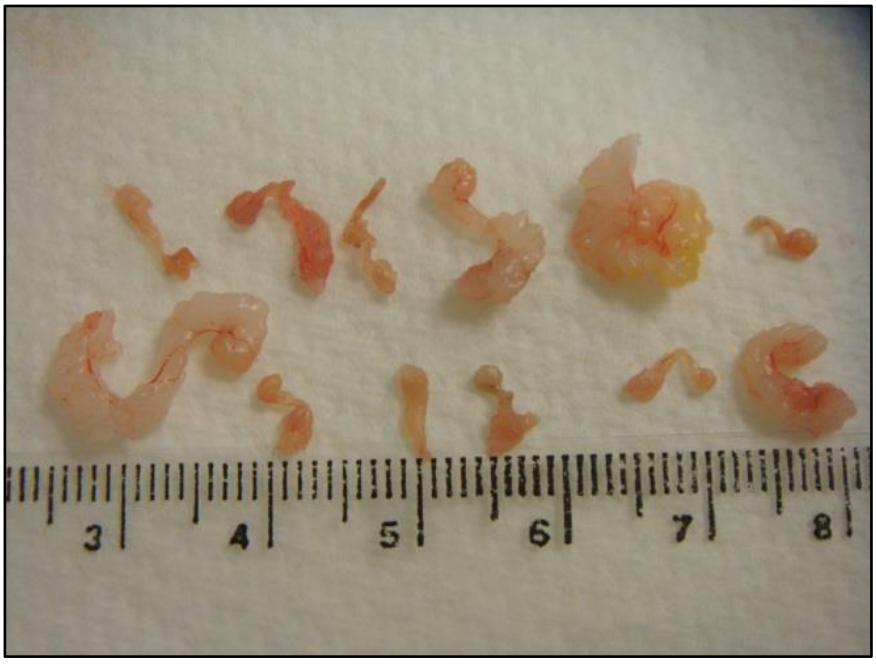


Figure 2: A. Full mature seminal vesicle at the time of dissection, B. Different seminal vesicle sizes used as indicator of testosterone concentration.

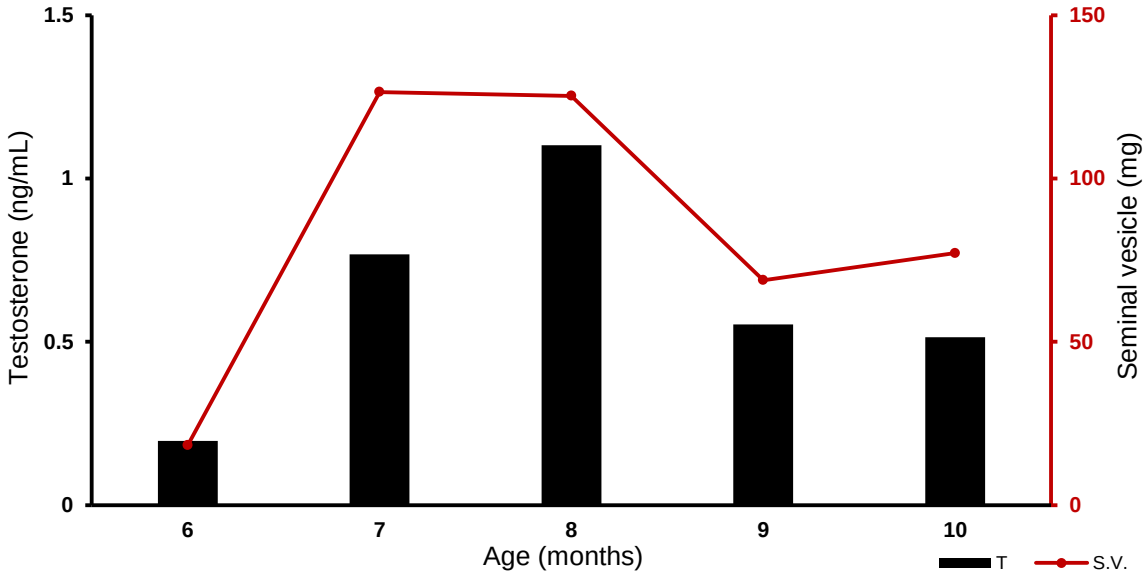
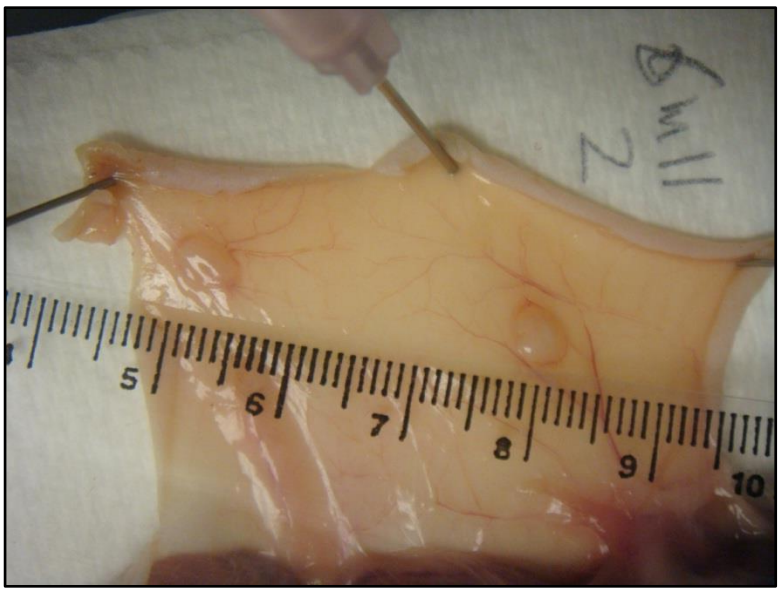


