THE INFLUENCE OF RETINOIC ACID ON CELL PROLIFERATION AND DIFFERENTIATION IN LAMB TESTIS TISSUE

by

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DEDICATION

I dedicate this thesis to my friends and family. I dedicate this thesis to my oldest friends, Ashley Anne Philogene and Katelyn Rose Moore. I dedicate this thesis to Christopher Harrison Cheek for his love and support before my acceptance into graduate school and throughout this whole journey. Most importantly, I dedicate this thesis to my parents, Belinda Martina Molloy and Alan Gerard Molloy, for showing me what it means to be a hard worker and a strong woman, and for always providing love, support, and encouragement throughout my life.

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LIST OF ABBREVIATIONS

Proliferating cell nuclear antigen ..... *PCNA*
Proto-oncogene c-Kit ..... *c-Kit*
Stimulated by retinoic acid gene 8 ..... *STRA8*
3β-Hydroxysteroid dehydrogenase *HSD3-β*
Seminiferous tubule ..... ST
Retinol ..... RE
Retinoic acid ..... RA
Spermatogonial stem cells ..... SSCs
Minimum Essential Medium ..... MEM
Handling media ..... HM
Fetal bovine serum ..... FBS

ABSTRACT

THE INFLUENCE OF RETINOIC ACID ON CELL PROLIFERATION AND DIFFERENTIATION IN LAMB TESTIS TISSUE

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The majority of wild ungulate species are threatened or endangered by extinction. Populations managed in zoos and breeding centers serve as 'insurance' for species sustainability and future reintroductions. However, 10-15% of animals born in *ex situ* collections die before reaching puberty and therefore fail to contribute to conservation breeding. Understanding the *in vitro* culture requirements for producing gametes from gonadal tissues could facilitate the rescue of germplasm from genetically valuable individuals. Most studies on this topic have focused on the laboratory mouse, with limited information on larger animal models. Retinoic acid (RA) is the biologically active form of vitamin A and is involved in the early steps of spermatogenesis by promoting meiosis to occur in progenitor cells. Furthermore, RA is also involved in Sertoli cell and gonocyte proliferation and/or differentiation in various species. It was hypothesized that RA would promote Sertoli cell and gonocyte proliferation and/or differentiation in testicular explants that were cryopreserved and thawed before *in vitro* culture. Testicular

pieces (1-2 mm^3) from 6-7 week-old lambs (n = 6) were cryopreserved using the slow cool method. Tissues were thawed at room temperature (1 min), then in water (25°C; 1 min), followed by three washes (5 min each) in MEM containing 20% FBS, 25 mM HEPES, and antibiotics). Thawed explants (5 pieces/treatment/week/lamb) were cultured for 5 weeks in the absence (0 μM, control) or presence (1 μM, 2 μM, and 5 μM) of RA (RA1, RA2, and RA5, respectively). Tissues were cultured on agarose blocks in MEM supplemented with 10% (v/v) FBS, sphingosine-1-phosphate (2 μM; for the first 2 weeks), insulin (2 μg/ml), transferrin (1.1 μg/ml), selenium (1 μg/ml), pyruvate (0.1 mM), glutamine (2 mM) and antibiotics. Tissues were harvested weekly to be assessed histologically and for gene expression studies. Twenty tubules of uniform size per piece were evaluated for the number of gonocytes and Sertoli cells. Tissues were analyzed for the expression of *PCNA* (cell proliferation), *c-Kit* (cell differentiation), *Stra8* (synthesis of RA-responsive protein), and *HSD3-β* (testosterone synthesis). Analyses were performed with a linear mixed model followed by a Tukey's test for post-hoc comparisons. Gonocyte counts changed continuously over 5 weeks of culture, and responded the most favorably to RA1 ($p < 0.05$). Under all treatment groups, Sertoli cell counts were maintained for the first three weeks of culture ($p > 0.05$), before sharply declining by week 4 ($p < 0.05$). In addition, Sertoli cells did not respond differently to RA1 or RA2 compared to the control ($p > 0.05$), but declined when treated with RA5 ($p < 0.05$). Both *c-Kit* and *PCNA* expression changed over 5 weeks, but *PCNA* was negatively impacted by RA5 ($p < 0.05$) while *c-Kit* responded positively to either RA2 or RA5 ($p <0.05$). *Stra8* experienced dramatic changes over the culture period, and responded

positively to RA1 ($p < 0.05$) compared to the other treatments. *HSD3-β* had the highest expression at week 4 before declining by week 5 ($p < 0.05$), but there was no effect of RA. This study demonstrates for the first time that lamb testicular explants can be 1) cultured *in vitro* for up to five weeks and 2) retinoic acid stimulates pathways involved in germ cell differentiation while promoting cell proliferation and steroidogenesis. Although advanced stages of spermatogenesis were not achieved in this study, results addressed critical knowledge gaps pertaining to long term culture of testicular tissue and the role of retinoic acid in lamb spermatogenesis *in vitro*.

CHAPTER ONE: INTRODUCTION

There are multiple challenges involved in preventing species extinction. Species that have small population sizes suffer from inbreeding and a loss of genetic diversity. Therefore, zoological institutions world-wide manage numerous species to protect them against extinction. However, 10 to 15 percent of endangered ungulates born in captivity die before reaching puberty, further adding to a crisis in keeping valuable genes present in the population (Horiszny et al. 2015; Piltz et al. 2012; Wildt et al. 2013). Growing reproductive tissues *in vitro* to the point of producing gametes offers an additional tool and could assist with conservation efforts. With advances in cryopreservation and thawing techniques, reproductive tissues can be stored long-term until needed. Previous studies have supported the notion that growing testicular tissues in cultures is possible, along with keeping structural integrity and promoting cell growth (Baert et al. 2015; Cai et al. 2016a; Cai et al. 2016b; de Michele et al. 2017). If testicular tissue from neonatal organisms can be collected at death, cryopreserved, and successfully thawed and cultured to the point that spermatozoa are produced, their valuable genetic information can be recovered. Retinoic acid (RA; a metabolite of vitamin A) has been linked to meiotic activity within precursor cells which eventually leads to the production of spermatozoa (Hogarth and Griswold 2010; Busada et al. 2015b). The effect of RA on cell cultures has been extensively studied to better understand the complex mechanisms that control

spermatogenesis (Busada et al. 2015a; Endo et al. 2015; Hogarth et al. 2014; Lambrot et al. 2006). However, few studies have investigated the effects of RA on long-term cultures (over three weeks), and most published studies involve the murine model.

Mammalian spermatogenesis is a highly complex process that begins with stem cells and culminates in formation of mature spermatozoa. The time required for complete spermatogenesis is species-specific, but in larger, more complex mammals this occurs over the course of at least 40 to 54 days (Hogarth and Griswold 2010). For male sheep, this process takes up to 47-48 days with puberty reached at 5-6 months (Bilaspuri and Guraya 1986). This process involves the interaction of a diversity of paracrine and endocrine factors within the highly structured and compartmentalized microenvironment of the seminiferous tubule (ST) and its epithelium (Hogarth and Griswold 2010). The complexity of ST structure and function illustrate the challenges and new knowledge needed to achieve successful spermatogenesis *in vitro*.

To date, the murine model is most widely used for *in vitro* testicular tissue research (Hogarth and Griswold 2010), while research in other models such as pig (Cai et al. 2016b; Wang et al. 2014) and humans (Lambrot et al. 2006) mainly revolve around culturing spermatogonial stem cells (SSCs). The failure to achieve complete spermatogenesis *in vitro* in other animal models highlights species-specific requirements for supporting testicular tissue proliferation and differentiation. There is a knowledge gap with respect to the minimum requirements for maintaining testicular explants and/or to achieve sperm production in various species. Therefore, there is an urgent need to

understand mechanisms regulating testes function *in vitro* in animal models other than the laboratory mouse.

From a conservation perspective, understanding the culture conditions to promote *in vitro* testicular tissue survival and differentiation could permit the rescue of germplasm from genetically valuable endangered species that die prematurely. To date, most efforts have focused on retrieving spermatozoa directly from the cauda epididymis from adult donors (Abu et al. 2016; Bruemmer 2006; Okazaki et al. 2012). Few studies have successfully grown testicular tissues *in vitro* to harvest spermatozoa. This is particularly important for neonatal animals wherein spermatogenesis has yet to begin. However, a major challenge to developing these technologies is maintaining tissue viability long enough to stimulate proliferation and differentiation of SSCs, leading to production of mature sperm.

If the technologies to grow sperm from testicular tissue recovered from neonate donors is developed, then this *in vitro* produced germplasm could be used to prevent inbreeding depression as the rescued gametes can still contribute to the population's gene pool (Andrabi and Maxwell 2007). The challenges with this concept is that long-term culturing of testicular tissue is yet to be successful due to oxidative stress and tissue necrosis (Bustamante-marín et al. 2012). Previous research has focused on the mechanisms behind differentiating precursor sperm cells *in vitro* (Arkoun et al. 2015), while there is still a need to investigate the growth of intact testicular explants for sperm harvesting.

This present study used the ovine model, contributing to the need for more research directed at tissue explant culture in vertebrates larger than a mouse. The challenges of keeping tissue explants alive *in vitro* to support complete spermatogenesis is a major area of investigation. Most studies have focused on understanding the effect(s) of different supplements on mitigating oxidative stress and preventing necrosis (Orth et al. 1998; Zhang et al. 2015; Zhao et al. 2014). The potential beneficial effects of vitamin A and its metabolites on mammalian reproductive systems (both male and female) also is currently being investigated to promote gamete viability. Most studies have focused on RA as it is the biologically active form of vitamin A and is efficiently processed compared to other metabolites (Busada et al. 2015b). Few studies have investigated the effects of RA on promoting spermatogenesis in tissue cultures over an extended period of time.

Spermatogenesis

The production of spermatozoa in the testis is a tightly regulated process with mechanisms that are not yet fully understood. Spermatogenesis involves three key processes: the renewal of stem cells and progenitor cells through mitosis, the creation of haploid progenitor cells through meiosis, and the differentiation of these haploid cells through spermiogenesis (Hogarth and Griswold 2010). Progenitor cells are the early precursors to gametes and are commonly known as A spermatogonia. When a progenitor cell starts the differentiation process, they become A1 spermatogonia and are ready to enter meiosis to eventually produce spermatocytes. Specific genes correlate to this process of spermatogonia entering meiosis. The expression of *c-Kit* within testicular

tissue is a marker for spermatogonial differentiation (Busada et al. 2015a). Progenitor cells undergoing differentiation express high amounts of *c-Kit* expression compared to other cell types.

The ST within the testes are highly organized with defined groups of germ cells in various stages of spermatogenesis. Histological analysis of a tubule will reveal a systematic progression of germ cells to the next developmental stage, also referred to as the spermatogenic wave (Hogarth and Griswold 2010). While the general steps involved in spermatogenesis is similar in most mammalian species, the number of developmental stages in each cycle of the seminiferous epithelium is species-dependent (Hogarth and Griswold 2010). In the ST of a mouse, the transition of A spermatogonia to A1 spermatogonia and subsequently into meiosis occurs every 8.6 days, whereas for humans the same cycle takes 16 days (Hogarth and Griswold 2010). Spermatogenesis starts when gonocytes migrate from the lumen of the seminiferous tubule towards the basement membrane to go through a series of mitotic divisions, transforming into spermatogonia at the basement membrane (Hogarth and Griswold 2010). The resulting spermatogonia are now ready to enter the next stage of cellular development. Once spermatogenesis is complete, spermiogenesis then occurs as each round spermatid elongates and morphologically transforms into a motile spermatozoon. After the completion of spermiogenesis, the spermatozoa are released from the seminiferous epithelium and enter the tubule lumen.

