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Lepidium sativum Effects on Reproduction and Visceral Organ Development in Sprague-Dawley Rats

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Lepidium sativum Effects on Reproduction and Visceral Organ Development in Sprague-Dawley Rats

By

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Abstract

Plants have been utilized as herbicides, insecticides, antimicrobials, antifungals, antivirals, cosmetics and therapeutic agents. Traditional medicine plays a pivotal role in health care around the globe. *Lepidium sativum* (LS) has been utilized in cooking for its peppery, tangy flavor and aroma and in traditional medicine. LS has been used to treat inflammation, bone fractures, hypertension, microbial infections, diabetes, bronchial asthma, osteoarthritis, constipation and other diseases. There have been no studies investigating the effects of LS seed extracts on preovulatory surge secretion of gonadotropin releasing hormone (GnRH), luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The highest level of inclusion of LS seeds at 50% has shown lethal effects. At 10% LS seed inclusion has shown ambiguous toxic and non-toxic effects. There is a paucity of information on LS effects on female and male reproductive function. To date, LS has been shown in females to act as a galactagogue, abortifacient and contraceptive. There are ambiguous results of LS having anti-ovulatory properties. In males, LS has been shown to increase testicular and epididymal sperm concentration and act as an aphrodisiac. The objectives of the study were to determine the effect of aqueous LS seed extract on the development and magnitude of surge releases of GnRH, LH and FSH and secondly, to determine the acute and chronic effects of 15% LS seed supplementation on gross organ morphology and histomorphometric indices, testosterone secretion and spermatogenesis in the Sprague-Dawley rat.

Thirty-two female Sprague-Dawley rats were ovariectomized using standard procedures for Experiment 1. Ten days post-ovariectomy, all rats were injected with estradiol-17β (10 μg/0.2 mL/rat, s.c. in corn oil) for three days. On the fourth day at 0800 h, sixteen rats were treated with

LSE (40 mg/Kg BW, i.p.) while the remaining sixteen received normal saline diluent at the same rate. At 1000 h, eight rats that received LSE and eight rats that received normal saline were treated with corn oil (0.2 mL/rat, s.c.). The remaining sixteen rats were treated with progesterone (2.5 mg/0.2 mL/rat, s.c. in corn oil). At 1300 h, the rats were euthanized using an overdose of 6 mL 2.5% tribromoethanol. The hypothalamus and pituitary glands were extracted and perifused in artificial cerebrospinal fluid for seven hours at a constant temperature of 37°C. Hypothalamic GnRH and pituitary LH and FSH concentrations were determined utilizing radioimmunoassays. There were no significant differences in GnRH, LH or FSH secretions. There was an observed GnRH surge in rats receiving saline and progesterone. Rats that received LSE and progesterone observed an earlier and diminished secretion of GnRH, but was not significant. Progesterone administration had a suppressive effect on LH average secretion. GnRH administration had no effect on LH or FSH secretion. Total hormonal secretions for GnRH, LH and FSH had no significant changes.

Forty-eight male Sprague-Dawley rats were utilized for Experiment 2. Upon arrival, rats were given feed and water *ad libitum* for two weeks for acclimation. The rats were weighed and assigned to either a control group (0% LS seed inclusion; n=24) or a treatment group (15% LS seed inclusion; n=24). At 0800 h rats were offered either normal rat chow or the 15% LS seed included rat chow for 8 weeks. At 2, 4, 6 and 8 weeks, six rats from each the Control or the Treatment groups were euthanized with carbon dioxide asphyxiation for a minimum of 10-minutes. All rats were weighed on the first day of the experiment, every other day and immediately after euthanization. Every day feed intake and refusal was measured. Immediately post euthanization, trunk blood was collected for hormone assays. Further, the paired testicles,

epididymides, prostate gland, seminal vesicles, kidneys, adrenal glands, heart, liver, spleen, lungs, brain and pancreas were harvested and weighed. The relative organ weights were normalized to weight per 100 g BW. One kidney and one testis was collected and used for histological analysis. The other testes were utilized for *in vitro* testosterone production. One cauda epididymis per rat was isolated and used for epididymal sperm count. Our results indicated a decrease in Treatment group's body weight during the first few days of the experiment. This occurred concurrent with a strong feed aversion for the Treatment group during the first few days. This effect is explained by the novel food source being offered to the rats. There was no effect of LS seed inclusion on adrenal gland, heart, liver, and spleen or lung weight. There was a significant increase in brain weight possibly due to hydrocephalus. There was also a significant increase in pancreatic weight possibly due to pancreatitis. There were no effects of LS supplementation on paired testicular, epididymides or seminal vesicle weights. The prostate gland did show a significant decrease in weight. The mechanism for this significant weight decrease is unknown. The testis histological analysis had no significant results except for an increase in week two Leydig cell diameter. There was no significant changes for *in vitro* testosterone production or plasma testosterone. Therefore, our results suggest no conclusive data for the aphrodisiac claims. The epididymal sperm density did not have significant changes, although they did increase concurrent between the groups through the experiment. Interestingly, there was an increase in renal weight through the experiment for the Treatment group. Histological analysis showed a significant change in the diameter of the Bowman's capsule, glomerulus and Bowman's space for the Treatment group. There was also an increase in glomerulosclerosis, metaplasia and hyperplasia in rats fed 15% LS seed inclusion. In the

proximal and distal tubules there was a significant increase in tubular degeneration throughout the experiment. These results paired together show a significant toxic effect for rats fed 15% LS seed. Overall, LS seed consumption for medicinal purposes should be consumed with caution because of the possible narrow therapeutic index.

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Chapter I: Introduction

The first synthesized drug did not appear until 1869. The drug chloral hydrate was utilized as a sedative-hypnotic (Jones, 2011). Prior to 1869 medicinal drugs were plant-derived. The usage of plants in traditional medicine dates back nearly 60,000 years ago. At a Neanderthal burial site in Shanidar, Iraq eight species of medicinal plants were exhumed with Neanderthal bodies, seven of which are still utilized today for their medicinal properties (Solecki, 1975). Apart as a food source, plants have been utilized as herbicides (Dayan & Duke, 2014), insecticides (Zoubiri & Baaliouamer, 2014), antimicrobials (Upadhyay, Upadhyaya, Kollanoor-Johny, & Venkitanarayanan, 2014), antifungals (Trakranrungsie, 2011), antivirals (Babar, Zaidi, Ashraf, & Kazi, 2013), cosmetics (Gediya, Mistry, Patel, Blessy, & Jain, 2011) and therapeutic agents (Veeresham, 2012). Important drugs that have been derived from plants include digoxin, quinine, quinidine, vincristine, vinblastine, atropine, morphine and codeine (Rates, 2001). Approximately 80% of pharmaceuticals are derived from natural compounds or natural products (Maridass & John De Britto, 2008). It is estimated that 50% of pharmaceuticals were first identified from plants and other organisms (Krief, Martin, Grellier, Kasenene, & Sévenet, 2004).

It is estimated that there are 450,000 angiosperm species (Pimm & Joppa, 2015) and about 1000 gymnosperms species (Christenhusz, et al., 2011). Of the existing plant species only 6% have been investigated for pharmacological properties and 15% have been investigated for phytochemical properties (Atanasov, et al., 2015). That means only about 27,000 plants have been systematically investigated for pharmacological properties and 68,000 plants for phytochemical properties. When utilized in traditional medicine, plants first need to be extracted to separate the medicinally-active chemicals utilizing selective solvents (Handa, Khanuja,

Longo, & Rakesh, 2008). Extraction techniques include maceration, infusion, percolation, decoction, Soxhlet, microwave-assisted, ultrasound-assisted, accelerated solvent and supercritical fluid extraction (Azwanida, 2015). Depending on the solvent used different compounds can be extracted. Nonpolar compounds can be extracted efficiently by hexane, 1-chlorobutane, dichloromethane, chloroform, isopropyl ether, ethyl ether, ethyl acetate and 1-butanol. Polar compounds need a polar solvent to efficiently extract the compounds (Siek, 1978). Alcohols like ethanol and methanol utilized as solvents in extractions have proven to be more efficient than water (aqueous extraction) in extracting phytochemicals (Gberikon, Adeoti, & Aondoackaa, 2015).

Plant and animal-derived medicines are important because of the 252 essential chemicals distinguished by the World Health Organization, 11.1% originate from plants while 8.7% originate from animals (Costa-Neto, 2005). In 2007, it was reported that nearly 38% of adults and 12% of children used some form of traditional medicine in the USA within a twelve month period (Barnes, Bloom, & Nahin, 2008). In a recent survey, estimates for adults who have utilized homeopathic remedies was 3.9% among USA, United Kingdom, Australia, Israel, Canada, Switzerland, Norway, Germany, South Korea, Japan and Singapore (Relton, Cooper, Viksveen, Fibert, & Thomas, 2017). Interestingly, it is estimated that at least 50% of the populations in Europe, North American and other developed regions have used traditional medicine at least once. In developing countries it is estimated that up to 80% of the population utilizes traditional medicine as their primary health care (World Health Organization, 2003). Traditional medicine is advantageous because of the ease of access and low cost. In many parts of the world traditional medicine is also used in regular cultural traditions.

Lepidium sativum (LS), commonly known as garden cress, is an annual and fast growing herb (Panwar & Guha, 2014). LS belongs to the Brassicaceae family. It is utilized as a spice for its peppery, tangy flavor and aroma. The plant grows up to 50 cm tall originating from 4 mm long and 2 mm wide seeds. LS has been utilized in traditional medicine to treat inflammation, bone fractures, hypertension, microbial infections, diabetes, bronchial asthma, osteoarthritis, constipation and to increase milk production (Falana, Nofal, & Nakhleh, 2014). Although there is a dearth of information on the efficacy, safety and mechanism of action for the utilization of LS in traditional medicine there is a paucity of information on LS and its role in the control of reproduction.

Reproduction in all mammals is regulated by the hypothalamic-pituitary-gonadal axis. The hypothalamus sits atop the axis and secretes the decapeptide gonadotropin-releasing hormone (GnRH). In both mammalian males and females, GnRH is secreted in a pulsatile fashion. This pulsatility in turn leads to pulsatile secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) which function to stimulate production of ovarian estrogen and progesterone and testicular testosterone. LH and FSH also play a pivotal role in modulating gametogenesis. In mammalian females only, there is an increased release of GnRH called the surge secretion of GnRH. As with the pulsatile mode of secretion, the GnRH surge causes the surge secretions of LH and FSH. The LH surge occurs immediately before and causes ovulation. The neuroendocrine regulation of both pulsatile and surge secretions of GnRH and the gonadotropins has been well-described (Plant & Zeleznik, 2015). Generally, it is known that different hypothalamic areas regulate these two modes of hormonal synthesis and secretion and

that all influences on reproduction, both internal and external, impact reproduction via these hypothalamic areas.

