FACTORS AFFECTING TESTICULAR GROWTH AND DEVELOPMENT IN THE BEEF BULL

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

At present no single parameter can predict potential daily sperm production (DSP) in bulls. Sertoli cells (SC) are responsible for nourishing and providing physical support to germ cells (GC) throughout spermatogenesis. The size of the SC population established before puberty is one of the major factors determining DSP in the bull. Fine needle aspiration (FNA) is a technique used for diagnostic purposes in many species but its application to determine the size of the SC population remains unexplored. Our objectives were to compare two stains (immunohistochemistry vs. conventional HE) and three techniques to determine SC density in neonatal and prepubertal bulls [22G FNA (SMEAR), 14G needle with vacuum (CORE) and tissue section (HIS)], and the effects of three different interventions on testicular growth, development and cytology in neonates, pre and peripubertal bulls: corn supplementation during mid to late gestation of dams, the age of the dam and the administration of vitamin A.

The significant correlation observed between GC and SC counts in neonatal and prepubertal bulls, highlighted the relationship between both cell populations established in the early gonad. Although no significant correlation existed between techniques for SC density, the significant relationship established between SC counts and GATA4+ cell density implies the process can be automated.

Corn supplementation to multiparous dams during mid to late gestation did not have any effect on testicular gross parameters and cytology in neonatal bulls coincident with the lack of differences in fetal growth between treatments.

No significant differences in scrotal circumference, testicular weight or cytology existed between prepubertal bulls from primiparous heifers or adult multiparous cows. Nevertheless, bulls from heifers had lower bodyweights at 194 days of age compared with bulls from cows.

The administration of vitamin A to peripubertal bulls had no effect on scrotal circumference, testicular weight or cytology. Nevertheless, epididymis in treated bulls were significantly heavier than non-treated animals.

The data generated in the present experiments highlights the need for more research to generate new interventions aiming to influence the size of the SC population in the testis and the potential daily sperm production in the beef bull.

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Genesis 1:25 "And God made the beasts of the earth according to their kinds and the livestock according to their kinds, and everything that creeps on the ground according to its kind. And God saw that it was good".

''Two cows, mildly mooing; No bull; nothing doing''. Ogden Nash, 1949

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This dissertation is dedicated to the great and only love of my life, my wife Clara Elena. I wouldn't have done it without your love and support, you are my model of bravery and courage.

To my parents and family back in Uruguay, I love you and owe you the most important treasures I inherited: faith and education.

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LIST OF ABBREVIATIONS

ADG.....Average daily grain.

ADHC1 .....Alcohol dehydrogenase C1.

Akt.....Protein kinase B.

AMH .....Anti-Müllerian hormone.

ARKO .....Androgen receptor knock out.

BCS .....Body condition score.

BMP .....Bone morphogenic protein.

BSA.....Bovine serum albumin.

BTB.....Blood testis barrier.

BSE .....Breeding soundness examination.

BW .....Body weight.

cAMP .....Cyclic adenosine monophosphate.

CDKI.....Cyclin dependent kinase inhibitor.

CE .....Coelomic epithelium.

CK18.....Cytokeratin 18.

CP.....Crude protein.

CX43.....Connexin 43 protein or Gap junction alpha 1 protein.

CYP19.....Aromatase cytochrome P450 gene.

DAPI .....4’, 6’-diamidino-2-phenylindole.

Dax1.....Dosage sensitive sex reversal adrenal hypoplasia critical region on chromosome X gene 1.

DDGS.....Distiller's dried grains with solubles.

DHH.....Desert hedgehog.

DHT .....Dihydrotestosterone.

DM ..... Dry matter.

Dpc ..... Days post-coitus.

DSP ..... Daily sperm production.

E2 ..... Estrogen.

EGF ..... Epidermal growth factor.

ERK ..... Extracellular signal–regulated kinases or MAP kinase.

FGF-9 ..... Fibroblast growth factor 9.

FNA ..... Fine needle aspiration.

FOG-1 ..... Friends of GATA1.

FOG-2 ..... Friends of GATA2.

FNS ..... Fine needle (14G) with suction.

FSH ..... Follicle stimulating hormone.

FSHr ..... Follicle stimulating hormone receptor.

GAPDH ..... Glyceraldehyde-3-phosphate dehydrogenase.

GATA4 ..... GATA4 transcription factor.

GATA+ Den ..... Total automatic count of all GATA4 positive cells present on the mirror section image divided by the total automatic count of all DAPI positive cells present in the field.

GATA+ Den A ..... Total automatic GATA4 positive sum area on the section image divided by the total automatic DAPI positive sum area present in the field.

GC ..... Germ cell.

GC HE Man ..... Manual GC count within individualized ST on HE stained mirror section image.

GC IHC ..... Automatic GC count within individualized ST on IHC-GATA4 stained tissue section image.

GC IHC Man.....Manual GC count within individualized ST on IHC-GATA4 mirror image.

GDNF.....Glial cell line derived neurotropic factor.

GnRH.....Gonadotropin releasing hormone.

GSR.....Germ: Sertoli cell ratio.

HE.....Hematoxylin and eosin.

HIS.....Conventional tissue section for histology.

IGF-1.....Insulin like growth factor 1.

IGF-2.....Insulin like growth factor 2.

IHC.....Immunohistochemistry.

IL-1.....Interleukin-1.

INSR.....Insulin receptor.

IP3K.....Inositol-trisphosphate 3-kinase.

LC.....Leydig cell.

LH.....Luteinizing hormone.

MAPK.....Mitogen activated protein kinase.

ME.....Metabolizable energy.

MIB-1.....E3 ubiquitin-protein ligase MIB-1.

mRNA.....Messenger RNA.

NGS.....Normal goat serum.

PDGF.....Platelet derived growth factor.

PGD2.....Prostaglandin D2.

PGH2.....Prostaglandin H2.

PGC.....Primordial germ cell.

PMC.....Peri myotubular cell.

PTU.....Propyl thiouracil.

P27KIP1 ..... P27 cyclin dependent kinase inhibitor 1 B.

P21cip1 ..... P21 cyclin dependent kinase inhibitor 1.

RA ..... Retinoic acid.

RATIO ..... Germ cell to SC ratio within individualized ST on IHC-GATA4 stained tissue section image.

RATIO A ..... Ratio of the total DAPI positive cell sum area divided by the total GATA4 positive cell sum area within each individual ST.

RATIO ..... Germ cell to SC ratio within individualized ST on IHC-GATA4 stained tissue section images using cell counts determined by the corresponding cell nucleus sizes.

RARα ..... Retinoic acid receptor alpha.

RARβ ..... Retinoic acid receptor beta.

RARγ ..... Retinoic acid receptor Gamma.

RXR ..... Retinoic acid X receptor.

RE ..... Retinol.

SC ..... Sertoli cell.

SCARKO ..... Sertoli cell androgen receptor knock out.

SC HE Den ..... Automatic total cell count minus the total manual count of all SC within all ST in the field by the total automatic count of all cells present in the field.

SC HE Man ..... Manual SC count within individualized ST on HE stained mirror image.

SC IHC ..... Automatic SC count within individualized ST on IHC-GATA4 stained tissue section image.

SC IHC Den ..... Sertoli cell density determined by total SC automatic count within all ST present in the IHC-GATA4 stained mirror section image divided by the total automatic count of all DAPI positive cells present in the field.

SC IHC Den N	Sertoli cell density determined by the automatic determination of the total GATA4 positive cell sum area within each ST present in the field divided by the average SC nucleus size on the total automatic count of all DAPI positive cells present in the field divided by the average GC nucleus size.
SC IHC Man	Manual SC count within individualized ST on IHC-GATA4 stained mirror section image.
SC IHC N	Sertoli cell count calculated as the automatic determination of the total GATA4 positive cell sum area within each individual ST divided by the average SC nucleus size.
Sf1	Steroidogenic factor 1.
SMR	Smear produced using a 22G needle with vacuum.
Sox9	SRY– Box 9 gene.
SRY	Sex determining region of Y chromosome.
ST	Seminiferous tubule.
T	Testosterone.
TBST	Tris-buffered saline and Polysorbate 20.
T3	Triiodothyronine.
TDN	Total digestible nutrients.
TGF-α	Transforming growth factor alpha.
TGF-β	Transforming growth factor beta.
VEGF	Vascular endothelial growth factor.
WNT4	Wingless-type MMTV integration site family member 4.
WT	Wild type.
WT1	Wilms's tumor gene 1.
14G VAC	14G needle with vacuum.

TESTICULAR DEVELOPMENT

Introduction

Reproduction in livestock, either assisted or natural, is the pre-requisite of production. Natural breeding is the global predominant form of reproduction in beef production systems (Chenoweth, 2004). In beef cow enterprises, the calf is the main product therefore reproductive efficiency determines profitability regardless of the system employed (Diskin and Kenny, 2014). Efficient and profitable management of a herd depends on maintaining a short calving season (Barth, 2018). The length of the calving season is determined by the length of the breeding season. In efficient beef herds, most of the cows are expected to produce a calf every 12 months. To achieve this goal, cows must conceive in a very short time considering the average gestation length for British breeds is 283 days (Casas et al., 2012). It is fundamental that the full potential of the herd fertility is achieved without being compromised by low fertility bulls (Barth, 2018). The bull is the individual within a beef herd that bears the greatest responsibility in overall herd fertility. One single bull is capable of siring 25 to 60 calves while a cow can only produce, under the best management conditions, one calf per year (Walker et al., 2009). The most commonly referred to and easily detected consequence of using sub-fertile bulls is the loss of pregnancies. However, delayed conception rate and extended calving season are further consequences of using sub-fertile bulls (Parkinson, 2014; Kastelic and Thundathil, 2017). It is calculated that for every 21-day period a beef cow remains open during the breeding season, there is a potential loss of 23 to 27 kg of weaning weight the following year for the calf she finally conceives (Barth, 2018). Detection of those individuals with higher fertility within a group of bulls is fundamental for efficient beef herd management. At the same time, the accurate detection of those animals

with lower fertility is key to avoid putting at risk the herd's full fertility potential. Besides, cows that conceive late, tend to do so in subsequent years (Barth, 2018).

Breeding soundness examination is a valuable tool to assess the potential reproduction capacity in bulls but within the group of bulls classified as apt, a whole range of undetected fertility levels exists among individuals (Kastelic et al. 2008). With the incorporation of genomics as a selection tool in cattle, bulls are being selected and incorporated in AI centers at a very early age (Dance et al. 2015) but bulls younger than one year of age have poor semen production and sperm motility compared with older bulls (Murphy et al. 2018). At present, there is no single parameter that allow us to predict the potential daily sperm production in the bull (Rajak et al. 2016b).

Since Enrico Sertoli published his first paper in 1863 about the *Celluli Madri*, the recognition of the importance and relevance of Sertoli cells (SC) in the testis has been studied (França et al. 2016). Sertoli cells are responsible for the differentiation of most cells in the testis during gonadal development and for the coordination and efficiency of spermatogenesis in the adult individual (Skinner and Griswold 2005). The number of SC present in the testis determines the potential daily sperm production in the bull by providing the special milieu inside the seminiferous tubule and the physical support necessary for the progression and differentiation of germ cells (Berndston et al. 1987a). Sertoli cells replicate during a limited time, extending from the fetal to the neonatal stage, ceasing at puberty with no further cell divisions in adulthood (Rawlings et al., 2008). In this manner, the ceiling of daily sperm production is fixed by the final size of the SC population established in the testis before puberty. Nevertheless, recent data defies the immutability dogma of SC multiplication after puberty in mammals (Zakhidov and Marshak 2015). This finding unveils the possibility of influencing the rate of Sertoli cell division due to

the plasticity observed in Sertoli cells under different hormonal and nutritional scenarios during cell division, opening new and exciting roads for research (Bollwein et al. 2017).

The development of low invasive techniques to determine testicular cytology, a more precise determination of the window time during which SC replicate, and the study of those factors that influence testicular growth and development are necessary in the bull. The information generated will help us identify those periods of greater Sertoli cell susceptibility during cell replication and the development of new interventions aiming to increase the final size of the Sertoli cell population and achieve greater potential daily sperm productions in bulls. Future studies will provide us a better understanding of the testis physiology filling the actual information gap on testicular growth and development in the bull.

Early Gonad Differentiation and Development

In mammals, five different stages are recognized in the development of the male gonad: formation of the genital ridge, formation of the bipotential gonad, sex determination, formation of the testicular cords and the development of the functional testis (Skinner and Griswold, 2005). The first two stages are independent of the future sex of the individual and are not under gonadotropin control (O'Shaughnessy et al., 2009).

The genital ridges form as two mounds at each side of the dorsal mesentery of the embryonic hindgut deriving from the intermediate mesoderm of the urogenital system (Skinner and Griswold, 2005). The genital ridges are composed of the coelomic epithelium (CE) and the primordial germ cells (PGC) that migrate via the hindgut and mesonephros from extraembryonic mesonephros at the base of the allantois (Swain and Lovell-Badge, 1999). Sertoli cells have a different origin than the other cell types present in the testis, deriving from the CE in the genital ridges (Karl and Capel, 1998).

According to the specie considered, the formation of the genital ridges (also known as urogenital ridges) appear between days 27 and 31 of gestation in the bovine embryo (Wrobel and Sub, 1998), 20 days of gestation in the sheep (McNatty et al, 1995), 18 to 20 days in the pig (McCoard et al., 2001), 9 to 10 days post-coitum (dpc) in the mouse (Karl and Capel, 1998), and 10 to 11 dpc in the rat (Skinner and Griswold, 2005).

For a better understanding of the development of the male gonad, the urogenital ridges are divided into three main regions. The first region or pronephros, situated at the cranial end of the ridges, is where the adrenal primordium is located. The second region or central, also known as mesonephros, is where the gonads will arise (Swain and Lovell-Badge, 1999). Finally, a third region or metanephros, is where the kidneys will form (Capel, 2000).

Male Gonad Differentiation

Our present knowledge of the molecular mechanism behind the early formation of the male gonad is incomplete, with proteins acting in different roles at different stages, sometimes in coordination with other proteins or in solitude, sometimes appearing for brief periods of time (Swain and Lovell-Badge, 1999). Most of the information generated about the early differentiation of the gonad was produced using the mouse and rat as animal models. Whenever available, time milestones and references from other animal species are presented with emphasis in the bull.

At embryonic days 10 to 11 in the mice, 11.5 to 12.5 dpc in the rat, 4th week in humans, 23 days of gestation in the sheep and 32 to 39 days in the bull, the gonad is still a primordial organ, being fully bi-potential and identical regardless of the XX or XY genotype (Skinner and Griswold, 2005; Hung-Chao Yao et al., 2015). Jost et al. in 1947 reported that the formation of female ducts and external genitalia in XX and XY rabbit fetuses castrated in utero occurred

before sex differentiation. He introduced the concept that in males, femininity is repressed, and maleness imposed by the testis (Jost et al., 1970). The reason behind this observation is that the transcriptome of somatic cells at this stage of the gonad is inclined towards the expression of genes associated with ovarian development (Jameson et al., 2012). Thus, the female is the default pathway, being the male pathway responsible of diverting the female gonad towards a male gonad. This event occurs independent of gonad hormones (Wilhelm et al., 2007).

The Y chromosome acts through the sex determining region gene (*Sry*) that triggers testis differentiation in the indifferent genital ridges that would otherwise become ovaries (Jost et al., 1970; Swain and Lovell-Badge, 1999; Sekido and Lovell-Badge, 2008). Furthermore, the expression of the *Sry* gene in XX gonads results in the formation of a testis, while in mouse XY embryos where the *Sry* gene is deleted or in XY humans carrying *Sry* gene coding mutations, ovaries are developed (Wilhelm et al., 2007; Hung-Chang Yao et al., 2015).

The *Sry* gene is activated by a subset of somatic XY cells at 10.5 dpc in the mice (Hacker et al., 1995), 12 dpc in the rat (Skinner and Griswold, 2005), week 7 of gestation in humans (Hanley et al., 2000), and day 23 of gestation in the sheep (Skinner and Griswold).

At the early stage of gonad development, a germ cell lineage and three additional cell lineages are present (McLaren, 2000). First, a supportive cell lineage that will give origin to SC in the testis and granulosa cells in the ovary. Both SC and follicular cells share the mission of surrounding germ cells while creating the necessary environment for germ cell differentiation and development. Second, a steroidogenic cell lineage, that in males develops into Leydig cells and the corresponding theca cells in females, producing those hormones responsible for the secondary sexual characteristics of the embryo. Finally, a connective tissue cell lineage, which is

the major contributor of endothelial and peri myotubular cells (PMC), surrounding the testicular cords and forming the basal lamina in conjunction with SC (McLaren, 1998).

Sry is necessary and self-sufficient to direct gonadal somatic progenitor cells towards a SC fate. In XX or XY embryos where *Sry* gene is absent or dysfunctional, somatic cells will differentiate into granulosa cells and the gonad will follow an ovarian pathway (Hung-Chao Yao et al., 2015). Interestingly, the expression of the *Sry* gene follows an anterior-posterior wave pattern along the gonad (Bullejos and Koopman, 2001).

The timing of expression of *Sry* is fundamental for the fate of the male gonad. In mice, if *Sry* is not expressed before E11.25 an ovary will develop (Capel, 2000). The window of time can be as brief as 6 h after the onset of *Sry* activation in mice (Hiramatsu et al., 2009). This highlights the short period of time in which the testis pathway needs to be activated to silence the ovarian pathway after which the gonad will result in an ovary or ovotestis (Capel, 2000; Hung-Chao Yao et al., 2015).

The early gonad development and CE outgrowth relies on several transcription factors including steroidogenic factor 1 (*Sf1*), Wilms's tumor 1 gene (*WT1*), Empty Spiracles Homeobox 2 (*Emx2*), GATA4, and the insulin family of growth factors (IGF1 and IGF2) and receptors (INSR and IGF1R; Capel, 2000). The loss of function of any of these factors will lead to the failure to form or maintain the primordial gonad (Hung-Chang Yao et al., 2015).

Steroidogenic factor 1, belongs to the family of orphan nuclear receptors, to which no ligand has yet been identified (Schimmer and White, 2010). This gene was typified first as a transcription factor for cytochrome P450 steroid hydroxylase, but later, using knockout mice, Sf1 was discovered as necessary for the development of the adrenal glands, gonads, pituitary, and ventromedial hypothalamus (Luo et al., 1994; Schimmer and White, 2010). Steroidogenic factor

1 plays an essential role as a regulator of the endocrine system development at multiple levels and is necessary for the differentiation and growth of the somatic cells present in the early gonad (Swain and Lovell-Badge, 1999). In mice, the gonads of embryos lacking Sf1 cease to develop between 11-11.5 dpc and degenerate via apoptosis while the adrenal glands fail to form (Luo et al., 1994). Before the time of *Sry* expression in the male, the expression of *Sf1* is identical for XX and XY genital ridges (Chang Yao et al., 2015). Later, higher levels of *Sf1* expression are associated with the testis owing to the differentiation of Leydig cells in the male gonad, where *Sf1* is required for steroidogenesis (Karpova et al., 2015).

The development of the Műllerian and Wolffian ducts differ between male and female embryos. In the female, the Műllerian ducts (or paramesonephric ducts) originate within each mesonephros as invaginations of the CE between days 11.5 to 12.5 dpc (Swain and Lovell-Badge, 1999). This duct runs parallel to the Wolffian duct turning towards the midline at the posterior end of the mesonephros fusing with the companion contralateral duct forming the future oviduct, uterus, cervix, and the cranial portion of the vagina in the female (Sanger, 2003). Under the influence of anti-Müllerian hormone (AMH) and Testosterone in the male, the Müllerian ducts regress while the mesonephric duct (or Wölffian duct) will give rise to the male ductal system forming the epididymis and vas deferens (Capel, 2000).

In humans, a *Sf1* mutation causes a complete XY reversal with the conservation of the Müllerian structures either directly suppressing AMH where *Sf1* acts as a promoter or indirectly through an abnormal SC differentiation or function (Achermann et al., 1999). Furthermore, it has been demonstrated that *Sox9* can synergize *Sf1* activation of the AMH promoter suggesting that both factors act in concert to bring about tissue specific expression of AMH (Sekido and Lovell-Badge, 2008).

The Wilms's tumor associated gene, or *WT1*, has been related in humans with mild genito-urinary malformation and a predisposition for the development of kidney tumors (Loo et al., 2012). In mice, deletions of this gene are associated with the absence of gonads and kidney malformations (Swain and Lovell-Badge, 1999). Wilms's tumor-associated gene contributes to the growth of the genital ridges through the ingression of cells from the CE, which are predecessors of SC. In accordance to this, mutations of *WT1* cause sex reversal due to a deficiency in SC precursors (Karl and Capel, 1998). Using knock out mice, *Sf1* gene expression was demonstrated to be *WT1* dependent, where binding sites for *WT1* have been identified and characterized in *Sf1* gene promoters (Wilhelm and Englert, 2002).

Another key player is dosage-sensitive sex reversal adrenal hypoplasia critical region on chromosome X gene 1, or Dax1, a nuclear receptor encoded by the NR0B1 gene. Coincidently, with the expression of the *Sry* gene, *Dax1* is expressed in the genital ridges of both sexes in the mouse on day 11.5 dpc (Swain et al., 1996). Progressively, *Dax1* is downregulated in the testis and remains active in the ovary. Some experiments in mice have shown that Dax1 acts antagonistically to *Sry* working as an "anti-testis" gene (Swain et al., 1998). *Dax1* acts as a repressor of *Sf1* through a mediated activation exerted by a possible protein-protein interaction mechanism. Additionally, *Dax1* also represses the synergistic action of *Sf1* and *WT1* on the AMH promoter acting as an inhibitor of testicular development (Goodfellow and Camerino, 1999). It is believed that *Dax1* acts in the same pathway as *Sf1*, as their patterns of expression correlate and include other organs than the gonad, like the adrenal, hypothalamus and pituitary, implying both genes in the reproductive function (Swain and Lovell-Badge, 1999).

Sertoli Cell Development and Differentiation

Sertoli Cell Ontogenesis

Sertoli cells are derived from the Coelomic epithelium as a difference with the rest of cell types in the testis (Karl and Capel, 1998). They are of mesenchymal origin, going through a mesenchymal to epithelial differentiation cell type process. This transformation implies the polarization of the cell, by the accumulation of substances in the basal pole like extra cellular matrix proteins Collagen IV, laminin, fibronectin, and heparin sulfate proteoglycan (Brehm and Steger, 2005; Barrionuevo et al., 2011).

Sertoli Cell Differentiation

Sertoli cells are the first testicular cells to differentiate in mammals (Wilhelm et al., 2007). Sex-determining region Y gene (*Sry*) is the specific gene of the supportive cell lineage that directs SC differentiation between days 10 to 12.5 dpc in mice (McLaren, 2000). Sertoli cell differentiation coincides with the arrival of PGC to the gonad from their extragonadal origin (McLaren, 1998). Once SC differentiate, they subsequently direct differentiation of the rest of the cell types in the gonad. Thus, the fate of the testis is the fate of SC differentiation by *Sry* expression that would otherwise become granulosa cells (Swain and Lovell-Badge, 1999; McLaren, 2000). After differentiation, SC replicate rapidly coinciding with the peak of *Sry* gene expression (Waites et al., 1985; O'Shaughnessy and Fowler, 2001). By 13.5 dpc in the rat, SC are actively dividing, reaching a maximum cell division at 20.5 dpc, declining gradually during the first 2 to 3 weeks postnatally (Migrenne et al., 2003).

Thirty-six hours after the initiation of *Sry* expression in the mouse XY gonad, three main events take place: the organization of the somatic supporting SC lineage surrounding the PGC to form the testis cords, a notorious growth of the male gonad in comparison to the female gonad,

and the appearance of characteristic blood vessels on the surface of the male gonad (Swain and Lovell-Badge, 1999).

At this time of the gonad differentiation, a group of cells that express *Sry*, start expressing *Sox9*, becoming later SC. Therefore, the expression of *Sry* marks the pre-SC lineage (Wilhelm et al., 2007). *Sry* -box containing gene 9 (*Sox9*) is one of the mostly studied genes in the developing gonad and is up regulated by the *Sry* gene. *Sry*-box containing gene 9 has been related to the secretion of anti Müllerian hormone (AMH) by SC.

Once *Sox9* is activated, its transcription is sustained by a mechanism independent of *Sry*, probably by auto regulation and another mediated by prostaglandin D2 (PGD2) signaling (Chaboissier et al., 2004). At the same time, there is growing evidence that *Sox9* is responsible for the down regulation of *Sry* transcription (Wilhelm et al., 2005).