The process of spermatogenesis is made possible through the Sertoli cells. These complex cells line the tunica propria of the ST and serve many functions, one of which is

to provide structural support to the tubules while providing nourishment to the spermatozoa (França et al. 2016), and also facilitating spermatogenesis through contact with germ cells (Hogarth and Griswold 2010). In addition, Sertoli cells also breakdown the cell-cell junctions binding the spermatozoa to the spermatids to facilitate their release **(**Korhonen et al. 2015). Immature spermatozoa then passively move from the testis to the epididymis via the rete testis, a network of interconnecting tubules located in the mediastinum testis that carries sperm from the ST to the efferent duct (Hogarth and Griswold 2010. Spermatozoa gain motility and the ability to fertilize an oocyte as they pass through the epididymis (Hogarth and Griswold 2010). The processes involved in the transformation of spermatogonia to motile spermatozoa is tightly regulated with many complex mechanisms involving hormonal influence and cell-to-cell communication (Sofikitis et al. 2008).

Regulation of Spermatogenesis

Spermatogenesis requires hormone regulation and direct cell-to-cell interactions between multiple cell types, such as Sertoli cells and germ cells. Leydig cells are localized among the ST and synthesize testosterone under the control of luteinizing hormone (LH) from the pituitary. Sertoli cells are regulated both by follicle-stimulating hormone (FSH) directly from the pituitary and testosterone from the Leydig cells (Hogarth and Griswold 2010). Testosterone binds to the androgen receptor (AR) and works with FSH as it binds to the FSH receptor (FSHR) to promote Sertoli cell function – supporting spermatogenesis.

Gene mutations that affect these hormones or their receptors can have negative implications for sperm production and viability. Male mice with mutations in the gene that encodes for FSHR have been reported as having a reduced testis size, correlating with the lower Sertoli cell numbers and therefore lower sperm counts (Hogarth and Griswold 2010). Testosterone helps regulate the release of germ cells so that they can begin the cell division process after meiosis (O'Shaughnessy et al. 2009). Along with having a direct influence on Sertoli cells, testosterone also works through the surrounding peritubular myoid cells to indirectly influence the Sertoli cells (Hogarth and Griswold 2010). Testosterone also is important for the regulation of spermatocyte differentiation through spermiogenesis.

FSH is responsible for regulating undifferentiated A spermatogonia and differentiated A1 spermatogonia by stimulating mitosis (Griswold 1998). However, previous studies have confirmed that while FSH and testosterone are important for their actions on Sertoli cells in initiating spermatogenesis, neither hormone is important in the initial entry of germ cells into meiosis (O'Shaughnessy et al. 2009). However, it is known that the A1 spermatogonia are transformed into spermatocytes via meiosis, which requires retinoic acid (Hogarth and Griswold 2010). The exact mechanism of retinoic acid in regards to regulation of meiotic division is unknown but is under investigation.

Retinoic Acid

Retinoic acid (RA) is the biologically active form of retinol (RE), also known as vitamin A. In contrast with RA, RE is converted into retinyl esters in the Sertoli cells for storage, instead of being provided directly to the A spermatogonia (Tedesco et al. 2013).

This is because within the body, the transport of RA via serum to the target tissues is difficult. As a result, RE is stored in the liver and mobilized to various parts of the body as needed before it is oxidized to RA (Hogarth and Griswold 2010). RA is also responsible for triggering A spermatogonia (undifferentiated cells) to undergo meiosis, which is the first step towards differentiation (Hogarth & Griswold, 2010). Sertoli cells are the main site of RA synthesis in the testis and may be responsible for providing RA to germ cells (Busada and Greyer 2015).

RA activity can be measured by the expression of *Stra8* (Stimulated by Retinoic Acid Gene 8). *Stra8* is responsible for regulating meiosis in both the ovaries and testes (Anderson et al. 2008). This gene is induced by RA, which in turn codes a 45-kD protein found in the cytoplasm (Oulad-Abdelghani et al. 1996). Currently there is little known about this protein, other than its role in promoting meiosis for spermatogenesis and that it is specifically expressed as the testes develop during embryogenesis (Miyamoto et al. 2002). Furthermore, in both embryonic and adult testes RA signaling causes *Stra8* expression in germ cells but not in somatic cells (Koubova et al. 2006). *Stra8* also promotes spermatogonial differentiation, which decreases when *Stra8* is not present (Endo et al. 2015). RA has a positive influence on both meiotic initiation and spermatogonial differentiation, and may act directly within the spermatogonia or indirectly on the surrounding Sertoli cells (Endo et al. 2015). Based on these previous studies, *Stra8* is a reliable marker for measuring the influence of RA in testes.

Retinoic Acid's Role in Spermatogenesis: *In Vitro* Studies

RA determines when germ cells enter meiosis, and as a result it has a strong influence on the seminiferous epithelium cycle. Our current understanding of RA's role in the testes is based on studies conducted on laboratory mice. In vitamin A-deficient mice, only undifferentiated spermatogonia and Sertoli cells are present and no spermatozoa are produced (Li et al. 2010). Germ cells failed to differentiate, leading to their accumulation in the testes of these mice. Once the mice are given vitamin A, they experience spermatogenic activity again (Li et al. 2010). This further supports that RA plays an important role in initiating meiosis within the testes. However, the extent of RA's mechanism most likely varies by species as a similar study conducted in rats showed that some preleptotene (early meiosis) spermatocytes are present, indicating that the inhibition of differentiation is not as complete in rats compared to mice (Hogarth and Griswold 2010).

A method of observing RA activity *in vitro* is by stimulating all available A spermatogonia to become A1 spermatogonia in vitamin A-deficient mice at the same time by reintroducing RA, which results in synchronized differentiation throughout the testis (Hogarth and Griswold 2010). Synchronicity causes the normally continuous spermatogenetic wave to disappear, resulting in spermatozoa to be released only at one point in time within seminiferous epithelium cycle (Pelt et al. 1996). While synchronizing spermatogenesis through vitamin A manipulation is not ideal for a living organism, it provides an easier way to study the different stages of the cycle and the complicated mechanisms between each stage. Recent studies have found that RA improves the

amount of spermatozoa production from testicular explants derived from pre-pubertal mice (Arkoun et al. 2015). Along with promoting the differentiation of germ cells, RA also has promising implications for tissue viability within cultures. Recently, Travers et al. (2013) evaluated different concentrations of RA and RE on mice testes explants and found that retinoic acid led to a reduction in apoptosis within the tissues while also promoting intratubular cell proliferation and cell differentiation.

Studies with Non-Murine Models

Reproductive technologies have been improved for a wide variety of species using mammalian testicular tissues from models other than the mouse. A previous study examined the effects of cryopreservation, freezing, and thawing on adult (Cai et al. 2016b) and neonatal bovine testes (Cai et al. 2016a). In both studies tissue integrity remained stable post-thaw. Spermatogonial stem cells (SSCs) proliferated quickly when cultured with Sertoli cells (Cai et al. 2016b), while gene marker *c-kit* increased in neonatal testes, tissues also exhibited signs of cells dispersing from their colonies along with cyst formations in some cells after 9 days of cell culture (Cai et al. 2016a).

Another study used porcine spermatogonial germ cells and transplanted them successfully into immune deficient mice, showing successful colonization and localization on the seminiferous tubule basement membrane in the recipient (Lee et al. 2013). Although individual cells and not full tissue explants were used, it shows the successful growth of transplanted cells in an *in vitro* culture system. Lee et al. (2013) cultured at different temperatures, with 31ºC being the most successful based on expression patterns of pluripotency marker genes. Menegazzo et al. (2011) used porcine

Sertoli cells to improve human sperm quality in long-term culture. SSC culturing can only occur in short periods of time as long-term cultures cause damage to the cells. Fresh human spermatozoa were co-cultured with Sertoli cell layers from prepubertal pig testes and found that the viability and motily of the sperm cells were maintained after 7 days, eliminating the need for cryopreservation for that period of time. Pramod and Mitra (2014) reported that isolated goat SSCs were stable after 1.5 months of *in vitro* culture in the presence of Sertoli cells. These studies demonstrate that Sertoli cells are able to preserve germ cell function, sperm viability, motility, and mitochondrial function.

Few studies have examined the influence of RA in larger vertebrate models. Recently, a study on young boars examined *Stra8* expression in SSCs, demonstrating testis-specific responses with most expression occurring in the cytoplasm (Wang et al. 2014). This study is one of the few that have attempted to examine *Stra8* expression outside of the mouse model. In contrast to similar experiments done in mice (Arkoun et al. 2015), germ cells in fetal human testes cultured with RA undergo increased apoptosis with a decrease in germ cells (Lambrot et al. 2006). Past research focused on using murine testicular tissues for studying RA activity, though there is currently an increased trend in studying its effects on other species such as pigs (Wang et al. 2014), cattle (Barendse et al. 2007), and humans (Griswold et al. 2015). Currently RA inhibition is also being investigated as a potential male contraceptive (Paik et al. 2014). Most studies focus on understanding the mechanisms of RA activity within the testis in short-term culture, particularly for in the murine model.

Challenges with *In Vitro* Testicular Tissue Cultures

Previous studies have demonstrated that growing testicular tissue in cultures can maintain the structural integrity of tubules while also supporting intercellular communication (Baert et al. 2015; de Michele et al. 2017). Considering the effects of cryopreservation on *in vitro* culture of testicular tissue is important to develop reliable technologies for collection, storage, and use of cryopreserved gonadal tissues. Success in maintaining seminiferous tubule integrity and preserving both Leydig and Sertoli cell function has been achieved in human testicular tissue (de Michele et al. 2017). While maintenance of tissue and cell integrity is important, the cultured tissues must also be able to proliferate and differentiate *in vitro.*

Objective and Hypotheses

Preserving genetic diversity of endangered populations is a challenge as genetically valuable individuals may experience reproductive issues or early death. Each species under the Species Survival Plan® (SSP) has a studbook – a database containing information pertaining to the genetic relatedness of the breeding members within each population. Captive breeding programs are important for creating self-sustaining populations while also increasing genetic diversity through careful breeding management. The early death of those individuals before the chance to reproduce results in the loss of genes in the population, further adding to the issue of inbreeding that impact many endangered ungulates. The ability to preserve and grow the testicular tissues of a deceased male so that their sperm can be harvested and used to create offspring would be

revolutionary in the efforts for eventually creating self-sustaining populations of endangered species.

This study aimed to investigate the effect of RA on stimulating *in vitro* spermatogenesis in cryopreserved testicular explants derived from pre-pubertal lambs. There were two main objectives to this study. The first was to understand the effect of RA on spermatogenesis in cryopreserved testicular explants from pre-pubertal lambs while also understanding the ideal RA concentration for cell proliferation and differentiation. The second objective was to understand the conditions needed to promote tissue viability in a multi-week culture system. It was hypothesized that increased concentrations of RA would ultimately result in increased cell proliferation of gonocytes and Sertoli cells, and cell differentiation was expected to occur through the transformation of gonocytes to spermatogonia and to more advanced stages of spermatogenesis with increased concentrations of RA over the course of a 5-week culture.

CHAPTER TWO: METHODS

The RA treatment groups were 1 μM, 2 μM, and 5 μM. There was a control with no RA, and a treatment group containing DMSO at 5 μM RA (0.05% DMSO in media) to assess the influence of DMSO alone on testicular tissue. The tissues were cultured for five weeks with half the media replaced every 48 hours. Tissue samples were collected on Day 0 for comparison with treatment groups, with the other tissues collected at the start of each week.

Sample Preparation

Testes from 6-7 weeks old lambs (n = 6 animals) were used. The testes were opportunistically collected from the University of Maryland- Eastern Shore Campus following routine castration per university guidelines and were processed within 24 hours. Testes from individual donors were placed in transport medium which consisted of Minimum Essential Medium (MEM; Sigma-Aldrich, Cat. #M2279) with Earle's salts supplemented with 6.38 mg/ml Penicillin and 13.12 mg/ml Streptomycin sulfate (Sigma-Aldrich, Cat. #P4333) and shipped at 4˚C via overnight delivery. Immediately upon receipt, testes were washed in Handling Medium (HM) consisting of MEM (Sigma-Aldrich, Cat. #M2279), Fetal Bovine Serum (FBS; Sigma-Aldrich, Cat. #F4135)

supplemented with HEPES buffer (Sigma-Aldrich, Cat. #M2279) and Penicillin Streptomycin (Sigma-Aldrich, Cat. #P4333) and processed for preparation of explants. Testes from each donor were processed individually. For preparation of explants, the testes were separated from the epididymis and then the tunica albuginea was removed using a pair of fine forceps and scissors (Pukazhenthi et al. 2015). The testicular parenchyma was then cut into long, thin strips and further cut into 1-2 mm^3 explants.