Figure 3: Testosterone level was strongly correlated to seminal vesicle weight (r = 0.92) in all five group ages.

A.



B.

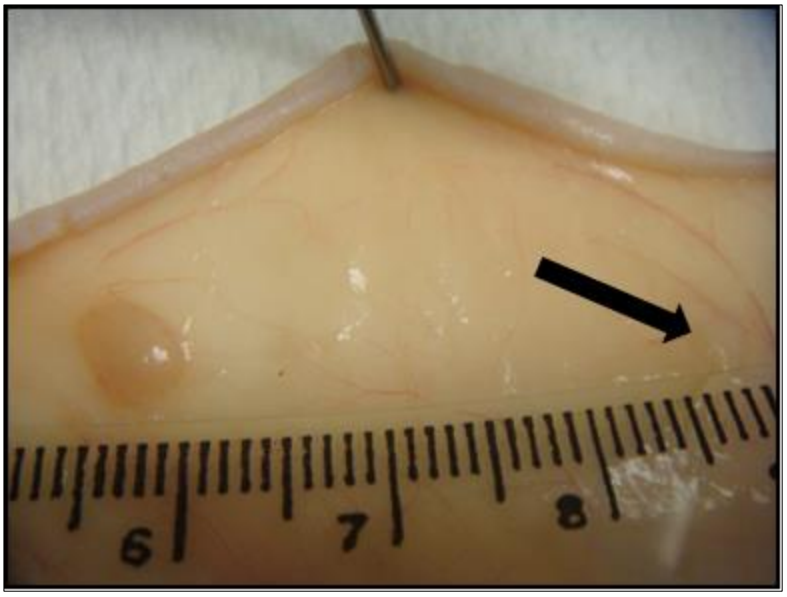


Figure 4: A. Photomicrographs of angiogenic development of xenografted testis tissue. The image is for 6-month-old alpaca donor testis onto an immunodeficient mouse. A clear Layer of capsule covered the graft tissue and vascular tissues are extensively extended