In the bipolar gonad, two main signaling molecules are secreted: FGF-9 and WNT4. XY mutants for FGF-9 showed reduced SC proliferation, loss of expression of *Sry* and *Sox9* and went through a male to female sex reversal. It has been demonstrated that both FGF-9 and *Sox9* work together in a forward loop (Hung-Chao Yao et al., 2015). Fibroblast growth factor-9 suppresses WNT4 in XY gonads establishing the male pathway while in XX gonads, WNT4 dominates. Steroid production in the early gonad is related to WNT4. Leydig cells produce testosterone as early as testis cord formation on days 12.5-13 dpc, while in the ovary, theca cells do not start producing steroids until birth (Vainio et al, 1999). XX mice lacking WNT4 show activation of genes that produce testosterone in the ovary and the masculinization of the Wolffian ducts. The genital ridges and mesonephros express WNT4, being later downregulated in the testis and activated in the ovary (Swain A. and R. Lovell-Badge, 1999).

Prostaglandin D_2 secreted by SC can induce the expression of *Sox9* in neighboring cells. Simultaneously, *Sox9* induces the expression of prostaglandin-D synthase (PgDs), which then catalyzes the conversion of PGH_2 to PGD_2, and hence secretion of *Sox9* in XX/XY chimeras (O' Shaughnessy and Fowler, 2001; Svingen and Koopman, 2013). Several extracellular molecules are implicated in testis differentiation like FGF-9, PGD_2, desert hedgehog (DHH), and PDGFα. Within this group, the absence of FGF-9 and PGD_2 suppresses the expression of *AMH* or *Sox9* (Wilhelm et al., 2005).

GATA4, a Zinc-finger transcription factor, is highly expressed at 11.5 dpc in the mouse and precedes the secretion of AMH by SC. GATA4 binds to a consensus in the AMH promoter to activate its expression. Anti-Műllerian hormone is a transforming growth factor beta (TGF-β) family member and is one of the earliest known products of SC (Al-Attar et al., 1997). Anti-Műllerian hormone was first characterized as the factor in serum that induces masculinization of the female twin in freemartin cattle (Rota et al., 2002). Later, AMH was shown to have a masculinization effect on ovaries in culture (Capel, 2000).

Anti-Műllerian hormone production starts on day 14.5 dpc in the rat, 8.5 gestation week in humans and 42 to 43 days of gestation in the bull, reaching a peak concentration between days 50 to 80 that corresponds with the regression of the Műllerian ducts (O'Shaughnessy and Fowler, 2001; Waites et al., 1985). The expression of AMH is under the coordinated control of the following transcription factors: Sox9, SF1, *WT1*, and GATA4. Anti-Műllerian hormone expression is subject to a negative regulation by *DAX1*, FOG1, and FOG2 (Hung-Chang Yao et al., 2015).

Sertoli Cell Replication and Sex Cord Formation

One interesting aspect to consider in the early gonad developmental stage is that all cellular events are gonadotropin independent. At this gonadal time there is still no secretion of FSH or LH as the beta subunit is not produced until days 16-17 dpc in the rat and mouse and days 50 and 70 dpc in the pig and sheep respectively (O'Shaughnessy and Fowler, 2001; Skinner and Griswold, 2005). This was confirmed in hypogonadal mice (hpg) which lack GnRH and in FSH receptor KO mice where SC numbers are normal during fetal development (O'Shaughnessy et al, 2009). A similar case occurs in humans, where SC activity during fetal development is independent of hormonal stimulation as the hypothalamic-pituitary axis is not functional during the early testis development. Later, SC proliferation is clearly dependent on gonadotropin support, where high levels of FSH present in the human male fetus plays a fundamental role in testis development. In anencephalic human fetuses, the testis is smaller presenting a lower number of SC compared with normal fetuses (O' Shaughnessy and Fowler, 2014).

Using the hypogonadal model in mice, Baker and O'Shaughnessy 2001, showed that even though cell replication control in both SC and LC was independent of gonadotropins during the early fetal stage, it differed between both cell types after birth. In LC, there was no difference between normal and hypogonadal mice between 5 and 20 days postpartum with a 10% decrease in LC numbers between 20 days and adulthood. In SC however, this change was more dramatic, with no difference between normal and hypogonadal mice during the fetal stage but a much slower and extended period of cell replication was observed later, resulting in a 50% reduction in SC at day 20 and a 65% reduction in the adult. Furthermore, a lower rate of mitosis observed in gonocytes was signaled as the consequence of the slower rate of SC proliferation observed previously, accompanied with a decrease on SC activity due to the loss of FSH and testosterone

stimulation (Baker and O'Shaughnessy, 2001). A similar effect was observed in the decapitated pig, with no difference in SC numbers between decapitated and control pig fetuses (Van Vorstenbosch et al., 1984).

The formation of the testicular cords is a crucial event in the formation of the testis, characterized by an intense SC mitotic activity (Angelopoulou et al., 2008). The sex cords formation occurs between 11.5 to 12 dpc and is completed by 12.5 dpc in the mouse, 13 to 13.5 dpc in the rat, 26 days of gestation in the pig, and 7 to 8 weeks of gestation in humans (Waites et al., 1985; Ross and Capel, 2005; Skinner and Griswold, 2005; O'Shaughnessy and Fowler, 2014). Fetal cord morphogenesis is a *de-novo* process meaning that it does not happen from invagination or branching. Fetal cord formation requires a threshold of SC numbers for the process to occur, either due to the mechanics of cell aggregation or a minimum concentration of necessary factors (Hung-Chao Yao et al., 2015). During the formation of the testicular cords, SC differentiate in the XY gonad and surround PGC (Ross and Capel, 2005). Coincident with the formation of the testicular cords is the migration of mesonephric cells that surround the SC-PGC aggregates, constituting the peri-myotubular cells (PMC; McLaren, 2000). One important event in the formation of the testis cords is the synthesis of the basal lamina, which is deposited between SC and PMC and is composed of laminin, an external cytoplasmic matrix secreted by SC; fibronectin, synthesized by peritubular myoid cells; and collagens produced by both cell types (Skinner et al., 1985b). Sertoli cells are responsible for the differentiation of PMC and LC through the Desert Hedgehog (Dhh) factor, secreted by SC on E11.5, pointed as the necessary signal for the formation of the testis cords (Clark et al., 2000).

During the cell aggregation that occurs in the formation of the testicular cords, SC differentiation from mesenchymal to epithelial cells occurs characterized by the secretion of

extracellular matrix proteins (Brehm and Steger, 2005; Barrionuevo et al., 2011). It is important to note that testis cord formation is the result of an orchestrated action between the different cell types that form this structure, where the Sertoli cell *WT1* gene is involved in peri-myotubular cell differentiation while both Sertoli and peri-myotubular cells interact to produce the basal membrane and the deposition of the extracellular matrix (Wen et al., 2016).

One crucial event that occurs simultaneously with testicular cord formation, is the development of a profuse vasculature derived from mesonephric endothelial cell migration. Between days 11.5 and 12 dpc, there is an intense endothelial cell migration from the mesonephros that coalesce to form a large vessel beneath the coelomic epithelium (Ross and Capel, 2005; Skinner and Griswold, 2005).Vessel branches grow and intercalate between the newly formed testicular cords (Hung-Chang Yao et al., 2015). The development of the testicular vasculature at this stage is fundamental for two reasons: the supply of the necessary oxygen for the rapidly proliferating tissue and the transport of testosterone from the newly differentiated LC (Capel 2000; Ross and Capel, 2005). Two angiogenic factors have been described in SC: vascular endothelial growth factor (VEGF) and platelet derived growth factors (PDGF) A, B and C. (Skinner and Griswold, 2005).

Interestingly, at this stage of testis formation, the addition of retinoic acid (RA), disrupts the newly formed testis cords and the basal membrane under in vitro culture (Skinner and Griswold, 2005). Preventing the occurrence of this disturbance at this stage, SC hydrolyze RA secreting the enzyme CYP26B1, blocking the premature entry of germ cell in meiosis (Svingen and Koopman, 2013; O'Shaughnessy and Fowler, 2014).

Testicular Development

Late Embryonic and Fetal Testicular Development

The main factors determining SC numbers in the testis are the duration of the cell multiplication period and the proportion of replicating cells (McCoard et al., 2003). Two important moments of SC proliferation are described in the mammalian testis: late embryonic development, declining at birth and a postnatal SC replication period coincident with testicular growth (Orth., 1982; Cortes et al., 1987; Hochereau de Reviers et al., 1995; Skinner and Griswold, 2005).

The proliferation of testicular somatic and germ cells in the sheep occurs at a high rate of cell division without cell differentiation throughout fetal growth before day 100 of gestation. Furthermore, fetal SC in the sheep undergo at least eight mitotic divisions of which six occur before day 110 of gestation. Between day 110 of gestation and birth, SC replication diminishes, increasing again after birth (Hochereau-de Reviers et al., 1995).

In humans, between weeks 7 and 19 of gestation, fetal SC duplicate every two weeks reaching 13 x 10^6. At birth the number of SC is 260 x 10^6 cells, reaching a total number of 3,700 x 10^6 in the adult (O'Shaughnessy and Fowler 2001). It is interesting to note that during the period of fetal SC replication, the proportion of cell apoptosis remains low, implying that it is the rate of SC replication the one responsible for reaching the final size of SC population rather than the balance between proliferation and cell death (Helal et al., 2002).

In mice, SC increase rapidly during the fetal stage as shown by the high proportion of cells incorporating 3H thymidine by day 16, and in the early neonatal stage with a 6.5-fold increase. Between birth and day 5 of age, there is a 4-fold increase in SC numbers, decreasing afterwards to 1.9-fold between day 5 and 20 of age, with no further increment in SC numbers

after day 20 of age (Baker and O'Shaughnessy, 2001). By day 14 postpartum, SC start showing the adult appearance characteristics of the differentiated cell, like the typical prominent nucleolus. By day 17 pp, no more labelled SC can be seen (Vergowen et al., 1991).

In the pig, there is an increase in SC numbers per ST cross sections between days 35 and 52 dpc, and from then on, SC numbers follow a linear trend, diminishing close to and during the early postnatal stage (Van Vorstenbosch et al., 1984). For most animal species, the period of SC proliferation shows a low activity in gene expression (O'Shaughnessy et al., 2007).

Postnatal Testicular Development

Compared with the early embryonic and fetal stage, the early postnatal stage is characterized by a decrease in the rate of SC replication and a concomitant increase in cell transcription (O' Shaughnessy et al., 2009). In neonatal rats, suppression of FSH reduced the final number of SC by 40%, while a rise in FSH produced by the administration of the hormone or hemicastration, increased SC numbers by 18-49% (Sharpe et al., 2003). It is interesting to note that at this stage, in spite of the lack of FSH, the animals remain fertile, while the lack of androgens will result in a complete arrest in meiosis and infertility, reflecting the importance androgens have on spermatogenesis regulation through SC activity (Chang et al., 2004).

In the bull, three periods of testicular growth are described in relation to the levels of gonadotropins and testosterone: the infantile period situated between birth and 8 weeks of age characterized by low levels of gonadotropins and corresponding low levels of testosterone. The second period, located between 8 and 20 weeks of age marked by a transient rise in gonadotropins levels, in particular LH with a concomitant increase in testosterone, and a third period of rapid testicular growth after week 25 of age, where gonadotropins reach a baseline while testosterone concentration continue to increase gradually (Evans et al., 1994; Brito et al.,

2007; Bollwein et al., 2017). Wrobel et al. (2000), utilized MIB-1, a monoclonal antibody against Ki-67 epitope to recognize proliferating cells in the bull testicle. The diameter of the ST and cytology were added as references. When ST reached a diameter of 50 µm (50-80 dpc), a high percentage of SC and PMC cells were stained by MIB-1. When the ST diameter reached 50 to 70 µm, a remarkably high MIB-1 activity in SC was observed (5 to 10% of SC per ST cross section), decreasing steadily when ST reach more than 80 µm (7-15 weeks of age) where most SC present were MIB-1 negative.

Prepubertal Testicular Growth

Using as a reference the growth rate of the postnatal bull testicle, two periods are recognized: a slow growth period until 25-weeks of age and a posterior rapid testis growth phase until puberty between 37 to 50 weeks of age (Figure 1). During the first phase, pre-spermatogonia and spermatogonia are established, adult LC appear and immature SC replicate. During the second and rapid postnatal phase, there is an increase in ST diameter and length with a dramatic proliferation of GC (Rawlings et al., 2008) In the bull, a rise in LH starting at 4-5 weeks of age occurs, peaking at 12 weeks of age and declining around 25 weeks of age (Rawlings et al., 2008). During this period, FSH concentrations remain high, declining at approximately 25-weeks of age, when testosterone serum concentrations increase coincident with the period of rapid testicular growth (Figure 1). It is well documented how the early gonadotropin rise in the bull will determine the time at which the onset of puberty occurs (Brito et al., 2007; Barth et al., 2008). Coincident with the transient rise in gonadotropins, the neonatal period of high SC proliferation rate in the bull occurs between 4 to 25 weeks of age (Rawlings et al., 2008; Figure 1). It has been demonstrated that bulls that have larger SC populations have greater testis weight and scrotal circumference in comparison with those males with fewer SC

(Bagu et al., 2004). Furthermore, a correlation between the final number of SC established in the adult mammalian testis and daily sperm production has been established (Berndston et al., 1987a). This correlation is determined by the physical limit in the number of germ cells that each SC can support within the seminiferous tubule, imposing a logical restriction to sperm production (Orth et al., 1982; Chojnacka et al., 2016). Sertoli cell proliferation cease at puberty, when the final size of the cell population is established in the testis and therefore the ceiling of daily sperm production in the bull is fixed (Moura et al, 1997; O'Shaughnessy et al, 2011).The magnitude of the early rise of FSH and LH is one of the major factors determining the age at puberty in the bull (Evans et al., 1994). Early maturing bulls have a higher serum LH concentration during the early postnatal increase compared with late maturing bulls, presenting higher testis volume, scrotal circumference and paired testis weight (Bollwein et al., 2017). This is in accordance with the delay in testicular development observed in young bulls when the occurrence of the early rise in LH is deferred using a GnRH agonist (Chandolia et al., 1997a). On the other hand, the administration of LHRH in Hereford bulls between 4 and 6 weeks of age resulted in increased LH pulse amplitude and testosterone levels, increased spermatogenesis and higher numbers of SC per seminiferous tubule determined at 24 weeks of age (Chandolia et al., 1997b).

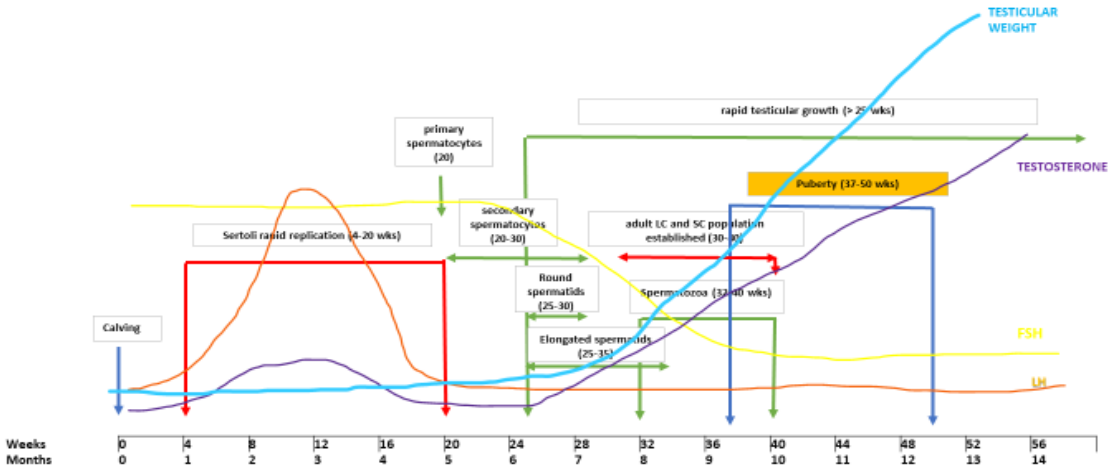

Figure 1. Testicular growth, development and cytology in the bull (Based on information from Curtis and Amman, 1981; Moura and Erickson, 1997; Wrobel, 2000; Bagu et al., 2004, Brito et al., 2007; Barth et al., 2008; Brito et al., 2012 and Rawlings et al., 2008).

Similar findings were reported by Bagu et al., (2004) after the administration of either bLH or bFSH to young bulls between 4 and 6 weeks of age. Bulls that received bFSH, reached puberty at a younger age, had a scrotal circumference greater than 28 cm, had higher numbers of SC per ST cross-section and advanced spermatogenesis at 56 weeks of age compared with non-treated bulls. On the contrary, the lack of significant effects on the bLH treated bulls could be due to the lack of a pulsatile pattern in the administration of LH.

The rapid testicular growth after 25 weeks of age in the bovine occurs when gonadotropins are low and after the occurrence of the early rise. For this reason, it has been suggested that the early rise in gonadotropins is necessary to “prime” the later rapid testicular growth, which occurs independent of gonadotropin levels (Barth et al., 2008).

Factors that Affect SC Replication and Testicular Development

Endocrinological Regulation of Sertoli Cell Replication

Several local growth factors and hormones have been reported that influence the number of SC such as IGF-1, FSH, thyroid hormone, activin, fibroblast growth factor (FGF), epidermal

growth factor (EGF), transforming growth factor α (TGF α), androgens, estrogens, and RA (Griffeth et al., 2014; Lucas et al., 2014; Meroni et al., 2019).

Androgens

Androgens were traditionally regarded as having a direct effect on immature SC replication, probably under the light of their indispensable role in spermatogenesis. In recent years, it has been demonstrated how its role in SC multiplication is exerted through other testicular cell types. Two main mechanism of actions are described for androgens and AR: one is the so called classical pathway of androgen action with a 30 to 45 minutes time period required for productive gene transcription after AR activation, and a so called non-classical testosterone signaling, which translates signals into alterations in cellular function within seconds to minutes through phosphorylation of second messenger cascades like MAPK, Akt and ERK (Smith and Walker, 2014). This non-classical mechanism has been shown to be mediated through a scaffold protein called modulator of nongenomic actions of the estrogen receptor (MNAR), which facilitates the activation of Src kinases and MAPK by androgen, forming a complex with AR and Src. The transcriptional activity of the androgen-bound AR complex is modulated by specific proteins called coregulators, either enhancing or repressing the target gene through chromatin remodeling and histone modification (Davey and Grossman, 2016).

Using three different mouse models, complete androgen receptor knock-out (ARKO), specific SC AR knock-out animals (SCARKO) and wild type (WT), Tan et al., (2005) reported how the ARKO model had a reduction in SC numbers and testis size while SCARKO animals only had minor changes in SC numbers when compared with the WT mice. The experiment suggested that the effects of AR on SC proliferation might be exerted in an indirect way. Furthermore, this finding is in agreement with the reported effects of androgens on SC

proliferation observed during this period when AR are not expressed in SC, suggesting the effects of androgens on SC replication are exerted through PMC which have a strong expression of AR at this stage (Skinner and Fritz 1985a; Tan et al., 2005). This is in accordance with the findings of De Gendt et al, (2004), that observed a reduction in SC numbers in ARKO mice but not in SCARKO individuals, although spermatogenesis was arrested later at late spermatocyte / early spermatid stage showing the essentiality of androgens through SC action on spermatogenesis and the possible role PMC play in mediating androgen effects on SC replication at earlier fetal stages (Welsh et al., 2009). In agreement with this, Rojas-Garcia et al. 2013, treating pregnant ewes with testosterone during pregnancy reported an increase in SC numbers in their lambs at 4 weeks of age with a concomitant reduction in AMH, TGF- β and TGFβ1 receptor, suggesting the mitogenic action of androgens on SC replication was done through PMC as lambs at this age do not express AR in their SC population.

On the other hand, the premature induction of postnatal AR immunolocalized in SC nuclei using a gain-of-function transgenic mouse model resulted in a decrease in the total number of SC in developing and mature transgenic testes, despite having normal or higher FSH receptor (FSHr) mRNA and circulating FSH levels (Hazra et al., 2013; Lucas et al., 2014). This early activation of AR expression in SC nucleus is due to the precocious activation of transcription factors involved in SC differentiation and cease of cell division and the early appearance of ST lumen, blood testis barrier formation through the expression of proteins involved in SC tight junctions and phagocytosis proteins (Sharpe et al., 2005).

Estrogens

Although estrogen (E2) concentrations are low in blood of the male, E2 concentrations in semen and in the *rete testis* can be higher than that detected in female blood, suggesting an

important role for E2 in the testis. The main source of testicular E2 is the conversion of testosterone, by the action of P45019 A1 aromatase enzyme encoded by the *CYP19* gene (Meroni et al., 2019). In the immature rat, the main origin of E2 are SC while in mature rats, Leydig cells become the main source of E2, although aromatase is present in both cell types (Lucas et al., 2011). Moreover, the presence of both E2 receptors (ER1 and ER2 or ERα and ERβ respectively) has been reported in the nuclei of immature rat SC in vitro. It has been reported how the activation of ERα results in the proliferation of immature rat SC involving the upregulation of cyclin D1. Furthermore, the phosphorylation and translocation of both receptors to the cell membrane has been described activating the mitogenic pathway MAPK3/1 (Lucas et al., 2008). On the other side, the activation of ERβ promotes the exit of the cell cycle, maturation of SC and cease of cell replication (Meroni et al., 2019). Nevertheless, Berger (2019) using an aromatase inhibitor in bulls between 2 to 22 weeks of age to produce a decrease in systemic and testicular estradiol, did not observe differences in the absolute numbers of SC per testis, testis weight or changes in FSH, LH and T.

Insulin Like Growth Factor-1

There is mounting evidence in most mammals of the key role of IGF-1 in SC proliferation during the fetal and neonatal period. Villalpando et al. 2008 showed in the mouse how the expression of both genes for IGF-1 and IGF-1 receptor was coincident with the fetal and perinatal peaks of SC replication at E16 and E18 days respectively. A similar finding was done on LC, coinciding with the peak of cell proliferation between E14-18, suggesting that at this stage of the fetal testis, the synthesis of more steroids is necessary to maintain the male phenotype (Villalpando et al., 2008).

With the advent of gene knock out animal models, Pitteti et al., 2013, using mutant mice for IGF-1 and Insulin receptors in SC, reported a 75% decrease in testis size and daily sperm production caused by a reduced proliferation rate of immature SC during the late fetal and early neonatal testicular period. They demonstrated how the insulin/IGF signaling pathway is essential in mediating the FSH proliferative action on immature SCs, where the rise in FSH produced by unilateral castration in the Insulin/IGF-1 receptors KO mice did not have any effect on SC numbers and testis size as occurred in the hemi-castrated control animals (Pitetti et al., 2013). This complementarity observed between FSH and IGF-1 was later countersigned by Dance et al. (2017), studying the combined effect of FSH and IGF-1 when added simultaneously in cultured immature SC collected from 8-week old bulls. They observed a significant difference in SC proliferation under the combined treatment of FSH and IGF-1 (1.5-fold) compared with an FSH-only and control treatment. The combined effect of FSH and IGF-1 demonstrated FSH requires IGF-I signaling pathway to mediate its action. Two major pathways have been described as activated by the Tyrosine-kinase family (Insulin/IGF-1) of receptors: the IP3K and the MAPK pathways, both involved in cell growth, proliferation and apoptosis (Griffeth, 2014). Akt, in the PI-3 kinase pathway has been suggested as the location where FSH and IGF-I exert their interaction in agreement with the observation that FSH increases Akt phosphorylation concomitant with an increase in IGF-1 (Khan et al., 2002; Dance et al., 2017).