To date, there have been no studies investigating the effects of LS seed extracts on preovulatory surge secretion of GnRH, LH and FSH. Although it has been shown that 10% LS supplementation in Wistar rats was nontoxic (Datta, Diwakar, Viswanatha, Murthy, & Naidu, 2011), questions have been asked about the exact physiologic or pharmacologic level of inclusion of LS in animal feeds. Further, it is unclear what the consequences of this level of LS inclusion for reproduction and somatic development might be. The objectives of the study are to determine the effect of aqueous LS seed extract on the development and magnitude of surge releases of GnRH, LH and FSH and secondly, to determine the acute and chronic effects of 15% LS seed supplementation on gross organ morphology and histomorphometric indices, testosterone secretion, and spermatogenesis in the Sprague-Dawley rat.

Chapter II: Literature Review

Plant-Derived Medicine and their Contributions to the World

Plant-derived medicine plays a pivotal role in healthcare worldwide. Even though there has been a recent increase over the past few decades in molecular modeling, combinatorial chemistry and synthetic chemistry methods the plant derived medicinal compounds have played a crucial source in the pharmaceutical industry (Salim, Chin, & Kinghorn, 2008). In 2001, approximately 25% of all drugs prescribed originated from plants (Rates, 2001). On average it takes about ten to twelve years for a drug to travel from the discovery stage to the market. Interestingly, twelve years later about 45% of top selling drugs originated from natural products in the environment (Lahlou, 2013). Apart from plant derivatives being utilized in the pharmaceutical industry, they also play a pivotal role in primary health care in developing countries.

Lepidium sativum: Botany, Distribution and Chemical Composition

Lepidium sativum (LS), commonly known as garden cress, is an annual, edible, fast growing herb which belongs to the Brassicaceae family (Panwar, et al., 2014). LS is cultivated in Africa, North America, parts of Europe (Nuez & Hernandez Bermejo, 1994) and India (Nadkarni, 1976). The requirements for LS are simple and broad. This means the plant can grow in moist soil with semi-shade or even in the absence of shade (Falana, et al. 2014). After sowing the seeds, they will begin to sprout four to six days later. To ensure a continuous supply of LS leaves farmers will sow the seeds in intervals of eight days (Wadhwa, Panwar, Agrawal, Saini, & Patidar, 2012). LS is used as a culinary ingredient for its peppery, tangy flavor and aroma. The seeds are small with a length ranging from 3-4 mm long and a width of 1-2 mm. They are

smooth, oval-shaped, pointed and triangular at one end with a reddish brown color (Doke & Guha, 2014).

LS seeds contain 22.7% of oil from a hexane extraction. The oil constituents revealed oleic (30.6%), linolenic (29.3%), gondoic (11.1%), palmitic (9.4%) and linoleic acids (7.6%). There was a large amount of the antioxidants γ- (1422 ppm), δ-(356 ppm) and α-tocopherols (21 ppm). The primary phytosterols include sitosterol (5.82 mg/g), campesterol (3.95 mg/g) and avenasterol (3.44 mg/g). Other phytosterols include cholesterol (0.50 mg/g), stigmaterol (0.30 mg/g), dihydrolanosterol (0.25 mg/g) and β-amyrin (0.16 mg/g) (Moser, Shah, Winkler-Moser, Vaughn, & Evangelista, 2009). Presence of carbohydrate and major sugars include mannose (38.9%), arabinose (19.4%), galacturonic acid (8%), fructose (6.8%), glucuronic acid (6.7%), galactose (4.7%), rhamnose (1.9%) and glucose (1.0%) (Karazhiyan, et al., 2009). LS seeds have been shown to include the isoflavonoids 7-hydroxy-4',5,6-trimethoxyisoflavone, 7-hydroxy-5,6-dimethoxy-2',3'-methylenedioxyisoflavone and 5,6-dimethoxy-2',3'-methylenedioxy-7-C-β-D-gluco-pyranosyl isoflavone (Sakran, Selim, & Zidan, 2014). Gallic acid is utilized as a standard for determining total phenolic content. This yields a gallic acid equivalent (GAE). Total polyphenolic content for whole LS seeds are 2,081 mg GAE/100 g (Doke & Guha, 2015). Ethanolic extract had evidence of cardiac glycosides, anthroquinone glycoside, cyanogenetic glycoside, flavonoids, alkaloids, tannins, glycine, cysteine and glutamine (Yadav, Jain, Srivastava, & Jain, 2011). The seeds contain benzylglucosinolate ~170 μmol/g dry weight. Eight day old seedlings also contained isothiocyanate, thiocyanate and nitrile hydrolysis products (Burow & Bergner, 2007). The β-carotene content was 433.41 μg/100 g. Zeaxanthin content was 2.35 μg/100 g. Lutein content was 96.21 μg/100 g. The total carotenoids content was 1.00

μmol/100 g (Diwakar, 2010). Lepidine, an alkaloid, was found in the seeds at a content of 3.7 mg/g dry weight. Lepidine was highest in content within the plantlets during the vegetative stage at 10.4 mg/g (Pande, Malik, Bora, & Srivastava, 2002). Whole LS seeds yield a multitude of nutrients: moisture (4.14 %), protein (22.47 %), fat (27.48 %), carbohydrate (34.24 %) and crude fiber (7.01 %). The minerals in LS seeds include potassium (1,193.95 mg/100 g), phosphorous (514.59 mg/100 g), magnesium (315.25 mg/100 g), calcium (296.60 mg/100 g), sulfur (293.02 mg/100 g), sodium (24.64 mg/100 g), iron (7.62 mg/100 g), copper (5.53 mg/100 g), zinc (5.05 mg/100 g), aluminum (2.82 mg/100 g), manganese (2.57 mg/100 g), boron (1.41 mg/100 g) and molybdenum (0.43 mg/100 g). The non-essential amino acid profile for LS seeds (g/100g protein) includes aspartic acid (9.76), glutamic acid (19.33), serine (4.96), glycine (5.51), alanine (4.83), tyrosine (2.69) and proline (5.84). The essential amino acid profile for LS seeds (g/100g protein) include histidine (2.66), threonine (4.51), arginine (8.04), valine (5.67), methionine (0.97), phenyl alanine (5.65), isoleucine (5.11), leucine (8.21) and lysine (6.26) (Gokavi, Malleshi, & Guo, 2004). The antioxidant properties of LS seeds had a 94.18% DPPH inhibition and a reducing power assay absorbance of 0.749 (Panwar, et al., 2014).

Lepidium sativum and Medicinal Utility

LS has been utilized to treat various human ailments such as seminal weakness, cold, bronchial asthma (Paranjape & Mehta, 2006), hyperglycemia (Eddouks, Maghrani, Zeggwagh, & Michel, 2005), osteoarthritis (Raval & Pandya, 2009), fracture healing (Juma, 2007), cough, dyspepsia, diarrhea (Manohar, et al., 2009), dysentery, leukorrhea, liver disease (Sakran, et al., 2014), renal diseases, skin, eye diseases and scurvy (Billore, Yelne, Dennis, & Chaudhari, 2005). It is also considered having antihypertensive effects and acts as a diuretic (Maghrani, Zeggwagh,

Michel, & Eddouks, 2005), thermogenic, depurative, ophthalmic, tenesmus, secondary syphilis and leprosy (Manohar, Viswanatha, Nagesh, Vishal, & Shivaprasad, 2012). LS has also been shown to have a high inhibitory effect against the pathogenic organisms *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumonae* and *Proteus vulgaris*. LS acted similar to standard antibiotics Gentamicin and Ketoconzol when tested for inhibitory effects on *Pseudomonas aeruginosa* and *Cadida albicans* (Adam, Salih, & Abdelgadir, 2011). It has been observed that 2.25% LS seed supplementation in broilers had no adverse effects (Shawle, Urge, & Animut, 2016) and 10% LS seed supplementation was nontoxic in Wistar rats (Datta, et al., 2011). Another study observed nontoxic effects at 2% LS seed supplementation, toxic effects but nonfatal at 10% and lethal and depressed growth rate at 50% in albino rats (Adam, 1999).

Lepidium sativum and Reproductive Regulation

To date, there have only been a handful of studies performed investigating the effects of LS on reproduction. LS seeds have been shown to act as a galactagogue (Kumar, Baghel, & Khare, 2011), abortifacient (Nath, Sethi, Singh, & Jain, 1992) and contraceptive (Sharief & Gani, 2004) in females. LS has been cited in many articles for anti-ovulatory properties but in one study with an ethanolic extract of LS seeds there was 0% inhibition of ovulation in rabbits (Vohora, Khan, & Afaq, 1973). Interestingly, LS seeds in males have been shown to increase testicular and epididymal sperm concentration (Naji & Abood, 2013a, 2013b) and act as an aphrodisiac (Mali, Mahajan, & Mehta, 2007). Therefore, contrasting results exist regarding the use of LS seeds as a reproductive medicinal supplement.

Hypothalamic Regulation of Reproduction

Reproduction in all mammals is regulated by the hypothalamic-pituitary-gonadal axis. The highest level of control happens at the hypothalamus. The hypothalamus is part of the diencephalon that lies below the thalamus. It forms the walls and lower part of the third ventricle of the brain. The rostral boundary of the hypothalamus is the optic chiasm while on the posterior end is the mammillary bodies. At the lower part of the third ventricle is the tuber cinereum. Located on the tuber cinereum is a small swelling known as the median eminence. This structure is part of the anatomical link between the hypothalamus and anterior pituitary gland. The interior region of the median eminence is responsible for housing the axonal fibers of neurons responsible for secreting oxytocin and vasopressin. The external region of the median eminence is responsible for containing the terminals of hypophysiotropic neurons. The hypophysiotropic neuron terminals release hypothalamic hormones into the hypophyseal portal system which will then travel to the anterior pituitary gland. The hypothalamus is responsible for secreting gonadotropin-releasing hormone (GnRH). GnRH is secreted in either a pulsatile or surge fashion. Pulsatile secretion is regulated by the arcuate region while surge secretion is regulated by the pre-optic area. Pulsatile secretion of GnRH is pivotal to maintaining the responsiveness of the gonadotroph. Constant infusion of GnRH has been shown to lead to a down-regulation of the processes responsible for gonadotropin release. The mechanism of GnRH pulsatile secretion has been attributed to intrinsic factors such as autocrine regulation (Karazhiyan, et al., 2009). Within the hypothalamic GnRH neurons are estrogen receptors alpha and beta. The expression of these receptors dictate that estrogen may directly modulate GnRH neuronal function. It has been shown that estrogen receptor beta activation led to increased GnRH secretion, cyclic adenosine

monophosphaste production, and action potential firing. Interestingly, when estrogen receptor alpha is activated subsequent activation of G protein-activated inwardly rectifying potassium channels. These channels then decrease because of the hyperpolarization of the cell membrane thus decreasing secretion of GnRH (Lüscher & Slesinger, 2010). Once GnRH has been secreted from the hypothalamus it travels through the hypophyseal portal system towards its target, the anterior pituitary gland.