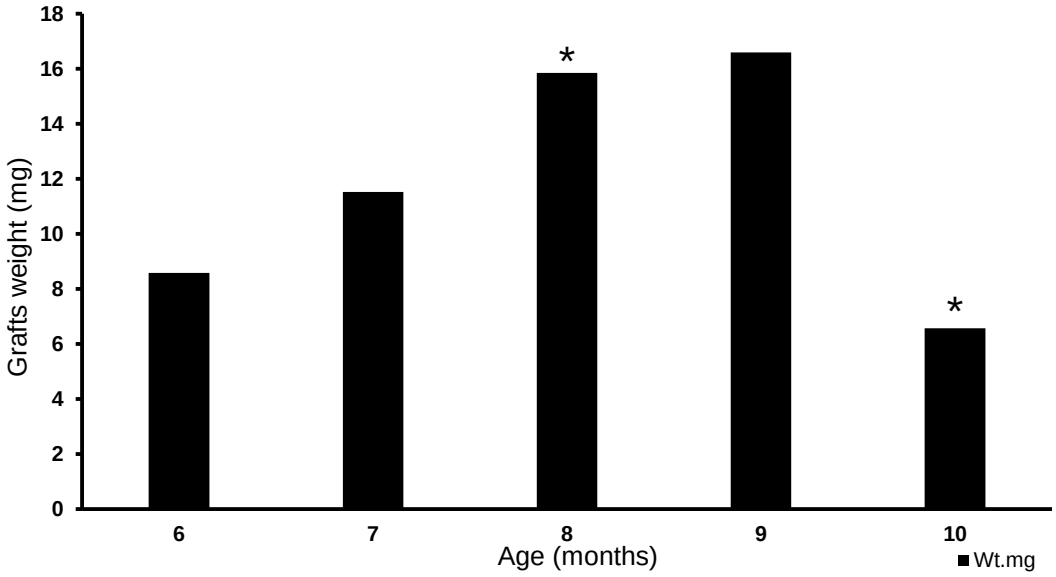


Figure 5: Grafts weight from five groups with significant (*) between 8 and 10 month groups.

Age, months	Grafts weight, mg (±SD)	Seminiferous tubules number (±SD)	Largest tubule, µm (±SD)	Tubules with meiotic germ cells	Mouse seminal vesicle, mg (±SD)	Testosterone, ng/mL (±SD)	Grafts collection % (±SD)
6	8.58 (±3.4)	72.70 (±53.0)	193.87 (±41.6)	13.53%	18.33 (±15.9)	0.20 (±0.1)	83.3 (±1.2)
7	11.53 (±5.4)	93.17 (±24.3)	192.50 (±32.6)	9.27%	126.50 (±185.1)	0.77 (±0.8)	79.1 (±1.3)
8	15.86 (±3.7)	104.01 (±55.9)	163.03 (±8.1)	2.60%	125.34 (±126.0)	1.10 (±1.4)	85 (±0.9)
9	16.60 (±6.8)	120.53 (±54.4)	134.95 (±11.7)	2.49%	68.90 (±62.3)	0.55 (±0.6)	83 (±0.6)
10	6.58 (±3.4)	77.71 (±51.5)	130.92 (±31.9)	5.79%	77.14 (±92.9)	0.51 (±0.4)	82.1 (±1.3)

Table 2: Recipient testosterone analysis and seminal vesicle weights, and grafts measurements.