Follicle Stimulating Hormone

Follicle stimulating hormone (FSH), is long known as a mitogenic factor in immature SC both *in vivo* and *in vitro*, (Lucas et al., 2014). Almiron and Chemes (1988), working with early neonatal rats (less than 10 days of age) demonstrated how when FSH levels were reduced by the administration of testosterone, there was a 25-30% reduction in the mitotic index of tubular cells

and in the labeling index of SC. Furthermore, when FSH levels were returned to normal or to higher than normal levels, the mitotic and labeling SC indexes returned to normal values. Moreover, Meachem et al. 1996, demonstrated that the administration of recombinant FSH in neonatal rats between birth and 20 days of age increased testicular weights, length and volume of ST, and resulted in a proportional increase in germ and SC producing a testicular hypertrophy that persisted into adulthood, increasing the spermatogenic potential of the individual. This is in agreement with the findings of Loveland and Robertson, 2005, who reported in 3-day old neonatal rats, that SC proliferation was FSH-dependent coinciding with the peak of SC replication for the specie, and how the maintenance of cell replication by day 9, required the addition of activin A to FSH, while on day 18, no cell proliferation was observed. It has been shown that the suppression of FSH in rats, reduces the final number of SC by 40% while the occurrence of a hormonal rise caused by neonatal hemicastration, produces an increase in SC between 18-49% (Sharpe et al., 2003). Furthermore, the FSHR disruption in transgenic rodents (FSHRKO), causes an important reduction in SC and germ cell numbers although individuals remain fertile (Lucas et al., 2014). In rat gonads, FSH receptor (FSHr) activity can be detected on days 14.5 of gestation with a four-fold increase in activity during the peri natal stage, between days 19.5 to 21.5, coincident with the peak of SC proliferation in the rat. In another experiment, a rise in FSHr after birth coincident with SC proliferation was reported between days 2 to 21 of age and two more FSHr increments on days 40 and 60 after birth (Walker and Cheng, 2005). In agreement with these findings, Allan and Handelsam 2005 reported that the exposure of neonatal rats to supraphysiological levels of recombinant FSH, resulted in testicular hyperplasia caused by an increase in SC numbers.

Bagu et al. (2004) injected bulls between 4 to 8 weeks of age with bovine FSH every two days observed an increment in serum FSH, scrotal circumference, earlier onset of puberty and an increase in the number of SC and germ cells per ST cross section vs. no treated animals. This is in agreement with the findings of Harstine et al. 2017 who treated bulls between 35 to 91 days of age injected with a slow release FSH formulation, observed an increase in the number of SC per ST cross section although there was no concomitant increment in Activin A or changes in testosterone concentrations in serum. Overall, FSH constitutes an important perinatal SC mitogenic factor in most animal species, determining the final number of SC established in the testis.

Thyroid Hormone

It has been known for some time that thyroid hormones inhibit both *in vivo* and *in vitro* SC cell proliferation and induce cell maturation in the prepubertal testis (Meroni et al., 2019). The high levels of expression of T3 receptors in proliferating SC suggest that SC are a major target for thyroid hormone regulation in most mammals (Santos Wagner et al., 2008). Thyroid hormones have the particularity of altering the period at which SC cells replicate by regulating cell differentiation. Thyroid hormones will produce a delay or advance on the age at which SC multiplication stops according to lower or higher concentrations of the hormone resulting in an increase or decrease in the final size of the SC population established in the testis (Sharpe et al., 2003). In humans, hypothyroidism during the neonatal stage results in an increase in testicular size and a delay in the onset of puberty (Lucas et al., 2014). Thyroid hormone receptors in human SC are present at week 17 of gestation with the expression of both the protein and the transcript as early as week 12 of gestation, coincident with the exponential replication of SC (O'Shaughnessy and Fowler, 2001). Auharek and França 2010, induced a transient hypo and

hyperthyroidism in neonatal mice using propylthiouracil (PTU) and T3, resulting in an increase and decrease respectively in the number of SC. A concomitant rise in the number of spermatogonia, and the number and volume of capillaries was observed associated with the increment in the number of SC in PTU treated mice. Furthermore, PTU mice had higher SC counts and greater daily sperm production at 100 days of age. On the other side, those mice treated with T3 had at the same age, lower daily sperm production when compared with control animals. A similar effect was observed in Meishan pigs, which have a shorter SC proliferative period when compared with other breeds, resulting in smaller testis and smaller seminiferous tubules in the adult. The suggested cause is a transient period of hyperthyroidism described during the late fetal stage (Skinner and Griswold, 2005). In the bull, Waqas et al. 2019 showed how an induced transient hypothyroidism using methimazole during the period of SC replication in the bull resulted in higher numbers of spermatozoa with no difference in morphology, motility, post-thaw motility, cleavage and embryo yield in IVF than control bulls at 28 months of age. Furthermore, the extension of the SC replication period for one month in treated bulls, resulted in higher testicular weights and SC counts per ST cross sections in adulthood.

The mechanism suggested for the control of SC proliferation by thyroid hormones is the effect on two specific cyclin dependent kinases inhibitors (CDKIs) of the cell cycle: $p27^{Kip1}$ and $p21^{Cip1}$, and by another mechanism involving Cx43, a structural gap junction protein (Santos Wagner et al., 2008). Gap junctions form intercellular channels among SC and between SC and germ cells, playing a key role in the regulation of cell differentiation and replication.

In vitro studies done in rats showed that FSH and T3 exert an additive effect on SC proliferation, inducing the gradual expression of AR and the consequent decrease in AMH (Sharpe et al., 2003). It has been postulated that the absence or weak expression of AR in SC

cells during the early neonatal period in humans and marmosets in the presence of high concentrations of FSH is part of a regulatory mechanism that prevents the precocious differentiation of SC and early entry in puberty (Sharpe et al., 2003). Later in puberty, with higher FSH, T3 will enhance androgen binding and AR mRNA expression, inducing puberty. Furthermore, the addition of FSH and T3 to 5 and 20-day old rat SC culture increased the expression of AR mRNA. The simultaneous addition to the culture media of both FSH and T3 resulted in a higher increase in AR mRNA than with any of the factors alone (Arambepola et al., 1998). Another component of SC maturation where T3 is known to have an effect is the remodeling of the ST basal membrane, where T3 increases the expression of laminin and entactin with a concomitant reduction in the production of collagen IV (Santos Wagner at al., 2008).

Transforming Growth Factors

The extensive growth of the testis observed in most mammals during late embryonic age, reflects the needs for growth factors for the sustainment of testicular development during this period. Two families of growth factors have drawn attention during the last years as playing a significant role in SC replication: EGF/TGF-α and TGF-β. The Epidermal Growth Factor (EGF) family includes Transforming growth factor α (TGFα) and epidermal growth factor (EGF), which have been demonstrated to affect SC proliferation during the testis cord formation and the late embryonic testis stage (Levine et al., 2000). The addition of antibodies against TGF-α severely affected testis growth in rats with a decrease in SC and interstitial cell replication under culture conditions. As a difference, interstitial cell division was more severely affected in EGFR KO animals suggesting a cell specificity for both growth factors (Levine et al., 2000). Petersen et al. 2001, observed a dose dependent increase in SC cell replication when TGF-α and EGF were

added to the culture media. Although, no increment in the stimulatory effect of SC replication was observed when both factors were added simultaneously, suggesting both growth factors might share common receptors.

Within the TGF-β superfamily, more than 40 members have been described. The most studied are: AMH, TGF-β1, TGF-β2, TGF-β3, Activin A, Activin B, Inhibin, Bone morphogenic proteins (BMP), growth and differentiation factor 9 (GDF9) and glial cell line-derived neurotropic factor (GDNF), (Poniatowski et al., 2015). Inhibin is produced by SC and to a lesser extent LC and was first identified due to their FSH suppressing activity both in vitro and in vivo. Both testosterone and inhibin regulate FSH production, where FSH activates cAMP which in turn activates the production of inhibin by SC, suppressing FSH by pituitary cells in a classic feed-back mechanism. On the contrary, activins are characterized by their FSH stimulation properties on pituitary cells (Klaij et al., 1992). One important difference between activin and inhibin is that the α subunit is only produced by SC and LC within the testis while both βA and βB activin subunits are produced by several cell types like SC, LC, PMC, and germ cells forming dimers in the cytoplasm (Meroni et al., 2019). In the murine, activin A is the product of fetal LC acting on SC, promoting their proliferation during late embryogenesis (Archambeault and Yao, 2010). The paracrine effects among testicular cells have been shown using genetic disruption of the activin A gene in fetal LC, resulting in the failure of fetal testis cord elongation and expansion, due to a decreased SC proliferation (Archambeault and Yao, 2010). Activin A has also been shown to regulate AR expression and FSH-induced aromatase activity (Loveland and Robertson 2005). In addition, Buzzard et al., 2003 demonstrated another synergistic effect of activin A on FSH action through the increment in the expression of FSH receptors in SC cells under in vitro conditions (Buzzard et al., 2003). This agrees with the findings of Fragale et al.,

2001 who described the temporal and spatial association between activin and the appearance of FSH receptors in SC during early postnatal gonad development in the rat coincident with the period of rapid SC. A precise temporal change in concentrations and localization of activin and inhibin in mice was described by Barakat et al., 2008, showing high concentrations of activin in SC during the proliferation stage with a gradual descent in concentration and concomitant rise in inhibin and FSH associated with SC maturation in the postpartum rat and mice. Furthermore, by the time of SC differentiation, cell production of the cell cycle inhibitor $P27^{kip1}$ increase alongside with an increment in the affinity between $P27^{kip1}$ with cyclins D2/E1 and CK4/2, caused by inhibin, inducing the arrest of the SC cell cycle (Loveland and Robertson, 2005). Another member of the TGF-β superfamily GDNF, has been shown to increase the rate of cell division of SC in early neonatal rats. Hut et al. 1999 demonstrated that SC culture of 6-day postpartum rat testicles increased cell replication rate when cultivated with GDNF and FSH simultaneously. The stimulation was dose and time dependent and was specifically inhibited when cultured with anti-GDNF and anti-GDNF receptor (Hu et al., 1999).

Other members of the TGF super family described as having regulatory effects on activin and inhibin are GATA4 which has been characterized as a regulatory factor in the synthesis of the β subunit along with IGF-1 and IL-1. As a difference with the other superfamily members, BMP proteins are key to germ cell formation, responsible for the proliferation and migration of germ cells to the genital ridge during the early gonad development, and in cell aggregation during the process of sex cord formation (Loveland and Robertson, 2005).

Retinoic Acid

Although the fundamental role of vitamin A in spermatogenesis was demonstrated many decades ago, very scarce recent information exists about the role of vitamin A on gonadal

development in the bull. Most of the experiments were conducted in rodents and under in vitro conditions.

Two main biological active compounds for vitamin A have been described: retinoic acid (RA) and retinol (ROL). Sertoli cells are responsible for the uptake, oxidation, signaling, degradation and storage of retinoids in the testis (Livera et al., 2002; Griswold, 2015). When retinol reaches the Sertoli membrane, the Stra6 receptor is internalized and converted in a two-step enzymatic process into the active metabolite all-trans RA, which is later hydroxylated and degraded.

Vitamin A exerts its effect through two different intracellular receptors families: RAR with its variants α, β, γ and RXR. Retinoic acid receptors RARα, β and γ have been described in the nucleus of SC (Galdieri and Nistico 1994; Livera et al., 2002). Retinoic acid receptors belong to the nuclear receptor super family. RAR bind to both all-trans and 9-cis RA while RXR only binds to 9-cis RA. The conversion of all-trans to 9-cis RA occurs by spontaneous isomerization or by the action of isomerases (Marill et al. 2003). Once bound, both homo and heterodimers receptors recruit coactivator proteins initiating transcription by binding to specific response elements situated in the promoter region of the gene (Duester, 2000).

In 1946, Erb et al., fed suboptimal levels of vitamin A to young dairy bulls and using testicle biopsies described the atrophy suffered by the germinal epithelium and the development of night blindness in some of the deprived animals. Rode in 1994, studying the effects of hypovitaminosis A in 16-month old bulls, reported a decrease in paired testicular weight, daily sperm production and sperm epididymal reserves. In vitamin A deficient rats, Thompson et al. 1964, reported a complete halt in spermatogenesis where the ST were populated only by SC and pre-meiotic germ cells.

The effects of RA reported on rat SC cultures *in vitro* can either be pro-differentiation causing the exit from the cell cycle ceasing replication, or a pro-proliferative effect, increasing SC numbers and the secretion of SC specific growth factors. It seems these antagonistic effects depend on the age of the cell culture at which RA is added to the medium.

The addition of RA to 13.5 to 14 dpc fetal SC rat culture resulted in the disruption of the formation of the sex cords and a decrease in the number of gonocytes (Cupp et al., 1999). The disorganization caused on the sex cords by adding RA to the culture media is thought to be the consequence of the disruption of the deposition of laminin and collagen IV in the basal lamina. Interestingly, when RA was added to day 3 post-partum rat SC culture, the mitotic index of SC was enhanced via RARβ (Livera et al., 2000). Moreover, retinoids have been reported to stimulate the secretion of transferrin, androgen binding protein, insulin like growth factor binding protein 4 and inhibin α by SC (Livera et al., 2002).

Nicholls et al., 2013 aded all trans-RA to immature neonatal SC rat culture and reported a pro-differentiation effect of RA neutralizing the pro proliferative effect of activin, blocking the entry of the SC into the cell cycle, and initiating the formation of the BTB. Furthermore, Cupp et al., 1999 showed how RA inhibited the proliferative effects of FSH and EGF on day 0 postpartum rat SC cultures and suggested the effect was mediated through the stimulation of the production of the inhibitor factor TGFβ. This is in accordance with the findings of Livera et al., 2000 who reported a decrease in the production of FSH-stimulated cAMP and reduced mitosis when RA was added to rat SC cultures.

Fetal Nutritional Programming

The intrauterine environment can be a major factor affecting fetal growth (Wu et al., 2004). This influence exerted in utero can have significant effects in the offspring wellbeing

extending after birth into adulthood (Wu et al., 2004; Funston et al., 2010; Reynolds and Caton, 2012). The early implantation period and the rapid phase of placental growth are described as periods of greater embryo and fetal susceptibility to maternal undernutrition. It is not surprising that the effect of the mother's nutritional restriction may be due to an underdeveloped placenta and consequently lower fetal oxygen and nutrient uptake (Reynolds et al., 2005).

Another proposed mechanism for the altered embryo and fetal growth is the change in the expression of the fetal genome by the uterine environment, also known as "fetal programming". Epigenetics provides a mechanism of action where the nutritional status of the mother can alter the expression of the fetus genome through DNA methylation and histone modifications (Reynolds and Caton, 2012). Placental angiogenesis was affected in sheep that were nutritionally restricted during early pregnancy (days 30 to 125) and re-alimented on days 125 to 150 of gestation (Funston et al., 2010). This period coincides with placenta differentiation and growth. During the early stages of fetal development, fetal organogenesis coincides with maximum placental growth and differentiation, being critical for normal fetal development (Funston et al., 2010). During the fetal life, the individual goes through a series of critical periods of development that coincide with phases of rapid cell division (Barker et al., 1997). The lack of oxygen or low nutrients can slow down cell multiplication acting either as a direct undernutrition effect on the cell or through the altered concentrations of hormones and growth factors (Barker et al., 1997). These effects can reduce permanently the number of cells in specific organs like the testis (Da Silva et al., 2001). Robinson et al. 1999 reported how lambs from ewes that gained 17% in bodyweight during the second half of pregnancy had more SC in their testes at birth than those lambs from ewes that just maintained their bodyweight over the same period. This is in accordance with the findings of Bielli et al. 2002 where lambs from mothers that were restricted

in their ME intake (70% requirements) from week 10 of pregnancy to parturition had fewer Sertoli cells than those lambs from mothers that received high ME intake (110% requirements). Furthermore, Kotsampasi et al., 2009 showed how lambs born from ewes that were nutritionally restricted between 31 and 100 days of pregnancy, had higher FSH and LH levels with lower Sertoli cell numbers and seminiferous tubule diameters when subjected to a GnRH challenge at 10 months of age. This suggested that some of the *in-utero* effects on the fetal male gonad can be manifested later in the individual's reproductive life, after puberty. Nevertheless, Asmad et al. 2013 found no differences in SC numbers and testis size in lambs whose mothers were fed *ad libitum* or at maintenance during pregnancy. Also, similar effects were found in other species like the rat where pups from undernourished mothers during pregnancy and lactation presented lower numbers of Sertoli cells, affecting their sperm production as adults (Genovese et al., 2010).

In one of the few studies conducted in cattle about the effects of different planes of nutrition in pregnant dams, *Bos Indicus* crossbred heifers were subjected to high and low protein and energy diets during the first and second trimester of pregnancy. Lower protein and energy levels during gestation resulted in higher prepubertal FSH concentrations and paired testicular weights. Interestingly, an association between IGF-1 and prepubertal FSH concentrations was established (Sullivan et al., 2010).

Postnatal Nutrition

It has been demonstrated how GnRH and ultimately gonadotropin release can be regulated by "metabolic sensors" which translate the signals provided by circulating concentrations of specific hormones and nutrients into neuronal signals. Hormones such as Insulin and IGF-1 have been implicated in metabolism regulation acting as modulators of GnRH release by the hypothalamus (Blache et al., 2000). Other metabolic factors suggested as

modulators of GnRH and gonadotropin secretion are glucose, leptin, volatile fatty acids and, some amino acids. Brito et al. 2007, grain-supplemented young beef bulls from 10 to 30 weeks of age resulting in a sustained and higher LH secretion during the early rise in gonadotropins. To discard a glandular effect, the bulls were subjected to a GnRH challenge showing that gonadotropin secretion capacity by the pituitary was unaffected by the nutritional treatment. Furthermore, they observed an increase in Testosterone and a concomitant temporarily associated IGF-1 rise in supplemented bulls, resulting in higher testicular weights and sperm production when the bulls reached maturity (Brito et al., 2007). In a similar series of experiments designed to study the effect of different planes of nutrition during the pre-pubertal period in the bull, Barth et al. 2008 reported that the temporal association observed between IGF-1 and increased GnRH/LH secretion reached a plateau once sexual development was completed. This association suggested a possible regulatory role of IGF-1 on GnRH and so on testicular growth and development in prepubertal bulls. It is interesting to note that in the experiment, no difference was observed between treatments in the concentration of other hormones like Insulin, Leptin and Growth hormone which have been proposed as potential metabolic sensors. Following the same line of research in dairy bulls, Dance et al. 2015 subjected three groups of animals to three different planes of nutrition (low, medium and high) between 2 and 31 weeks of age after which all bulls were put under a medium plane diet. They reported that those bulls under the high nutritional plane reached puberty at an earlier age and had heavier testicles at 71 weeks of age. Furthermore, bulls on the high nutritional plane showed an earlier and more sustained level of LH temporarily associated with higher levels of IGF-1. In a similar experiment aiming to compare two dairy breeds and two planes of nutrition (high and low) during the infantile period in bulls, higher systemic levels of IGF-1, insulin, LH and resulting

testosterone levels were reported in those bulls under a higher plane of nutrition with no changes in leptin levels between groups (Byrne et al., 2017).

Sertoli Cells and the Energy Metabolism of Germ Cells

Sertoli cells provide the physical support, growth factors and nutrients for GC development and constitute the BTB controlling the milieu inside the ST creating the microenvironment necessary for GC differentiation (Skinner and Griswold 2005). The BTB is one of the tightest blood barriers in the body, excluding all molecules bigger than 1,000 Da (Alves et al., 2013). For this reason, the situation of those GC beyond the BTB makes them completely dependable on SC for their nutrient supply (Regueira et al., 2015).

While SC can utilize glucose as an energy substrate and convert it to lactate, spermatocytes and spermatids are unable to use glucose as their energy source, depending on the exogenous lactate provided by SC (Regueira et al., 2015). The preference by some cells to use fermentative metabolism of glucose instead of the more efficient oxidative metabolism is known as the “Warburg effect” and is characteristic of tumor cells and SC in the testis (Vander Heiden et al., 2009; Oliveira et al., 2014). This is an intriguing fact, as aerobic glycolysis is an inefficient way to produce energy, where only two ATP molecules are produced from glucose to lactate, in comparison to mitochondrial oxidative phosphorylation which can produce up to 35 ATP per glucose molecule (Voet and Voet, 2010). One postulate of the Warburg effect is that proliferating cells use aerobic glycolysis as a protective mechanism against oxidative stress, as the activation of the pentose-phosphate pathway (PPP) in glycolysis produces high levels of the anti-oxidative by-product NADPH. Furthermore, it has been shown that the PPP is necessary for maintaining the biosynthesis of nucleotides and ribose-phosphate essential for RNA synthesis in proliferating cells (Oliveira et al., 2014; Alves et al., 2013).

It has been shown that when isolated spermatocytes and spermatids are treated with lactate in their culture media, an increase in mRNA and oxygen utilization is observed which does not occur when glucose is added to the media (Boussouar and Benahmed, 2004). Spermatogonia constitute an exception, as being seated against the basal membrane of the ST allows them to uptake glucose directly from blood circulation (Boussouar and Benahmed, 2004). Lactate dehydrogenase isoenzymes are formed by random association of four subunits to make homo or hetero tetramers. These subunits are encoded by three genes: *Ldha, Ldhb and Ldhc*. *Ldhc* is exclusively expressed in germ cells, and as expected, the disruption of the Ldhc gene results in male infertility caused by a decrease in sperm motility produced by the failure to develop the hyper-activated motility necessary for fertilization and a reduction in ATP production (Alves et al., 2013). Glucose is taken up from the extracellular space via glucose transporters (principally GLUT1 and GLUT3) and converted to lactate, constituting lactate dehydrogenase (LDH) fundamental for the conversion of glucose to lactate. Lactate is transported across the plasma membrane by monocarboxylate transporters (MCT), (Riera et al., 2009).

The exposure of isolated spermatids to glucose as the only energy source, results in ATP depletion, showing that glucose metabolism is not adequate to supply spermatids with energy (Grootegoed et al., 1986). Sertoli and cancer cells can use alternative routes to glucose utilization like glutamine and glycogen for ATP production (Oliveira et al, 2014). Furthermore, SC maintain a high acetate production for the *de novo* synthesis of phospholipids, fundamental for membrane synthesis of proliferating cells in processes like spermatogenesis (Alves et al., 2013). β-oxidation in SC is important to produce high ATP levels necessary for spermatogenesis while playing a key role in re-cycling lipids as one important role of SC is to phagocytize and degrade

apoptotic germ cells producing lipids that will be used as ATP sources (Xiong et al., 2009; Alves et al., 2013).

The production of lactate production is under endocrine control, principally by FSH, Insulin and IGF-1, while it has been reported the increase in lactate production by paracrine and autocrine factors such as epidermal growth factor (EGF), transforming growth factor β (TGFβ), basic fibroblast growth factor (bFGF), tumor necrosis factor α (TNFα) and interleukin 1α (IL-1α) (Boussouar and Benahmed, 2004). Three mechanism have been described for the control of lactate production by growth factors: the activation of glucose uptake, LDH activity and *Ldha* gene transcription.

Rato et al., 2012 showed how the addition of DHT increased the utilization of glucose by SC under in vitro culture while decreasing the production of lactate through a decrease in the expression of LDH-A and MCT-4 which is fundamental for the export of lactate from SC. This finding suggested glucose is re directed by DHT towards the use of glucose by the Krebs cycle increasing the efficiency of glucose utilization and highlighting the role of DHT as an energy modulator in SC. On the other hand, deprivation of human SC culture of insulin produces a decrease in the expression of LDH-A and MCT-4 mRNA (Alves et al., 2013).

FSH controls spermatogenesis through the phosphorylation of PIK3 which in turn activates mitosis, oxidative burst and glycolysis. When a PIK3 antagonist was added to the SC medium, it was shown that the increase in lactate production and LDH activity by FSH was blocked (Alves et al., 2013). Another mechanism proposed for the stimulation of lactate production by FSH is through the activation of hypoxia inducible factors (HIF) expression. When SC were incubated under the presence of LW6 which degrades the HIFα subunit, FSH was unable to increase lactate production through a decrease in glucose uptake, GLUT-1,

pyruvate kinase M ½ (Pkm2) and LDH activity (Galardo et al., 2017). On the other hand, Oliveira et al., 2012 reported how human SC cultures deprived of insulin for 48 h exhibited a decrease in GLUT-1, MCT4 and LDH mRNA, showing how insulin acts as an important energy modulator in SC.

The addition of T3 to the medium of SC cultures, increases protein synthesis and lactate production. Furthermore, in rat immature SC cultures, Carosa et al., 2005 reported an increase in mRNA expression of GLUT-1 with the addition of T3 although T3 did not affect GLUT-1 promoter activity suggesting a post-transcriptional regulation mechanism.

It has been known for some time the regulatory role that paracrine factors like bFGF and IL-1 play in the regulation of SC metabolism. Riera et al., 2002 demonstrated how the addition of bFGF to rat SC culture increased the production of lactate in a dose dependent manner. Furthermore, the addition of bFGF increased glucose transport, LDH activity and the production of mRNA for GLUT-1 and LDH-A.

On the other hand, IL-1α increased lactate production in a dose and time dependent manner when added to purified pig SC culture through an increase in glucose uptake, and the redistribution of LDH isoforms in favor of LDH-A expression and the activity of LDH-A4 which transforms pyruvate into lactate (Nehar et al., 1998).