The pituitary gland is located in the sella turcica. It is separated into the posterior lobe, neurohypophysis, and anterior lobe, adenohypophysis. The posterior lobe is consists of neural tissue while the anterior lobe consists of glandular tissue. The anterior pituitary gland is responsible for secreting the reproductive hormones luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in response to GnRH stimulation. LH and FSH then regulate gonadal functions: gametogenesis and steroidogenesis. The female gonads consist of the estrogen and progesterone producing ovaries while the male gonads consist of the testosterone producing testes. These gonadal steroids have negative feedback loops that act on the hypothalamus, long loop, or the pituitary gland, short loop. These negative feedback loops help maintain hormonal homeostasis. Ovaries and testes produce gametes: eggs and sperm, respectively. Eggs and sperm are created from undifferentiated gonadal cells called germ cells.

Female Reproduction

The ovaries consist of a tough fibrous capsule known as the tunica albuginea. The innermost layer is known as the medulla which is where the vessels are located. The layer between the medulla and tunica albuginea is the cortex. The cortex contains an abundance of ovarian follicles that are at various stages of development. Within each primary follicle contains

a primary oocyte. Surrounding the primary oocyte are granulosa cells, a basement membrane and an outer layer of theca cells.

Ovaries are attached to the uterus by the ovarian ligament. There is a small gap between the ovary and fimbriae connected to the fallopian tube. The fallopian tube then joins with the uterus. At the base of the uterus connects the cervix and then the vaginal canal. The uterus consists of three layers: innermost endometrium, middle myometrium and outmost perimetrium.

The female body produces gametes on a monthly cycle known as the menstrual cycle. The name stems from the 3-7 day bloody uterine discharge known as menses. The menstrual cycle is divided into two more cycles: the ovarian cycle and uterine cycle. The ovarian cycle is further divided into three phases: follicular phase, ovulation and luteal phase. The follicular phase consists of growth of follicles in the ovary which is regulated by FSH. This phase has a duration ranging from ten days to three weeks. During ovulation one or more follicles have matured leading to the release of an oocyte which is regulated by a preceding surge in LH. The surge in LH is produced by a surge of GnRH. One key enzyme involved with the expulsion of the ovum from the ovulatory follicle is cyclooxygenase 2 (COX-2). The LH surge propagates the synthesis of prostaglandins in the cumulus cells (Dinchuk, et al., 1995). COX-2 mediates the synthesis of prostaglandins from arachidonic acid (Ricciotti & FitzGeral, 2011). LH also modulates the luteal phase transforming the ruptured follicle into the corpus luteum. The corpus luteum then secretes progesterone that will prepare the uterus for pregnancy. If pregnancy does not occur after two weeks then the corpus luteum will regress into the corpus albicans and cease to secrete hormones. The ovarian cycle will then start over.

The uterine cycle refers to ongoing changes in the endometrial lining of the uterus regulated by ovarian hormones. The uterine cycle is divided into three phases: the menses phase, proliferative phase and secretory phase. The menses phase coincides with the beginning of the ovarian follicular phase. This is marked by the menstrual bleeding that occurs in the uterus. The proliferative phase occurs towards the end of the ovarian follicular phase. This is marked by an increase in the number of cells within the endometrium awaiting pregnancy. At the end of the proliferative phase ovulation occurs. The secretory phase coincides with the ovarian luteal phase. The hormones secreted from the corpus luteum convert the thick endometrial layer into a secretory structure. If pregnancy does not occur then the thickened endometrial layer that was converted to secretory structures are sloughed off during menses.

Female Rodent Reproductive Cycle

Female rodents are polyestrous, which includes *Rattus norvegicus*. The rats have an estrous cycle lasting four to five days. The cycle is composed of four phases: proestrus, estrus, metestrus and diestrus. The first phase, proestrus, is described as when the animal is coming into heat. The second phase, estrus, is characterized by a female animal's readiness to receive a male for coitus. If conception does not take place then the female will move into the next phase, metestrus. This phase is characterized by the estrus effects subsiding within the reproductive tract. The next phase is diestrus, comparable to the luteal phase in primates. The cycle will then repeat back to the proestrus stage. Vaginal smears are the most common way to determine the phase female rats are in. Proestrus is characterized by a predominance of nucleated epithelial cells. The estrus phase is characterized by anucleated cornified cells. Metestrus is characterized by the same proportion of leukocytes, cornified and nucleated epithelial cells. Diestrus is

characterized by a predominance of leukocytes (Yener, et al., 2007). After diestrus, LH and FSH levels start to increase from the anterior pituitary gland. FSH triggers expression of LH receptors in the ovaries while LH will propagate the synthesis and secretion of androstenedione which leads to the synthesis of estrogen. During proestrus there is an increase in estrogen secretion which inhibits GnRH release subsequently inhibiting LH and FSH secretion. This inhibition is also achieved by the secretion of the ovarian hormone inhibin. Estrogen levels peak during midday of the proestrus phase. This peak is important because the inhibition of GnRH ceases thus increasing GnRH secretion and LH and FSH responsiveness to GnRH. The effects of estrogenic modulation here is pivotal to the surge in preovulatory GnRH, LH and FSH levels. There is however a second FSH surge which is GnRH-independent and occurs during ovulation or soon after. The second surge in FSH helps to prepare a follicle for the next cycle. During proestrus, progesterone levels increase and peak during ovulation. After ovulation, LH and FSH secretion is inhibited by progesterone and estrogen. Progesterone levels also increase with estrogen during proestrus which stimulates GnRH secretion which reinforces the LH preovulatory surge. Estrus can be induced in rats by administering estradiol-17β and progesterone. Once estrus begins, eight to twelve hours later ovulation occurs (Plant, et al., 2015).

Neural Control during Estrous Cycle

Throughout the estrous cycle the LH secretion pattern is pulsatile. The frequency of these pulses are less than one pulse per hour. It has been shown that the mechanism for LH secretion is under a 24 h photoperiodic control. This means it can fall under the category of a circadian rhythm (Levine & Sawyer, 1950). Studies have shown the paired suprachiasmatic nuclei (SCN)

of the hypothalamus functions as a generator of the signal that is entrained to the light-dark rhythm. The SCN is responsible for generating 24 h rhythms. The SCN times the appropriate initiation of GnRH surge on the afternoon of proestrus. This surge in GnRH subsequently secretes a surge of LH. The estrous cycle ceases when the SCN is lesioned. As mentioned before, GnRH pulsatility is pivotal to maintaining responsiveness of the anterior pituitary gland. During the afternoon of proestrus, the GnRH pulses are interrupted by a surge in the secretion of GnRH. This surge will propagate the preovulatory surge in gonadotropin hormones. While the SCN provides daily neuronal signals for the surge secretion of GnRH, the anteroventral periventricular nucleus receives these signals. Combined with the ovarian hormones, a surge secretion of GnRH is initiated (Plant, et al., Zeleznik, 2015).

Male Reproduction

The testes consist of a tough fibrous capsule known as the tunica albuginea. Within the tunica albuginea are an abundance of seminiferous tubules. This is the location where spermatogenesis takes place. The interstitial tissues around the seminiferous tubules consist of blood vessels and testosterone producing Leydig cells. Inside the seminiferous tubules the germ cells or spermatogonia, mature towards the lumen into sperm cells. Sertoli cells regulate sperm development by secreting proteins to help nourish developing sperm cells. Spermatogonia whose fate is to become sperm cells mature towards the lumen of seminiferous tubules. The seminiferous tubule exits the testis and joins the epididymis. The epididymis is a worm like structure that wraps around the testis consisting of three sections: head, body and tail. The tail of the epididymis stores mature sperm. The tail then forms into the vas deferens. The vas deferens then joins into the urethra. Sperm contribute approximately 1% of the volume of semen that is

ejaculated. The other 99% of the semen volume come from fluid secreted by accessory glands: bulbourethral glands, seminal vesicles and prostate gland. The bulbourethral glands contribute a mucus-like fluid utilized for lubrication. The fluid also contains buffers that neutralize the residual acidic urine in the urethra and the acid environment of the vaginal. The seminal vesicles contribute prostaglandins and nutrients. The prostaglandins help influence sperm motility and transport in male and female reproductive tracts. The prostate gland also contributes nutrients with the seminal vesicles for sperm metabolism.

Metabolism

Metabolism begins with the intake of food. The behavioral mechanism of when to eat depends on hunger and satiety. These two functions are controlled by the hypothalamus by a tonically active feeding center and a satiety center that regulates feed intake by inhibiting the feed center. Peptides that have shown to increase food intake include ghrelin and orexins. Peptides that have shown to decrease food intake include cholecystokinin, glucagon-like peptide-1, peptide YY, corticotropin-releasing hormone, α-melanocyte-stimulating hormone and cocaine- and amphetamine-regulated transcript. Food begins to be broken down mechanically and chemically in the mouth then moves towards the stomach where the food is digested into smaller molecules via acid secretion. The chyme then moves into the small intestines where acinar cells from the pancreas further aid in digestion. The acidic chyme is then neutralized by sodium bicarbonate secreted by duct cells in the pancreatic duct. Bile is then secreted from hepatocytes concurrent with the pancreatic enzymes and sodium bicarbonate. The bile facilitates enzymatic fat digestion. The broken down smaller molecules are then absorbed in the intestines. After the

molecules are absorbed they flow through the hepatic portal vein to the liver. The liver functions as a filter to remove any potentially harmful xenobiotics before they enter the rest of body.

Apart from aiding in digestion the pancreas plays a key role in maintaining plasma glucose homeostasis. The pancreas secretes two key hormones: insulin and glucagon. Within the pancreas are structures called islets of Langerhans that contain beta cells and alpha cells. Beta cells are responsible for secreting insulin to lower plasma glucose levels by increasing uptake of glucose in the tissues. Alpha cells are responsible for secreting glucagon to increase plasma glucose levels by increasing the synthesis of glucose. These hormones are predominately responsible for plasma glucose homeostasis.

Mammals have two bean shaped kidneys lateral to the abdominal aorta. The functions of the kidneys are to regulate extracellular fluid volume and blood pressure, osmolarity, maintenance of ion balance, excretion of wastes and production of hormones. The kidney is divided into an inner medullary region and an outer cortical region. Within the regions is a vast network of tubules called nephrons. Each nephron is initiated by a Bowman's capsule that surrounds the glomerulus. The Bowman's capsule is comprised of simple squamous epithelium. Blood is filtered out of the glomerulus and into the Bowman's capsule. The filtrate then flows into the proximal convoluted tubule, distal convoluted tubule, and collecting duct on its way to the bladder to await excretion.

Chapter III: Hypotheses

Based on the evidence presented in the literature review section of this thesis, we hypothesize:

a. females exposed to LS seed extract and P_4 will have a lower amplitude GnRH surge
b. females exposed to LS seed extract and P_4 will have a lower amplitude LH surge
c. females exposed to LS seed extract and P_4 will have a lower amplitude FSH surge
d. males fed rat chow with 15% inclusion of LS seeds will have an increased sperm density
e. males fed rat chow with 15% inclusion of LS seeds will have an increased testosterone secretion in perifusate samples from testis parenchyma
f. males fed rat chow with 15% inclusion of LS seeds will have an increased plasma testosterone level
g. males fed rat chow with 15% inclusion of LS seeds will have histopathological evidence
h. males fed rat chow with 15% inclusion of LS seeds will have abnormal growth of their organs.