Approximately 15 pieces of testicular tissue were placed in a labeled cryovial (1.8 ml) with 0.5 ml of freezing medium consisting of TCM 199 (Sigma-Aldrich, Cat. #M4530) and 20% FBS and supplemented with ethylene glycol solution (Sigma-Aldrich, Cat.# 721972). A minimum of six separate vials per donor were cryopreserved. Vials were placed inside a CoolCell™ slow freezing unit (Biocision®, San Rafael, California), and stored in a -80˚C freezer overnight. The following morning, the vials were removed from the freezer and immediately plunged into liquid nitrogen, transferred to metal canes, placed inside a protective plastic sleeve, and stored in liquid nitrogen for at least 6-8 weeks.

Thawing Testicular Tissue for Culture

At the start of culture preparation, 2-3 vials per animal were removed from long-term liquid nitrogen storage and transported to the laboratory in a small liquid nitrogen dewar. Each vial was thawed for 1 minute at room temperature, followed by a 25°C water bath for 1 minute (Pukazhenthi et al. 2015). Two mL of HM was added to each vial to dislodge the tissue pieces, which were then emptied into a sterile culture dish, and incubated at room temperature for 2 min with occasional mixing. The tissues were then

transferred to another petri dish with 5 mL of fresh HM and incubated (5 minutes) with occasional stirring. This step was repeated two more times to remove the cryoprotectant from the tissue pieces. Thawed tissue pieces were then cut into smaller pieces and assigned to various treatments. To allow for the tissues to equilibrate in their new culture media, the tissues were evenly divided into the different treatment groups (RA, DMSO, and control), with no less than 30 pieces per treatment (5 tissues per well, 5 weeks). Each week, testicular pieces (2-4) were placed in 2 ml of Bouin's solution for histological assessments and were incubated overnight (4˚C). The Bouin's solution was then replaced with 70% ethanol and stored at 4˚C until processed for histological evaluations. In addition, explants (2-3) were placed in labeled cryovials, flash-frozen in liquid nitrogen, and stored at -20˚C until used for gene expression analyses.

Tissue Culture and Sample Collection

Agarose (1.5% w/v; AMRESCO; #0710-100G) was heated in sterile water using a microwave oven and poured into a 60 mm plastic petri dish to allow gel formation. Once cooled, the agarose gel was cut into uniform blocks and immersed in 10 mL of pre-culture media (MEM and 10% Knock-Out Serum Replacement, and 1% penicillin-streptomycin) before placed in the incubator at 30°C and 5% CO_2 overnight. The culture medium consisted of MEM with 10% KSR supplemented with 2 μM of sphingosine-1-phosphate for the first 2 weeks (Tocris Bioscience, Bristol, United Kingdom, cat. #1370), insulin (2 μg/ml), transferrin (1.1 μg/ml), selenium (1 μg/ml), pyruvate (0.1 mM), glutamine (2 mM), testosterone (10 μg/ml) 1% penicillin-streptomycin and antimycotic solution (Sigma-Aldrich, Cat. #A5955). The culture medium was then divided into

separate tubes with the different concentrations of retinoic acid (1 μM, 2 μM, and 5 μM; diluted with DMSO) and incubated for at least 1 hour before application to the well plates.

Two plates per animal (Costar® 24 Well Clear TC-Treated Multiple Well Plates, Sterile, Cat. #3527) was used for a total of 30 wells each (5 treatment groups, 6 weeks). A single agarose block was placed in each well and 250 μL of the corresponding culture medium (based on treatment) was added to each well. Five tissue pieces were placed and evenly spaced out on each agar block and incubated at 30°C with 5% CO_2. Every 48 hours, half the media was replaced for each treatment group. At the end of each week, tissue samples were removed from culture, weighed using a sterile petri dish, and placed in previously autoclaved 1.5 mL Eppendorf tubes (one for histology and one for gene expression assessments). Bouin's solution was placed in the tubes for histology before being stored at 4°C overnight. Tubes for gene expression were flash-frozen in liquid nitrogen and stored at -20°C.

Preparation for Histological Assessment

The tissue pieces were incubated in 70% ethanol at room temperature with gentle rotation, with the ethanol changed every half hour to remove the Bouin's solution (until the solution was clear of yellow coloration). The tissues were then removed from the tube and placed at the center of tri-folded lens paper which were snapped into tissue cassettes (Fisherbrand, #15182701K). Immediately after placement, the cassettes were submerged in a 70% ethanol bath for 10 minutes which was repeated for a total of 3 washes. The cassettes were then moved into ascending grades of ethanol (80%, 95% and 100%) using

the same washing method before being placed in two SlideBrite (Sasco Chemical Group, Inc.) baths that were 10 minutes each in duration. After the wash series, the cassettes were transferred through a series of three jars containing liquid paraffin wax (Parablast, SIGMA-ALDRICH, P3558) at approximately 55°C. The cassettes were placed in the first wax jar for an hour, transferred to the second jar with wax for another hour, and then incubated overnight in the third jar of liquid paraffin wax. The following day, the tissues were removed from the cassettes and evenly placed in a metal mold before hot wax was added from a dispenser. The molds were then quickly transferred to a cold plate and held for approximately 15 seconds to harden the wax, and then transferred to a cold plate (-20°C) for 30 minutes before long-term storage at 4°C.

Preparation of Slides

The wax blocks were removed from the refrigerator and kept on ice before sectioning (6 microns) using a microtome and placed on glass slides. A total of 21 serial sections were made from each tissue block. The sections were separated in consecutive groups of three and were placed in a water bath at approximately 45°C. Each section was then transferred to a premium superfrost microscope slide (Fisherfinest, 12-544-7) and labeled with Lamb ID, week, treatment, and section number. Slides were placed on a warm plate (42°C) to dry overnight before staining. The staining followed a standard eosin and hematoxylin protocol before adding a slide cover with permount (Fisher Chemical) and left to dry overnight. The slide labels were then covered and numbered to avoid bias when performing histological assessments. 20 tubules of uniform size per tissue were evaluated (quantitation) for numbers of gonocytes and Sertoli cells.

Gene Expression Assessments

The tissue samples for RNA isolation were removed from -20°C storage and processed immediately using the "RNeasy mini kit" (Qiagen, Germantown, Maryland; Cat. #74136). The tissues were disrupted and homogenized in 350 µL (<20 mg tissue) or 600 µL (≤ 30 mg tissue) lysis buffer RLT with β-mercaptoethanol (1 µL per 1 mL RLT), centrifuged for 3 minutes at maximum speed (13,000 x *g*) and the supernatant was saved. An equal amount of 70% ethanol was added to the supernatant and mixed by pipetting before being transferred to an RNeasy Mini spin column with collection tube and centrifuged for 15 seconds at 8,000 x *g*. The flow-through was discarded and 700 µL of wash buffer RW1 was added and centrifuged for 15 s at 8,000 x *g*. Five hundred µL of wash buffer RPE was added, centrifuged for 15 s at 8,000 x *g*, with the flow-through discarded, and the addition repeated for 2 minutes at 8,000 x *g*. The spin column was then placed in a new collection tube and centrifuged at full speed for 1 minute to dry the membrane. Finally, the spin column was placed in a new 1.5 mL Eppendorf tube with 30 µL of RNase-free water applied directly to the spin column membrane, centrifuged for 1 minute at 8,000 x *g*. This step was repeated with the flow-through to ensure a high concentration of RNA (per kit guidelines). The resulting RNA sample was assessed using a NanoDrop One (Thermo Scientific, Waltham, Massachusetts), with ideal purity ratios being approximately 1.8 for A260/A280 and between 1.8 and 2.2 for A260/A230.

For cDNA synthesis, the SuperScript® III First-Strand Synthesis SuperMix kit (Invitrogen™ Cat. #18080-400) was used, with the volume calculated for each RNA sample based on concentration to achieve 50 ng/uL of RNA for each sample. From the

kit, the following components were added to a 0.2-mL PCR tube (Thermo Scientific) on ice: the specific RNA amount (calculated based on concentration), 1μL of Oligo(dT) primer, 1 μL annealing buffer, and RNase/DNase-free water for a total of 8 μL. The tubes were mixed, centrifuged, and incubated in a thermal cycler at 65°C for 5 minutes, and then immediately placed on ice for at least 1 minute. The 2X First-Strand Reaction Mix (10 μL) and the SuperScript® III/RNaseOUT™ Enzyme Mix (2 μL) was then added to each tube before being mixed, centrifuged, and incubated for 50 minutes at 50°C (specific to the Oligo(dT) primer), then at 85°C for 5 minutes to terminate the reaction before storing on ice (-20°C) for polymerase chain reaction (PCR).

The genes selected for expression studies were *PCNA*, *c-Kit*, *Stra8*, and *HSD3-β*. *β-actin* was used as a reference gene. The expression of *PCNA* was chosen for cell proliferation (Zhang et al. 2017), *c-Kit* was chosen as a marker for spermatogonial differentiation (Busada et al. 2015a), *Stra8* (Anderson et al. 2008) was used as a marker for RA action, and *HSD3-β* (Rasmussen et al. 2013) was chosen for steroidogenesis activity. All genes were expected to be between 125-150 base pairs in size.

Table 1. Target genes with their corresponding forward and reverse primers.

Gene	Forward Primer	Reverse Primer
β–actin	5'-GCG GCA TCC ACG AAA CTA-3'	5'-TGA TCT CCT TCT GCA TCC TGT C-3'
PCNA	5'-GTG TTT TGC CTC TCG CTC TC-3'	5'-GAA GGG TTA GCT GCA CCA AG-3'
c-Kit	5'-GTT GGG CCA GTG GAT GTA CA-3'	5'-CCC ACA TCG TTG TAA GCC CT-3'
Stra8	5'-GAA TCG TAT TCA GGA ACT GGA-3'	5'-GCA TAT TCT TTC TTG GCC TC-3'
HSD3-β	5'-TTC CCG GAT GAG CCT TCC TA-3'	5'-GTC ACT AGG TGG CGG TTG AA-3'

Genes were tested through qualitative PCR using different RNA concentrations and different annealing temperatures to determine ideal primer conditions. A master mix was prepared for each primer set based on the amount of reactions (including one additional reaction for pipette error). In a 0.2 μL PCR tube, AmpliTaq Gold™ 360 Master Mix (12 μL) was combined with PCR-grade water (6.5 μL), the forward and reverse primer set (1 μL each) and 4 μL of cDNA for a 25 μL reaction. The tubes were then placed in a thermal cycler (Bio-Rad, C1000 Touch™, Hercules, California) and was denatured at 95°C for 10 minutes, followed by a repeat of 30 cycles of 95°C for 15 seconds, annealing temperature (based on primer) for 30 seconds, followed by extension at 72°C for 1 minute before repeating the cycle, and then a final elongation stage at 72°C for 10 minutes.

The resulting PCR products were analyzed via gel electrophoresis using a 2% agarose gel made with Tris/Borate/EDTA (TBE) buffer (Thermo Scientific, Cat. #B52) with SYBR™ Safe DNA Gel Stain (1 μL per mL TBE; Invitrogen; Cat. #S33102) and heated via microwave. The resulting gel was poured into the electrophoresis chamber with a gel comb inserted and left to solidify (approximately 30-45 minutes depending on gel size). TBE buffer was then poured over the gel to easily remove the comb. DNA loading dye (GelPilot, Qiagen Germantown, Maryland, Cat #239901) was used to load the ladder and PCR products into the wells via pipette after being evenly mixed on sterile parafilm. The gel was then run at 100 v and checked using GeneSys (GBOX-CHEMI-XX6, Syngene, Frederick, Maryland) for gel imaging and analysis. Gels were examined

for amplification product size, issues such as potential contamination in the PCR product or non-specific primer binding.