Sertoli Cells and the Immunological Protection for Germ Cells

Three distinctive processes are recognized during spermatogenesis: mitosis (where the spermatogonia goes through a series of cell divisions), meiosis (the spermatocyte goes through a process of DNA recombination, reduction and division) and spermiogenesis (spermatid formation). The resulting meiotic and post meiotic cells that appear around puberty, after the

establishment of the systemic immunotolerance, express novel surface and intracellular antigens that might elicit immune reactions against them (Tung and Fritz, 1978).

It has been shown how the BTB constituted primarily by the apposition of the bodies of adjacent SC and the formation of tight junctions between SC, isolate GC from the host immune system (Mital et al., 2011). Furthermore, it has been demonstrated under normal conditions the detection of immune cells like macrophages, T cells and dendritic cells in the interstitial space while their presence beyond the PMC could not be detected, being excluded from the basal and ad luminal compartments of the ST (Kaur et al., 2013).

In addition, SC are capable of secreting immune modulating factors. The culture of lymphocytes with the addition of interstitial and intra ST fluid, resulted in a decrease in lymphocyte proliferation (Kaur et al., 2014). This role was clearly demonstrated using NOD (non-obese diabetic models), which are type 1 autoimmune diabetes, where co-xenotransplantation of Langerhans islets with SC resulted in the prolonged survival of the transplanted islets compared to controls. This showed that the immune protection and modulation exerted by SC was not limited within the mechanical limits provided by the BTB in the testis (Suarez-Pinzon et al., 2000). Moreover, it was suggested that this protection was mediated through TGF-β which transformed immune cells from interferon-γ type that infiltrate and destroy islets cells to IL-4 nondestructive cell phenotype.

Cytology Assessment in the Bull Testicle

Introduction

Immunohistochemistry (IHC) uses the principle of antibody binding to specific proteins to detect precise cells in a tissue. The expression of specific SC markers is a changing process that varies with time, following the progression of cell growth and differentiation, reflecting

consequently a complex and extremely dynamic course. The selection of a specific SC marker expressed at the time of interest in the animal is crucial for an accurate assessment of the cell population at that specific age of the bull. Both AR and GATA4 proteins used in the present series of experiments are only expressed by SC within the ST micro-structure of the testicle at the age of the bull considered.

Fine Needle Aspiration

Implementation of the breeding soundness examination (BSE) has improved the ability to detect bulls that are satisfactory potential breeders from those not apt to breed. The addition of semen analysis to the BSE after the development of safe and reliable electro ejaculators to collect semen samples from unhandled bulls has improved the efficacy of this examination (Chenoweth, 2002; Chenoweth and McPherson, 2016). Nonetheless a significant range of fertility levels within the group of bulls classified as apt, remains undetected (Kastelic and Thundathil, 2008). A satisfactory potential breeder is a bull that has a BSE that reaches a minimum scrotal circumference (usually 28 cm), presents ≥ 70% morphological normal spermatozoa, and ≥ 30% progressive motility (Barth and Waldner, 2002). Nevertheless, data from AI centers show that bulls above the established baseline can yield pregnancy rates that differ by more than 20 to 25% (Kastelic and Thundathil, 2008). Bull breeding soundness examination is less effective at predicting fertility in the upper end than at the lower end of the bull fertility spectrum (Chenoweth, 2002). Bull fertility is affected by several factors and no single diagnostic test can predict an individual's fertility (Rodriguez-Martinez, 2003). The use of multi-sire breeding in extensive systems, low reproductive pressure, and prolonged breeding seasons contribute to disguise this situation where it is very difficult to assess each individual bull's contribution to the overall herd fertility (Coulter, 1997; Chenoweth, 2004).

One important criterion for bull selection and one of the most important measurements collected when performing a BSE is scrotal circumference. Scrotal Circumference is highly correlated with paired testicular weight, so what we are really doing when measuring scrotal circumference is determining the amount of potential sperm producing tissue within the testis (Coulter, 1997) as each gram of normal testicular parenchyma can potentially produce approximately 17 x 10^6 sperm per day (Amman and Almquist, 1962).

Scrotal circumference is extensively used as a reference to determine puberty of the bull regardless of age and body weight (Lunstra, 1982). It has also been demonstrated that heifers of bulls with higher scrotal circumference enter puberty at an earlier age. The Society for Theriogenology in 1992, established minimum thresholds for scrotal circumference that a bull needs to achieve in relation to age, to be classified as a satisfactory potential breeder. All bulls that do not comply with this minimum measurement, will either be classified as an unsatisfactory potential breeder or as classification deferred (Chenoweth, 2002).

With the increased application of genomics in AI sire selection, semen from bulls is collected and marketed at a younger age. Genomic selection is not only significant in livestock genetic improvement but also has an impact in AI center's profitability by increasing the total number of semen doses collected and marketed (Dance et al., 2016). Although, the assessment of a bull's ability to mate and semen quality is relatively easy to determine in the field, their value as predictors of a bull's fertility is limited (Parkinson, 2004). None of the actual methods of selection of future breeding bulls can predict the potential daily sperm production ability and the fertility of the individual (Rajak et al., 2013). Furthermore, at present, no estimates of heritability of the probability of a bull passing a BSE has been evaluated (Cammack et al. 2009).

Sertoli cells role in spermatogenesis has long been recognized for a long time (Sertoli, 1865) through the establishment of the blood testis barrier, providing physical contact support and contributing to the microenvironment necessary for germ cell development (Sharpe et al., 2003). Sertoli cell proliferation is confined to a specific window of time that extends from the fetal stage at mid-gestation to 20 weeks in the newborn calf (Moura and Erickson et al, 1997; O'Shaughnessy and Fowler 2011). The final number of Sertoli cells is established at puberty and will limit the number of germ cells that can be sustained, fixing the ceiling of daily sperm production in the adult bull (Berndston et al., 1987b; O'Shaughnessy and Fowler 2011). Furthermore, early events occurring during Sertoli and Leydig cell differentiation are potential indicators of testicular growth at later ages (Moura et al., 2011).

Accurate quantification of the final number of Sertoli cells in the adult bull could be an important tool to predict potential daily sperm production of the individual. To our knowledge, no studies have been conducted to determine the Sertoli cell population in the prepubertal individual as a predictor of its daily sperm production as an adult bull. Furthermore, the value of determining the size of the Sertoli cell population in the prepubertal bull as a selection tool for replacement bulls remains unexplored.

The first applications of the Fine Needle Aspiration (FNA) technique were for the diagnoses of suspected neoplasm in different human tissues (Martin and Ellis, 1930). The earliest report of the use of FNA to collect a testicular sample was performed by Stewart et al. 1933 for the diagnosis of testicular cancer in humans. Interestingly, both pioneers described the FNA technique almost exactly as it is applied today. An 18G needle was inserted into the tissue of interest using a reverse and forward motion while applying negative pressure using a syringe.

The main advantages of FNA are reliability, simplicity, safety, and low invasiveness and were recognized early by both Martin et al., 1930 and Stewart et al. 1933 as well as continuing to be endorsed by a long succession of subsequent researchers (Craft et al., 1997; Webb, 2000; Heath et al., 2001; Leme and Papa, 2010). The use of FNA to collect tissue samples was soon compared with the application of open biopsy and the first high correlations between diagnoses made from smears using FNA and conventional histology was established, leading to the rapid expansion in the application of FNA for oncology diagnosis in human patients (Godwin, 1956; Webb, 2000).

The interest in the use of testicular FNA rapidly expanded to other areas of human medicine outside oncology, like the diagnosis of male reproductive pathologies (Aridogan et al., 2003), the assessment of fertility in patients (Craft, 1997), and the retrieval of spermatozoa in assisted reproductive techniques for intracytoplasmic sperm injection (Dohle et al., 2012). In patients with suspected occlusive pathology of testicular efferent ducts with normal FSH concentrations, FNA is widely used to assess spermatogenesis through the success or failure of retrieving spermatozoa (Aridogan et al., 2003).

Craft et al., 1997 obtained intact seminiferous tubules (ST) from patients using FNA with19 G or 21G needles. This provided an additional advantage for the technique as the preservation of the ST structure, and consequently the germinal epithelium, enabled a precise assessment of all stages of spermatogenesis. Furthermore, FNA offers the possibility of sampling different tissue areas for a better parenchyma representation or when a first attempt does not yield enough tissue (Craft et al., 1997; Leme et al., 2018).

On the contrary, using finer needle calibers results in the disruption of the ST structure and therefore, loss of topographical references and the dispersion of the different testicular cell

types, requiring analysis of the smears by an experimented testicular cytologist (Leme et al., 2018). The need for a trained technician is one of the reasons signaled as a restraint to the widespread adoption of FNA for the assessment of spermatogenesis in most animal species, including the bull (Craft et al., 1997). Other disadvantages generated by the disruption of the testicular microanatomy structure in FNA smears is the impossibility of assessing other parameters like ST diameters, lumen areas, basement membranes, and interstitial space (Craft et al., 1997; Aridogan et al., 2003).

Although FNA advantages surpass those of conventional histology tissue sampling, some unfounded fears are described and signaled as responsible for the slow adoption of FNA as a routine technique for testicular function assessment. The most frequently reported problems described as potential sequelae of the application of FNA are the risk of hematomas, trauma, and autoimmune response against spermatozoa due to the disruption of the BTB, inflammation, increased testicular pressure and infection (Craft et al., 1997; Heath et al., 2001; Leme and Papa., 2010).

In contrast, reported problems of open biopsy include scrotal pain and discomfort to more severe surgical complications like bleeding, inflammation, infection, and testicular atrophy due to permanent devascularization of the testis caused by accidentally hitting a major testicular artery (Aridogan et al., 2013).

Nevertheless, the safety of FNA has been demonstrated in humans, bulls (Heath et al., 2001; Rajak et al., 2013; Rajak et al., 2016a), horses (Leme and Papa, 2000), dogs (Dahlbom et al., 1997), and cats (Leme et al., 2018) with a series of experiments designed to compare its use with conventional open biopsy.

In bulls, Heath et al. (2001) reported a transient change in the isothermal pattern of the scrotum after FNA application with the appearance of small hyperechoic areas corresponding to the aspirated locations. Furthermore, the altered isothermal pattern corresponded with an increase in the appearance of cytoplasmic droplets in spermatozoa, typical of increased testicular temperature. The hyperechoic lesions subsided after a few days with no loss of germinal epithelium within the ST. The absence of changes in testicular biometry and semen quality after FNA application in bulls has also been documented (Heath et al., 2001; Rajak et al., 2013).

In 1987a, Berndston et al. first established a strong correlation between Sertoli cell population size in the testis and daily sperm production in the bull. This concept, supported by the works of Sharpe et al. 2003, established the concept of spermatogenesis efficiency defined as the proportion of spermatids per Sertoli cell, underlying the fundamental role that Sertoli cells have in nourishing and supporting germ cells in the testis. They further introduced the notion of a potential physical limitation in the actual number of germ cells supported by each Sertoli cell within the seminiferous tubule.

Recently, the application of FNA to determine testicular cytology differences between Holstein-Friesian and Holstein Friesian x Tharparkar crossbred bulls was reported and correlated with semen quality and cryotolerance (Rajak et al. 2016b). Researchers reported that those bulls that held a higher proportion of SC per germ cell presented better semen quality and cryotolerance of spermatozoa and suggested, for the first time, the significance that cytology determination might have for predicting semen quality in adult bulls (Rajak et al., 2016b). Furthermore, Tripathi et al. 2015 reported that *Bos Indicus* x *Bos Taurus* crossbred bulls compared with exotic purebred bulls, presented lower testicular weights, volumes, scrotal

circumference, ST diameters, ST area, and a smaller proportion of Sertoli cells determined by FNA.

Sertoli Cell Markers

Androgen Receptors

Androgens exert their function binding to androgen receptors (AR), which are proteins of 110 KD localized in the cytoplasm and nucleus of cells, also known as NR3C4 (Smith and Walker, 2014). The AR is transcribed by a gene located in the X chromosome and belongs to the steroid hormone nuclear transcription factor family, with more than 100 members described so far. Two main compounds bind to AR: testosterone (T) and dihydrotestosterone (DHT) converted from testosterone by 5α-reductase. They bind to the AR with different affinities, where DHT does it with a two-fold stronger binding and dissociates with a 5-fold decreased rate than T (Davey and Grossman, 2016). Androgen receptors have a wide range of localization in different tissues not limited to the testis like musculoskeletal, cardiovascular, immune, neural and hemopoietic systems (Takeda et al., 1990). Within the male reproductive system, AR have a strong presence in the ductal system like epididymis and rete testis while in the testis parenchyma they are highly expressed by LC, arteriole smooth muscle and vascular endothelial cells (Smith and Walker, 2014). In the seminiferous tubule, they are abundant in PMC and SC, not being expressed in GC (Takeda et al., 1990; Bremner et al., 1994; Hill et al., 2004; Walker and Cheng 2005; O'Hara et al., 2015; O'Shaughnessy et al., 2011). Nevertheless, AR are not expressed in the immature SC during the fetal and neonatal period although LC are actively secreting androgens. This transient physiological time is known as the "androgen insensitivity period", constituting a protective mechanism preventing the precocious initiation of spermatogenesis in the prepubertal immature testis (Rey et al., 2009). For this reason, AR protein

expression is used as a specific cell marker for mature peri and postpubertal SC (Sharpe et al., 2003).

GATA4

GATA4 is an evolutionary conserved transcription factor that belongs to the GATA family of six different transcriptional regulators with two Zn fingers in their constitution. During fetal testicular development, GATA4 is expressed in different cell types: SC, fetal LC, fibroblast-like interstitial cells and PMC while postnatally, GATA4 is strongly expressed in the somatic cell population of developing gonads (Lourenço et al., 2011). For this reason, GATA4 is used as a nuclear cell marker in immature proliferating SC in the fetus and neonate in the Hamster and other species (Sharpe et al., 2003; Tarulli et al., 2006). In the mouse, GATA4 can be found mainly in SC and adult LC (Kyronlahti et al, 2011). GATA4 is important for regulating the expression of several proteins necessary for gonad differentiation and development, including NR5A1 (encoding for Sf1) and FOG2, to regulate the expression of the sex-determining genes *Sry*, *SOX9* and *AMH* as well as key steroidogenic factors (Lourenço et al., 2011). Furthermore, GATA4 is fundamental for the formation of the blood testis barrier (BTB), a key feature of SC maturation and in the production of SC derived lactate, primary energy substrate of spermatocytes and spermatids (Schrade et al., 2015). Both GATA4 and GATA6 have been used as early SC markers in the developing mouse gonad (Ketola et al., 1999), and in bulls from birth to 19 months of age (Jimenez-Severiano et al., 2005; Harstine et al, 2017).

In the present dissertation, a series of studies have been developed with the main objective of comparing the use of two stains (IHC-GATA4 vs. conventional HE) and three sampling techniques [22G FNA (CORE) vs. 14G with vacuum (CORE) vs. conventional tissue section (HIS)] to determine the size of Sertoli cells in the testis of neonatal, pre and peripubertal

beef bulls. In addition, a series of interventions were performed to assess the effects of corn supplementation in dams in mid to late gestation, the age of the dam and the administration of vitamin A on testicular development, growth and cytology in neonatal, pre and peripubertal beef bulls.

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COMPARISON OF FINE NEEDLE ASPIRATION AND TISSUE SECTIONS TO DETERMINE SERTOLI AND GERM CELL COUNTS IN NEONATAL AND PREPUBERTAL BEEF BULLS[1]

Abstract

Fine needle aspiration (FNA) is used for many decades as a diagnostic tool in different animal species and humans but its value to determine Sertoli cell density in bulls remains unexplored. GATA4 is a specific SC marker and is expressed only by Sertoli cells (SC) within the seminiferous tubule (ST) in prepubertal bulls. The objectives were to compare the use of two stains [immunohistochemistry with GATA4 (IHC-GATA4) vs. hematoxylin and eosin (HE)] and three techniques [22G needle FNA (SMEAR) vs. 14G needle applying vacuum (CORE) and tissue sections (HIS)] to determine SC density in neonatal and prepubertal beef bulls. For the stain comparison, prepubertal Angus bulls (n = 14; 90-97 days of age; BW = 159.82 ± 3.67 kg) were surgically castrated with an open knife procedure. At castration, body weight, scrotal circumference, testicular, dissected and epididymis weights were determined. Mirror tissue sections were produced for individualized ST cell counts and stained with IHC-GATA4 and HE. For the tissue sampling technique comparison, neonatal (n = 44; 12h of age; BW = 40.25 ± 0.91 kg) and prepubertal Angus bulls (n = 14, 90 to 97 days of age, BW = 159.82 ± 3.67 kg and n = 24, 182-207 days of age, BW = 255.1 ± 6.01 kg) were castrated using the same procedure as in the stain comparison study. The following cell parameters were obtained on testicular samples: Sertoli (SC), germ cell (GC), and GC:SC ratio per ST, and SC and GATA4+ cell densities.

[1] The material in this chapter was co-authored by Nicolas Negrin Pereira*, [1], Pawel Borowicz*, Kevin Sedivec [†], Jordan Flaten *, James D. Kirsch *Cody Wieland [†], Stephanie Becker [†] and, Carl R. Dahlen*. (*Department of Animal Sciences, North Dakota State University, Fargo, ND 58108. [†]Central Grasslands Research and Extension Center, Streeter, ND 58483.). Nicolas Negrin Pereira had primary responsibility for collecting samples in the field and for interviewing users of the test system. Nicolas Negrin Pereira was the primary developer of the conclusions that are advanced here. Nicolas Negrin Pereira also drafted and revised all versions of this chapter. Carl Dahlen served as proofreader and checked the math in the statistical analysis conducted by Nicolas Negrin Pereira

All data was analyzed using Pearson's product moment correlations, linear regression and PROC MIXED for repeated measurements. A high correlation was observed between stains for SC (r = 0.94; $P < .0001$) and GC (r = 0.95; $P < .0001$) counts performed on individualized ST cross sections. Germ cell counts were highly correlated with SC cross section counts (r = 0.59; $P = 0.03$) and SC density (r = 0.57; $P = 0.032$) in IHC sections. However, when comparing IHC SC density across techniques, no correlations were observed ($P \leq 0.20$). The high correlation observed between SC count done in the same ST cross sections using IHC and HE confirms the value of GATA4 as a specific SC marker. The significant correlation detected between SC counts done in individual ST cross sections or as SC density with GC counts indicates the important relationship between both cell populations within the ST. The lack of significant correlations for SC density between the three techniques suggests the use of SMEAR and CORE are not reliable sampling technique to determine SC density in prepubertal bulls.

Key words: fine needle aspiration, GATA4, germ cell, neonatal, pre pubertal bull, Sertoli cell.

Introduction

Although breeding soundness examinations (BSE) in beef bulls are valuable tools to assess the aptitude of potential breeders, a wide range of subfertile individuals remain undetected (Kastelic and Thundathil, 2008). None of the current methods of selection of breeding bulls can predict the potential daily sperm production (DSP) of individual bulls (Rajak et al., 2013). Potential DSP of the bull is determined by the size of the Sertoli cell (SC) population in the testicle (Berndston, 1987a). In the neonate, SC replicate at a high rate between 4 and 20 weeks of age (Moura and Erickson, 1997). Once puberty is reached in the bull, SC stop multiplying and therefore the ceiling of daily sperm production is fixed (O'Shaughnessy and Fowler, 2011). The incorporation as a selection tool of an accurate assessment method of SC population size could

identify those individuals that have a higher potential capacity of sperm production and currently cannot be detected.

Fine needle aspiration (FNA) is used for many decades as a diagnostic technique for neoplasms (Martin, 1930), reproductive pathologies (Aridogan et al., 2003), and infertility in humans (Craft et al., 1977) and animals (Leme and Papa, 2010). When compared with open biopsy, FNA presents several advantages, like low invasiveness and almost no complications (Heath et al., 2001). While the use of a finer needle caliber means smaller tissue samples lowering the risk of complications, the loss of the ST microstructure represents a challenge for accurate SC recognition (Aridogan et al., 2003). On the other hand, the use of higher caliber needles (14G) while preserving the structure of the ST augments invasiveness. To overcome this obstacle, the combined use of SC markers like GATA4 can provide an alternative to cytological recognition in smears (Jimenez-Severiano et al., 2005).

We hypothesize that SC density in neonatal and prepubertal beef bulls can be determined using FNA and GATA4 as a specific SC marker. The objectives of the present experiments were:

1. To compare the use of two stains [Immunohistochemistry with GATA4 (IHC) vs HE]
2. To compare three sampling techniques [22G fine needle aspiration (SMEAR) vs 14G needle aspiration with vacuum pressure (CORE) vs. tissue section (HIS)] to determine SC density in neonatal and prepubertal beef bulls.

Materials and Methods

Tissue Sampling

Stain Comparison (IHC-GATA4 vs. HE)

The North Dakota State University Animal Care and Use Committee (IACUC #A18077) approved all animal procedures in this experiment. Fourteen age-matched crossbred Angus bulls

(90 to 97 days of age, BW = 159.82 ± 3.67 kg) were surgically castrated using an open knife procedure. Bulls were restrained in a cattle chute during the procedure. Local anesthesia was injected into each spermatic cord using 4 ml of 2% lidocaine hydrochloride (Vet Tek, Blue Springs, MO). The scrotum was cleaned and a 10% iodine solution in alcohol was applied to prepare the incision site. The skin was opened at the bottom of the scrotum using a scalpel. The parietal layer of the *tunica vaginalis* was excised and the testicles exposed. An emasculator was used for cutting and crushing the spermatic cord to prevent excessive bleeding. Once the testicles were excised and trimmed of excessive connective tissue, the vas deferens was identified and cut at the union with the tail of the epididymis.

Each testicle was weighed with the epididymis attached. Next, the epididymis was dissected, and both the testicle and epididymis were weighed separately.

The right testicle of each bull was used to take 4 x4 mm parenchyma samples for mirror histology sections.

Techniques Comparisons (SMEAR vs. CORE vs. HIS)

The North Dakota State University Animal Care and Use Committee approved all procedures applied on the animals used in this study (IACUC #A18077, A16010 and A19031). Neonatal (n = 44, 12h of age, BW = 40.25 ± 0.91 kg) and prepubertal (n = 14, 90 to 97 days of age, BW = 159.82 ± 3.67 kg, and n = 24, 182-207 days of age, BW = 255.1 ± 6.01 kg) Angus bulls were surgically castrated using an open knife procedure as described previously. After castration, the following testicular tissue samples were collected:

1. Parenchyma samples using a 22-gauge fine needle aspiration (SMEAR),
2. Parenchyma samples using a 14-gauge needle applying vacuum pressure with a syringe (CORE), and

3. Tissue samples (4 x 4 mm) for histology (HIS).

Smears and testicular parenchyma collections using SMEAR and CORE were done on the upper half of the testis while HIS samples were collected from the bottom half of the testicle, avoiding regions that could be altered by previous sampling.

The technique of FNA consists of inserting a sterile needle (14G or 22G x 1 ¼'') connected to a 5- or 10- ml disposable syringe. The needle is gently inserted and advanced perpendicularly into the testicular parenchyma. Once the needle is fully introduced into the testis, the plunger is pulled back creating vacuum inside the syringe to draw a cellular sample into the needle. While the plunger is pulled back, the needle is moved forward and backward within the testis two to three times for approximately four seconds thereby dislodging the cells and facilitating their suction into the needle. The plunger is released before withdrawing the needle from the testis to avoid the aspirate being sucked into the syringe. Next, the syringe is detached from the needle, filled with air, and used to expel the aspirate on a clean glass slide.

Tissue Processing

Mirror Tissue Sections for Stain Comparison (IHC-GATA4 vs. HE)

To facilitate penetration of the formaldehyde solution, all tissue sections were devoid of albuginea for better parenchyma fixation. Tissue sections were placed inside previously labeled plastic cassettes and placed in jars containing 10% buffered formaldehyde solution.

Tissue sections were fixed for a minimum of 72h before tissue processing. Tissue sections were later embedded in paraffin using a tissue processor (Leica ASP300S, Leica Biosystems Inc., Buffalo Grove, IL) and placed in blocks individually while the four CORE samples were placed in one paraffin block. Using a microtome (HistoCore, Autocut, Leica), tissue was cut in 4.5 µ thickness sections.

To validate the specificity of GATA4 as a SC marker and the accuracy in the cytological recognition of SC and GC in prepubertal bulls, tissue mirror sections were produced cutting adjacent tissue sections from each paraffin block and placing them on clean glass slides with their complementary mirror planes of sections facing up. One of the complementary mirror sections was stained with hematoxylin and eosin, and the other one with IHC-GATA4.

Techniques Comparisons (SMEAR vs. CORE vs. HIS)

Glass slides for SMEAR were previously identified with the bull ID, date, and needle caliber used for the FNA aspirate. The slides were placed on a clean surface and exposed to air until dry. Once the smear dried, the slides were submerged in 95% alcohol and transported to the histology laboratory in Fargo for further processing.