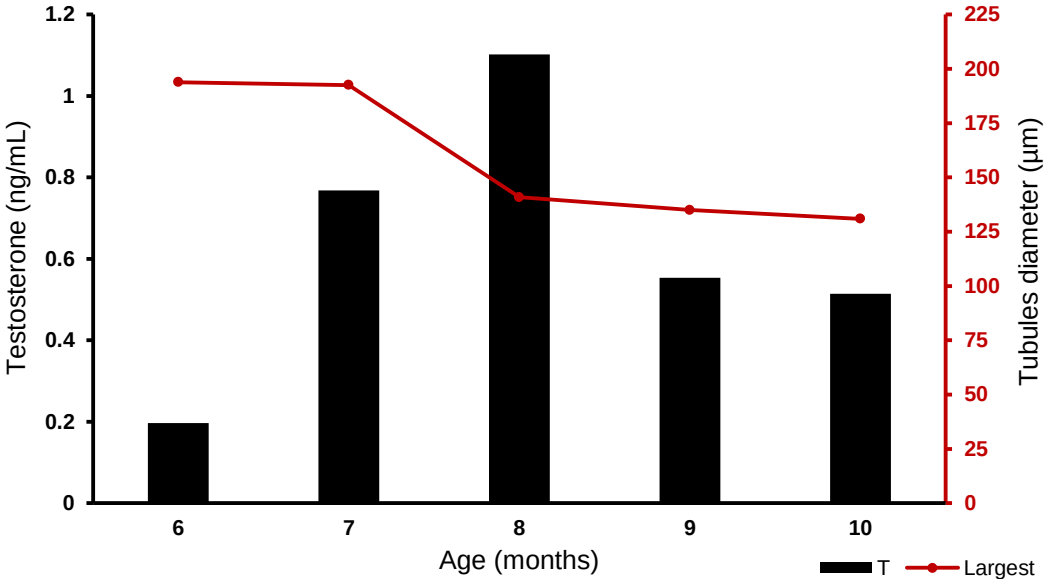


Figure 6: Testosterone levels in compare to seminiferous tubules diameter (r = -0.08).

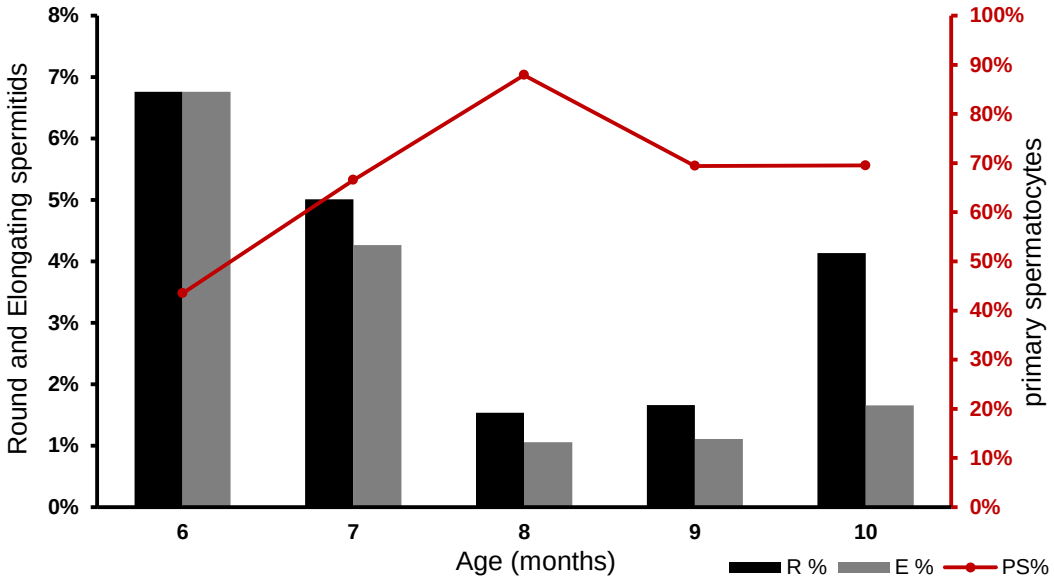


Figure 7: A. Percentage of tubules that round (R) and elongated (E) versus primary spermatocytes (PS) in all five groups.

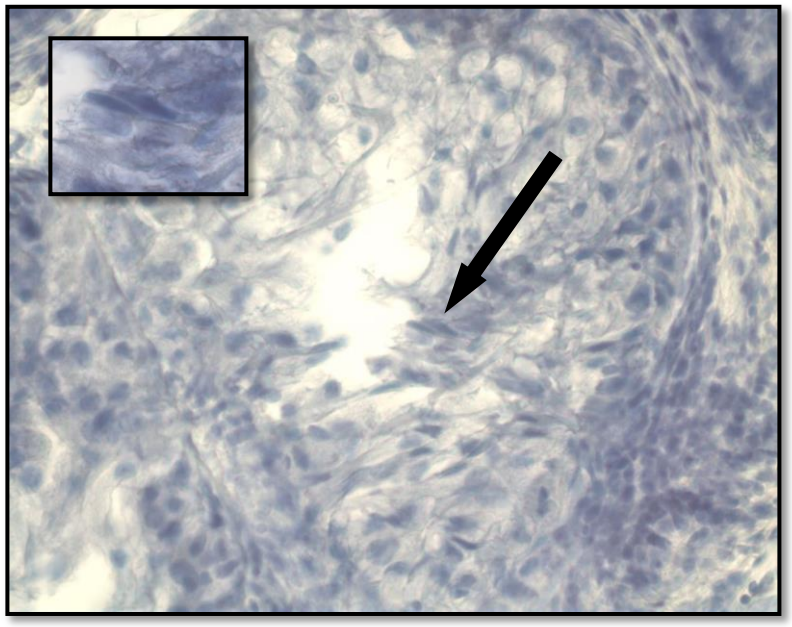


Figure 8: Graft tubule contained elongating spermatids. Insert is the same elongating spermatids at higher magnification.