All target genes were determined to amplify well at 50 ng/μL of RNA with a 60° annealing temperature. Quantitative Real-Time PCR (qPCR) was used to determine the amount of relative gene expression in each sample. Lightcycler 96-well plates (Roche; Cat. #04729692001, Indianapolis, Indiana) were used in conjunction with the LightCycler 96 Instrument. Each plate held eight individual samples at a time with each of the five genes in duplicates. Master mix was prepared for each primer using 10 μL of Fast Start Essential DNA green master (Roche), 7 μL of PCR-grade water, and 1 μL of each primer (per reaction). Nineteen μL of master mix was loaded into the corresponding wells, followed by 1 μL of cDNA which was thoroughly mixed with the master mix via pipette. The plates were then sealed, briefly vortexed and centrifuged before being placed in the LightCycler. The conditions for all plates were: a preincubation step of 95°C for 10 minutes, followed by a 3-step amplification period of 95°C for 10 seconds, 60°C for 10 seconds, and 72°C for 10 seconds. The final step (melting) involved 95°C for 10 seconds, 65°C for 10 seconds, and 97°C for 1 second. Melting curves were generated to confirm purity (single product) of amplification products. Ct values were compared between the reference gene (*β-actin*) and each target gene to calculate the fold change per treatment group using the ΔΔCt method. Duplicates for all samples were averaged to get one Ct value. Ct values were first separated based on the target gene (*PCNA, c-Kit, Stra8,* and *HSD3-β*) and then sorted by duration in culture (week). The target gene Ct value was

subtracted from the *β-actin* Ct value for each lamb per treatment group (ΔCt), and then ΔCt for each treatment group was subtracted from the ΔCt of the control group to get the final 2^-(ΔΔCt) value for each sample.

Statistical Analysis

All statistical analyses were performed using R statistical software (R Core Team, 2012). All histological analyses were performed with tissue nested within individual lamb and set as a random effect with a linear mixed model (Kuznetsova et al. 2017). There was no interaction effect of treatment and week. DMSO was determined to not have an effect on histology or gene expression and was also removed from the model. A Tukey's post-hoc comparative test was included to detect significant differences between the treatment groups and the weeks. Gonocytes and Sertoli cells did not fit a normal distribution and were skewed to the right, so a Poisson distribution was applied to the data set. Gene expression analyses were performed using a linear mixed model with a random effect of individual lamb. The fold change values from the qPCR data was also right-skewed, so data were transformed using an xbox transformation to account for the small values, before a Poisson distribution was applied. The model-estimate means and standard errors were used to construct the figures (mean ± SEM) and a p value of 0.05 was used to determine significance.

CHAPTER 3: RESULTS

Histological Evaluation of Testicular Tissue Explants

Explants were comprised of seminiferous tubules containing Sertoli cells, gonocytes and interstitial tissue (Figure 1). Tissue architecture appeared normal with no excessive space among seminiferous tubules. However, most explants expressed varying degrees of necrosis/fibrotic changes during the 5-week culture period (Figure 2). Fibrosis/necrotic changes were first observed in the center of the tissue and expanded outwards following extended culture. Despite these changes, most tissues expressed morphologically normal seminiferous tubules on the periphery of the testicular explants.

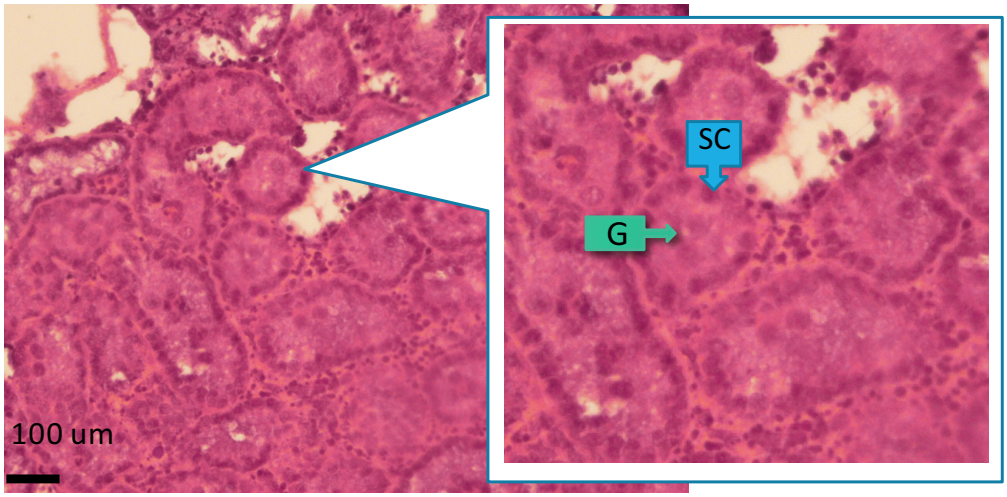

Figure 1. Histology of week 1 control tissue with a gonocyte (G) and Sertoli cell (SC).

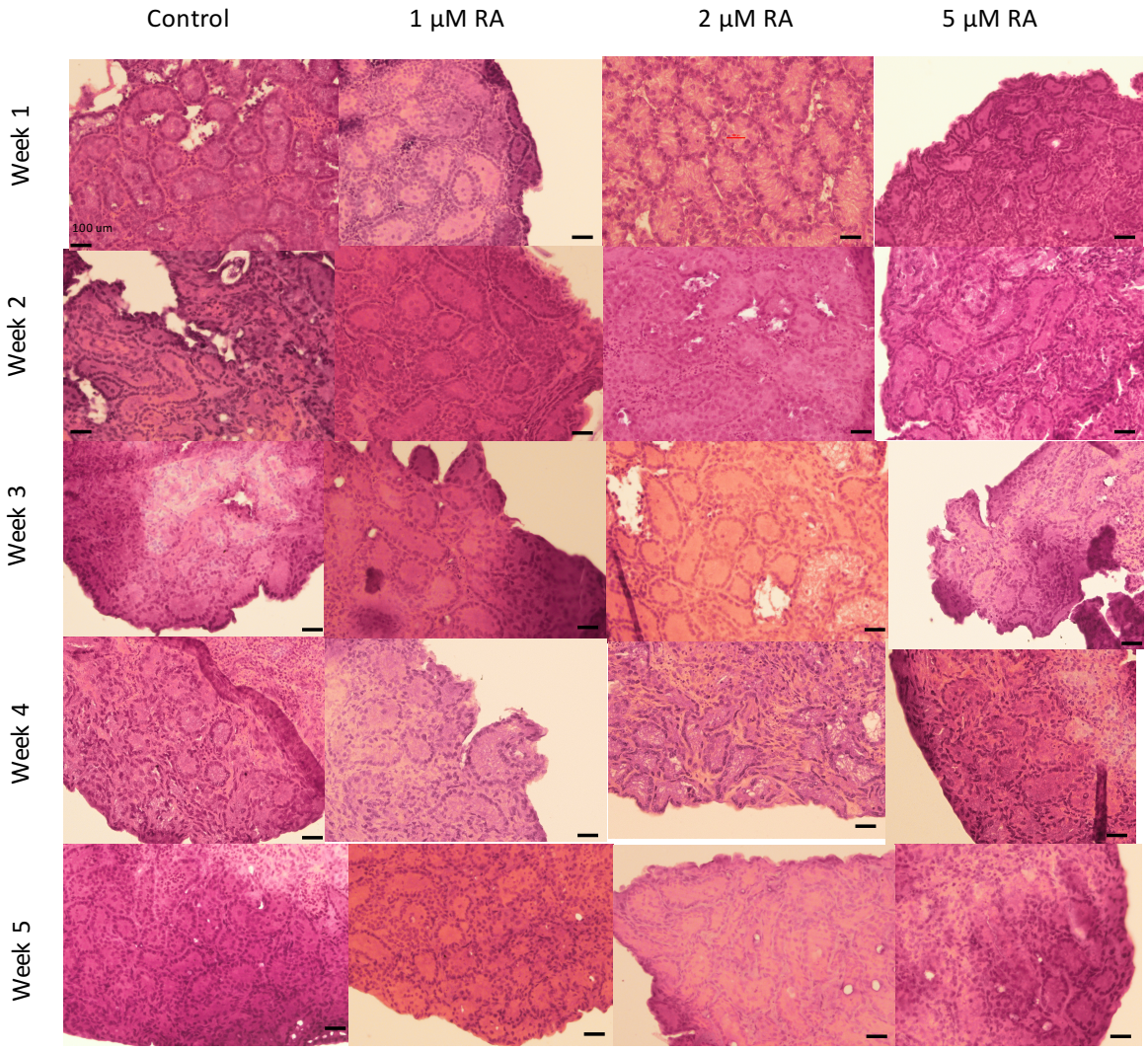

Figure 2. Histological comparison of tissue samples over 5 weeks and across treatment groups (control, RA1, RA2, and RA5).

Following *in vitro* culture on agarose blocks in a gas-liquid interphase, the number of gonocytes exhibited changes over time (Figure 3). Gonocyte numbers decreased ($p < 0.035$) from week 1 (48.80 ± 1.05) to week 2 (44.91 ± 1.05) but there was no ($p > 0.05$) further change in gonocyte numbers from week 2 to week 3 (42.64 ± 1.05). Thereafter, in week 4, the number of gonocytes increased slightly (50.73 ± 1.05) to return

to week 1 numbers but exhibited a further sharp ($p < 0.001$) decline (39.00 ± 1.05) by week 5.

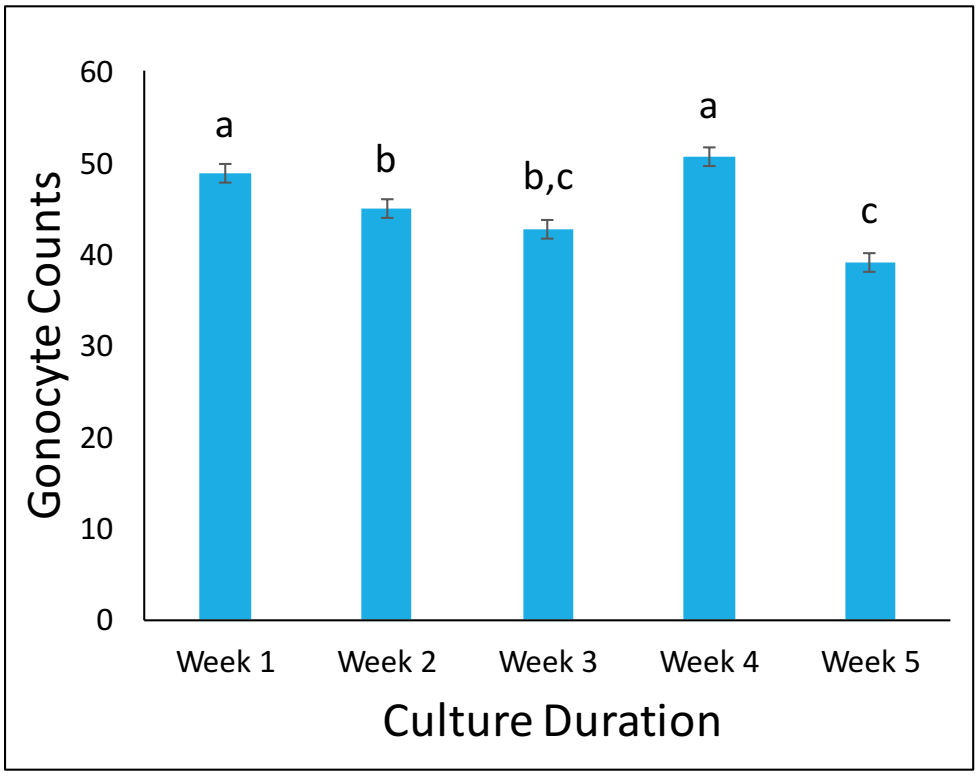

Figure 3. Changes in the number of gonocytes over a five-week culture period (per 20 tubules) in *in vitro* cultured pre-pubertal lamb testicular explants. Values represent mean ± SEM. Bars with different superscripts are statistically significant.

When comparing the different retinoic acid concentrations (Figure 4), there were no differences in gonocyte numbers among the control, RA1 (1 µM) and RA5 (5 µM). However, testicular explants cultured in the presence of 2 µM retinoic acid (RA2) exhibited a sharp decline in gonocyte numbers (42.52 ± 1.05) compared with the other treatment groups ($p < 0.001$).