Tissue collected using CORE and HIS were placed in previously identified plastic cassettes with the bull ID, date, needle caliber, and sample number written in pencil. The tissue samples obtained were placed between two blue histology sponges holding the tissue during processing to prevent any tissue loss through the cassette slots. Next, the cassettes were placed in plastic jars containing 10% buffered formaldehyde solution. The plastic jars were labelled with the date; bull ID, time of collection, animal species, tissue type, and operator.

Tissue sections and CORE samples were later embedded in paraffin using a tissue processor (Leica ASP300S, Leica Biosystems Inc., Buffalo Grove, IL) and placed in blocks individually while four CORE samples were placed in one paraffin block. Using a microtome (HistoCore, Autocut, Leica), tissue was cut in 4.5 µ thickness sections.

Staining

Mirror Tissue Sections for Stain Comparison (IHC-GATA4 vs. HE)

Mirror tissue sections for immunohistochemistry were previously deparaffinized through washes of xylene, and decreasing concentrations of alcohol (100%, 95% and 70%) for tissue rehydration. Samples were submerged in sodium citrate buffer and placed in an antigen retriever (2100 Retriever, Aptum Biologics, UK). Blocking time was established at 1 h and 15 min after which the samples were incubated sequentially using anti-rabbit polyclonal antibody to GATA4 (ab124265, Abcam, Cambridge, MA) at a dilution rate of 1:500 in 1% BSA. at 4^0 C for 16 h overnight, and then incubated for one hour at room temperature with goat anti-rabbit IgG (H+L) CF633 fluorescent antibody (Biotium, Freemont, CA), 1:250 dilution in 1% BSA. As a counterstain, 4′,6’-diamidino-2’-phenylindole (DAPI) was applied for 15 min. Negative controls were performed incubating samples with no primary antibodies to check for secondary antibody non-specific binding. The same corresponding number of complementary mirror tissue sections were stained using a conventional HE protocol.

Techniques Comparisons (SMEAR vs. CORE vs. HIS)

The same IHC protocol used for the stain comparison study, was used for the technique comparison. As a difference, tissues from neonatal bulls (12 h of age) were incubated sequentially 1h at room temperature, using rabbit polyclonal antibodies against GATA4 (ab84593, abcam, Cambridge, MA), 1:200 dilution in 1% bovine serum albumin (BSA) and goat anti-rabbit Ig G (H+L) CF633 florescent antibody (Fisher Brand, Waltham, MA), 1:250 dilution in 1% BSA, with DAPI as a counterstain. Corresponding numbers of SMEAR, CORE and HIS from 90-97 days of age bulls were stained with a conventional HE protocol.

Image Capturing and Processing

Mirror Tissue Sections for Stain Comparison (IHC-GATA4 vs. HE)

Fluorescent IHC-GATA4 and bright field HE images of smears and tissue sections were taken using a Zeiss Imager M2 epifluorescence microscope equipped with a Zeiss piezo automated stage and AxioCam HRm camera (Carl Zeiss International, Jena, Germany). Reference images were set up for both IHC and HE images and used as templates for the remaining samples

Within each complementary HE and IHC mirror tissue section, the same ST were located and individualized using micro anatomical structures as references and selected for individual SC and GC counts (Figure 2). Once the same ST was identified in both IHC and HE stained mirror sections, it was numbered and circumscribed using the selection tool option in the image processing software. GATA4 positive SC cells within the ST (red channel or C1) were counted first automatically using the Image Pro software. The split function was applied using the watershed option, separating individual cells for an accurate count. Once the automatic cell count was finished, blind manual counts on the same ST GATA4 positive cells was done. The same procedure was performed on DAPI stained cells within the same individual ST (blue channel or C2). Once the individualized ST were counted both automatically and manually, all ST within the field were circumscribed and both GATA4 and DAPI positive cells counted inside and outside the ST in the whole field for cell density determination. Vascular areas when present, were circumscribed, cropped and excluded from the field image for an accurate parenchyma cell density count.

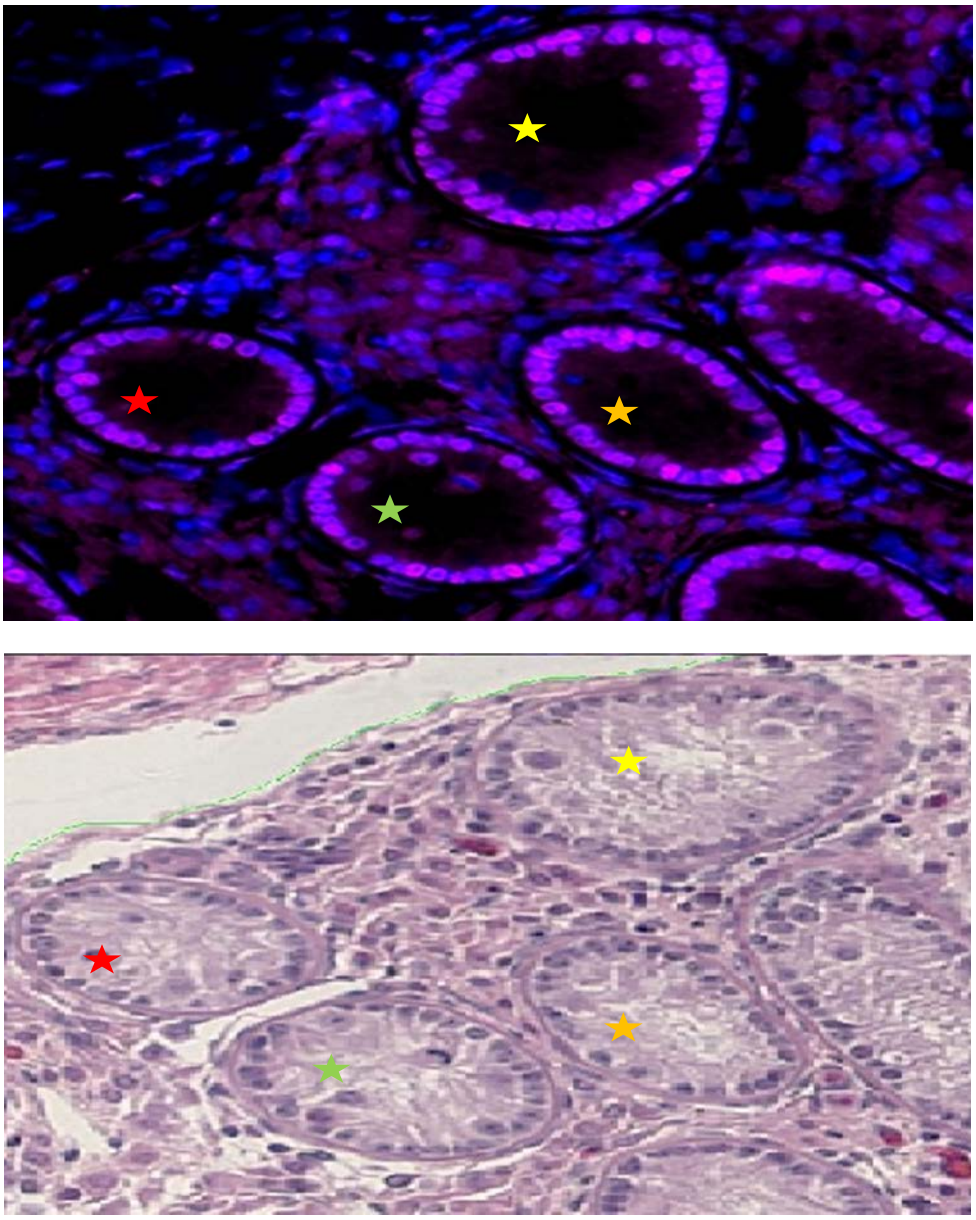

Figure 2. Images corresponding to complementary mirror tissue sections stained with IHC-GATA4 (top) and HE (bottom). Stars of the same color correspond to the same ST cross section.

A similar procedure was performed on the HE image, first counting cells within the corresponding individual ST and then inside and outside all ST present in the field. Sertoli cell counts were determined subtracting all germ cells present in the individual ST from the total cell count. Both SC and GC were determined following cytological criteria (cell aspect, nucleus size,

location within the ST, presence or absence of a prominent nucleolus, chromatin and cytoplasmic aspect, etc).

Techniques Comparisons (SMEAR vs. CORE vs. HIS)

SMEARS

From each sample, five different images from randomly chosen fields were captured at 20x magnification and saved as JPG and tif image files for cell counts.

Cell counts on SMEAR were performed using the Image Pro Premier 3D software (Media Cybernetics, Inc., Bethesda, MD) with the smart segmentation function as the main tool. Objects to be counted were selected within the field of interest, as well as the appropriate background. The cell split function was also applied, ensuring that all cells within the field were included in the final count. During counting, high cell density areas with nucleus apposition or clogged cells were avoided.

Cell counts in the IHC slides were conducted automatically by applying the smart segmentation function of the Image Pro software on the DAPI stained image first (blue channel or C1) and subsequently on the GATA4 positive cells (red channel or C2; Figure 2). Once cell counts were determined, they were exported and saved in an Excel file.

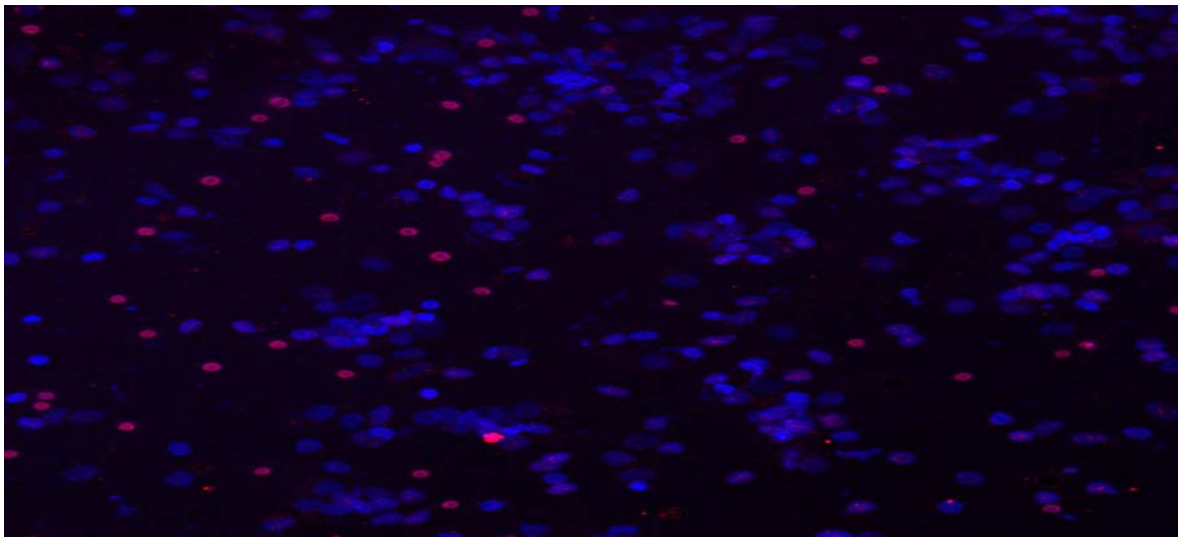

Figure 3. Image of pre-pubertal beef bull testicular parenchyma smear obtained using FNA with a 22G needle stained by IHC using anti-GATA4 antibody as a specific Sertoli cell marker and counterstained with DAPI (red = GATA4+ cells; blue = DAPI stained cells).

Similarly, cell counts in HE images (Figure 4) were performed automatically using the smart segmentation function of the Image Pro software by selecting the objects of interest (all cells present in the field) and the appropriate background correction. However, SC counts in HE SMEAR were performed using the manual selection option in the software. Each cell recognized as a SC was marked using a cross symbol and the final number of cells was counted automatically by the software. The cytology criteria for SC selection was based on the characteristics of the nucleus like chromatin aspect, presence of strong basophilic nucleolus, nucleus shape, size, position, etc.

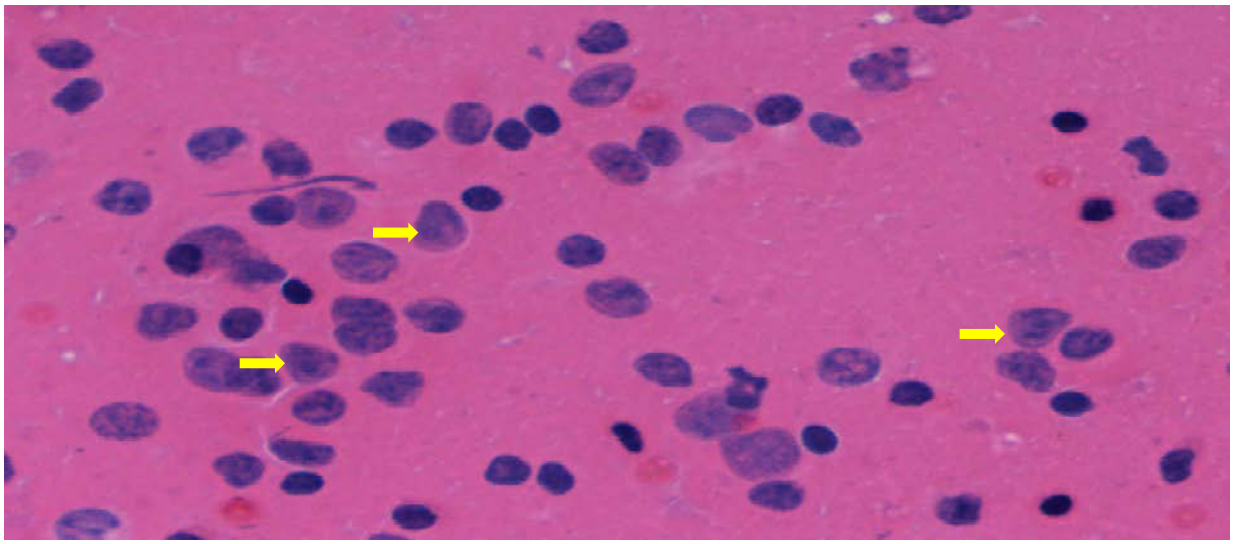

Figure 4. Image of pre-pubertal beef bull testicular parenchyma smear obtained by FNA using a 22G needle and stained with HE (yellow arrows = SC nucleus).

Sertoli cell density (SCD) per smear (slide) was calculated as the sum of all SC counted in all five images divided by the total number of cells counted in all five images.

SCD/slide = (total number of SC in all five images) / (total number of cells present in all five images).

Four SCD values were obtained per bull, corresponding to the two SMEAR stained with IHC and the two SMEAR stained with HE. The four values obtained for SCD were used in the statistical analysis.

CORE

One of the sections containing four samples was stained with IHC and the other with HE for comparison. As the ST structure is preserved in the CORE samples, the same cell parameters measured on tissue sections were obtained.

Cell Count Parameters

For the stain and techniques comparison, the following cell count parameters were determined:

1. Automatic SC count within individualized ST on IHC-GATA4 stained section image (SC IHC),
2. Manual SC count within individualized ST on HE stained section image (SC HE),
3. Manual GC count within individualized ST on IHC-GATA4 section image (GC IHC**),**
4. Germ cell to SC ratio within individualized ST on IHC-GATA4 stained tissue section image (RATIO).
5. Manual GC count within individualized ST on HE stained section image (GC HE),
6. Sertoli cell density determined by total SC automatic count within all ST present in the IHC-GATA4 stained section image divided by the total automatic count of all DAPI positive cells present in the field (SC IHC Den),
7. Total automatic count of all GATA4 positive cells present on the section image divided by the total automatic count of all DAPI positive cells present in the field (GATA4+ Den),

Statistical Analysis

Data for bodyweight, scrotal circumference, testicular weight, dissected testicular weight, epididymis weight, and cell counts were analyzed using the PROC CORR and PROC REG procedure in SAS (SAS version 9.4; SAS Inst. Inc, Cary, NC).

Cell counts for SC DEN on repeated samples, were analyzed using a mixed effects model ANOVA (MIXED procedure of SAS) for repeated measurements with the fixed effects of treatment (stain) with smear and bull and their interactions with treatment, as random effects. Means were separated using the PDIFF option (least significant difference) of the LSMEANS statement of SAS. The following covariance structures for repeated measures were tested: compound symmetry, unstructured, autoregressive, and ante-dependence. The covariance

structure was selected based on the lowest Bayesian information criteria in a comparison of all covariate structures considered. Significance levels for all data comparison were determined and considered significant when $P<0.05$.

Results and Discussion

Descriptive Statistics

Testicular morphological and cytological parameters are presented by age of bull in Table 1.

Table 1. Morphological and cytological measurements in neonatal (12 h of age) and prepubertal beef bulls (90-97 days of age and 182-207 days of age)

	Neonatal	Prepubertal	
	12h	90-97 days	182-207 days
	Mean ± SEM	Mean ± SEM	Mean ± SEM
Body weight (g)	$40.17^{a} \pm 0.56$	$159.82^{b} \pm 3.67$	$255.14^{c} \pm 6.02$
Scrotal circumference (cm)	n/a	$17.96^{a} \pm 0.36$	$22.43^{b} \pm 0.50$
Testicular weight (g)	$5.3^{a} \pm 0.14$	$32.5^{b} \pm 1.81$	$97.25^{c} \pm 6.86$
Dissected testicular weight (g)	$4.48^{a} \pm 0.13$	$29.17^{b} \pm 1.71$	$90.24^{c} \pm 6.36$
Epididymis weight (g)	$0.80^{a} \pm 0.02$	$2.95^{b} \pm 0.18$	$5.96^{c} \pm 0.39$
Sertoli cell count per ST	$15.87^{b} \pm 0.19$	$35.28^{a} \pm 1.76$	$38.54^{a} \pm 3.44$
Germ cell count per ST	$1.56^{a} \pm 0.08$	$2.4^{a} \pm 0.40$	$69.25^{b} \pm 3.90$
GC:SC ratio	$0.09^{a} \pm 0.004$	$0.06^{a} \pm 0.003$	$2.26^{b} \pm 0.24$
GATA4+ cell density	$0.76^{a} \pm 0.03$	$1.14^{b} \pm 0.09$	$0.63^{a} \pm 0.05$

ST = seminiferous tubule; SC = Sertoli cell; GC = germ cell; GATA4+ cell density = total number of GATA4 positive cells divided by all cell present in the field.
ST = seminiferous tubule; SC = Sertoli cell; GC = germ cell; GATA4+ cell density = total number of GATA4 positive cells divided by all cell present in the field.
Different superscript letters mean significant differences at a significance level of $P \leq 0.05$.

Testicular parenchyma density was determined in all samples on IHC-GATA4 mirror sections with a mean value of 0.002 ± .0001cells / px2.

Stain Comparison (IHC-GATA4 vs. HE)

SC counts done on the same individual ST cross section using IHC and HE were highly correlated ($r = 0.94$, $P < .0001$; Table 2; Figure 5). Instrumenting the methodology of mirror tissue sections was fundamental to locate the same individual ST and cross section in both

complementary tissue sections. This allowed us to apply two different stains on the same cells and compare cell counts using IHC or HE in the same cross section, validating the specificity and accuracy provided by GATA4 as a SC marker in prepubertal bulls. A similar scenario was found for GC, with high correlations established between counts done per ST cross sections using IHC and HE (r = 0.95; $P < .0001$; Table 2).

Table 2. Pearson's correlation coefficient and significance levels between Sertoli and germ cell counts per seminiferous tubule cross section on IHC-GATA4 or HE tissue sections in prepubertal beef bulls (90-97 days of age)

	Pearson's correlation coefficients and significance levels			
Parameter	SC IHC	SC HE	GC IHC	GC HE
SC IHC[a]		0.94***	0.59*	0.55*
SC HE[c]			0.37	0.45
GC IHC[d]				0.95***
GC HE[e]				

[a]SC IHC = SC automatic count per ST cross section on IHC-GATA4 mirror section.
[c]SC HE Man = SC manual count per ST cross section on HE mirrors section.
[d]GC IHC = GC manual count per ST cross section on IHC-GATA4 mirror section.
[e] GC HE = GC manual count per ST cross section on HE mirrors section.
*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.0001.

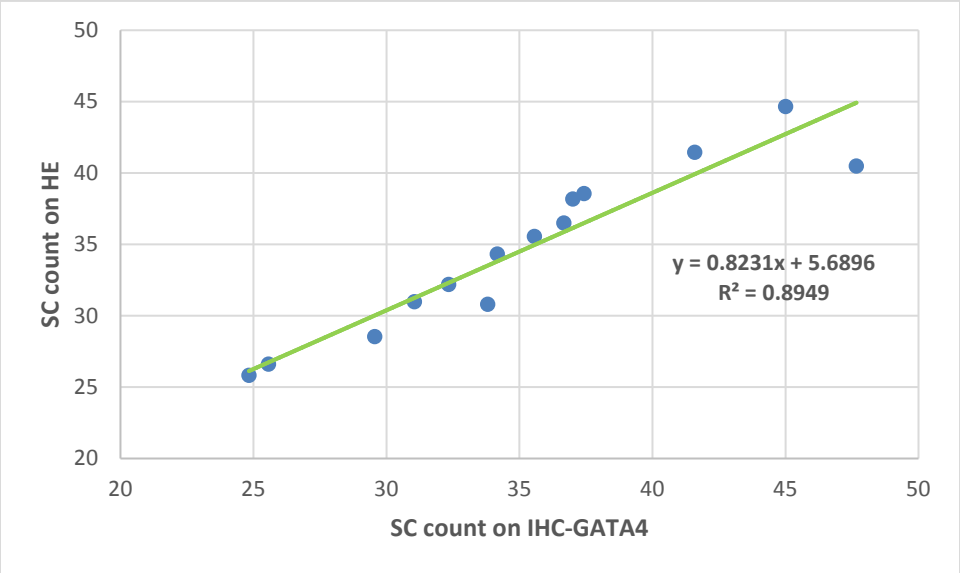

Figure 5. The relationship between SC counts done on ST cross sections stained with IHC-GATA4 and HE in prepubertal beef bulls (90-97 days of age)

In addition, Germ cell counts on IHC and HE presented a high correlation with SC counts per ST cross section on IHC (r = 0.59, *P* = 0.03 and r = 0.55, *P* = 0.04; Table 2).

This agrees with the early findings of Berndston et al. 1987, who established a strong correlation between the number of SC and GC in the testicle and the potential DSP of the adult bull. The relationship between GC and SC is established at the beginning of the formation of the testis cords on the 40-day old bull embryo, being the SC population size what determines the final number of primordial GC and consequently the potential DSP of the adult bull (O'Shaughnessy and Fowler, 2011). This is because each individual SC has a restricted physical capacity to sustain and nurse a fix number of germ cells, where the size of one cell population determines the size of the other cell population (Johnson et al., 2008).

Furthermore, GC counts showed a high correlation with SC density determined on IHC-GATA4 (r = 0.57, *P* = 0.032; Table 4). Moreover, GC counts on HE sections, presented a significant correlation with SC and GATA4+ cell densities determined on IHC slides (r = 0.65, *P* = 0.011 and r = 0.55, *P* = 0.039 respectively; Table 3).

GATA4 is a transcription factor expressed only by SC within the ST while expressed by other interstitial cell types like peri-myotubular cells, Leydig cells and fibroblast cells outside the circumscription of the ST (Ketola et al., 2000; Tarulli et al., 2013). In our study, within the circumscription of the ST, GATA4 was only expressed by SC in accordance with the findings of Harstine et al. (2017) using GATA4 in Angus bulls of similar age and Jimenez-Severiano et al. (2005) applying the same SC marker in Zebu bulls on testicle tissue sections obtained from birth and up to 15 months of age. Despite the presence of other than SC GATA4+ cell types in density measurements, a highly significant correlation existed between SC counts per ST cross section and GATA4+ cell density (r = 0.58, *P* = 0.02; Table 3). Furthermore, a highly significant

correlation was observed when SC and GATA4 positive cells densities were compared (r = 0.69, *P* = 0.005; Figure 6). This suggests GATA4+ cell density can be used as a reliable predictor for SC counts and therefore opens the possibility of automating the process of SC counts in prepubertal bulls.