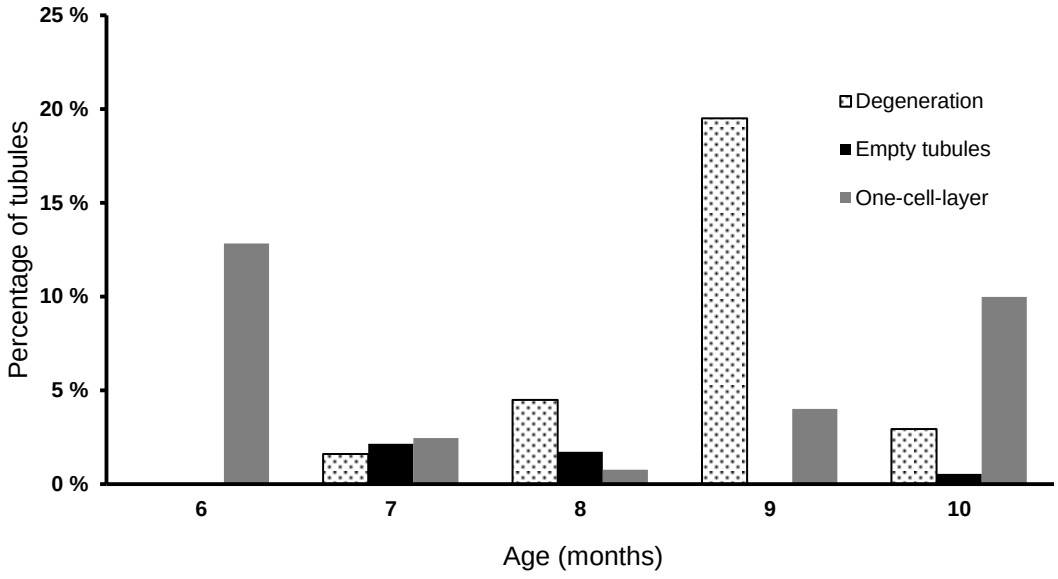


Figure 9: Percentage of tubules with abnormalities in all five groups

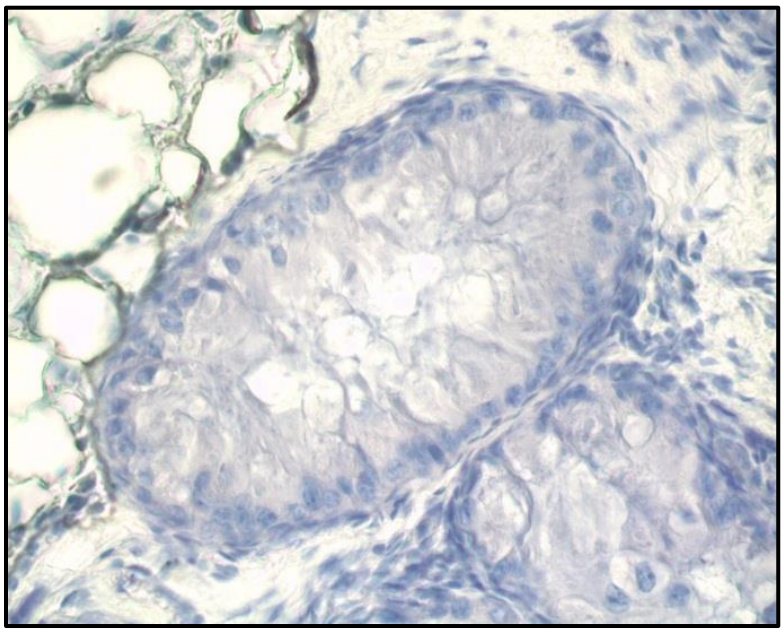


Figure 10: Tubules in grafts contain one layer cells.