Chapter IV: Materials and Methods

The research protocols utilized in this study were approved by the Institutional Animal Care and Usage Committee of St. Cloud State University, St. Cloud, Minnesota, USA. The research was conducted in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals (2011). All experiments were carried out in the vivarium at St. Cloud State University, St. Cloud, Minnesota, USA.

Plant Acquisition and Preparation

LS seeds were purchased from a local market in Gombe, Nigeria and confirmed in the Department of Forestry and Wildlife Management, Federal University of Agriculture, Abeokuta, Nigeria. The seeds were electrically blended to a fine powder using a single-speed food blender (Waring Products, Torrington, CT, USA). The LS seed powder (LSSP) was kept in an air tight container at 4°C until used for animal treatments.

Aqueous Extraction of LS Seeds

LSSP was mixed with deionized water (1 g/100 mL) for 24 h. The solution was filtered with a vacuum through Whatman qualitative filter paper grade 3 (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). The filtrate was then dehydrated in an oven for 48 h. The LS aqueous extract (LSE) was stored in an air tight container at 4°C.

Experiment 1: Effect of LS Seed Extract on the Surge of GnRH, LH and FSH in Ovariectomized Female Rats

Our objective of this experiment was to determine the effect of aqueous LSE on the development and magnitude of the surge releases in GnRH, LH, and FSH that lead to the process of ovulation in rats. Thirty-two female Sprague-Dawley rats (St. Cloud State University, St.

Cloud, MN, USA) weighing 200-250 g and between seven and ten weeks of age were used for this study. Rats were ovariectomized using standard methods and housed in individual plastic cages. They were maintained under a lighting regime of 12 hours under light and 12 hours in the dark at 25°C. Rat feed (Global Diets, Madison, WI, USA) and water were provided *ad libitum*. The rats were utilized seven to ten days after ovariectomy to allow time to heal. On days 1 to 3 at 1000 h, all rats were injected with estradiol-17β (10 μg/0.2 mL/rat, s.c. in corn oil). On day 4 at 0800 h, sixteen of the rats were treated with LSE (40 mg/Kg BW, i.p.) while the remaining sixteen received normal saline diluent at the same rate. At 1000 h, eight rats that received LSE and eight rats that received normal saline were treated with corn oil (0.2 mL/rat, s.c.) while the remaining sixteen rats, eight that received LSE and eight that received saline, were treated with progesterone (2.5 mg/0.2 mL/rat, s.c. in corn oil). These regimens of hormonal treatments produce physiological levels of plasma estradiol-17β and progesterone and consistently generate preovulatory-like surges of GnRH, LH and FSH. These treatments therefore produce the following treatment groups: Group 1: OVX+E_2+Saline, n=8; Group 2: OVX+E_2+LSE, n=8; Group 3: OVX+P_4+Saline, n=8; Group 4: OVX+P_4+LSE, n=8. At 1300 h, the rats were euthanized using an overdose of 6 mL 2.5% tribromoethanol. The hypothalamus was extracted by removing the brain from the skull. The brain was then inverted exposing the posterior side. Utilizing a scalpel and number 11blade, a cut was made immediately posterior the optic chiasm. Another cut was made anterior the mammillary bodies. Two more cuts were made on the ventral lateral ridges. The hypothalamus was then cut midsagittal to yield two hemi-hypothalamic fragments. The pituitary gland was extracted from the surface of the sella turcica with a scalpel

and number 11 blade. The hemi-hypothalamic fragments and pituitary gland from each rat were then perifused.

Perifusion of Hemi-Hypothalamic Fragments

The hemi-hypothalamic fragments obtained from the above treatment groups were perifused utilizing a 12-chamber Suprafusion 1000 (Brandel, Gaithersburg, MD, USA) with artificial cerebrospinal fluid (aCSF) at 37°C for a period of seven hours. The perifusate fractions were collected at 15-minute intervals yielding a collected volume of 2 mL per fraction. GnRH concentrations were determined in all perifusates using radioimmunoassays.

Perifusion of Pituitary Gland

Pituitary glands obtained from the above treatment groups were perifused using a 6-chamber Suprafusion 1000 (Brandel, Gaithersburg, MD, USA) with aCSF at 37°C for a period of seven hours. After 60 minutes, 10 ng/mL of GnRH was administered to the pituitary glands for 30 minutes. GnRH was added to assist in surging of LH and FSH because of the severed link between the hypothalamus and pituitary gland. The perifusate fractions were collected at 15-minute intervals yielding a collected volume of 2 mL per fraction. LH and FSH concentrations were determined in all perifusates using radioimmunoassays.

Radioimmunoassay of GnRH

GnRH concentrations were determined utilizing the technique by Ellinwood et al. (1985). GnRH (Sigma Aldrich, St. Louis, MO, USA) was iodinated using ^{125}I (Perkin Elmer Laboratories, Waltham, MA, USA) by the Chloramine-T method. The GnRH standards were 1, 5, 10, 25, 50, 100, 250, 500 and 1,000 pg/mL. The antiserum R1245 (Colorado State University, Fort Collins, CO, USA) was utilized at 1:12,000 dilution.

The radioimmunoassay (RIA) for GnRH is a three-day assay. On day 1, standards (200 μL), perifusate samples (200 μL) in duplicates and antibody (50 μL) were pipetted into 12x75 mm borosilicate glass tubes (VWR International, Radnor, PA, USA). The tubes were then vortexed briefly and stored at 4°C for 24 h. On day 2, the tracer (^{125}I-GnRH; 11,000 cpm/50 μL) was pipetted to all tubes, vortexed briefly and then stored at 4°C for 24 h. On day 3, 1.5 mL 95% ice cold ethanol was pipetted into all tubes as a precipitating agent and then vortexed. The tubes were then centrifuged at 3,000 rpm at 4°C for 20 minutes using the Sorvall RT7 plus centrifuge (Thermo Scientific, Waltham, MA, USA). The supernatant was discarded into a waste receptacle. The tubes were then loaded and counted in a Packard Cobra II gamma counter (Perkin Elmer, Waltham, MA, USA).

Radioimmunoassay of LH

LH concentrations were determined utilizing the techniques described by Bernard et al. (1983). LH was iodinated using ^{125}I (Perkin Elmer Laboratories, Waltham, MA, USA) by the Chloramine-T method. The LH (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD, USA) standards that were used include 0.8, 1.6, 3.1, 6.2, 12.5, 25 and 50 ng/mL. The first antiserum AFP 240580 was utilized at 1:40,000 dilution. The second antibody that was used is goat anti-rabbit immunoglobulin G (GARGG, Equitech-Bio, Inc, Kerrville, TX, USA) at a dilution of 1:1 with 0.01 M phosphate buffered saline (PBS) and EDTA.

Briefly on day 1, standards (200 μL), perifusate samples (100 μL) in duplicates, first antibody (100 μL) and tracer (^{125}I-LH; 20,000 cpm/100 μL) were pipetted into 12x75 mm borosilicate glass tubes (VWR International, Radnor, PA, USA). The tubes were then vortexed

briefly before being stored at 4°C for 48 h. On day 3, the second antibody GARGG (200 μL) was pipetted into the tubes along with 6% polyethylene glycol (500 μL). Afterwards, the tubes were vortexed briefly and centrifuged at 3,000 rpm at 4°C for 15 minutes using the Sorvall RT7 plus centrifuge (Thermo Scientific, Waltham, MA, USA). The supernatant were discarded into a waste receptacle. The tubes were then loaded and counted in a Packard Cobra II gamma counter (Perkin Elmer, Waltham, MA, USA).

Radioimmunoassay of FSH

FSH concentration was determined utilizing techniques described by Odell et al. (1968). FSH was iodinated using ^{125}I (Perkin Elmer Laboratories, Waltham, MA, USA) by the chloramine-T method. The FSH (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD, USA) standards that were used include 0.125, 0.25, 0.5, 1.5, 2.5, 5.0, 10.0, 20.0 and 50 ng/mL. The first antiserum AFP C528813 was utilized at 1:15,000 dilution. The second antibody that was used is GARGG (Equitech-Bio, Inc, Kerrville, TX, USA) at a dilution of 1:1 with 0.01 M PBS and EDTA.

The RIA for FSH is a 5-day assay. On day 1, standards (200 μL), perifusate samples (200 μL) in duplicates and first antibody (200 μL) were pipetted into 12x75 mm borosilicate glass tubes (VWR International, Radnor, PA, USA). The tubes were then vortexed briefly before being stored at 4°C for 24 h. On day 2, the tracer (^{125}I-FSH; 20,000 cpm/100 μL) were pipetted into all tubes, vortexed briefly, and then stored at 4°C for 60 minutes using a Sorvall RT7 plus centrifuge (Thermo Scientific, Waltham, MA, USA). The supernatant was discarded into a waste receptacle. The tubes were then loaded and counted in a Packard Cobra II gamma counter (Perkin Elmer, Waltham, MA, USA).

Experiment 2: Effect of LS Seed Inclusion on Reproductive Hormones, Sperm Concentration, Metabolic and Growth Indices in Male Rats

The objective of this experiment was to determine the acute and chronic effects of 15% LS seed supplementation on gross organ morphology and histomorphometric indices, testosterone secretion and spermatogenesis involved in metabolic and spermatogenic functions in the Sprague-Dawley rat.

LS Experimental Diet

Normal rat chow (Global Diets, Madison, WI, USA) was electrically blended using a single-speed food blender (Waring Products, Torrington, CT, USA) to a fine powder. The 15% LS experimental feed was produced by mixing 17 g of finely ground normal rat chow and 3 g of LSSP with 10 mL deionized water. The mixture was then pelleted by hand into six pellets. The pellets air-dried for 24-48 h before they were utilized as feed.

Experimental Animals

Forty-eight mature male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) weighing 250-350 g and between seven and ten weeks of age were used for this study. Rats were housed in individual plastic cages for two weeks prior to the experiment for acclimation. The rats were maintained under a lighting regime of 12 hours under light and 12 hours in the dark at 25°C. During the acclimation period rat feed and water were provided *ad libitum*.

After the acclimation period, each rat was weighed and assigned to either a control group (0% LS seed inclusion; n=24) or a treatment group (15% LS seed inclusion; n=24). The length of the study was eight weeks. This duration is important to enable us to determine the acute and

then chronic effects of feeding these seeds on metabolism and reproduction. Secondly, the 56-day study included a period of 48 to 52 days, the duration of one spermatogenic cycle in the rat (Hafez, 1970). At 0800 h daily, rats were offered either normal rat chow or the 15% LS seed included rat chow for 8 weeks. At 2, 4, 6 and 8 weeks, six rats from each the Control or the Treatment groups were euthanized with carbon dioxide asphyxiation for a minimum of 10-minutes. All rats were weighed on the first day of the experiment, every other day and immediately after euthanization to determine bodyweight changes. Every day feed intake and refusal was measured. Immediately post euthanization, trunk blood was collected for hormone assays. Further, the paired testes, epididymides, prostate gland, seminal vesicles, kidneys, adrenal glands, heart, liver, spleen, lungs, brain and pancreas were harvested and weighed. The relative organ weights were normalized to organ weight per 100 g BW. One kidney and one testis were preserved in 10% formalin buffer for histological analysis. The other testes were utilized for *in vitro* testosterone production determination. One cauda epididymis per rat was isolated and utilized for the epididymal sperm count.