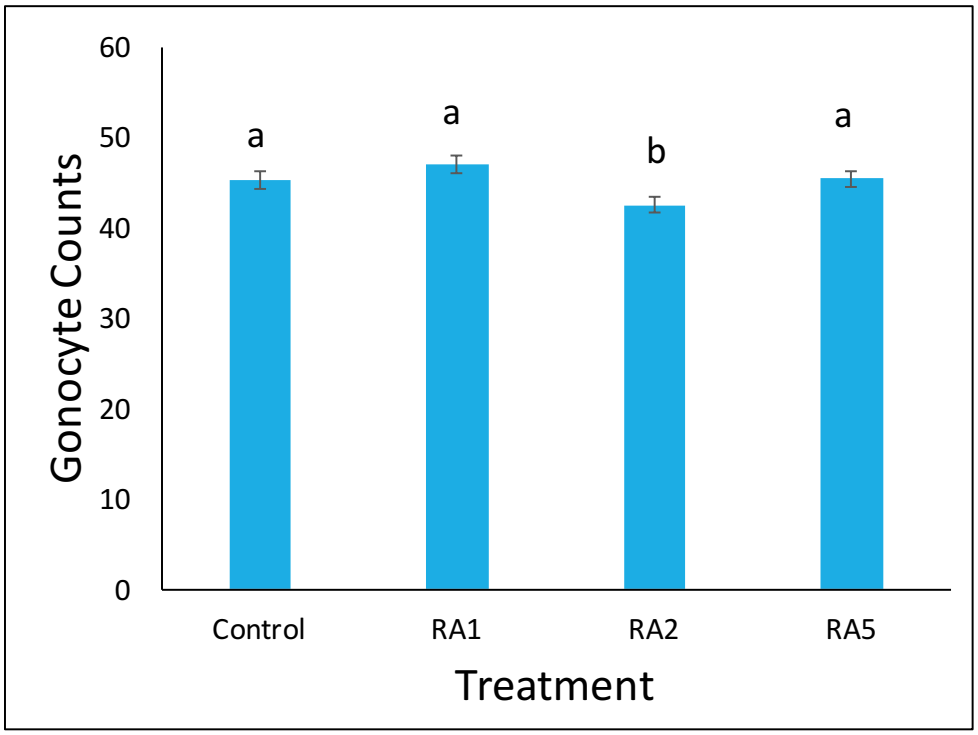

Figure 4. Changes in number of gonocytes following 5 weeks of *in vitro* culture of pre-pubertal lamb testes in the absence (control) and presence of RA (1 µM, 2 µM and 5 µM). Values represent mean ± SEM. Bars with different superscripts are statistically different.

Sertoli cell numbers remained unaltered ($p > 0.05$) for the first three weeks of *in vitro* culture, but declined ($p < 1x10^{-4}$) sharply by weeks 4 and 5 (Figure 5). Sertoli cell

numbers were lower ($p < 0.0001$) in week 4 (299.87 ± 1.03) and week 5 (295.76 ± 1.03) compared with weeks 1-3. Furthermore, there was no difference ($p > 0.05$) between week 4 and week 5.

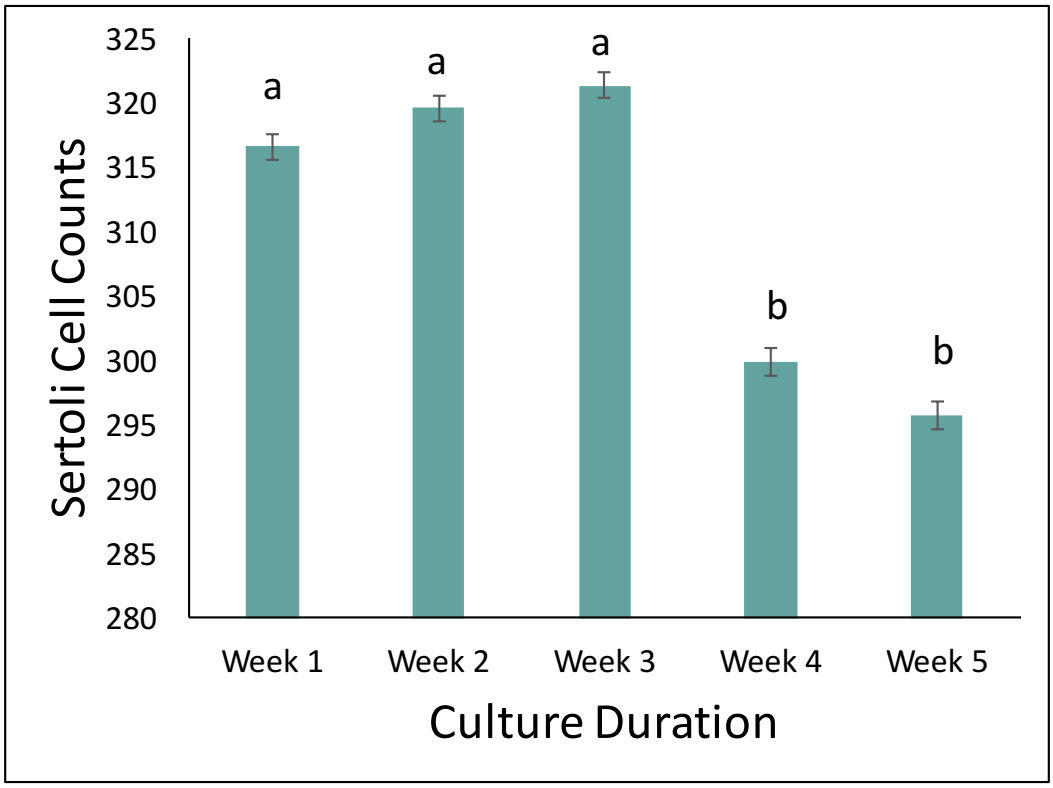

Figure 5. Changes in the number of Sertoli cells (per 20 tubules) following 5 weeks of *in vitro* culture of pre-pubertal lamb testes. Values represent mean ± SEM. Bars with different superscripts are statistically different.

Sertoli cell numbers decreased throughout *in vitro* culture in the presence of RA (Figure 6). Although there were no differences in Sertoli cell numbers between control and RA1 or RA1 and RA2, *in vitro* culture in the presence of higher concentration of RA

(RA5) resulted in a significant decline in Sertoli cell numbers (299.26 ± 1.03) compared with control ($p < 0.001$) and RA2 ($p = 0.0376$).

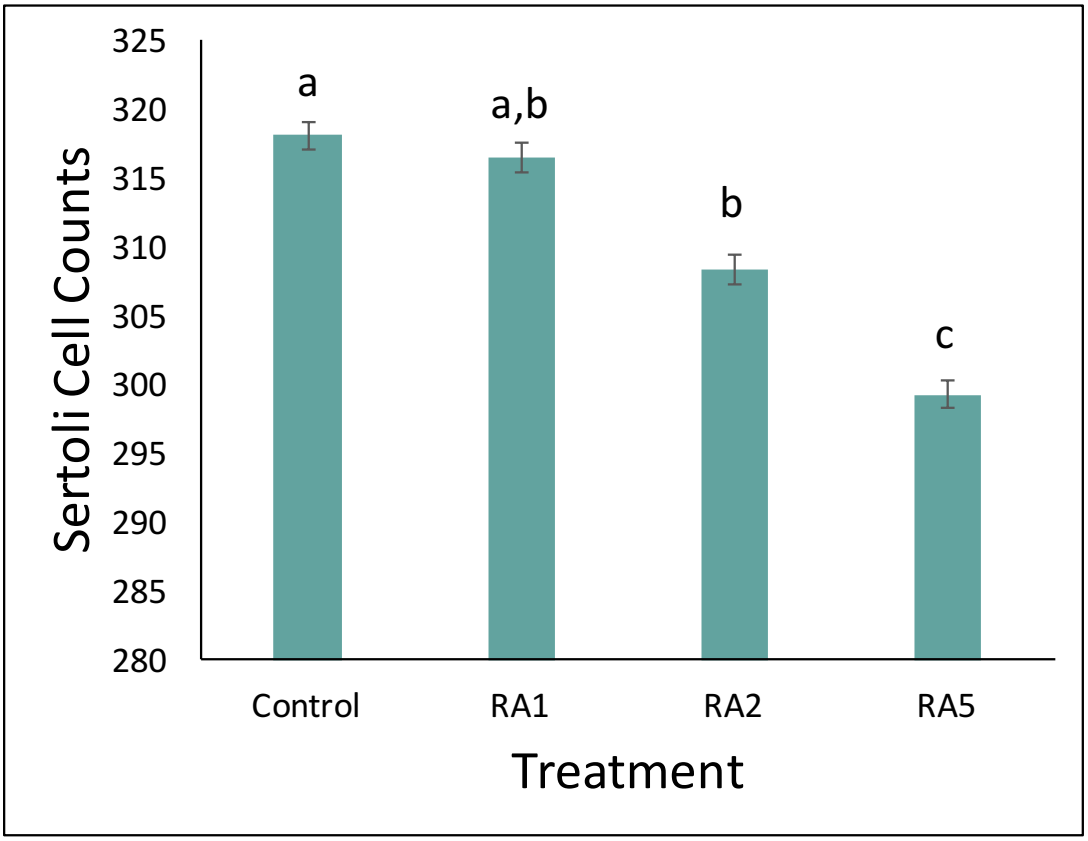

Figure 6. Changes in number of Sertoli cells following 5 weeks of *in vitro* culture of pre-pubertal lamb testes in the absence (control) and presence of RA (1 µM, 2 µM and 5 µM). Values represent mean ± SEM. Bars with different superscripts are statistically different.

Gene Expression Analysis

Gene-specific primers were designed using sequences from ENTREZ database, and were checked through qualitative PCR and gel electrophoresis (Figure 7). All genes yielded products of expected size (see Methods).

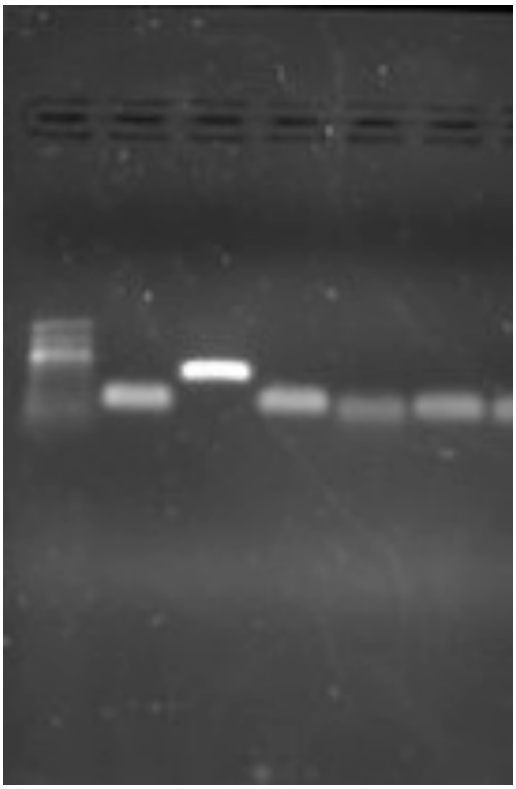

Figure 7. Gel electrophoresis image of a 50 base-pair ladder with *β-actin*, *PCNA, c-Kit, Stra8,* and *HSD3-β* (left to right) at an annealing temperature of 60°C.

Proliferating Cellular Nuclear Antigen (*PCNA*)

Overall, *PCNA* gene expression increased 1.28-fold ± 0.01 ($p < 0.001$) in week 2 and then declined ($p = 0.04163$) until week 4, before returning to week 1 levels by week 5 ($p > 0.05$) (Figure 8).