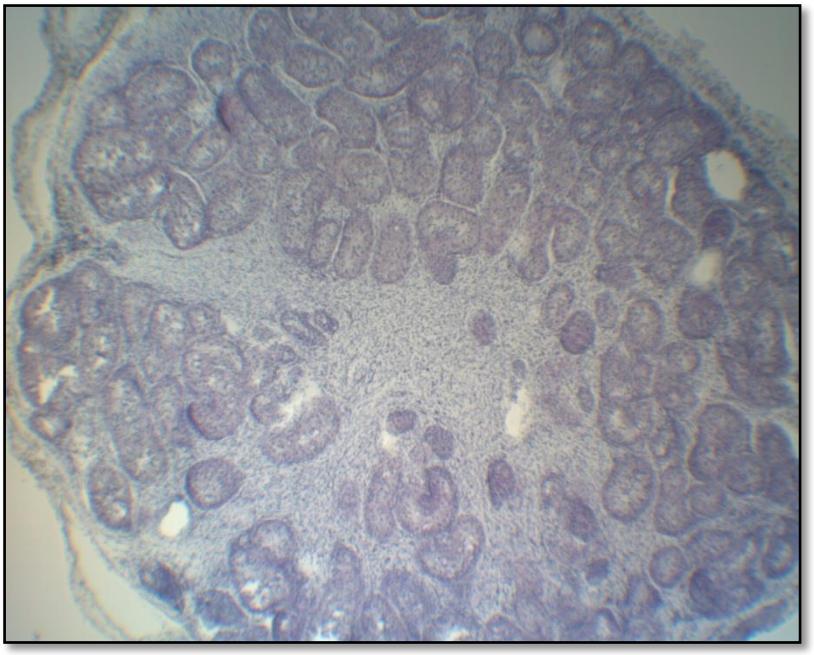


Figure 11: Degenerated tubules present in the center of most grafts.

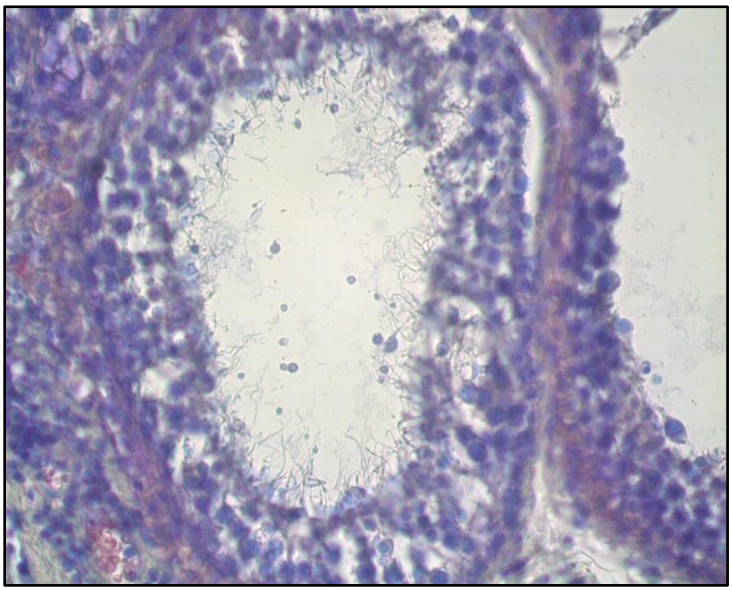


Figure 12: Tubule of piglet graft that contained mature spermatozoa.

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CONCLUSION

The alpaca, *vicugna pacos*, is a domesticated species of camelidae family that originate in South America. Male alpaca possess several reproductive unfavorable characteristics including late puberty and sexual maturity, slower testicular growth, higher incidence of testes abnormalities. Age at puberty age and factors regulating the pattern of puberty and sexual maturation are still a subject of controversy. The process of sperm production in terms of cell type population series that appear in particular seminiferous tubule and regulation of sperm production in terms of genes that expressed by germ and somatic cells and spermatogenesis in general remains poorly studied in this species. No studies have investigated in details the cellular events occurring in the seminiferous tubules during and around the time of puberty. This represents a gap in our knowledge base and a barrier for our ability to develop reproductive technology in these animals. The present dissertation is the most comprehensive investigation to date on the seminiferous epithelium development and investigation of testis function in alpaca. Testosterone hormone, a major key in puberty and sexual maturation, was studied in two blood samples in relation to interstitial cell changes. Moreover, testis tissues from prepubertal males were transplanted onto nude mice as controlled microenvironment (*in vivo* culture system) to evaluate testis cells dynamic.