Kidney Histological Analysis

The kidneys obtained for histological analysis were stored in 10% formalin until fixated in paraffin wax. Each kidney was sectioned at 5 μm superficially and then 100 μm deep. The tissue was then stained in Hematoxylin and Eosin. Pictures were taken of the Bowman's capsule, glomerulus, proximal tubule and distal tubule utilizing a light microscope DM750 (Leica, Buffalo Grove, IL, USA) with an attached camera. The diameters of the aforementioned structures were measured using ImageJ (National Institute of Health, Bethesda, MD, USA).

Within each structure evidence of disease presence was graded as 1, disease present, or 0, disease absent. The disease presence is presented as a percentage.

Testes Histological Analysis

The testes obtained for histological analysis were stored in 10% formalin until fixated in paraffin wax. Each testis was sectioned at 5 μm superficially and then 100 μm deep. The tissue was then stained in Hematoxylin and Eosin. Pictures were taken of the seminiferous tubules, germinal cell layer and Leydig cells utilizing a light microscope DM750 (Leica, Buffalo Grove, IL, USA) with an attached camera. The diameters of the seminiferous tubules, thickness of germinal cell layer and diameter of Leydig cells were measured using ImageJ (National Institute of Health, Bethesda, MD, USA).

***In Vitro* Testosterone Production**

Two 100 mg samples (A and B) of parenchyma from each testis were loaded into Suprafusion 1000 equipment (Brandel, Gaithersburg, MD, USA). The parenchyma was perifused for three hours collecting 4 mL of perifusate at 30 minute increments at a constant temperature of 37°C. During the first hour, all samples were perifused in 0.01 M phosphate buffered saline (PBS). During the second and third hours, sample A was perifused in 0.01 M PBS while sample B was perifused in 150 mIU/mL human chorionic gonadotropin (hCG) in 0.01 M PBS. The hormone hCG was utilized because of its stimulatory effect of testosterone secretion similar to luteinizing hormone. Perifusate fractions were stored at -80°C until assayed utilizing a double antibody testosterone ^{125}I radioimmunoassay kit for testosterone (MP Biomedicals, Costa Mesa, CA, USA).

Epididymal Sperm Density

The cauda epididymis was isolated by cutting approximately 1 mm down from the junction of the vas deferens and the cauda epididymis and also at the junction of corpus epididymis and cauda epididymis. The cauda epididymis weight was then obtained. The epididymal sperm concentrations were obtained using a Neubauer hemocytometer as described by Wang (2003). The sperm density was calculated as total number of sperm per cauda / cauda weight (mg).

Statistical Approach

Statistical analysis utilized the statistical software SPSS 24 (IBM, Armonk, NY, USA). The main effect of dose and time and their interactions on GnRH, LH and FSH secretions were determined using the multivariate analysis of variance (MANOVA) in experiment 1. Experiment 2 utilized a MANOVA to analyze the effect of the treatment of the seeds and the effect of time of feeding for the various aforementioned measurement variables (body weights, feed intake, organ weights, testosterone secretions, plasma testosterone, sperm concentration and various histological parameters: Bowman's capsule diameter, glomerular diameter, proximal and distal tubule diameter, disease presence, seminiferous tubule diameter, germinal cell layer thickness and Leydig cell diameter).

Chapter V: Results

Experiment 1

The profile of GnRH in hemi-hypothalamic fragments obtained from all treatment groups are shown in Figures 1-3. Ovariectomized, estrogen primed, progesterone treated rats showed increased GnRH secretion behavior at 180 and 240 minutes after progesterone administration. Although not statistically significant, this secretion corresponded to the afternoon and is indicative of a surge-like secretion. LSE treatment at 40 mg/Kg BW seemed to cause this surge-like secretion to occur earlier, around 40 and 90 minutes, in the progesterone-treated rats and the magnitude of the surge was also attenuated. Although pulsatile, no surge-like GnRH secretion was observed in either saline or LSE treated ovariectomized, estrogen-primed hemi-hypothalamic fragments.

Overall, average GnRH secretion was significantly greater in progesterone-treated, estrogen-primed rats compared to saline-treated, estrogen-primed rats (Figure 1). The total GnRH secretion for estrogen-primed, progesterone and saline-treated, ovariectomized rats was 96.5 pg/mL (Table 1). The estrogen-primed, progesterone and LSE-treated, ovariectomized rats was slightly increased at 98.9 pg/mL, but not significant. The estrogen-primed, saline-treated, ovariectomized rats had similar GnRH secretions of 92.5 pg/mL. Rats that were estrogen-primed, LSE-treated, ovariectomized had nonsignificant decreased total GnRH secretions of 74.3 pg/mL.

Averaged over time, perifusate LH secretion was significantly greater ($p<0.05$) in ovariectomized, estrogen-treated rats compared to ovariectomized, estrogen-primed, progesterone treated rats. LSE treatment had no effect on LH secretion in either the estrogen or progesterone-treated rats (Figure 6).

Temporally, LH secretion from the pituitary gland in all treatment groups decreased over time. Perifusate LH level was high varying from 15-36 ng/mL pre-GnRH administration (Figures 4 & 5). Administration of GnRH of 10 ng/mL had no stimulatory effect on LH secretion in any treatment group. Consequently, no surge-like LH secretion was observed. Total LH secretions were similar among the ovariectomized, estrogen-primed, progesterone and saline or LSE treated rats, 209.4 and 208.1 ng/mL, respectively (Table 1). Ovariectomized, estrogen-primed, saline or LSE treated rats had similar total LH secretions of 297.4 and 301.2 ng/mL, respectively.

The profile of FSH in perifusates obtained from all experimental groups are shown in Figures 7-9. Overall, FSH decreased from time 0 to about 180 minutes and then stabilized. Average perifusate FSH levels were significantly higher in ovariectomized, estrogen-primed, progesterone-treated rats compared to ovariectomized, estrogen-primed rats. LSE administration significantly increased ($p<0.05$) average perifusate FSH secretion in the ovariectomized, estrogen-primed rats but had no effect in the ovariectomized, estrogen-primed, progesterone-treated rats. Administration of GnRH at 10 ng/mL had no effect in perifusate FSH secretion. Total FSH secretion for ovariectomized, estrogen-primed, progesterone-treated rats were similar to ovariectomized, estrogen-primed, LSE-treated rats, 21.4 and 24.2 ng/mL, respectively (Table 1). The ovariectomized, estrogen-primed rats had a nonsignificant decrease in total FSH secretion, 17.0 ng/mL, compared to ovariectomized, estrogen-primed, LSE-treated rats, 35.7 ng/mL.

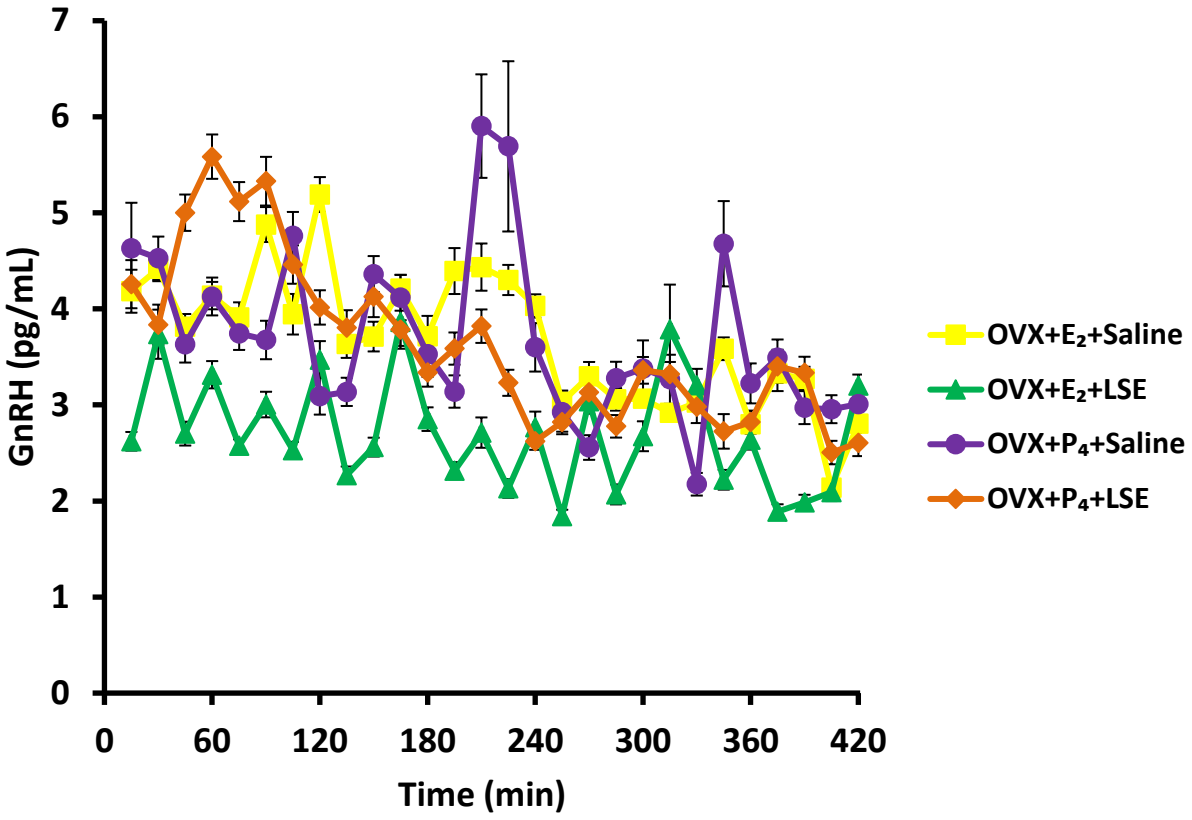

Figure 1. Perifusion of hemi-hypothalamic fragments from ovariectomized female Sprague-Dawley rats for gonadotropin releasing hormone. Control groups receiving corn oil + saline, corn oil + LSE and progesterone + saline: yellow square, green triangle and purple circle, respectively. Experimental group receiving progesterone + LSE, orange diamond.