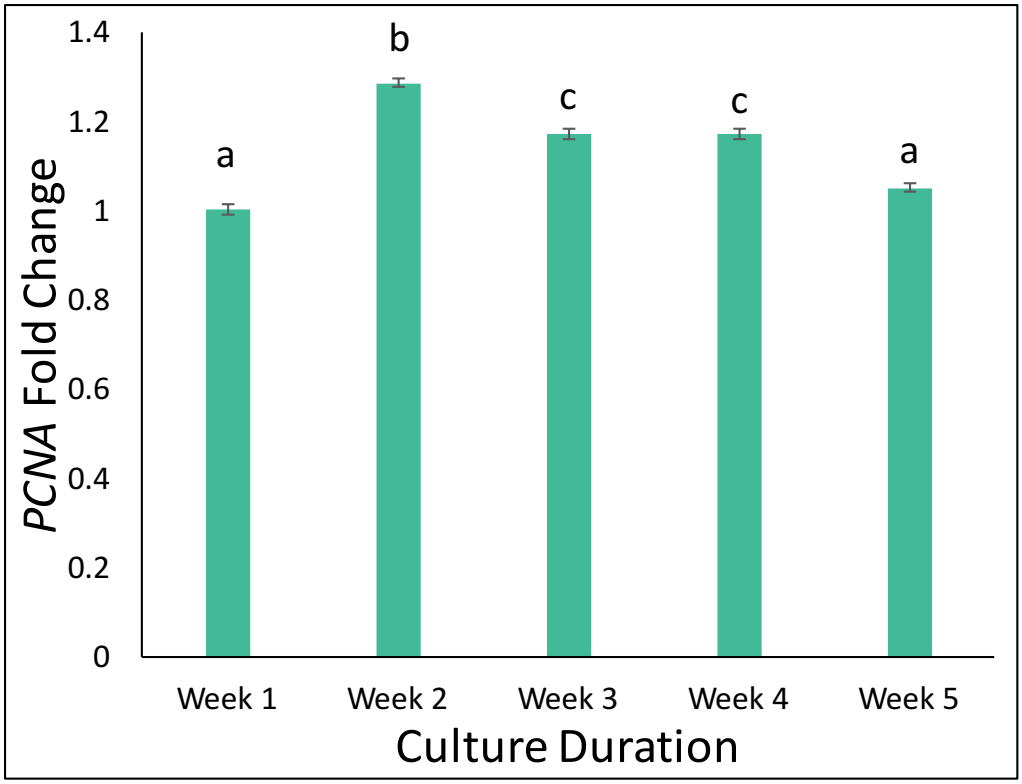

Figure 8. *PCNA* gene expression (fold change) following 5 weeks of *in vitro* culture of pre-pubertal lamb testes. Values represent mean ± SEM. Bars with different superscripts are statistically significant.

Retinoic acid failed ($p > 0.05$) to increase PCNA gene expression but at the highest concentration (RA5) tested, retinoic acid exerted a negative effect (0.96-fold ± 0.01) on PCNA gene expression (Figure 9).

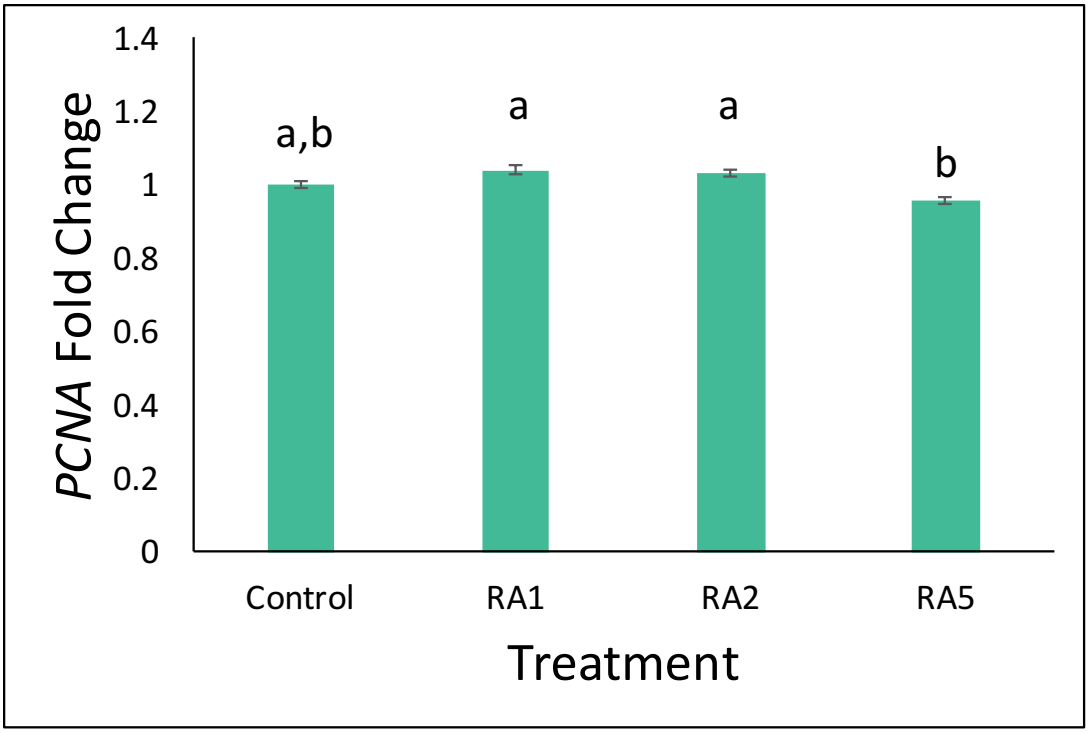

Figure 9. *PCNA* gene expression (fold change) following 5 weeks of *in vitro* culture of pre-pubertal lamb testes in the absence (control) and presence of RA (1 µM, 2 µM and 5 µM). Values represent mean ± SEM. Bars with different superscripts are statistically different.

Proto-oncogene c-Kit (*c-Kit*)

Expression of *c-Kit* increased ($p < 1x10^{-4}$) 1.45 ± 0.01-fold until week 3 where it returned to week 1 levels before increasing again in week 5 to 1.14 ± 0.01-fold (Figure 10). However, gene expression was different ($p < 1x10^{-4}$) between week 2 and 3 (1.27 ± 0.01-fold) as well as week 5 compared to week 1.

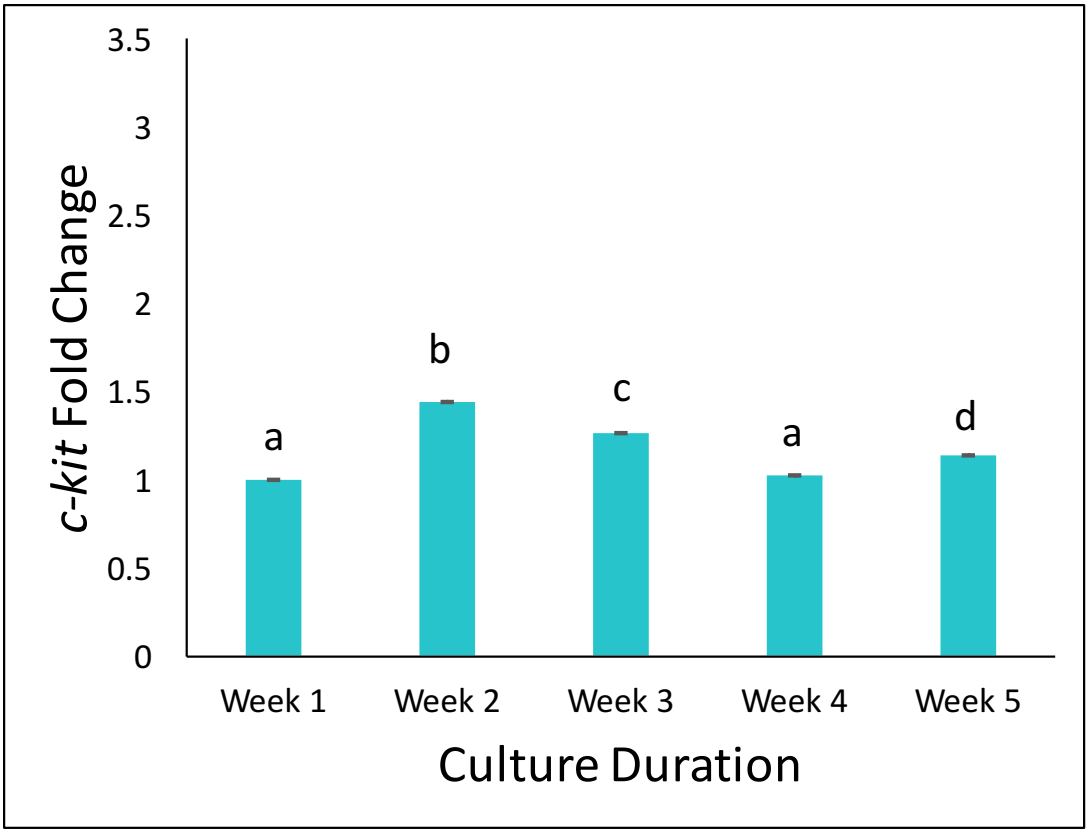

Figure 10. *c-Kit* gene expression (fold change) following 5 weeks of *in vitro* culture of pre-pubertal lamb testes. Values represent mean ± SEM. Bars with different superscripts are statistically significant.

In contrast, *c-Kit* expression increased 2.45± 0.01-fold (RA1; $p < 1x10^{-9}$), and 3.31± 0.01-fold with RA2 ($p < 1x10^{-9}$) and RA5, $p < 1x10^{-9}$) compared to the control (Figure 11). There was no difference ($p > 0.05$) in gene expression between RA2 and RA5.

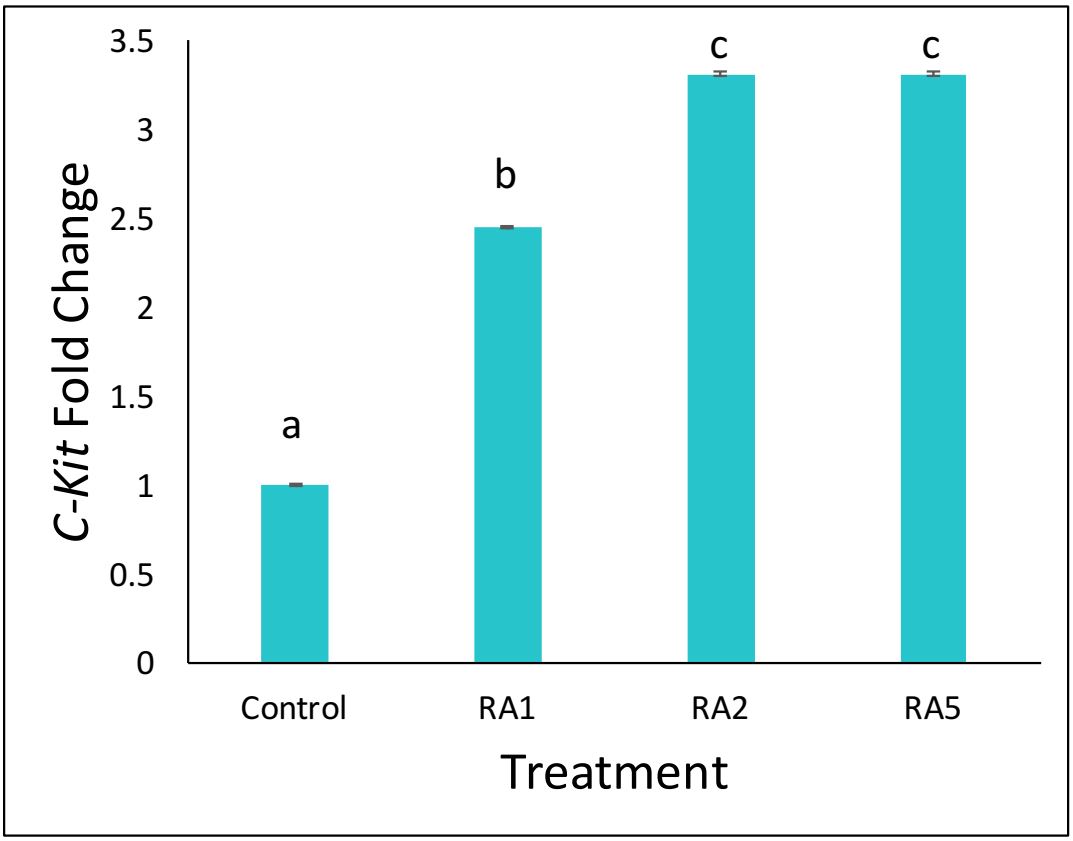

Figure 11. *c-Kit* gene expression (fold change) following 5 weeks of *in vitro* culture of pre-pubertal lamb testes in the absence (control) and presence of RA (1 µM, 2 µM and 5 µM). Values represent mean ± SEM. Bars with different superscripts are statistically different.