Basal testosterone concentration in serum increased with advancing age. Concentration of the testosterone in prepubertal ages (6 to 11 months) was less than 0.1 ng/ml and in adult ages was about 3.0 ng/ml. Administration of 3000 IU of hCG resulted in a significant sharp increase in serum testosterone level ($P = 0.05$) after two hours. This response varied according to age. Mean serum testosterone level increased, 2.6 folds, in 6 to 8 months group (from 0.09 to 0.24 ng/mL).

First sharp increase of testosterone concentration, 3.4 folds, was seen between 12-14 months of age (3.81ng/mL) whereas second increase in response to hCG was observed at 21-24 months of age (6.89 ng/mL). The aromatizing ability of the testicular tissue was undetectable in young males until the age of 14 months where the serum estrogen was detected in few males. Interestingly, only samples showed testosterone concentration of 3ng/mL or higher contained measurable estrogens. Those animals were also recognized with high number of Leydig cells. It is possible that the Leydig cell number and aromatase enzymes have an effect on estrogen production which could mediate by the high testosterone concentration. Histological evaluation of testes at all ages in this study revealed that testosterone production is correlated to Leydig cell numbers. At ages of 6-8 months, average number of Leydig cell per field examined was 7.06 and the testosterone concentration was 0.24 ng/mL, whereas the quantitative distribution and the functional characteristics of these Leydig cell populations at 3-5 years was 97.4, and the concentration level of testosterone was 6.74 ng/mL. In contrast, in 15-17 months group where the number of mature Leydig cells was 43.53, testosterone level was 1.8 ng/mL. Great variations in testosterone concentration have been noticed among individual alpaca even in the same age group.

Testosterone is the androgen that essential for male fertility and is responsible for supporting and sustaining spermatogenesis. In the absence of testosterone, males are infertile because spermatogenesis rarely progresses to produce mature sperm. Sertoli cells are the major cellular target for the testosterone signaling and these cells are important for supporting germ cells development. Part of the dissertation was performed to study testis morphology and the spermatogenesis process in the alpaca. Specific chronological patterning of the seminiferous epithelium is a key element of spermatogenic regulation in animals, and plays an important role in male fertility. Spermatogenic activity starts with the migration of gonocytes from the center of seminiferous cords

to relocate at basement membrane and differentiate to spermatogonia and ends by formation of mature sperm that released into the lumen. Interstitial compartment begins with compact mesenchymal-like-cells to recognizable function cells such as mature Leydig cells. It is well known that spermatogenesis process is a function of age. Histological and morphometrical investigation was achieved with high cautious and linked to male ages. Males in this work were divided into five groups with close male ages together based on information from previous literature studies. Furthermore, in seek of additional accurate results, RT-PCR was performed on selected samples and was compared to histological findings. Spermatogenesis is a well-organized process that is under the control of a highly ordered gene expression culminating with the formation of mature spermatozoa. Each feature of testicular development involves specific genes. Group one which involved ages, 6, 7, 8, 9, and 10 months was highly important group because we believe from our previous study that these ages are the transitional period from prepubertal age entering puberty changes. As was expected, males of this group had many common features, for instance all these animals had gonocytes barely started migration to basement membrane and testosterone level was low. Tubules diameter was 67.5 µm and lumen observed in 10.4% of total tubules examined. Seminiferous epithelium activity has been observed in 8.3% tubules of only six males which confirm the idea of unity of these animals. Overall no significant differences were observed between these ages in this group. Changes in seminiferous epithelium and cells in interstitial area continued developing slowly in group 2, 3, and 4 with non-significance difference. This might be attributed to slow development of testis and the delay of puberty in alpaca. In adult ages (group5), full testis function has been obviously seen. It was interested to know that basal testosterone concentration (2.1 ng/ml) was significantly correlated to all parameters examined especially with Sertoli cells number and tubules with mature spermatozoa. This finding gives a strong

evidence of the effect of testosterone in testis development. Cell markers were specific in vast majority of males included in this group.