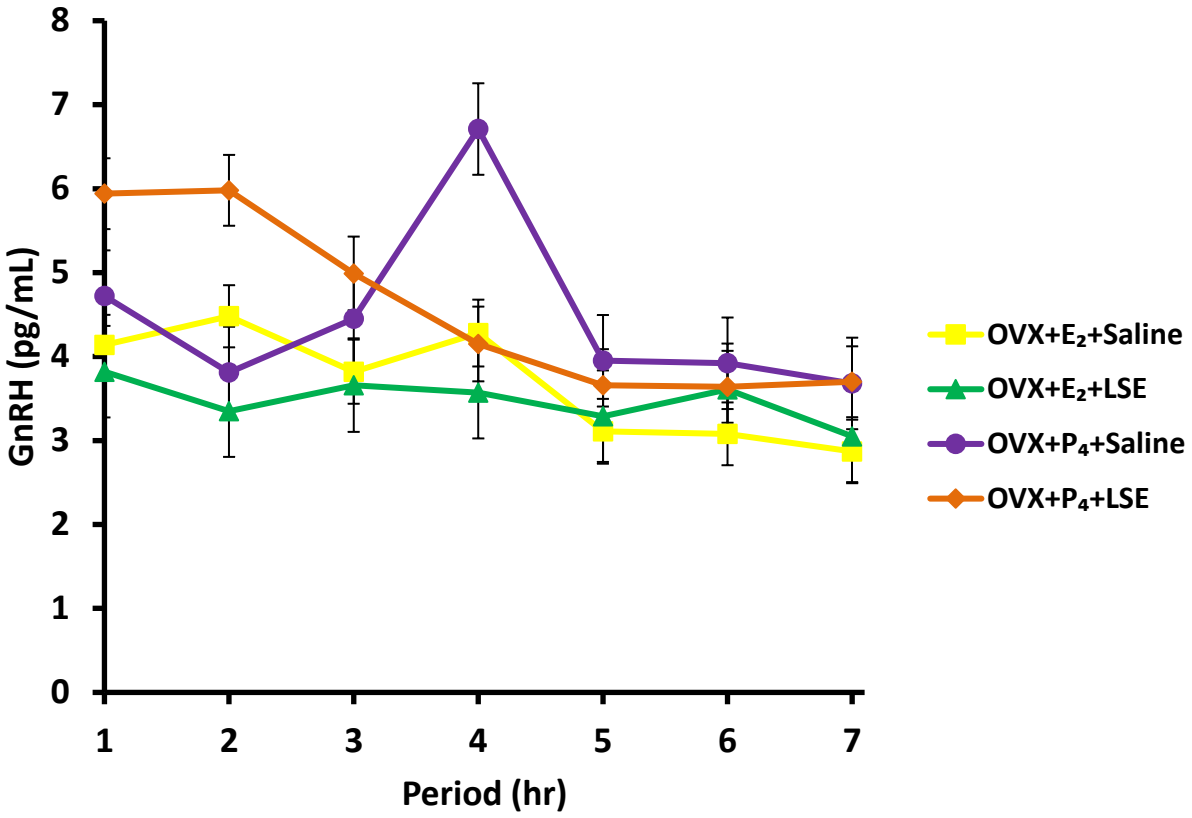

Figure 2. Perifusion of hemi-hypothalamic fragments from ovariectomized female Sprague-Dawley rats for gonadotropin releasing hormone by period. Control groups receiving corn oil + saline, corn oil + LSE and progesterone + saline: yellow square, green triangle and purple circle, respectively. Experimental group receiving progesterone + LSE, orange diamond.

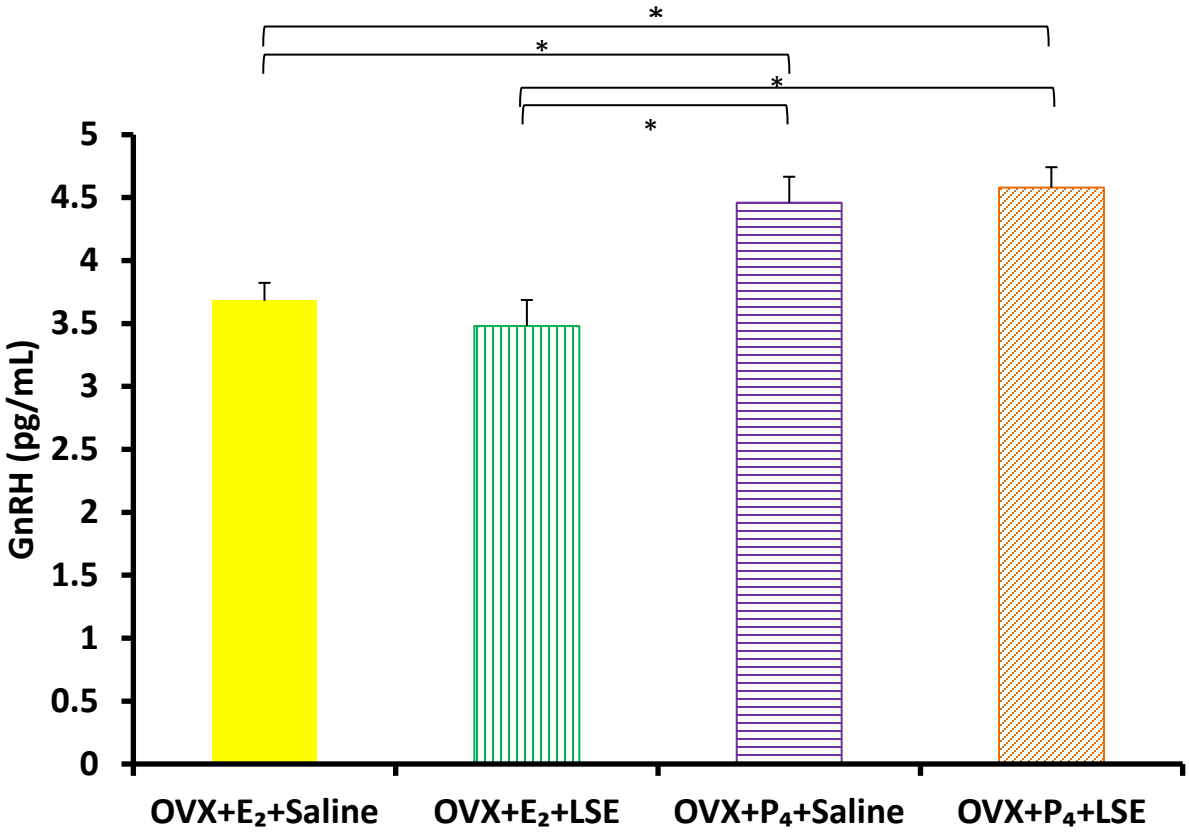

Figure 3. Perifusion of hemi-hypothalamic fragments from ovariectomized female Sprague-Dawley rats for average gonadotropin releasing hormone secretion. Control groups receiving corn oil + saline, corn oil + LSE and progesterone + saline: yellow solid, green vertical stripes and purple horizontal stripes, respectively. Experimental group receiving progesterone + LSE, orange diagonal stripes. * indicates significantly different at $p<0.05$.

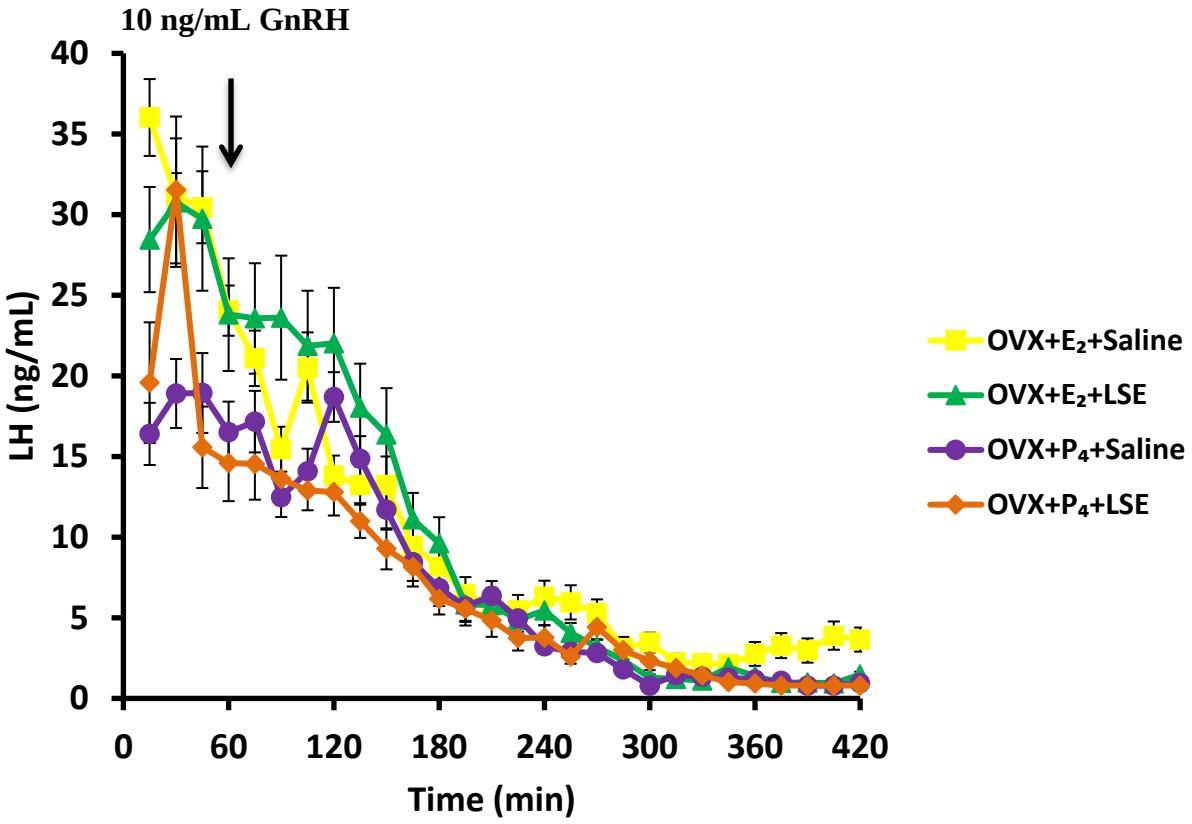

Figure 4. Perifusion of pituitary gland fragments from ovariectomized female Sprague-Dawley rats for luteinizing hormone. Control groups receiving corn oil + saline, corn oil + LSE and progesterone + saline: yellow square, green triangle and purple circle, respectively. Experimental group receiving progesterone + LSE, orange diamond.