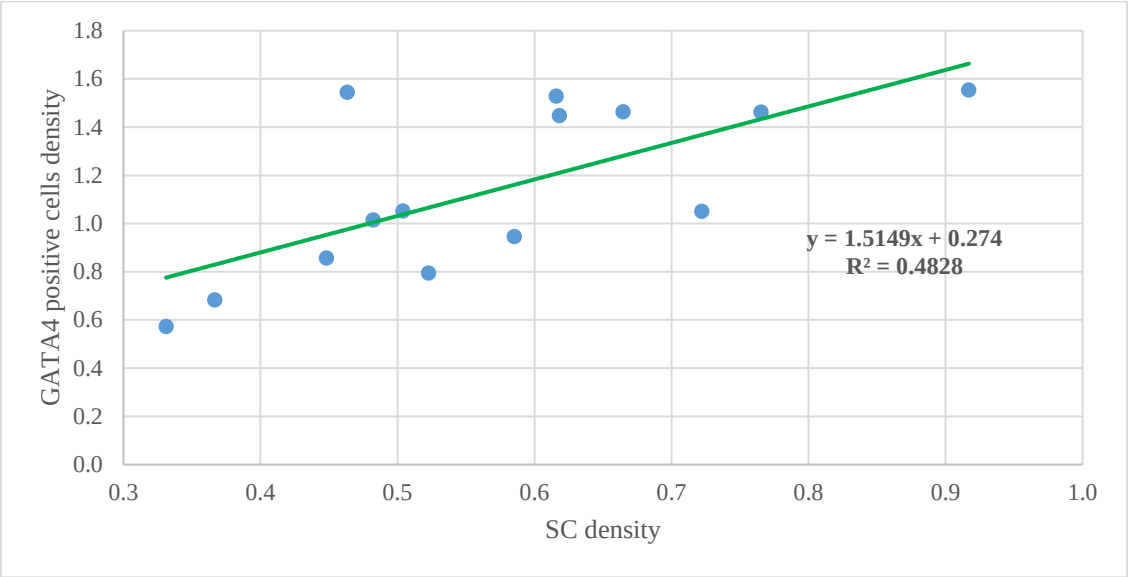

Figure 6. The relationship between SC and GATA4 positive cell densities in prepubertal beef bulls (90-97 days of age)

Table 3. Pearson's correlation coefficients and significance levels between Sertoli and germ cell counts per seminiferous tubule cross section and cell densities prepubertal beef bulls (90-97 days of age)

	Pearson's correlation coefficients and significance levels					
Parameter	SC IHC	SC HE	GC IHC	GC HE	SC IHC Den	GATA4+ Den
SC IHC[a]		0.94***	0.59*	0.55*	0.45	0.58*
SC HE[b]			0.37	0.45	0.33	0.53*
GC IHC[c]				0.95***	0.57*	0.48
GC HE[d]					0.65*	0.55*
SC IHC Den[e]						0.69**
GATA4+ Den[f]						

[a]SC IHC = SC automatic count per ST cross section on IHC-GATA4 mirror section.
[b]SC HE = SC count per ST cross section on HE mirror section.
[c]GC IHC = GC manual count per ST cross section on IHC-GATA4 mirror section.
[d]GC HE = GC manual count per ST cross section on HE mirrors section.
[e]SC IHC Den = SC automatic count within all ST divided by all cells present in the image.
[f]GATA4+ Den = automatic count of all GATA4+ cells divided by all cells present in the image.
*P<0.05; **P<0.01; ***P<0.0001.

Techniques Comparison (SMEAR vs. CORE vs. HIS)

When GATA4+ density in SMEAR was compared with SC density in CORE and HIS, no correlation was established (r = -0.41; *P* = 0.20 and r = 0.15; *P* = 0.60, Table 4).

The use of yearling bulls as primary breeders has become more common, representing a great economical advantage as the inclusion of younger bulls signifies lower production costs, a shorter generational interval, and thus, potentially great genetic improvement (Barth et al., 2008). The extensive application of genomics in cattle selection results in the choice and inclusion of much younger bulls in AI genetic improvement programs (Dance et al.,2016) while representing a challenge as yearling bulls produce semen of poor quality compared with older bulls (Barth et al., 2002). Therefore, it is important that those bulls with a potentially higher DSP are detected accurately at an early age.

Fine needle aspiration (FNA) is used as a diagnostic tool for infertility in humans (Aridogan et al., 2003), dogs and cats (Menard et al., 1986), horses (Leme and Papa, 2012), and bulls (Heath et al., 2002). It represents a low invasive, low cost, simple technique with minimal negative consequences. It has been used as a complement to the breeding soundness exam (BSE) in adult bulls to aid in the diagnosis of suspected fertility problems (Chapwanya et al., 2008) and to distinguish between bulls with different sperm freezing capacity (Rajak et al., 2016). Nevertheless, the use of FNA as a potential tool to determine SC and GC counts in newborn and prepubertal bull testicles remains unexplored.

The absence of correlations between techniques for SC and GATA4+ cell densities in the present study might be the result of a combined effect of a series of factors. One of these factors is the lack of ST micro-anatomical references in smears hindering the cytological reconnaissance of immature SC considering the heterogeneous distribution and mixture of different type of loose

cells in smears. This characteristic causes the need of a trained technician in specific testicular cytology to evaluate the smears. This factor is reported as one of the obstacles against the massive adoption and inclusion of FNA for SC counts in BSE protocols (Craft et al., 1997; Leme et al., 2018).

When SC density was analyzed within and between repeated SMEAR samples stained with IHC and HE, no significant correlation ($P = 0.479$) was established, showing the inconsistency and high variation observed between samples. Mean SC density (0.315 ± 0.017) for SMEAR stained with HE was significantly greater ($P < .0001$) than the mean SC density determined in IHC SMEAR (0.134 ± 0.017).

Haseler et al. 2011 suggested many factors interplaying in conventional FNA compared with automatic systems of cell aspirates like uneven application of vacuum, syringe and needle control, hand force and differences in biopsy yield affecting the proportion of testicular cell types harvested at each sampling event.

Nevertheless, highly significant correlations were established between CORE and HIS for SC ($r = 0.49$; $P < .0001$, Table 4), GC ($r = 0.93$; $P < .0001$) and the GC: SC ratio ($r = 0.84$; $P < .0001$). This finding suggests the preservation of the ST, common in both CORE and HIS might be one of the reasons for the correlations observed in cell counts per ST cross sections.

In addition, a significant correlation was observed between GC and SC per ST cross sections in CORE ($r = 0.25$, $P = 0.03$) being highly correlated in HIS ($r = 0.56$; $P <.0001$).

Furthermore, GC counts per ST were highly correlated with the GC:SC ratio for both CORE ($r = 0.98$; $P <.0001$) and HIS ($r = 0.88$; $P < .0001$). The ratio of GC per SC is used by many authors as an indicative of the relationship between the two main cell types that populate the ST established early during the gonad development (Grinswold 1995) and is used later as

predictive of the potential daily sperm production in the pubertal and adult bull and as an indicative of spermatogenesis efficiency later in the life of the adult individual (Orth 1982, Berndston et al. 1987a, 1987b, Bagu et al., 2004).

Table 4. Pearson's correlation coefficients and significance levels between techniques (SMEAR vs. CORE vs. HIS) for SC, GC, and the GC:SC ratio per ST cross section and SC density in neonatal and prepubertal beef bulls

	Pearson's correlation coefficients and significance levels								
	SC DEN SMEAR	SC CORE	GC CORE	RATIO CORE	SC DEN CORE	SC HIS	GC HIS	RATIO HIS	SC DEN HIS
SC DEN SMEAR[a]		- 0.46**	0.76***	0.64***	-0.41	0.13	0.66***	0.51**	0.15
SC CORE[b]			0.25*	0.12	0.22	0.49***	0.17	0.17	-0.08
GC CORE[c]				0.94***	-0.05	0.55***	0.93***	0.84***	0.047
RATIO CORE[d]					-0.15	0.56***	0.94***	0.84***	0.12
SC DEN CORE[e]						0.23	0.19	0.06	0.06
SC HIS[f]							0.55***	0.25*	-0.08
GC HIS[g]								0.88***	-0.02
RATIO HIS[h]									0.01
SC DEN HIS[i]									

[a] SC DEN SMEAR = SC density determined on IHC-GATA4 smears using a 22G needle aspiration
[b] SC CORE = SC counts per ST cross section on IHC-GATA4 using a 14G needle with vacuum
[c] GC CORE = GC counts per ST cross section on IHC-GATA4 using a 14G needle with vacuum
[d] RATIO CORE = GC:SC ratio on IHC-GATA4 using a 14G needle with vacuum
[e] SC DEN CORE = SC density determined on IHC-GATA4 using a 14G needle with vacuum
[f] SC HIS = SC counts per ST cross section on IHC-GATA4 conventional tissue sections
[g] GC HIS = GC counts per ST cross section on IHC-GATA4 conventional tissue sections
[h] RATIO HIS = GC:SC ratio on IHC-GATA4 in conventional tissue sections
[i] SC DEN HIS = SC density determined on IHC-GATA4 conventional tissue sections
*P<0.05; **P<0.01; ***P<0.0001.

Conclusions

The high correlation observed between SC counts done on mirror images of the same ST cross sections (one stained with IHC-GATA4 and one with HE), confirms the value of GATA4 as a specific SC marker and reflects the high accuracy of the automatic cell count using an image processing software tool applied with the appropriate settings for the specific cell type count.

The significant correlation established between SC counts done in individual ST cross sections or as SC density with GC counts observed in neonatal and prepubertal bulls shows the importance of the relationship between SC and GC cell populations established at a very early age in the bull, with the formation of the sex cords during the male gonad development.

Furthermore, the high correlation present between SC counts per ST cross sections for both IHC and HE, and the strong and significant correlation between SC and GATA4+ cell densities within IHC HIS mirror sections infers SC density determination through GATA4+ cell density can be automated. In addition, the relationship between both SC and GATA4+ cell densities suggest it is possible to avoid the need of a specialized technician for individual SC recognition.

Nevertheless, the lack of significant correlations for SC density across techniques observed at different bull ages reflects differences in cell counts among the sampling methods, preventing the application of SMEAR and CORE as reliable sampling technique to determine SC counts in neonatal and prepubertal bull testis.

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THE EFFECT OF CORN SUPPLEMENTATION IN BEEF COWS DURING MID TO LATE GESTATION ON GONAD DEVELOPMENT OF THE NEWBORN BULL CALF[2]

Abstract

The objective of the current experiment was to assess the effects of corn supplementation in multiparous beef cows during mid-to late gestation on testicular growth and development, and Sertoli cell (SC) density in newborn male calves. Forty-four multiparous beef cows carrying bull calves were randomly assigned to one of the following treatments: 1) corn supplementation at 0.2% of BW (SUP; n = 22) or 2) no supplement (CON; n = 22). Bulls were surgically castrated before 12 h of age and testicular parenchyma samples were collected using a 14G needle applying vacuum pressure (CORE) and conventional tissue sections (HIS) and fixed in a 10% buffered formaldehyde solution. Samples were stained using anti-GATA4 as a specific SC marker and DAPI as counterstain. Image analysis was performed using Image Pro Premier software. No differences existed between CON and SUP cows for birth weight ($P > 0.50$) and testicular weight ($P=0.98$). Furthermore, no difference was observed between treatments for SC ($P = 0.31$), germ cell counts ($P = 0.29$), SC density ($P = 0.27$) and GATA4 density ($P = 0.76$). Although no significant correlations existed between the dam and bull offspring plasma hormone and metabolites concentrations determined in a concurrent experiment using the same animals, a significant correlation was established between glucose concentrations of the mother and GATA4 positive cells in the bull testicle ($r = 0.45$, $P = 0.002$) suggesting a positive effect of the dam nutrition on testicular cytology of the neonatal bull.

[2] The material in this chapter was co-authored by Nicolás Negrin Pereira*, Pawel Borowicz*, Alison Ward*, Jordan Flaten*, Bryan Neville§, and Carl R. Dahlen* (*Department of Animal Sciences, North Dakota State University, Fargo 58108; §Central Grassland Research Extension Center, North Dakota State University, Streeter 58483.). Nicolas Negrin Pereira had primary responsibility for collecting samples in the field and for interviewing users of the test system. Nicolas Negrin Pereira was the primary developer of the conclusions that are advanced here. Nicolas Negrin Pereira also drafted and revised all versions of this chapter. Carl Dahlen served as proofreader and checked the math in the statistical analysis conducted by Nicolas Negrin Pereira

Key words: bull, GATA4, newborn, Sertoli cells, supplementation.

Introduction

Under and overnutrition of the dam can result in alterations in fetal growth that extend to later in adult life (Wu et al., 2004). Both nutritional scenarios might be common in beef cows grazing extensive systems worldwide (Wu et al., 2006). Some of these nutritional effects can be mediated through alterations in the placenta and vascular development (Reynolds et al., 2005). Moreover, maternal nutritional status can alter the epigenetic state of the fetal genome and gene imprinting expression impacting fetal gonadal growth and development (Dupont et al., 2012).

During late gestation, fetal testis development in the bull is associated with GnRH, FSH, and LH pulse frequencies. The fetal hypothalamic-pituitary-gonad axis can be affected by severe nutritional restriction mediated through the placenta (Rhind et al., 2001). In the fetal gonad, Sertoli cells (SC) have a key role in the differentiation and development of most testicular cells (Skinner and Griswold 2005). Sertoli cells rapidly proliferate in the mammalian embryonic testis. This proliferation extends into the postnatal period until approximately 20 weeks of age in the bull calf (Rawlings et al., 2008). During cell proliferation, SC are susceptible to alterations of the hypothalamus-pituitary-gonad axis during fetal life (Dupont et al., 2012). Furthermore, supplementation of ewes during late pregnancy resulted in an increase in the number of SC in the testis of their male lamb offspring (Robinson et al., 1999). Conversely, disturbances in SC development can affect Leydig and fetal germ cells (GC), altering potential fertility in the adult bull (Sharpe et al., 2003).

The final number of SC established in the male gonad will determine testicular size, GC numbers, and spermatozoa output (Berndston et al., 1987; Johnson et al., 2008). At present there

is very little information about the potential effects of dam supplementation on testicular growth and development of the fetus.

We hypothesize that diet supplementation during the second half of gestation in the cow will influence fetal gonad development. The objectives of the current experiment were to evaluate the effects of corn supplementation during mid to late gestation beef cows on testis development, and SC and GC count in their newborn male offspring.

Materials and Methods

All procedures described in the current experiment were approved by the North Dakota State University Institutional Animal Care and Use Committee (IACUC #A16010). Forty-four Angus cross multiparous beef cows (661±7.8 kg; 5.2±0.1 BCS on a 9-point scale; 7.5±0.2 years of age) carrying male calves determined by ultrasound from the NDSU Central Grassland Research Extension Center, Streeter ND, were transported to the NDSU Beef Cattle Research Complex in Fargo. Cows were stratified by body weight and body condition score (BCS) and randomly assigned to one of two dietary treatments; 1) Control (CON; n=22), cows had *ad libitum* access to a diet based on low quality forage (57.54% TDN 6.4% CP) ; or 2) Supplemented (SUP; n=22) cows received CON diet with access to a corn supplement up to 0.2% BW (94.5% TDN; 7.64% CP). The dietary treatments were applied after a 3-week adaptation period and intake was individually monitored for the duration of the experiment using electronic feeders to measure feed intake (Hokofarms, Insetec, B.V., Markenesse, Netherlands). Treatments started at day 100 of gestation and extended for 22 weeks. A basal forage diet was offered *at libitum* to each cow consisting of 45% hay, 45% wheat straw and 10% concentrated separator byproduct (CSB) based on a DM basis (57.54% TDN, 6.4% CP). At day 154 of gestation, the basal diet was adjusted to 60% hay, 30% wheat straw and 10% CSB (58.63%

TDN, 7.11% CP). Corn was offered to the SUP group from day 100 to day 265 of gestation at 0.2% of BW. On day 265, at approximately two weeks before expected parturition, all cows were fed the same diet *ad libitum* consisting of 45% straw, 25% distiller's dried grains with solubles (DDGS), and 30% corn silage (62.4% TDN and 11.6% CP) based on DM. All cows had free access to water and trace mineralized salt blocks. Cows were monitored 24 h/d from day 265 of pregnancy until the end of calving. Calf weights were recorded at birth (0 h) and all calves were surgically castrated using an open knife procedure before 12 h of age.

Once the testicles were removed, any excessive connective tissue was trimmed and the *vas deferens* identified and excised at the union with the tail of the epididymis. Whole testicles were weighed, epididymis dissected and detached from the testicles and weighed separately. All testicular parenchyma samples were collected from the right testicle, using two different techniques: 14G needle sampling applying vacuum pressure (CORE) and conventional tissue section (HIS) as described in Experiment II in Chapter 1.

Hormonal and metabolite determination to assess the effects of corn supplementation in dams and calves was done as part of another experiment that paralleled the present, using the same animals. Plasma concentrations of progesterone (P4), estradiol (E2), cortisol, triiodothyronine (T3), thyroxine (T4), T3:T4 ratio, non-esterified fatty acids (NEFA), blood urea nitrogen (BUN) and glucose of the dams, and plasma concentrations of cortisol, T3, T4, T3:T4 ratio and glucose of the calves at birth were determined. Cow blood samples were collected via jugular venipunction on days 100, 125, 150, 180, 210, and 240 of pregnancy, and at calving. In calves, blood samples were obtained at birth and at 24 h of age. Plasma concentrations of hormones and metabolites were determined using the laboratory procedures and techniques described by Tanner et al., 2017. Mean concentrations for each hormone and metabolite was

obtained averaging the concentrations obtained in all collection days, resulting in a single mean value for each respective hormone and metabolite. This value was used to determine the relationships between hormone concentrations and metabolites, with testicular growth and development parameters and cell counts in calves.

Statistical Analysis

Mean differences between treatments (supplemented or not supplemented) for scrotal circumference, intact testicle, dissected testicle and epididymis weights, SC and GC counts were analyzed with the PROC GLM procedure of SAS with treatments as fixed effect and bull as random effect (SAS version 9.4; SAS Inst. Inc., Cary, NC).

Bodyweight, scrotal circumference, testicular weight, dissected testicular weight, epididymis weight, cell counts, hormone and metabolites concentrations were analyzed using the PROC CORR and PROC REG procedure in SAS (SAS version 9.4; SAS Inst. Inc, Cary, NC).

For all parameters studied in this experiment, significant differences were considered when $P \leq 0.05$.

Results and Discussion

As previously reported our experimental diets did not influence uterine blood flow or placental morphology in dams of our bulls (Tanner et al., 2017). This is in accordance to the lack of differences observed in birth weights between CON and SUP groups (40.09 vs. 40.25 ± 0.8 kg respectively; $P>0.50$, Table 5).

In the present experiment, no differences were observed in testicle ($P=0.95$, Table 5), testicular parenchyma ($P=0.81$) and epididymis weights ($P=0.52$) between bulls from CON and SUP cows at 12h of age.

Table 5. The effect of the dam corn supplementation (0.2% DMI) during late gestation on testicular development and growth of newborn beef bulls (12h of age)

	Control (n = 22)	**Supplemented** (n = 22)	
Parameter	Mean ± SEM	Mean ± SEM	*P*-Value
Birth weight, kg	40.6 ± 0.91	40.2 ± 0.92	0.78
Testicular weight, g	5.46 ± 0.27	5.45 ± 0.18	0.98
Testicular parenchyma weight, g	4.60 ± 0.27	4.65 ± 0.17	0.88
Epididymis weight, g	0.85 ± 0.04	0.79 ± 0.04	0.34

SEM= standard error of the mean.

Nevertheless, in the study run simultaneously with the present, Tanner 2017 observed a significant greater body weight in steers from cows supplemented with corn when they reached 21 days of age. Furthermore, steers from supplemented dams showed a tendency for greater ADG from birth to 3 weeks of age but no significant difference in body weight was seen at weaning. It is interesting to note that this temporary effect on steers growth from supplemented dams could have caused potential alterations in testicular growth and development that might have been observed later in the bulls if remained intact.

Supplemented dams increased their BW and BCS during the study and presented a tendency for greater rump fat suggesting they were in a better energy status than non-supplemented cows (Tanner et al. 2017). In addition, they had lower NEFA and BUN plasma concentrations, suggesting better ruminal nitrogen utilization and a probable better CP supply, this difference in nutritional and energy status between cows was not sufficient to elicit changes in the testicular cytology of the male fetuses as seen in other studies where energy and protein differences between groups was greater. In one of the few experiments conducted in composite beef heifers to study the effects of energy and protein supplementation during the first and second trimesters of pregnancy in testicular growth and development, Sullivan et al., 2010 observed larger paired testicular volumes and increased FSH prepubertal concentrations in those

bulls under lower maternal dietary protein (175% less CP) and energy levels (44% less ME) during early gestation compared with those dams under the high CP and ME diet.

Most of the studies conducted to assess the nutritional effects during pregnancy on fetal testicular development and growth include diet restrictions and were done in other species like humans and sheep. Da Silva et al. 2001, working with male lambs whose mothers were offered high and moderate nutrient intake during pregnancy, reported lower placental and birth weights for the high nutrient intake group. Furthermore, growth restricted male lambs presented lower testosterone levels and delayed puberty suggesting possible alterations on the hypothalamus-pituitary-gonad axis. In addition, Deligeorgis et al. 1996 showed how lambs from energy restricted dams presented altered pituitary response when challenged with GnRH at 55 days of age.

In contrast to supplementation, the effect of diet restriction during the dam pregnancy on ST and the number of SC in the neonate testis has been reported. Bielli et al. 2002 described greater ST volumes and SC numbers in newborn lambs from ewes that received 110% of their metabolizable energy (ME) requirements for maintenance and normal fetal growth in comparison with those dams that received only 70% of their ME requirements. Similarly, Kotsampasi et al. 2009 observed a reduction in the diameter of ST and number of SC at 10 months of age in ram lambs born from dams subjected to a harsh diet restriction between 31 and 100 days of gestation of 50% vs. 100% of their ME and crude protein requirements.

Sertoli cells have a restricted physical capacity that limits the number of GC that can be supported by each individual cell (Orth et al., 1982). Consequently, the GC to SC ratio is used as an indication of the potential daily sperm production (DSP) in the mammalian testis and is related to the bull's freezing ability (Rajak et al., 2016). In the present experiment in neonatal

bulls, no difference was observed between CON and SUP groups for SC (P = 0.31) and GC numbers (P = 0.29) per ST cross section (Table 6). Furthermore, no difference between treatments was observed for the GC to SC ratio (P = 0.84). In addition, no significant difference was established between treatments for SC and GATA4 positive cell densities (P = 0.27 and 0.76 respectively).

Table 6. Effect of the dam corn supplementation (0.2% DMI) during late gestation on Sertoli and germ cell counts determined by immunohistochemistry with anti-GATA4 as a specific Sertoli cell marker in newborn beef bull testicles (12 h of age)

	Control (n = 22)	**Supplemented** (n = 22)	
Parameter	Mean ± SEM	Mean ± SEM	*P*-Value
Sertoli cells	16.16 ± 0.35	15.72 ± 0.26	0.31
Germ cells	1.66 ± 0.13	1.48 ± 0.12	0.29
Germ cells / Sertoli cells ratio	0.10 ± 0.01	0.09 ± 0.01	0.43
Sertoli cells density	0.55 ± 0.01	0.97 ± 0.37	0.27
GATA+ cells density	0.77 ± 0.05	0.75 ± 0.07	0.76

SEM = standard error of the mean.

The rate of SC multiplication in the bull is maximal during the late fetal stage, extending up to approximately 20 weeks of age in the neonate (Hochereau-De Reviers et al., 1995). Several factors have been identified as having a positive effect on Sertoli cell rate of division (Lucas et al., 2014), and many of them can be affected by hormonal and nutritional treatments suggesting SC size population can be influenced through interventions during the cell replication period.

No significant correlations were established between SC, GC or the GC:SC ratio in IHC-GATA4 tissue sections and plasma concentrations of P4, E2, cortisol, T3, T4, T3:T4 ratio, BUN, NEFA and glucose in dams, and cortisol, T3, T4, T3:T4 ratio and glucose in calves at birth. Nevertheless, a strong correlation was observed between glucose concentration in plasma of the dams and GATA4+ cell density in testicular tissue sections of the offspring (r = 0.45; P = 0.002; Figure 7).