Stimulated by Retinoic Acid Gene 8 (*Stra8*)

Stra8 gene expression increased in testicular explants over five weeks of *in vitro* culture (Figure 12). Expression was highest ($p < 0.001$) in weeks 3 (3.8± 0.01-fold) and 4 (3.6± 0.01-fold) compared to the control. Although *Stra8* gene expression decreased in weeks 3 and 5, expression remained significantly higher ($p < 0.001$) compared to the control.

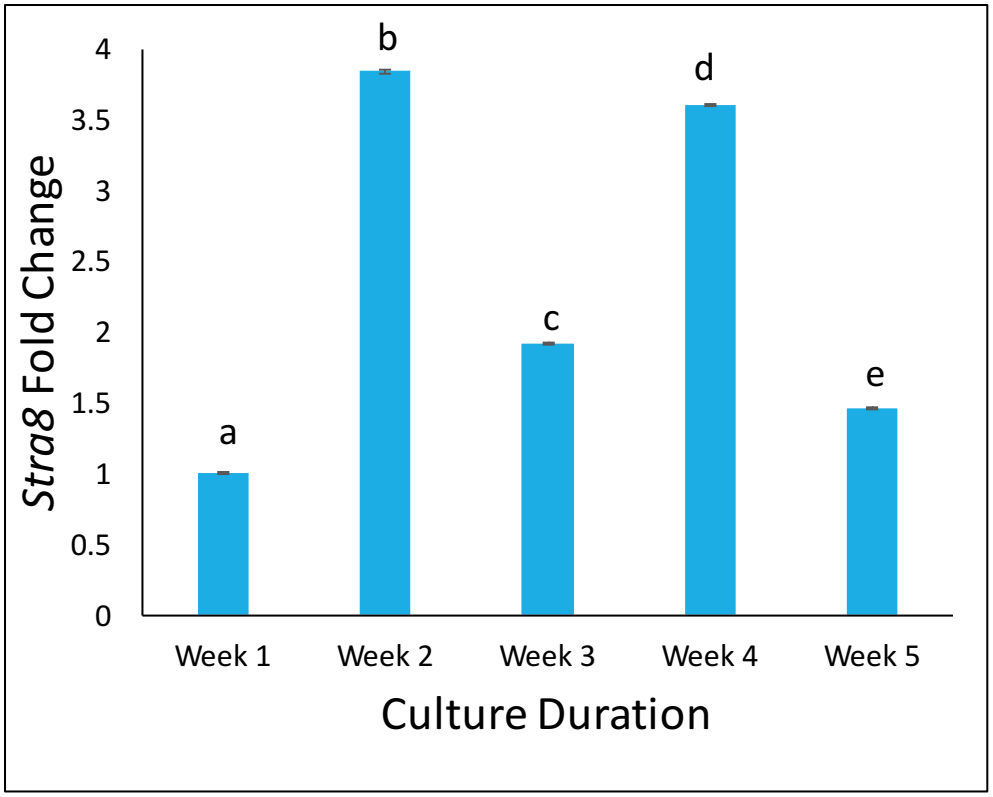

Figure 12. *Stra8* gene expression (fold change) following 5 weeks of *in vitro* culture of pre-pubertal lamb testes. Values represent mean ± SEM. Bars with different superscripts are statistically significant.

Retinoic acid exerted a stimulatory effect on *Stra8* gene expression following *in vitro* culture (Figure 13). Gene expression increased 2.8± 0.01-fold compared to control in RA1 ($p < 0.001$) but declined to 1.4± 0.01-fold in RA2 ($p < 0.001$). In the presence of RA5, *Stra8* expression was not different from the control but was lower than RA1 or RA2 ($p > 0.05$).

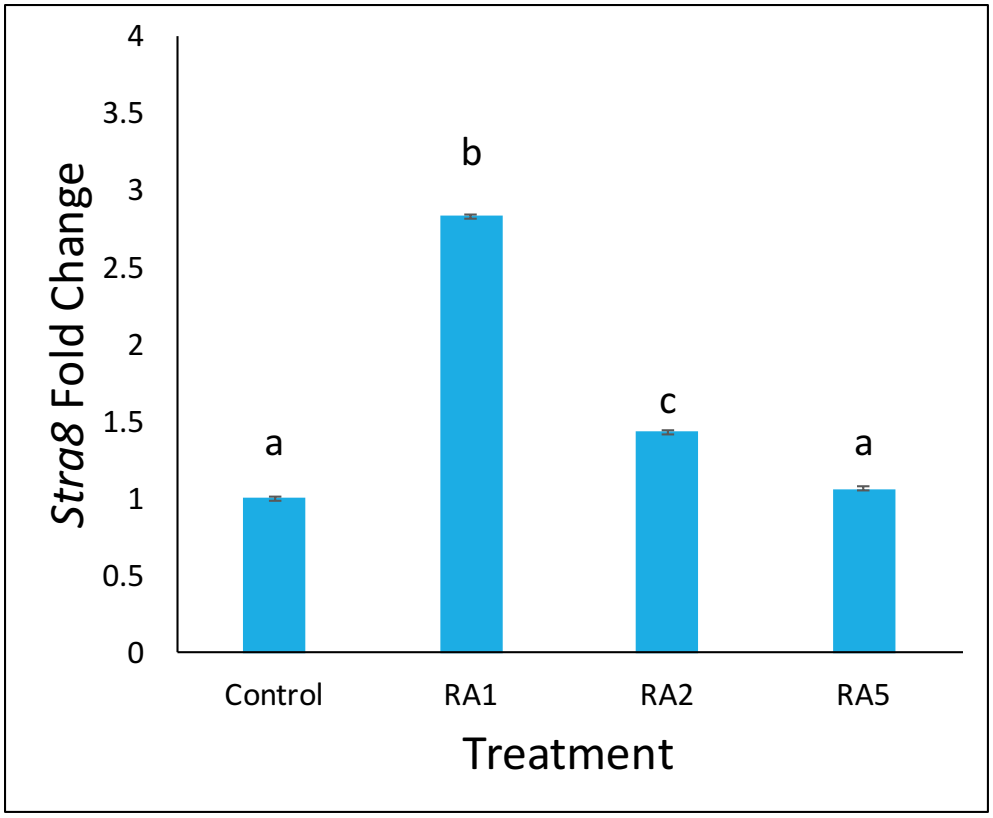

Figure 13. *Stra8* gene expression (fold change) following 5 weeks of *in vitro* culture of pre-pubertal lamb testes in the absence (control) and presence of RA (1 µM, 2 µM and 5 µM). Values represent mean ± SEM. Bars with different superscripts are statistically different.

3-Beta-Hydroxysteroid-Dehydrogenase (*HSD3-β*)

HSD3-β gene expression decreased ($p < 0.0298$) in week 2 to 0.90 ± 0.01-fold (Figure 14). By week 3, expression level returned ($p > 0.05$) to week 1 levels. However, gene expression was higher ($p < 0.001$) in weeks 4 (1.3± 0.01-fold) and 5 (1.1± 0.01-fold) compared to week 1, 2 or 3.

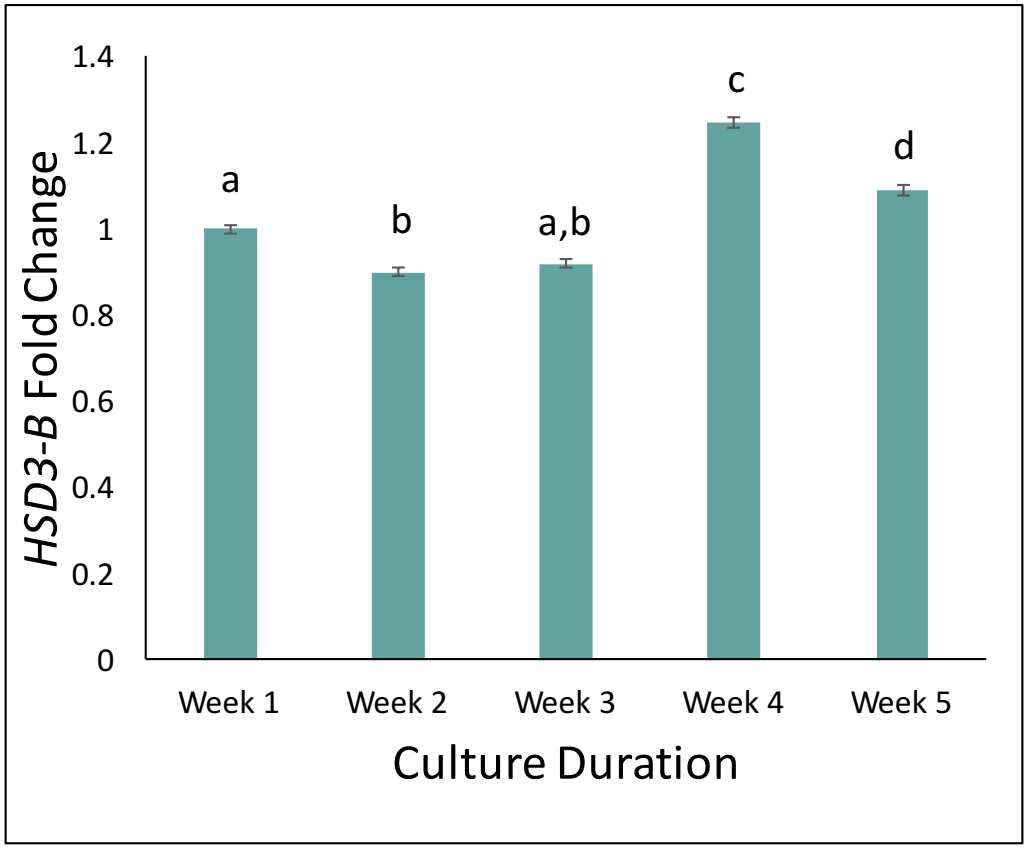

Figure 14. *HSD3-β* gene expression (fold change) following 5 weeks of *in vitro* culture of pre-pubertal lamb testes. Values represent mean ± SEM. Bars with different superscripts are statistically significant.

Retinoic acid exerted a minimal effect on *HSD3-β* gene expression (Figure 15). Although gene expression was marginally increased in RA1 by 1.10± 0.01-fold ($p = 0.00233$) and RA5 by 1.08± 0.01-fold (0.01202) compared to control, there was no difference ($p > 0.05$) in gene expression among the three RA concentrations evaluated.