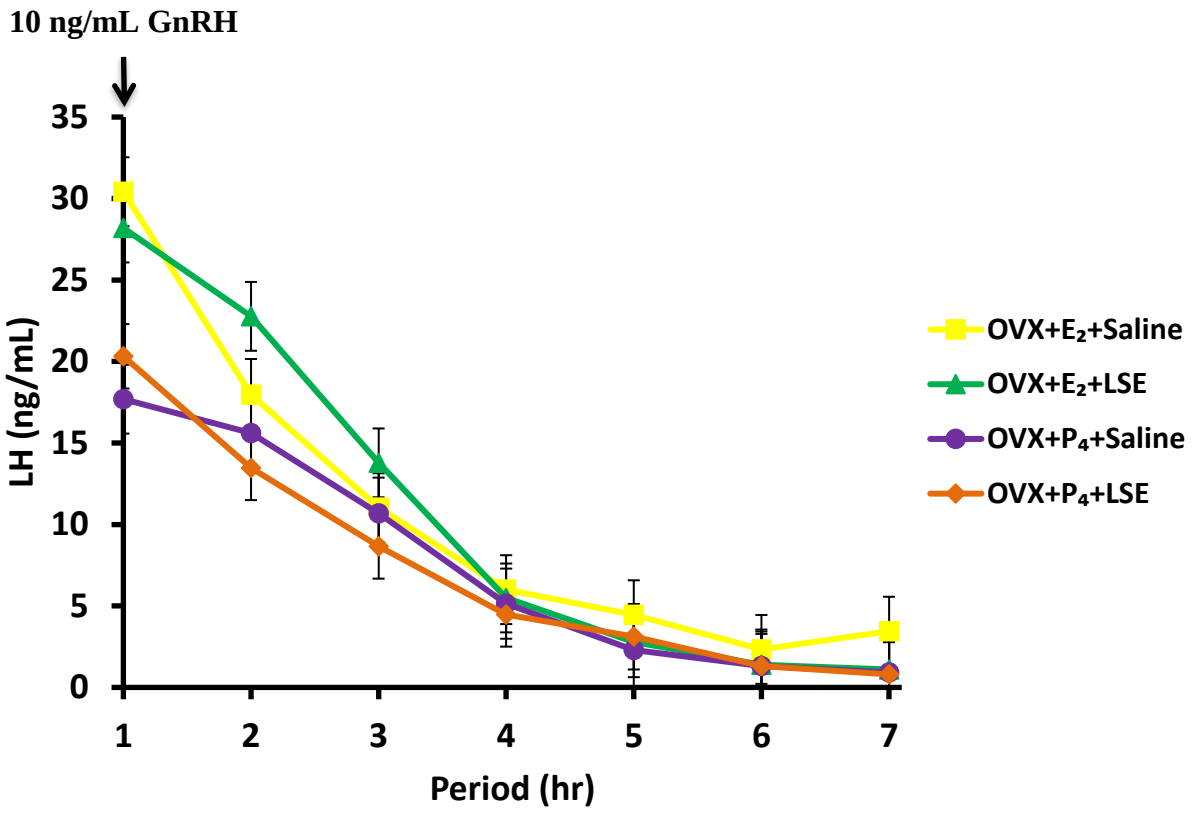

Figure 5. Perifusion of pituitary gland fragments from ovariectomized female Sprague-Dawley rats for luteinizing hormone by period. Control groups receiving corn oil + saline, corn oil + LSE and progesterone + saline: yellow square, green triangle and purple circle, respectively. Experimental group receiving progesterone + LSE, orange diamond.

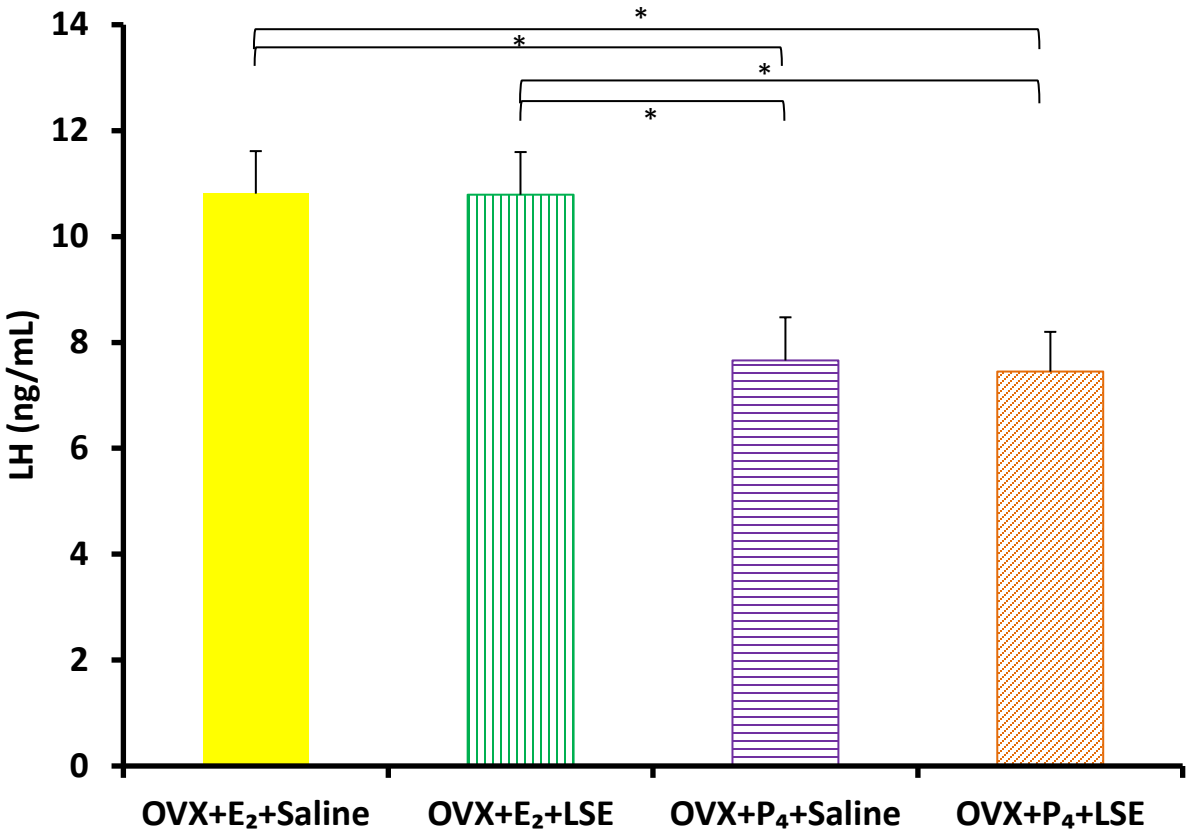

Figure 6. Perifusion of pituitary gland fragments from ovariectomized female Sprague-Dawley rats for average luteinizing hormone secretion. Control groups receiving corn oil + saline, corn oil + LSE and progesterone + saline: yellow solid, green vertical stripes and purple horizontal stripes, respectively. Experimental group receiving progesterone + LSE, orange diagonal stripes. * indicates significantly different at $p<0.05$.

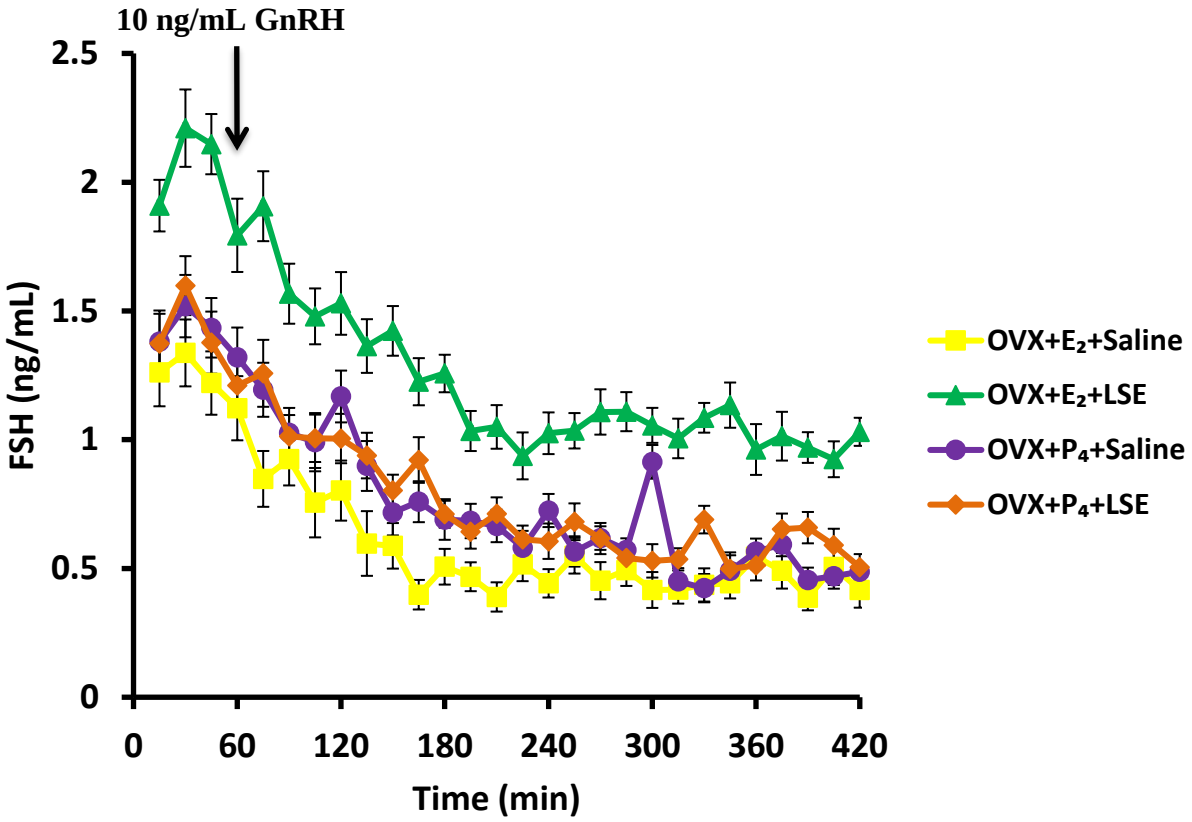

Figure 7. Perifusion of pituitary gland fragments from ovariectomized female Sprague-Dawley rats for follicle-stimulating hormone. Control groups receiving corn oil + saline, corn oil + LSE and progesterone + saline: yellow square, green triangle and purple circle, respectively. Experimental group receiving progesterone + LSE, orange diamond.