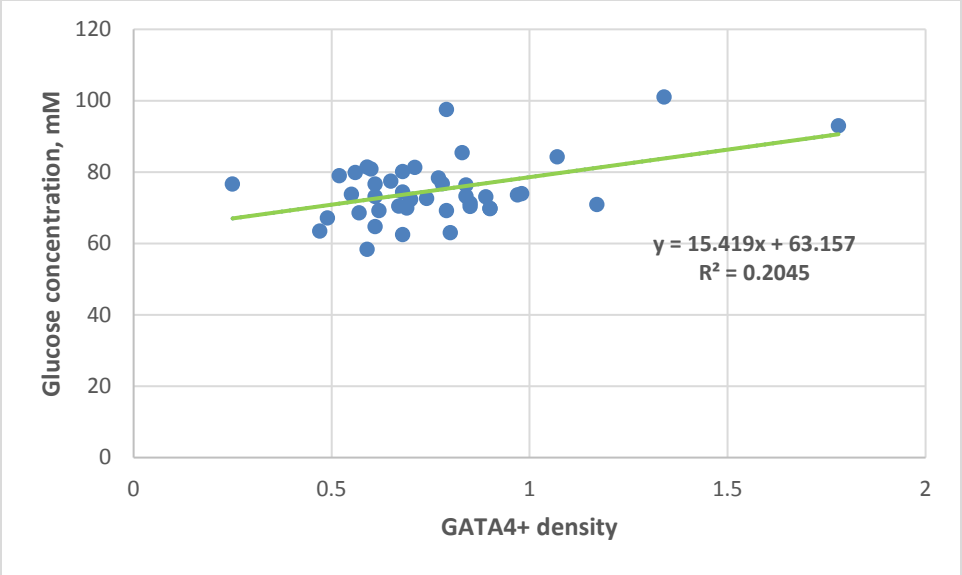

Figure 7. The relationship between glucose concentration of the dam and GATA4+ cell density of the offspring (12h of age).

This relationship is very interesting to observe as it has been shown the key role GATA4 plays in the production of lactate from glucose by SC as the principal energy source of GC in the testis (Schrade et al. 2016).

Conclusions

The inclusion of a corn supplement at 0.2% of BW during mid to late gestation in beef cows did not influence fetal testicle development and growth of the bull calf at birth.

A temporary effect of the supplement observed on the neonate bull calves body weights at 3 weeks of age suggests supplementation of the dam during mid to late gestation can potentially affect neonatal testicular growth and development later in the neonate life.

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THE EFFECT OF THE AGE OF DAM ON TESTICULAR GROWTH AND DEVELOPMENT IN PREPUBERTAL BEEF BULLS[3]

Abstract

Lower testicular weights and scrotal circumferences in bulls from heifers compared with bulls from cows has been observed at one year of age. The objective of the present study was to evaluate the impact of dam age on testicular growth, development, and cytology in beef bulls. Twenty-four age-matched prepubertal Angus bulls (182-207 days of age; BW = 255.1 ± 6.01 kg) from primiparous (HEIFERS, n = 12; 2 years of age) and multiparous (COWS, n = 12; 6 years of age) dams were surgically castrated with an open knife procedure and testicular parenchyma samples collected for cytology comparison using immunohistochemistry with GATA4 as a specific SC cell marker. Conventional tissue sections (HIS) and 14G needle aspirations with vacuum (CORE) were fixed in 10% buffered formaldehyde solution while smears produced using a 22G needle with aspiration (SMEAR) were air dried and fixed in 95% alcohol. At castration, body weight, scrotal circumference, testicular, and dissected testicular weights were determined. Data were analyzed using Pearson's product moment correlations, linear regression and mixed linear models for repeated measurement. The following cytological parameters were determined: SC and GC counts per ST cross sections, GC: SC ratio, and GATA4+ cell density.

Body weights from HEIFERS bulls were lower than body weights from COW bulls (240.89 ± 7.73 vs. 269.39 ± 7.39; $P = 0.006$) while no significant differences were observed for scrotal circumference, intact and dissected testicular and epididymis weights. Nevertheless,

[3] The material in this chapter was co-authored by Nicolás Negrin Pereira*, Pawel Borowicz*, James D. Kirsch*, Jordan Flaten*, Cody Wieland§, Stephanie Becker§, Kevin Sedivec§, and Carl R. Dahlen* (*Department of Animal Sciences, North Dakota State University, Fargo 58108; §Central Grassland Research Extension Center, North Dakota State University, Streeter 58483.) Nicolas Negrin Pereira had primary responsibility for collecting samples in the field and for interviewing users of the test system. Nicolas Negrin Pereira was the primary developer of the conclusions that are advanced here. Nicolas Negrin Pereira also drafted and revised all versions of this chapter. Carl Dahlen served as proofreader and checked the math in the statistical analysis conducted by Nicolas Negrin Pereira.

HEIFERS bulls presented a numerical difference for lower SC counts in heifers vs. COWS although not significant (34 ± 1.65 vs. 45 ± 2.83 respectively, $P = 0.31$). No significant differences existed for GC, GC: SC ratio and GATA4+ cell density. No effect of the age of the dam was observed in the present study on testicular growth and development and cytology on prepubertal beef bulls.

Key Words: cows, GATA4 density, heifers, scrotal circumference, Sertoli cells.

Introduction

Beef heifers are usually bred at 55-65% of their mature bodyweight implying significant needs for continued growth during pregnancy (Funston and Deutscher 2004). For this reason, nutrient demands during gestation are proportionally much less in beef cows than in heifers (Lalman and Richards, 2017). In addition, depending on the physiological status and age of the animal, forage-based diets may not meet the nutritional requirements needed, resulting in body weight loss of the dam affecting the offspring (Funston et al., 2012). The difference in nutrient requirements for both dam categories can result in different nutritional likely situations for possible *in utero* effects on the fetus (Funston et al., 2010). It has been reported how alterations in hormone concentrations like T4 and metabolites like BUN and NEFA in the dam might cause alterations in the calf performance manifested later in life (Tanner 2017).

It has been shown that when heifers enter their first pregnancy with 70% of their mature body weight, the birth weight of their offspring is 10 to 15% lower compared with offspring from mature adult body weight dams. On the contrary, when primiparous heifers are bred at a more mature body weight, no difference in birth weights is present, showing that it is maturity and not parity what is affecting fetal growth (Wu et al., 2006). Furthermore, Lunstra et al., 1985 reported that bull calves raised by first parity dams, presented smaller testicles as yearling bulls compared with those raised by older cows. This difference was present across breeds at age and

weight constant. It is well documented how different planes of nutrition of the mother can have an effect on the developing gonad of the male fetus as reported by different researchers in different animal species like the rat (Genovese et al., 2010), sheep (Kotsampasi et al., 2009); humans (Wu et al., 2004) and cattle (Sullivan et al., 2010). Three possible mechanisms have been proposed to explain the effects of different nutritional planes of the mother on the fetal gonad: alterations in the placenta growth and development (Reynolds et al. 2019), disruptions on the hypothalamus-pituitary-gonadal axis (Deligeorgis et al., 1996; Sullivan et al., 2010) and epigenetic effects (Dupont et al., 2012).

We hypothesize that the age of the dam affects testicular growth, development and cytology of prepubertal bulls. The objective of the present study was to assess the effects of the age of the dam on scrotal circumference, testicular and epididymis weights, and Sertoli and germ cell counts in prepubertal beef bulls.

Materials and Methods

The North Dakota State University Animal Care and Use Committee (IACUC #A19031) approved all procedures in the present experiment. Twenty-four age-matched crossbred Angus bulls (182 to 207 days of age, BW = 255.1 ± 6.01 kg) from the Central Grasslands Research Extension Center near Streeter ND, were used to evaluate the impact of age of dam on testicular growth, development and cytology. Bulls were born from either primiparous (HEIFERS; n = 12, 2 years of age) or multiparous (COWS; n = 12, 6 years of age) dams. Cows and heifers were located during the whole experiment at the Central Grassland Research and Extension Center (NDSU – CGREC) close to Streeter, ND (latitude 46.74, longitude -99.47). After breeding in June, pregnant cows and heifers were on grasslands with *ad lib* access to salt and minerals for the following five months. In November, management differed with cows starting a corn residue

grazing study while heifers were put on crops aftermaths having access to cereal and forage grains regrowth. From January to April, cows and heifers start receiving a TMR diet consisting of corn silage, hay, DDGS and corn with monensin and minerals included in the ration. In May, cow and calf pairs returned to grazing (June to September). In October, bulls were surgically castrated using an open knife surgical procedure. Body weight, scrotal circumference, testicular, dissected and epididymis weights were determined at castration. Testicular parenchyma tissue for cytology was collected using SMEAR, CORE and HIS sampling methods and processed as described in Chapter 2. Immunohistochemistry combined with the use of GATA4 as a specific SC marker and DAPI as a counterstained was used to determine the following cytological parameters: SC and GC cell counts per ST cross section, GC:SC ratio and GATA4+ cell density.

Statistical Analysis

Mean differences between age of the dam (heifer or cow) for scrotal circumference, intact testicle, dissected testicle and epididymis weights, and SC and GC count per ST cross sections were analyzed with the PROC MIXED procedure of SAS with age of the dam as fixed effect including body weights and age as covariates (SAS version 9.4; SAS Inst. Inc., Cary, NC).

Cell counts on samples obtained using SMEAR, CORE and HIS were analyzed using a mixed effects model ANOVA (MIXED procedure of SAS) for repeated measures with the fixed effects of dam age (heifer or cow) including body weights and age of the calves as covariates. Means were separated using the PDIFF option (least significant difference) of the LSMEANS statement of SAS. The following covariance structures for repeated measures were tested: compound symmetry, unstructured, autoregressive, and ante-dependence. The covariance structure was selected based on the lowest Bayesian information criteria in a comparison of all

covariate structures considered. Significance levels for all data comparison was determined and considered significant when $P < 0.05$.

Results

Body Weight and Testicular Gross Morphology

A significant difference existed for body weights, with bulls from cows being heavier than bulls from heifers (269.39 ± 7.39 vs. 240.89 ± 7.73 respectively, $P = 0.006$; Table 7). Nevertheless, no significant differences were observed between bulls from cows or heifers for scrotal circumference ($P = 0.634$), testicular ($P = 0.393$), dissected ($P = 0.404$) and epididymis weights ($P = 0.236$).

Table 7. The effect of the age of dam on body weight (kg) and testicular gross parameters in prepubertal beef bulls (182-207 days of age)

	Heifer	Cow	*P*-Value
Parameter	Mean ± SEM	Mean ± SEM	
Body weight, kg	240.89 ± 7.73	269.39 ± 7.39	0.006
Scrotal circumference, cm	21.83 ± 0.65	23.04 ± 0.75	0.634
Testicular weight, g	91.93 ± 8.82	102.59 ± 10.66	0.393
Dissected testicular weight, g	85.12 ± 8.28	95.37 ± 9.78	0.404
Epididymis weight, g	6.07 ± 0.56	5.87 ± 0.58	0.236

SEM = standard error of the mean.

Age of Dam and Testicular Cytology

There was a tendency for a significant difference between bulls from heifers and cows for GC counts in ST cross sections ($P = 0.06$) and GATA4+ density ($P = 0.07$; Table 8). Nevertheless, there were no significant differences observed between heifers and cow bulls for SC ($P = 0.31$), and the GC:SC RATIO ($P = 0.26$).

Table 8. The effect of age of dam on testicular cytology in prepubertal beef bulls (182 to 207 days of age)

	Heifer	Cow	*P*-Value
Parameter	Mean ± SEM	Mean ± SEM	
Sertoli cells	34 ±1.7	45 ±2.8	0.312
Germ cells	73 ± 2.8	68 ± 2.9	0.06
Germ: Sertoli cell ratio	2.42 ± 0.12	1.96 ± 0.18	0.265
GATA4+ cell density	0.52 ± 0.03	0.71 ± 0.04	0.07

Sertoli cells = Sertoli cell automatic count per ST cross section in IHC-GATA4 tissue cuts.

Germ cells = Germ cell automatic count per ST cross section in IHC-GATA4 tissue cuts.

Germ: Sertoli cell ratio = total germ cell count divided by total Sertoli cell count per ST cross section in IHC-GATA4 tissue cuts.

GATA+ cell density = total GATA+ cell count divided by all DAPI+ cells in the field in IHC-GATA4 tissue cuts.

SEM = standard error of the mean.

Discussion

The Age of Dam and Body Weight, Scrotal Circumference and Testicular Parameters

Nutrient restriction in beef cattle is quite common in extensive grazing systems where the access to grass and roughage of quality is very limited with little or no supplement provision (Wu et al., 2006). Pregnant cow and heifer nutrient requirements differ, with heifers requiring supplementary nutrients to accommodate additional growth (Funston and Deutscher 2004). Duncan et al. (2018) reported higher placental and calf weights in multiparous cows compared to primiparous dams when placed under the same plane of nutrition. Among the different factors that contribute to the intrauterine environment, maternal nutrition is well documented as one of the major factors that determines placental and fetal growth (Barker, 1997). Alterations in the vascular development of the placenta has been proposed as one of the mechanisms mediating the effects of different planes of nutrition of the dam in fetal growth and development (Reynolds et al., 2005). A timing overlap between the rapid phase of placental growth and development, and

fetal organogenesis is described, implying that alterations in placental development at this time might affect normal organogenesis in fetus including the testis (Funston et al., 2010).

It is normal management practice to feed primiparous heifers with greater relative volumes and quality feeds trying to neutralize the negative effects that an equal plane of nutrition with multiparous dams might cause on heifer performance and fetal growth. Although a differential diet was implemented in heifers in the present study, a significant difference existed for body weight between bulls from heifers and cows ($P = 0.006$, Table 7). This is in accordance with the reports of Da Silva et al. 2003, where overfed immature ewes presented restricted placental growth and a numerical difference for decreased fetal weights although not significant. In a previous study, the same author (Da Silva et al., 2001) reported restricted placental growth, lower birth weights in lambs from adolescent ewes kept on a higher plane of nutrition compared with those kept at a moderate plane, presenting lower testicular weights, testosterone concentrations and delayed puberty suggesting an alteration in the hypothalamus-pituitary axis in the growth restricted lambs.

Deligeorgis et al., 1966 reported a lower pituitary response in undernourished fetal lambs to a GnRH challenge done at 55 days of age. In cattle, Sullivan et al., 2010 demonstrated how different planes of nutrition can alter the hypothalamus-pituitary-gonad axis in the bull fetus. In pregnant *Bos Indicus* cross heifers subjected to lower protein concentrations during the first trimester of pregnancy, bull calves presented higher pre-pubertal FSH concentrations and paired testicular volumes compared with those male calves from heifers subjected to higher protein and energy concentrations during the same period of gestation.

Lunstra et al., 1985 reported lower testicular weights and scrotal circumference in yearling bulls at age and weight constant from heifers vs. cow dams suggesting the effects of an

undefined *in utero* factor. This effect was observed across breeds, establishing a correction factor for scrotal circumference in bulls from heifers.

The data produced by several authors show us that the testicle growth and development of the fetus from heifers is more exposed to potential negative effects caused by alterations in the nutritional status of a more nutritionally vulnerable dam category mediated through alterations in placental growth, the hypothalamus pituitary axis or epigenetic effects.

Nevertheless, in the present experiment, no significant difference existed for scrotal circumference or testis weight between heifers and cows ($P = 0.63$ and $P = 0.39$ respectively; Table 7).

The Age of Dam and Testicular Cytology

In the present study, a numerical difference existed although not significant for lower SC counts per ST cross sections in HEIFERS vs. COWS bulls (34.13 ± 1.65 vs. 45.16 ± 2.83 respectively; $P = 0.31$) and a tendency for higher GC counts per ST cross sections and lower GATA4+ cell density in bull calves from heifers vs. cows ($P = 0.06$ and $P = 0.07$ respectively; Table 8).

In the overfed adolescent ewe model, Da Silva et al. 2003, reported no difference in SC counts per ST in the testicles of 103-day old male fetuses from dams on a higher plane of nutrition although a placental growth restriction was observed. A similar situation was observed in the present experiment, where bulls from heifers presented smaller birth weights compared to bulls from multiparous cows. Furthermore, it has been documented how different planes of nutrition in pregnant dams can affect testicular cytology of their male neonates (Robinson et al., 1999; Da Silva et al, 2001; Bielli et al,2002).

In sheep, Kotsampasi et al., 2009 showed how male lambs from pregnant ewes under a 50% metabolizable protein and energy requirements regime during their second half of gestation responded with greater FSH concentrations to a GnRH challenge at 10 months of age compared with non-restricted ewes. Furthermore, lambs from the restricted ewes presented lower SC counts and smaller ST diameters compared with control lambs from non-restricted dams. Deligeorgis et al., 1996 reported a lower pituitary responsiveness in lambs undernourished during their fetal stage at 55 days of age showing a clear disruption of the hypothalamus-pituitary axis. In rats, Genovese et al., 2010 reported lower testis weights, SC counts per ST cross sections and absolute numbers of SC per testis in undernourished mothers from pregnancy to puberty.

Nevertheless, the higher nutritional requirements imposed on heifers, along with normal physiological intake limitations described during the last weeks of pregnancy in cattle (Stanley et al., 1993) suggests the risk of a potential setup of a diet restriction like scenario in primiparous dams under extensive conditions if differential nutritional management is not implemented.

Conclusions

Bulls from heifers had lower body weights than bulls from cows, although no differences were observed in scrotal circumference or other gross testicular parameters.
Nevertheless, a numerical difference although not significant statistically for lower SC counts and a tendency for higher GC counts and GATA4+ cell density was observed in bulls from heifers. The lack of significant differences in cytology and testicular growth and development parameters in bulls from heifers vs. cows in the present study, might be reflecting the benefits of the application of differential nutritional managements for heifers and cows providing with their different nutritional requirements.

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THE EFFECT OF VITAMIN A ON TESTICULAR GROWTH AND DEVELOPMENT IN PERIPUBERTAL BEEF BULLS[4]

Abstract

Daily sperm production potential in the bull is determined by the size of the Sertoli cell population (SC) established in the testis. Sertoli cells replicate from the fetal stage until puberty when the cell population reaches its final size fixing the ceiling of potential sperm production in the bull. Vitamin A through its active compound retinoic acid (RA) has been reported as one of the factors that regulate SC division. The objectives of the present study were to assess the effects of the administration of vitamin A on scrotal circumference, intact and dissected testicular weights, and SC and germ cell (GC) counts in peripubertal bulls. Fourteen aged-matched Angus crossbred bulls (287±3.3 days, 310 ± 10 Kg) were randomly assigned to one of two treatments: 1) one single intramuscular injection of 1,000, 000 IU of vitamin A (Vitamin A D, Durvet laboratories, Blue Springs, MO) or 2) control (no treatment). Scrotal circumference was measured in all bull calves at the time of treatment administration and 11 days later at castration. Testes and epididymis were dissected and weighed, and testicular parenchyma samples were collected from each testicle, fixed in formaldehyde, embedded in paraffin and cut. Tissue samples for histology were stained with immunohistochemistry using androgen receptor (AR) as specific SC marker and DAPI as counterstain. Relative expression of genes related to RA metabolism and regulation (*ADHIC, RARα, RARβ* and *RARγ*) and SC maturation and differentiation (*CDKIB1, CK18, AMH* and *GATA4*) was determined using RT-qPCR. No

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differences were observed among treatments for scrotal circumference, testicular weight and testicular parenchyma weight, although bulls treated with vitamin A had heavier epididymis (g) (12.00±1.09 vs 8.35±1.13; $P = 0.042$). Furthermore, no differences were observed between treatments for SC and GC counts, and the GC: SC ratio. No differences were observed in the relative expression of those genes involved in RA metabolism and regulation, and SC maturation and differentiation. Administration of vitamin A had no effect on the growth, development and cytology of the testis in peripubertal beef bulls. Nevertheless, an increase in epididymis weight was observed in those bulls treated with vitamin A.

Key words: beef bulls, gene expression, germ cells, retinoic acid, Sertoli cells.

Introduction

Berndston et al (1987a) demonstrated that bulls sustaining a larger Sertoli cell population have greater Daily Sperm Production (DSP), testicular weight and scrotal circumference. In addition, bulls that produce good quality semen with greater higher numbers of viable spermatozoa after thawing present a higher proportion of SC numbers (Rajak et al. 2016). Sertoli cells proliferate during a specific window of time that extends from the fetal stage at mid-gestation, up to 20 weeks in the neonatal calf (Moura and Erickson 1997; Rawlings et al. 2008; O'Shaughnessy and Fowler, 2011). Once puberty is reached, Sertoli cells cease to replicate fixing the ceiling of potential daily sperm production in the adult bull (Berndston et al. 1987b).

Several factors are reported as affecting SC replication like thyroid hormone T3, testosterone (T), follicle stimulating hormone (FSH), insulin-like growth factor 1 (IGF-1), and vitamin A through its active compound retinoic acid (RA; Buzzard et al. 2003, Lucas et al. 2014). Retinoic acid has been implicated in the proliferation and differentiation of several tissues other than the testis like the prostate and tumors, interacting with factors like transforming

growth factor beta (TGF-β), (Cupp et al., 1999). Furthermore, genetically engineered mice lacking RA receptors show a delay in SC differentiation, progressive spermatogenic degeneration and infertility (Nicholls et al. 2013).

Recent papers refute the immutability dogma of SC quiescence after puberty, suggesting that under special circumstance SC can resume cell replication (Johnson et al. 2008; Zakhidov and Marshak, 2015). There is very little available information about interventions executed during the peri-pubertal period in the bull and their ramifications in adult daily sperm production.

We hypothesize that the administration of vitamin A can influence testicular growth and development and the establishment of the SC population in peripubertal bulls. The objectives of the present study were the assessment of the effects of vitamin A on scrotal circumference, testicular weight, SC and GC counts, and testicular gene expression of *RARα, RARβ, RARγ, ADHC1, CDKN1B, AMH, CK18,* and *GATA4* in peripubertal beef bulls.

Materials and Methods

The North Dakota State University Animal Care and Use Committee (IACUC #A16012) approved all procedures in the present experiment. Fourteen aged matched bull calves (287±3.3 days, 310 ± 10 Kg) from the North Dakota State University Beef Unit were used in the present study. Bulls were randomly assigned to one of two treatments:1) one single intramuscular injection of 1.000.000 IU of Vitamin A (Vitamin AD Injectable, Durvet laboratories, Blue Springs, MO), following the dose prescribed in the label of 2 ml to yearling cattle (500,000 IU/ ml), (VIT A; n = 8) or 2) no treatment (Control; n = 6). It has been shown that one single parenteral administration of vitamin A to bulls was of clearly higher effectiveness compared to an oral application considering an 11.6% higher liver recovery of vitamin A from fresh tissue

and a higher and faster plasma concentration achieved after the injection of a low dose in deficient animals Flachowsky et al., 1991).

The bulls diet consisted of 20% hay, 40% corn silage, 20% DDGS, and 20% corn. Scrotal circumference was measured at the time of treatment administration and at castration 11 days later.

Prior to castration, bulls were restrained and given an epidural anesthesia (8 ml of 1% lidocaine hydrochloride). The scrotum skin was opened using a Newberry knife and the testicles exposed. Pampiniform plexus, testicular artery and vas deferens were crushed and severed using an emasculator to prevent excessive bleeding. Once the testicles were excised, the excessive connective tissue was trimmed, and the spermatic cord sectioned at its testicular attachment. Next, the vas deferens was identified and sectioned at the union with the epididymis tail. The epididymis was dissected from the testicle and weighed separately. Finally, the tunica albuginea was removed from each testicle and the testicular parenchyma weighed.

Within ten minutes of collection, testicular parenchyma samples for gene expression determination were flash frozen in liquid nitrogen and stored at -80^0C until processing. Testicular parenchyma samples for histological examination (4 x 4 mm) were collected, fixed in a 10% buffered formaldehyde solution, embedded in paraffin and cut in 5 µ thickness sections using a microtome (Leica Biosystems Inc., Buffalo Grove, IL). Tissue sections for immunohistochemistry were deparaffinized through washes of Xylene, and decreasing concentrations of alcohol (100%, 95% and 70%). Samples were submerged in sodium citrate buffer and placed in an antigen retrieval (2100 Retriever, Aptum Biologics, UK) and incubated sequentially using mouse monoclonal antibody to androgen receptor (ab9474, abcam, Cambridge, MA) at 4^0C overnight with agitation, and then incubated for one hour at room

temperature with goat anti-mouse IgG (H+L) CF633 fluorescent antibody (Biotium, Freemont, CA). For each tissue section, images were taken at 20x magnification using a Zeiss Imager M2 epiflfluorescence microscope equipped with Zeiss piezo automated stage and AxioCam HRm camera (Carl Zeiss International, Jena, Germany). Image analysis (Image-Pro Plus, Media Cybernetics, Inc., Bethesda, MD, USA) was performed on images taken from five randomly chosen fields. Within each image, four to six seminiferous tubules were randomly selected for Sertoli and germ cells individual cell counts. The germ cell to SC ratio (RATIO) was calculated as the total number of DAPI positive cells (GC) within all ST divided by the total number of AR positive cells (SC) within all ST in all five images.