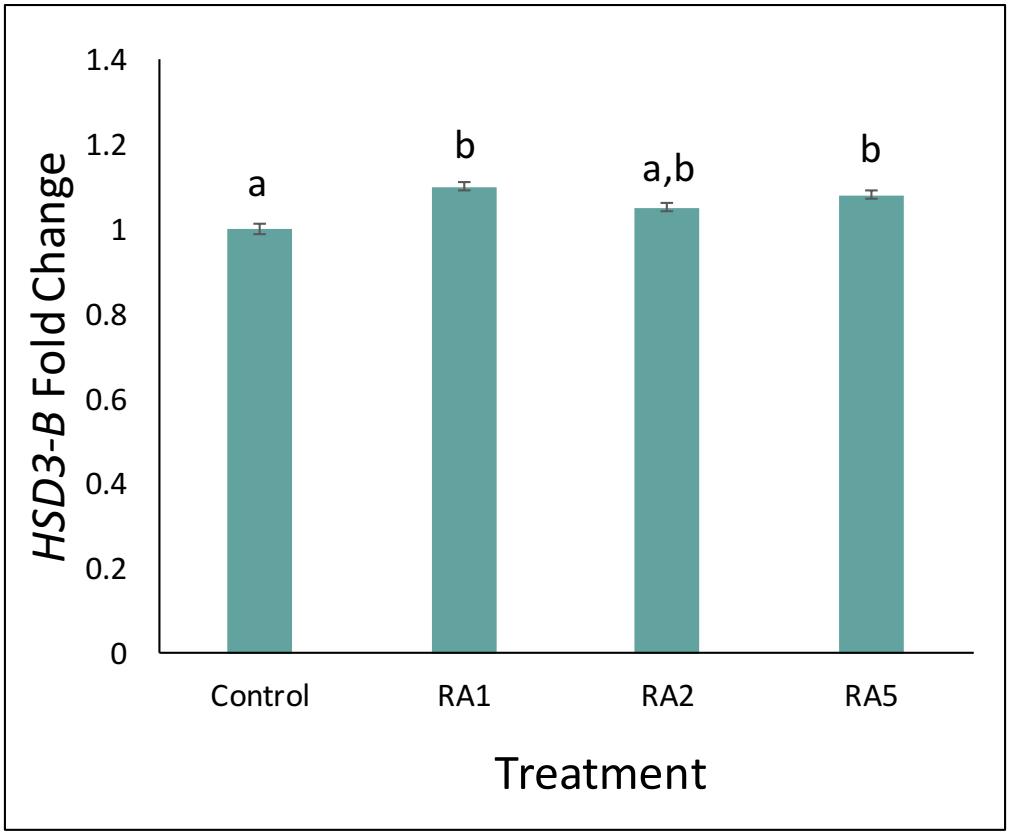

Figure 15. *HSD3-β* gene expression (fold change) following 5 weeks of *in vitro* culture of pre-pubertal lamb testes in the absence (control) and presence of RA (1 µM, 2 µM and 5 µM). Values represent mean ± SEM. Bars with different superscripts are statistically different.

CHAPTER FOUR: DISCUSSION

The goal of this study was to fill a knowledge gap with respect to the minimum requirements for maintaining lamb testicular explants and/or to achieve sperm production *in vitro*. There were two main objectives to this study: 1) to understand the effect of RA on spermatogenesis and to find the RA concentration that supports cell proliferation and differentiation in cryopreserved testicular explants; and 2) to understand the conditions needed to promote tissue viability in a multi-week culture system. It was hypothesized that the addition of RA would lead to an increase in cell proliferation and differentiation in testicular explants during *in vitro* culture for five weeks. Based on the results of this study, the hypothesis was partially supported in that we observed cell proliferation and differentiation in tissues treated with RA compared to the control (no RA). However, *in vitro* culture for five weeks itself resulted in poor tissue survival and a concomitant decrease in both cell counts and expression of various target genes.

The Influence of Culture Duration on Cell Proliferation and Gene Expression

While there was not an overall increase in gonocyte counts over the 5-week culture period, gonocytes slightly decreased the first three weeks but increased by week

4, indicating that the culture conditions used were able to maintain germ cell viability (Figure 3). This is supported by the consistent expression of *PCNA* from week 1 to week 4, with values significantly dropping by week 5 (Figure 8). A similar result was seen in the testis tissue explants from prepubertal bulls that were cultured for at least two weeks, with an apparent increase in gonocytes before being xenografted into mice and continuously maintaining SSCs (Cai et al. 2016a). Efforts have been made to isolate seminiferous tubules from cats and dogs to culture, with cell viability evident for up to 4 weeks (Comizzoli and Wildt 2012).

Similar to gonocytes, Sertoli cell numbers also were successfully maintained for the first 3 weeks of in vitro culture, but then dramatically declined by weeks 4 and 5 (Figure 5). Around this time, tissues also expressed varying degree of necrosis/fibrotic changes which suggests a failure of the extant culture conditions to support Sertoli cell viability or function (Figure 2). Similar observations in Sertoli cell declines have been reported in mice (O'Shaughnessy et al. 2009), bovine (Cai et al. 2016a), and cats (Silva et al. 2004). Declining Sertoli cell numbers hold implications for gonocyte proliferation, as NOTCH1 signaling in Sertoli cells promotes gonocytes to start the differentiation process (Garcia and Hofmann 2013). A decrease in Sertoli cells implies a lack of gonocyte regulation, resulting in the absence of SSCs and a decrease in the ability of the tissue to promote spermatogenesis.

The above results were supported by gene expression analyses. Markers for cell proliferation (*PCNA*), cell differentiation (*c-Kit*), retinoic acid signaling (*Stra8*), and steroidogenesis (*HSD-3β*) exhibited similar expression patterns. Specifically, *PCNA*

expression was only minimally increased in week 2 with this pattern maintained up to week 4 (Figure 8). Then in week 5, there was no difference in expression of P*CNA* in testicular explants compared to week 1. In contrast, *c-Kit* expression exhibited a cyclic pattern with a slight increase for the first three weeks followed by a significant drop in week 4 and then a gradual increase in week 5 (Figure 10). It cannot be ruled out that with optimized culture systems, there would be increased support for cell viability as well as differentiation.

Another interesting finding from this study is that retinoic acid aids in maintaining tissue structure and function *in vitro*. In the presence of retinoic acid, *Stra8* expression was periodically elevated during *in vitro* culture (Figure 12). Furthermore, expression appeared to be cyclic – increasing over 3-fold at week 2 and 4 but then declining by week 5. Spermatogenesis is naturally asynchronous to supply a continuous amount of spermatozoa, but when disrupted the processes can become synchronous with varying levels of spermatogonia produced (Hogarth et al. 2015). *Stra8* changes are potentially caused from a feedback mechanism orchestrated by the Sertoli cells, which are responsible for providing RA to the cells (Hogarth and Griswold 2010). *Stra8* expression was high at week 4 of culture compared to weeks 1 and 3 (Figure 12), even though that is when tissues begin to exhibit necrosis/fibrosis (Figure 2). Possible reasons for these seemingly erratic changes could be a type of feedback mechanism involving *Stra8* expression, which little is known about but warrants further investigations.

In contrast, expression of the marker for steroidogenesis (*HSD3*-β) exhibited a very small, yet, significant increase over time (Figure 14). However, it is not clear if this

increase was adequate to maintain testosterone synthesis in the testicular explants. Increased amounts of *HSD3-β* expression may be indicative of the remaining Sertoli cells responding to hormone-dependent signaling cascades in the ST (Alves et al. 2013). Therefore, Sertoli cells may be attempting to maintain suitable amounts of testosterone within the remaining tubules as testosterone is supplied from the Leydig cells. Gonocytes from mouse testis were shown to be sensitive to androgens while proliferating and while the exact mechanism is unknown, gonocytes were found to be easily inhibited in the direct presence of androgens (Merlet et al. 2007). This may explain the observed temporal changes in gonocyte numbers if they were repetitively inhibited by the fluctuating amounts of testosterone in their environment. However, in the present study we did not analyze testosterone production in the medium since the basal culture medium contained exogenous testosterone (10 μg/mL).

Retinoic Acid on Cell Proliferation and Gene Expression

There is little evidence from previous studies to suggest that spermatogonial stem cells respond to RA before their development into gonocytes, as an increase in RA did not cause an increase in SSCs, which result from differentiated gonocytes (Agrimson et al. 2017). Sertoli cells are responsible for harboring RA until needed to initiate meiosis in SSCs (Hogarth and Griswold 2010), so this trend may indicate that RA1 is the ideal concentration for initiating spermatogenesis.

When analyzing the cell numbers as a function of RA concentration, 2 μM RA exerted a negative effect on gonocyte numbers. However, both 1 μM and 5 μM RA were not significantly different from the control in regards to gonocyte proliferation (Figure 4).

In contrast to gonocytes, RA failed to exert an effect on Sertoli cells although concentrations higher than 1 μM appeared to be detrimental to Sertoli cells (Figure 6). In mice, retinoic acid helped maintain tissue integrity while limiting cell apoptosis at 1 μM (Travers et al. 2013), although it is possible that there are other negative effects on the tissue culture system that would prevent a beneficial response from RA. Interestingly, RA signaling has been associated positively with Sertoli cell numbers (Nicholls et al. 2013), but is also known to suppress Sertoli cell proliferation in favor of maturation in rat testis tissue (Buzzard et al. 2003). Most of the regulatory mechanisms involving the Sertoli cell cycle are dependent on FSH, as disruption of FSH has been linked to testicular dysfunction (Sharpe et al. 2003). Therefore, it would be informative to examine the interplay between FSH and RA on the regulation of testicular function.

Although no clear patterns emerged from histological assessment of testicular tissue, gene expression analyses provide more insight into the probable involvement of RA on supporting *in vitro* spermatogenesis. Expression of *PCNA* showed a moderate, yet not statistically significant, increase in the presence of 1 μM and 2 μM RA, but 5 μM RA appeared to suppress *PCNA* gene expression (Figure 9). Based on these results, it remains unclear what role RA plays in gonocyte proliferation and this warrants additional studies. Although *PCNA* expression was inconclusive, *c-Kit* expression increased 2.4-3.3 fold compared to control in the presence of RA but there was no difference between RA2 and RA5 (Figure 11). Likewise, *Stra8* expression was significantly elevated in the presence of RA1 and RA2 suggesting that RA supports germ cell differentiation *in vitro*. These results also clearly demonstrate that RA pathways (via *Stra8*) are involved in

spermatogenesis in the lamb testis. These findings are also supported by previous reports that suggest the involvement of *Stra8* in spermatogenesis by initiating meiosis as seen in the murine model (Anderson et al. 2008).

CHAPTER FIVE: CONCLUSIONS

Semen collection and cryopreservation is often heralded as one of the most efficient options to preserve germplasm from rare and endangered animals. However, there are few options for rescuing the germ line of immature, prepubertal individuals. Earlier attempts focused on xenotransplantation of testicular tissue pieces under the skin of an immune-suppressed mouse to generate mature spermatozoa (Yildiz et al. 2013; Pukazhenthi et al. 2015). Recent studies have investigated *in vitro* culture of testicular explants using a gas-liquid interphase or microfluidic devices (Komeya et al. 2017). Most advances, such as the generation of mature sperm and production of live offspring, have been limited to the rodent model. Studies conducted on large animal models have repeatedly failed to either support long term culture of testicular tissue in vitro or support resumption of spermatogenesis. In the present study, we evaluated the neonatal sheep as a model for numerous other rare and endangered ungulates. Earlier, we reported on cryopreservation of lamb testicular tissue and resumption of spermatogenesis after xenografting (Pukazhenthi et al. 2015). However, efforts to culture testicular tissue *in vitro* have faced many challenges. This study clearly demonstrates for the first time that lamb testicular explants can be 1) cultured *in vitro* for up to five weeks and 2) RA

stimulates pathways involved in germ cell differentiation while promoting cell proliferation and steroidogenesis. Although resumption of spermatogenesis was not achieved in this study, results addressed critical knowledge gaps pertaining to long term culture of testicular tissue and the role of retinoic acid in lamb spermatogenesis *in vitro*.

The next steps to advance this study would be to investigate the effects of further improving the culture system to promote cell proliferation. It was found that the addition of stem cell factor and the hormone triiodothyronine promoted spermatogenesis and resulted in higher amounts of spermatogonial stem cells (Kim et al. 2015). Furthermore, the use of microfluidic devices in maintaining long-term tissue cultures is promising as the system mimics *in vivo* conditions with a porous membrane separating a tissue from a continuously flowing medium. A study conducted with mouse testis tissue with a microfluidics device found that spermatogenesis was maintained for 6 months with fertile spermatozoa (Komeya et al. 2016). It would beneficial to investigate the use of a microfluidics system with lamb testis tissue to examine how the tissue responds with retinoic acid in this more realistic environment. When examining the implications of the present study, it is important to consider the effects of reproductive physiological systems *in vivo* compared to *in vitro* culture systems. Various hormones may be secreted in pulses that vary in frequency (Brabant et al. 1992). The secretion of various hormones involved may follow a pulsatile pattern which may exert a beneficial effect on testicular tissues *in vitro*. It is possible that the continued presence of RA in the culture system may be detrimental to tissue viability and may fail to support spermatogenesis *in vitro* as RA was

added to the culture system in a non-pulsatile pattern. Future studies should focus on assessing the impacts of pulsatile release on gonadal tissues *in vitro*.

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BIOGRAPHY

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