The five ages in group 1 (6 to 10 months) in pervious study were selected to include in ectopic testicular xenografting experiment. This technique is a method that has potential use for investigating spermatogenesis in mammalian species. During the prepubertal phase of testicular development, somatic, Sertoli and Leydig cells undergo proliferation and differentiation. However, developmental stage of both the somatic and germ cells during the prepubertal period could have an impact on the establishment of spermatogenesis in grafted testicular tissue. To increase the efficiency of sperm production in grafted testicular tissue, it is important to gain an understanding of changes in testis cell differentiation throughout the early postnatal period of testicular development. Evaluation of recipient mouse serum testosterone level, vesicular gland weights, and histological examination of grafts after harvested have provided a clear understanding of testicular tissue growth in different donor alpaca ages after 24 weeks grafting period. Considerably low concentrations of testosterone (0.2 to 1.1 ng/ml) were observed in all serum samples collected from nude mice included in this study comparing with control uncastrated mice and in other donor species. Interestingly, no significant difference has been noticed in the ability of Leydig cells to produce detected testosterone level between all donor ages in current experiment. This observation reveals that number of both immature and mature Leydig cell populations in pre-grafting testis tissue in different ages had no significant effect on the testosterone production. Difference in grafts weight that obtained in this study could be attributed initially to vascularization around newly grafted tissues and the capability of Sertoli cells to respond to recipient mouse pituitary-released gonadotropins. The average percentage of tubules containing meiotic cells in 8 month grafts was 87.9%, whereas, meiotic cells were present in an average range of 43.5% of the tu-

bules in grafts from 6 month donor testes. Grafts from 8 month old donor contained the highest percentages, but not significant, of tubules with meiotic germ cells than grafts from 6 month donors that contained lowest percentage. Spermatogenesis had progressed through meiosis as evidenced by the presence of elongating spermatids. The average percentage of seminiferous tubules in grafts with elongating spermatids was 6.7, 4.2, 1.0, 1.1 and 1.6% from donor alpacas aged 6,7,8,9, and 10 months, respectively. Overall, appearance of elongating spermatids clearly existed for all grafts regardless of donor ages. Differentiation of germ cells seem to be more sensitive to the hypoxic damage associated with testicular tissue collection, preparation, and grafting procedure but neither pre-grafting stages of germ cell development nor recipient hosts endocrine environment has any effect on tubule pattern. Degeneration and unclassified tubules phenomenon was 19.5, 4.3% in 9 months old and 0.0, 30.1% in 6 months respectively. This phenomenon is probably due to ischemia around and in grafting vesicular tissue that reduced the ability of germ cells to survive. Even though, our statistical analysis indicates that no significant difference in grafts from all five age groups, 6 month old origin was the best for grafting based on the most advanced germ cell stages that differentiated into round and elongated spermatids in 24-weeks period time. Remarkably, no significant differences have been observed in grafts from 6 month age group in term of the onset of meiosis compared with the intact age-matched males. Ectopic alpaca testis xenografting is a promising technique for the study of testicular physiology and pathophysiology in alpaca.

Finally, this dissertation has focused on alpacas' testes development. Primarily through histological examination and supported by testosterone analysis. Testis growth in alpaca progresses slowly with no significant difference between Pre-puberty ages. Real time PCR was also achieved to identify expression of genes that are involved in germ and Sertoli cells development

in alpaca testes and match the result with testis morphology. Five pre-puberty ages 6, 7, 8, 9, and 10 months were ectopically xenografted onto the backs of nude mice to take a full advantage to mimic the appearance of seminiferous tubules with elongating spermatids as seen in intact males. Seminiferous tubule cross sections with elongating spermatids have been seen in all grafts with no significant difference. The global result of current research recognized pre-puberty males (6, 7, 8, 9, and 10 months) as group that has no significant difference of almost all features studied. Expanded study is needed to identify specific factors control cellular changes and development. If pre-pubertal to pubertal transit period is completely understood, it will be straightforward to estimate sperm production and use the information in male breeding soundness evaluation and will allow better diagnosis of some causes of male infertility.