Gene expression was assessed using real-time quantitative polymerase chain reaction (RT-qPCR) for the following genes: retinoic acid receptors alpha (*RARα*), beta (*RARβ*), and gamma (*RARγ*), alcohol dehydrogenase C 1 (*ADHC1*), cyclin dependent kinase inhibitor 1 B (*CDKN1B*), anti-Műllerian hormone (*AMH*), cytokeratin 18 (*CK18*), GATA binding protein 4 (*GATA4*) and Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). RNA was extracted and purified using RNeasy Plus Mini Kit (Qiagen Inc, Valencia, CA). Concentration of extracted RNA was measured using a Take3 module of a Synergy H1 Microplate reader (BioTek, Winooski, VT). A total of 1 µg of RNA was used for cDNA synthesis using an iScript advanced cDNA Synthesis Kit (Bio-Rad laboratories Inc, Hercules, CA). Serial 1:10 dilutions of cDNA were done for primer validation for optimal cDNA concentration and primer efficiency.

Gene expression was quantified using a 7500 Fast, Real-Time PCR System (Applied Biosystems, Grand Island, NY) with SYBR Green Master Mix (Bio-Rad) in duplicate. The thermal-cycling conditions were as follows: hold for 20 s at 95^0 C, cycle for 3 s at 95^0 C, 30 s at

60 ^{0}C for a total of 40 cycles. Melt Curves for efficiency assessment were performed after each run under the following conditions: 15 s at 95^{0} C, 60 s at 60^{0} C, 15 s at 95^{0} C and 15 s at 60^{0} C.

Relative gene expression was calculated using the ΔΔCt method (Livak and Schmittgen, 2001) with *GAPDH* as the reference gene chosen for its consistency of expression and lowest M value (M = 0.196) after calculating and comparing the expression stability with four other candidate reference genes: *YWHAZ* (Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta), *ACTB* (β-actin), *GAPDH* (Glyceraldehyde-3-Phosphate Dehydrogenase), and *HPRT1* (Hypoxanthine Phosphoribosyl transferase 1) using the geNorm function within qbase+ software (Biogazelle, Zulte, Belgium) according to Vandesompele et al. (2002). Primer sequences were obtained using NCBI Primer Blast (Table 9).

Table 9. Primers used for determination of relative gene expression of retinoic acid receptor alpha, beta and gamma, anti-Műllerian hormone, cytokeratin 18, cyclin-dependent inhibitor 1B, transcription factor GATA4 and glyceraldehyde phosphate dehydrogenase in peripubertal bull testis (287±3.3 days)

Gene	Genbank Reference sequence accession number	Direction	Sequence
RARα[a]	XM_005220738	F	GCTCAGAACAGCGTGTCTCT
		R	CTTGGCGAACTCCACAGTCT
RARβ[b]	XM_015460896.1	F	GAGAGCTACGAGATGACGGC
		R	GCGCTGGAATTCGTGGTGTA
RARγ[c]	NM_001130756.1	F	AGCTCATCACCAAGGTCAGC
		R	GCACTGGAGTTCGTGGTGTA
ADH1C[d]	NC_037333.1	F	AAGGAAGCTGCACGCTAGTT
		R	CTCCTTCAGCCTGGTCAGTG
AMH[e]	NM_173890	F	TGGAAATGGTGCGCTCCTGA
		R	GTCTTTCGTGTGAAGCAGCG
CK18[f]	NM_001192095.1	F	CCTGAGGGCTCAGATTTTTGC
		bR	GCAGCAAGACGGGCATTATC
CDKN1B[g]	NM_001100346.1	F	AGGACACGCATTTGGTCGAT
		R	GGAGAGGAATCATCTGCGGC
GATA4[h]	NM_001192877.1	F	TCAAAGACACCAGCAGGTCC
		R	GTTTTGATGGGGCGCATCTC
GAPDH[i]	NM_001034034	F	AGGTCGGAGTGAACGGATTC
		R	ATGGCGACGATGTCCACTTT

[a]*RARα* = retinoic acid receptor alpha.
[b]*RARβ* = retinoic acid receptor beta.
[c]*RARγ* = retinoic acid receptor gamma.
[d]*ADHC1*= alcohol dehydrogenase C1.
[e]*CDKN1B* = cyclin-dependent kinase 1 B.
[f]*AMH* = Anti- Műllerian hormone.
[g]*CK18* = cytokeratin 18.
[h]*GATA4* = transcription protein GATA4.
F = forward; R= reverse.

Statistical Analysis

Mean differences between treatments for differences in scrotal circumference, intact testicle, dissected testicle and epididymis weights, and SC and GC count per ST cross sections were analyzed with one-way ANOVA using the PROC GLM procedure of SAS (SAS version 9.4; SAS Inst. Inc., Cary, NC). Treatment differences in relative quantification of gene

expression for *RARα, RARβ, RARγ, ADH1C, CDKN1B, AMH, GATA4, CK18* and *GAPDH*, were analyzed using the *t*-test procedure of SAS. Significant differences were considered when P < 0.05.

Results and Discussion

No significant difference for SC counts between groups ($P = 0.480$) was observed in the present study, suggesting a most possible scenario of an already established SC population in the testis at the time of vitamin A administration (Table 15). This agrees with the age of the animals of approximately 41 weeks at the moment of the treatment administration, which is above the limit reported by most researchers for the period of replication in Sertoli cells (Rawlings et al., 2008) and past the estimated time of occurrence of the early gonadotropin rise in the bull (Evans et al., 2005). Furthermore, no significant differences existed between groups for GC counts per ST cross section ($P = 0.12$; Table 10) and for the GC:SC ratio ($P = 0.14$).

Table 10. The effect of the administration of vitamin A on testicular development and growth of peripubertal Angus cross bulls (287 ± 3.3 days)

	Vitamin A (n = 8)	Control (n = 6)	*P* value
Parameter	Mean ± SEM	Mean ± SEM	
Difference in scrotal circumference, cm	1.62 ± 0.26	2.17 ± 0.17	0.135
Testicular weight, g	228.17 ±17.00	217.76 ±24.21	0.722
Testicular parenchyma weight, g	141.02 ±11.82	154.57 ±30.59	0.615
Epididymis weight, g	12.00 ±1.09	8.36±1.13	0.042
SC IHC	45.24 ± 3.16	48.16 ±1.99	0.169
GC IHC	216.46 ± 26.60	160.28 ±15.30	0.121
RATIO	5.09 ±0.88	3.38 ±0.38	0.139

[a]SC IHC = SC automatic count per ST cross section in IHC-AR tissue cuts.
[b]GC IHC = GC automatic count per ST cross section in IHC-AR tissue cuts.
[c]RATIO = total GC count divided by total SC per ST cross section in IHC-AR tissue cuts.
SEM = standard error of the mean.
P < 0.05.

The period of SC replication in the testicle has been studied exhaustively in many species like the human (Franke et al., 2004), rat (Livera et al., 2000), pigs (Berger et al., 2012) and sheep

(Rhind et al., 2001) but not as much in the bull. This is reflected in a lack of consensus among authors about the exact duration of this period of SC division in the bull (Rawlings et al., 2008; Moura et al., 2011). Although the accepted concept for most mammals is the cease of SC multiplication when puberty is reached (Berndston et al., 1987b) species like the golden hamster and the stallion present normal seasonal fluctuations in Sertoli cell populations size (Johnson et al., 2008). Recent findings defy the dogma of SC immutability and suggests mature SC are not terminally differentiated, and can be reprogrammed to their undifferentiated state, resuming mitosis under in vitro conditions (Zakhidov and Marshak, 2015).

The role of RA in testicular function has been studied for some time with several effects reported like the disruption of the sex cords during fetal development caused by high doses of vitamin A, and a halt in spermatogenesis during germ cell meiosis under vitamin A deficiency (Livera et al., 2002). Furthermore, RA acts as a modulator of the seminiferous epithelium cycle, regulating the initiation of meiosis in germ cells through a mechanism involving RA synthesis enzymes, RA receptors, and degradative cytochrome P450 enzymes (Griswold et al., 2012). Retinoic acid receptors have been localized within the Sertoli cell nucleus, playing a crucial role in the establishment and maintenance of the blood testis barrier (Hasewaga et al., 2012). The establishment of the BTB coincides with the induction of SC differentiation and the cease of SC replication, marking the onset of puberty (Sharpe et al., 2003).

Retinoic acid has been described as a key regulating factor of SC differentiation in equilibrium with activin, co-existing both factors for a short period of time around puberty (Nicholls et al., 2013). Furthermore, it has been reported that retinol and RA can modulate the effect of FSH on the Sertoli cell by decreasing levels of cAMP in vitro (Galdieri and Nistico, 1994), being FSH along with IGF-1, important mitogenic factors affecting SC replication in the

bull (Harstine et al., 2017). Furthermore, a decrease in paired testicular weight, daily sperm production and epididymis reserves has been observed in bulls subjected to a diet low in vitamin A concentration (Rode et al., 1994).

Interestingly, bulls treated with vitamin A had greater epididymis weights ($P = 0.042$; Table 10). This effect agrees with previous reports about the abundant presence of vitamin A receptors, in particular *RARα* in the epididymis, (Akmal et al., 1996). Using double negative dominant mutant transgenic mice for *RARα,* Costa et al. 1997 reported the presence of aberrant epididymis epithelial differentiation and the expansion of the connective tissue surrounding the ductus presenting different grades of male infertility, highlighting the importance of RA in the regulation of epididymal development and function (Ghanamm et al., 1969). Possible effects of vitamin A on the epididymis growth and development can be of great importance considering the epididymis is the organ responsible for the sperm transport, concentration, storage, and maturation. Furthermore, the maturation of sperm in the epididymis involves the acquisition of the forward motility and the ability to fertilize (Caballero et al. 2010).

Table 11. Testicular gene expression as relative quantification normalized to *GAPDH* for *RARα, RARβ, RARγ, ADHC1, CDKN1B, AMH, CK18* and *GATA4* in relation to vitamin A treatment in peri pubertal bulls (287±3.3 days)

	Control (n=6)	Vitamin A (n=8)	*P* value
	Mean±SEM[a]	Mean±SEM	
RARα[a]	0.96 ± 0.09	0.97 ± 0.06	0.93
RARβ[b]	1.62 ± 0.28	1.82 ± 0.20	0.58
RARγ[c]	0.96 ± 0.11	0.95 ± 0.47	0.98
ADHC1[d]	0.29 ± 0.12	0.17 ± 0.06	0.10
CDKN1B[e]	0.79 ± 0.08	0.88 ± 0.06	0.43
AMH[f]	0.63 ± 0.05	0.58 ± 0.05	0.62
CK18[g]	1.11 ± 0.14	1.10 ± 0.10	0.98
GATA[h]	0.97 ± 0.05	0.92 ± 0.06	0.61

[a]RARα = retinoic acid receptor α.
[b]RARβ = retinoic acid receptor β.
[c]RARγ = retinoic acid receptor gamma.
[d]ADHC1 = alcohol dehydrogenase 1 C.
[e]CDKN1B = cyclin dependent kinase inhibitor 1 B.
[f]AMH = anti-Műllerian hormone.
[g]CK18 = cytokeratin 18.
[h]GATA4 = GATA4 protein.
SEM = standard error of the mean.

The absence of differences observed between treatments for the expression of parenchymal RA receptors *RARα* (*P* = 0.93), *RARβ* (*P* = 0.58) and *RARγ* (*P* = 0.98), suggests no modulation effect was produced by the administration of vitamin A (Table 11). Although the period elapsed between vitamin A treatment and tissue sampling at castration was of only 11 days, it is enough to observe any effects on *RAR* and *ADH1C* expression. In previous experiments, changes in testicular tissue expression of *RAR* were observed within 24 h of the administration of vitamin A in vitamin A deficient rats (Van Pelt et al., 1992). Nevertheless, recent findings show that RA can also act through extranuclear, non-genomic effects through mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (Erk)

broadening the extension of vitamin A actions in SC, not restricted to the genes analyzed in the present study (Al Tanoury et al., 2013).

The lack of effects observed on the expression of RA receptors agrees with the absence of difference between groups for *ADHC1* ($P = 0.10$) expression, the enzyme responsible for the conversion of retinol to retinaldehyde and RA. Alcohol dehydrogenase 1 C is used as indicative of retinoid signaling during development and cell differentiation in adult epithelial tissues. (Duester et al., 2000).

Cyclin dependent kinase inhibitor 1B (*CDKN1B*) translation reaches its maximum during quiescence and early G_1 phase of the cell cycle (Besson et al., 2008). Extracellular growth factors which promote cell division reduce transcription and translation of $p27^{Kip1}$ (Holsberger et al., 2005). In the present experiment, no difference was observed between treatments for *CDKN1B* gene expression ($P = 0.43$; Table 11), suggesting a probable quiescent Sertoli cell population present in the testicle at the time of vitamin A treatment, in agreement with the lack of cytological effects observed in the testis.

Anti-Műllerian Hormone (*AMH*) is a glycoprotein secreted exclusively by undifferentiated SC and is responsible for the regression of the Műllerian ducts in the male (Capel, 2000). The expression of *AMH* is switched on during testis differentiation, extending postnatally when it is switched off once SC reach maturity, coincident with the onset of meiosis in germ cells (Rajpert-De Meyts et al., 1999). For this reason, *AMH* is one of the most widely used biomarkers of SC maturation (Edelsztein et al., 2016). Nonetheless, no significant difference was found in the present experiment between control and vitamin A groups for the expression of *AMH* ($P = 0.62$).

Cytokeratin 18 (*CK18*) is an intermediate filament characteristic of immature epithelial cells that disappears with cell maturation but whose expression in conserved by those SC that maintain undifferentiated immature features (Maymon et al., 2000). The co-expression of *AMH*, and *CK18* in immature SC, validates the use of CK18 as a marker of SC differentiation in infertile human patients (Steger et al., 1996). In the case of CK18, one of the cell maturity markers used in the present experiment, no difference was detected between control and vitamin A treated bulls ($P = 0.98$).

GATA4 proteins belong to the family of Zinc finger transcription factors that regulate gene expression, differentiation and cell proliferation. The early, intense expression of GATA4 in SC coincides with the proliferative phase of these cells during the first 2 postnatal weeks in the mouse (Ketola et al., 1999). *GATA4* plays a fundamental role in the formation of the BTB, a key feature of Sertoli cell maturation and in the production of SC derived lactate, primary energy source of spermatocytes and spermatids (Schrade et al., 2015). No significant difference existed between control and vitamin A treated groups for the expression of *GATA4* ($P = 0.61$).

Conclusions

In the present study, no effects were observed in testicular cytology of peripubertal Angus bulls after the administration of 1,000,000 IU of vitamin A. Concomitantly, the absence of significant differences in the expression of those genes linked with SC cell maturation and differentiation like *CK18* and *AMH* suggests a most probably scenario of an already established and quiescent SC population at the time of vitamin A treatment.

The response observed in a highly RA regulated organ like the epididymis in treated animals highlights the importance of administering vitamin A at this age of the peripubertal beef bull.

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SUMMARY AND CONCLUSIONS

Within a herd, the bull is the individual that bears the major responsibility in the overall fertility as it is normal that a single sire breeds between 25 to 60 cows. Breeding soundness examination is one of the best tools we have at present to assess the potential reproductive capacity of a bull, presenting limitations to distinguish between different levels of fertility among those bulls included in the apt group. At present there is no single factor that allow us to predict potential daily sperm production in the bull while the use of multiple bulls under grazing conditions contributes to hide those bulls with a lower reproductive capacity. For this reason, the selection process of a replacement bull at weaning in a commercial farm or the incorporation of a young sire selected through genomics into an AI center are loaded with a great amount of uncertainty about the bull's future reproductive performance. The fact that yearling bulls are poor sperm producers compared to older bulls, adds difficulty to the correct assessment of its potential reproductive performance. The key role played by Sertoli cells in the nourishment and physical support of germ cells from the early formation of the primordial sex cords in the late embryonic stage and later throughout the whole spermatogenic process explains why the size of the cell population established before puberty is one of the major determinants of daily sperm production in the bull. The development of an accurate technique to assess the size of the cell population and the design of interventions aiming to influence the size of that cell population were the foundations behind the two lines of research presented in this program. In the first study, no significant correlation could be established for SC density across techniques, preventing the use of 22G FNA and CORE to determine SC density in prepubertal bulls. The lack of correlations shown between smears produced using a 22G FNA reflects a low repeatability that might be the consequence of intrinsic properties of the technique itself. The

impossibility of keeping uniform vacuum levels and the loss of needle control by the use of a hand-held syringe could be some of the factors against obtaining the same cell counts between samples (Haseler et al. 2011). Nevertheless, the development of mirror tissue sections and the performance of cell counts in the same seminiferous tubule cross section using IHC-GATA4 and HE demonstrated the value of GATA4 as a specific SC cell marker in neonatal and prepubertal bulls and the high accuracy of automatic cell counts done with the image software. The high correlation observed between SC counts in seminiferous tubule cross sections and SC density with GATA4+ cell density in the field infers SC density determination in tissue sections can be automated and done faster without the need of a cytology specialist. The significant relationship established between CORE and HIS on cell counts done within the microstructure of the seminiferous tubule which is preserved in both techniques, suggests the absence of the ST structure in smears might be one of the reasons contributing to the absence of correlations observed.

The lack of differences in birth weights, testicular weights and cytology between bulls from corn supplemented vs. non-supplemented dams was in accordance with the lack of effects observed between both groups for placental weight and blood flow parameters as reported in the simultaneous experiment developed by Tanner (2017). Nevertheless, the experiment contributed to fill the gap of information existent about the effects of diet supplementation during pregnancy in cattle and the effects on testicular development in the bull. Most of the studies have been done in other species and using a restriction model. Interestingly, a high correlation was observed between glucose concentrations of the dams and GATA4+ density in bulls, suggesting a relationship between glucose levels and testicular cell activity. The expression of GATA4 has been associated with the production of lactate as the main energy source for germ cells by SC

and with immature SC replication, opening a new interesting line for research. The difference in ADG and body weights in steers from supplemented dams observed at 21 days of age suggests a possible temporary effect of the corn supplement that was manifested later in the neonate. This observation opens the question if the delayed effect could have caused possible alterations in the testicles if the animals remained intact.

Although no differences were observed in scrotal circumference as reported by Lunstra et al. (1985) between bulls from primiparous heifers and cows, significant differences existed for birth weights. In addition, bulls from heifers had lower numbers of SC although not significant and tendencies for higher germ cells and lower GATA4+ cell density suggesting cytological differences could have been present if a differential diet was not implemented. The additional requirements of growth during pregnancy imposed on immature heifers puts them at risk of creating possible in utero nutritional effects on the fetal testicle, highlighting the importance of different diet provision for heifers and cows.

There are very few studies conducted on the effects of administering vitamin A to peripubertal bulls. The lack of differences in SC counts between the vitamin A group and control was in accordance with the lack of gene expression of those genes related to SC differentiation. Considering the pro differentiation effect of retinoic acid on the testis, the administration of vitamin A did not have any effect on the size of the cell population at this age of the bulls, providing a safe time frame for the use of vitamin A without alterations on the testicular cytology and most importantly, without inducing a premature SC differentiation causing a decrease in the final population size. The effect on the epididymis growth observed in vitamin A supplemented bulls suggests its administration on peripubertal bulls should be recommended.

Future Directions

When we consider the research developed in the present dissertation, we can clearly see room for improvement in the experiments and the opening of new exciting lines for research. In the technique comparison study, the use of an automated system of FNA using a reciprocating procedure device or RPD might improve needle and vacuum control, producing the same vacuum in all samples increasing the technique consistency (Haseler et al. 2011). Furthermore, the addition of a second antibody with GATA4 or the use of a different cell marker with expression exclusively in Sertoli cells like WT1 (Sharpe et al. 2003) might contribute to eliminate the potential interference in cell counts caused by the presence of other GATA4 positive cells in smears. In addition, it would be interesting to perform the techniques *in vivo* and not on the testicles post castration as it was done in the present experiment. Another element that should be considered is the use of different fixatives for SMEAR vs. CORE and HIS. Considering the different biochemical reactions on tissue proteins during the fixation process caused by formaldehyde and alcohol, the use of the same fixative in all samples might improve the design of the study (Denda et al. 2012).

One of the contributions of the present experiments was the identification of window times where it is possible to exert an effect on Sertoli cell replication. Although there were no significant effects on testicular growth and development and cytology in the corn supplementation study, the observation of a delayed temporary effect on calf growth at 21 days of age suggests corn supplementation effects might occur later in the prepubertal bull (Tanner 2017). It would be interesting to study the possible effects of energy and protein supplementation during the dam gestation at different ages of the bull, not limited to the neonate (Sullivan et al. 2010).

Sertoli cells are described as being susceptible to the action of several hormones and metabolites (Lucas et al. 2014). One of the most important mitogenic factors identified on SC are FSH and IGF-1 (Dance et al. 2017). The use of KO mice lacking IGF-1 receptors in Sertoli cells showed a dramatic decrease in the numbers of Sertoli cells and a concomitant effect on other testicular cell types (Griffeth et al. 2014). In addition, several reports show how both FSH and IGF-1 share common pathways and IGF-1 is proposed as the mediator of FSH effects during Sertoli cell replication (Khan et al. 2002). Furthermore, Brito et al. 2007 showed how IGF-1 played a key role as a metabolic mediator during the early rise of gonadotropins in the bull. It would be interesting to study the effects of induced increases in IGF-1 concentrations on prepubertal bulls by the administration of recombinant bST.

As a concluding remark, the plasticity of Sertoli cells during specific time frames in the late fetal, neonatal and prepubertal stage of the bull opens a wide range of possible interventions with the objective of increasing the size of the Sertoli cell population and daily sperm production in the bull.

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APPENDIX

Testicular Parenchyma Sampling Protocol for Conventional Histology and Fine Needle Aspiration

1. Bulls are restrained in a cattle chute during the surgical procedure of castration using the open knife procedure. It is recommended the use of a Newbery knife or a scalper.
2. Before commencing the procedure, local anesthesia is injected into each spermatic cord using 4 ml of 2% lidocaine.
3. It is important to allow enough time for the local anesthetic to diffuse in the tissue once injected and check the sensitivity of the area before cutting the scrotum.
4. While the anesthesia is working, the scrotum is cleaned thoroughly using warm water and soap, and a 10% iodine solution in alcohol is applied to prepare the incision site.
5. Once the testicles are excised and trimmed of excessive connective tissue, the same testicle size should be used for tissue sampling.
6. The skin is opened at the bottom of the scrotum using a scalpel or Newbery knife. The parietal layer of the *tunica vaginalis* is excised and the testicles exposed.
7. An emasculator is strongly recommended for cutting and crushing the spermatic cord, preventing excessive bleeding.
8. Once the testicles were excised and trimmed of excessive connective tissue, the vas deferens was identified and cut at the union with the tail of the epididymis.
9. Each testicle is weighed with the epididymis attached. Next, the epididymis is dissected, and both the testicle and epididymis were weighed separately.
10. The same side testicle is used to collect the samples (right or left testicle using the same side for all the samples).

11. For histology samples, testicular parenchyma of approximately 4 x4 mm are collected.
12. It is very important that the tissue sample is devoid of any albuginea tissue, allowing the fixative to penetrate better.
13. The tissue sample is put in a plastic jar containing a 10% buffered formaldehyde solution. It is recommended to use a fixative to tissue ration not less than 20:1.
14. The tissue should be allowed to stay in the fixative solution for a period not less than 72 h.

Tissue Collection Using Fine Needle Aspiration with 22G or 14G Needle

1. Tissue collections using 22G needle (SMEAR) or 14G needle (CORE) should be done on the other half of the testis not used for previous sample collections, avoiding regions that could be altered by previous sampling.
2. Syringes not bigger than 10 ml (5 ml syringes are preferred) should be used for the tissue collection as larger volume syringes have a lagging period before achieving maximum vacuum once the plunge is withdrawn.
3. The needle is attached to the syringe and gently inserted into the testis, while advancing perpendicularly into the testicular parenchyma.
4. Once the needle is fully introduced into the testis, the plunger is pulled back creating vacuum inside the syringe to draw a cellular sample into the needle.
5. While the plunger is pulled back, the needle is moved forward and backward within the testis two to three times for approximately four seconds, dislodging the cells and facilitating their suction into the needle.
6. The plunger is released before withdrawing the needle from the testis to avoid the aspirate being sucked into the syringe.

7. The syringe is detached from the needle, filled with air, and used to expel the aspirate on a clean glass slide previously identified with the bull number, needle caliber, date and time.
8. The slide with the smear is allowed to dry not less than 10-15 min.
9. Once the slide is dried, it is put into a 95% alcohol solution and processed in a histology laboratory.