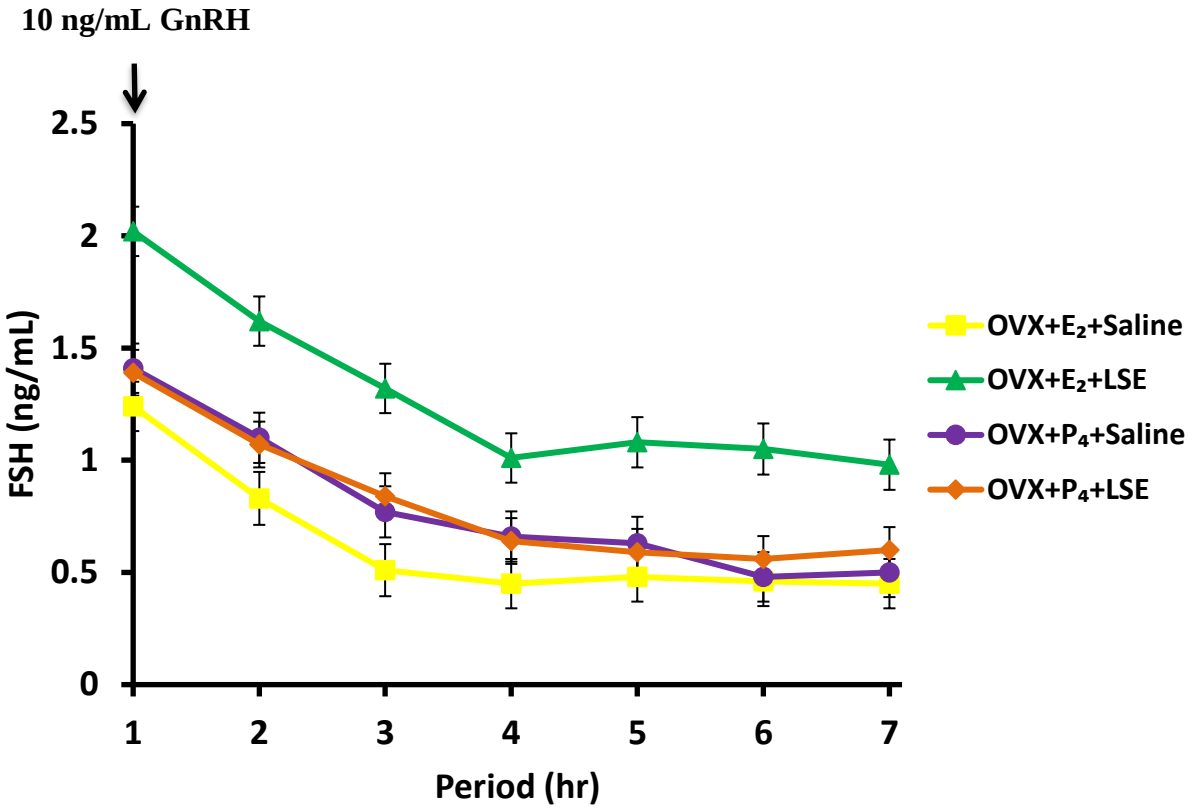

Figure 8. Perifusion of pituitary gland fragments from ovariectomized female Sprague-Dawley rats for follicle-stimulating hormone by period. Control groups receiving corn oil + saline, corn oil + LSE and progesterone + saline: yellow square, green triangle and purple circle, respectively. Experimental group receiving progesterone + LSE, orange diamond.

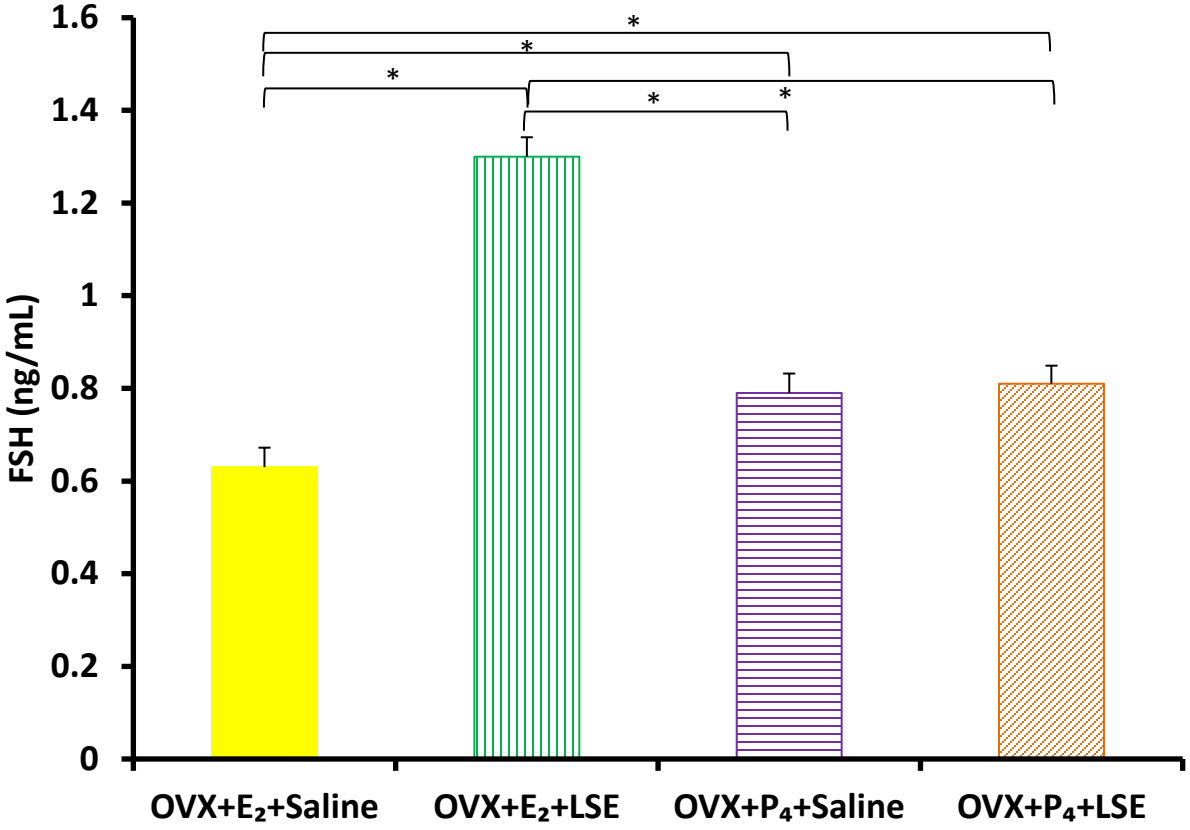

Figure 9. Perifusion of pituitary gland fragments from ovariectomized female Sprague-Dawley rats for average follicle-stimulating hormone secretion. Control groups receiving corn oil + saline, corn oil + LSE and progesterone + saline: yellow solid, green vertical stripes and purple horizontal stripes, respectively. Experimental group receiving progesterone + LSE, orange diagonal stripes. * indicates significantly different at $p<0.05$.

Table 1. Effect of aqueous LS extract on total hormonal secretion of GnRH, LH and FSH from the hypothalamus and pituitary gland for 7 hours

	Total Hormone Secretion		
Group	GnRH (pg/mL)	LH (ng/mL)	FSH (ng/mL)
OVX+E_2 +Saline	92.5 ± 2.36	297.4 ± 21.85	17.0 ± 2.01
OVX+E_2 +LSE	74.3 ± 2.10	301.2 ± 42.18	35.7 ± 2.25
OVX+P_4 +Saline	96.5 ± 3.56	209.4 ± 20.44	21.4 ± 1.89
OVX+P_4 +LSE	98.9 ± 3.59	208.1 ± 22.08	24.2 ± 1.90

Values are mean ± S.E.M. of 29 female Sprague-Dawley rats. No significance was observed.

Experiment 2

Over the duration of the experiment rats fed 0% LS seed supplementation increased in body weight. During the first week of the experiment, rats fed 15% LS seed supplementation decreased in body weight (Figure 10) due to the strong feed aversion (Figure 11). During the rest of the experiment the treatment group gained weight at a rate concurrent with the control group. The control group had a slight feed aversion but remained steady during the rest of the experiment. Rats fed 15% LS seed inclusion had a significantly strong feed aversion during the first few days of the experiment. Afterwards rats in both groups ate approximately the same amount of feed each day (Figure 11).

Visceral Organ Weights

There were no significantly different paired testicular weight differences throughout the experiment. Although, there was a non-significant increase in paired testicular weights in rats fed 15% LS seed inclusion during weeks four (Control: 0.840g, Treatment: 0.933 g) and six (Control: 0.862 g, Treatment: 0.940 g). Week two and eight paired testicular weights were similar (Figure 12). The epididymides weight differences were not significant (Figure 13). There was a significant decrease in the treatment groups prostate gland weight during weeks two (Control: 0.240 g, Treatment: 0.190 g) and six (Control: 0.220 g, Treatment: 0.190 g). There was however a nonsignificant decrease during weeks four (Control: 0.224 g, Treatment: 0.197 g) and eight (Control: 0.220 g, Treatment: 0.200 g) (Figure 14). There were no significant weight changes for the seminal vesicles weight (Figure 15). Interestingly, the control group had a significantly lower renal weight than the treatment group at each biological endpoint (Figure 16). During weeks two, four, six and eight the control group renal weights were 0.688, 0.628, 0.670

and 0.661 g, respectively. During weeks two, four, six and eight the treatment group renal weights were 0.749, 0.831, 0.810 and 0.890 g, respectively. There were no significant weight changes for the adrenals, heart, liver, spleen and lungs (Figure 17-21). The control group brain weight decreased during the experiment at each biological time point. There was a nonsignificant weight increase for the treatment group during week two (Control: 0.480 g, Treatment: 0.497 g). During weeks four (Control: 0.459 g, Treatment: 0.528 g), six (Control: 0.452 g, Treatment: 0.489 g) and eight (Control: 0.451 g, Treatment: 0.491g) the brain weight increased significantly (Figure 22). The pancreatic weight for the control group decreased over the duration of the experiment (Figure 23). Weeks two, four and six had similar pancreatic weights. The pancreatic weight during week eight was significantly increased in the treatment group (Control: 0.077 g, Treatment: 0.104 g).

Renal Histology

Dietary supplementation of LS seeds for eight weeks induced significant signs of toxicity (Tables 2 & 3). There was a trend of increasing toxicity as the experiment proceeded. The Bowman's capsule diameter was not significant except in week eight (Control: 0.164 mm, Treatment: 0.186 mm). The diameter of the glomerulus was significantly decreased in week four (Control: 0.158 mm, Treatment: 0.147 mm) and significantly increased in week eight (Control: 0.145 mm, Treatment: 0.155 mm). There was a significant increase in the difference in Bowman's capsule and glomerulus in week eight (Control: 0.0194 mm, Treatment: 0.0311 mm). Renal histopathological analysis yielded significant increase in presence of glomerulosclerosis (Control Total Average: 34.6%, Treatment Total Average: 65.0%), metaplasia (Control Total Average: 32.9%, Treatment Total Average: 74.6%) and hyperplasia (Control Total Average:

39.8%, Treatment Total Average: 79.4%) in the treatment group's renal corpuscle. Renal Bowman's capsule and glomeruli for rats that received 0% LS seed inclusion and 15% with glomerulosclerosis, metaplasia and/or hyperplasia are depicted in Figure 24 for weeks two and four. Renal Bowman's capsule and glomeruli for rats that receive 0% LS seed inclusion and 15% with glomerulosclerosis, metaplasia and/or hyperplasia are depicted in Figure 25 for weeks six and eight.

The kidneys displayed abnormal proximal and distal convoluted tubule diameter and presence of tubule degeneration (Table 4). The proximal convoluted tubule diameter was significantly larger in week eight for rats receiving 15% LS seed inclusion (Control: 0.0482 mm, Treatment: 0.0561 mm). There was significantly increased presence of proximal convoluted tubule degeneration throughout the eight week experimental period. During weeks two, four, six and eight the proximal tubule degeneration presence was low in the control group at 0.0, 2.5, 2.5 and 0.0%, respectively. During weeks two, four, six and eight the proximal tubule degeneration presence was high in the treatment group at 35.0, 60.0, 47.5 and 62.5%, respectively. The distal convoluted tubule diameter was significantly larger in week eight for rats receiving 15% LS seed inclusion (Control: 0.0396 mm, Treatment: 0.0507 mm). There was significantly increased presence of distal convoluted tubule degeneration throughout the eight week experimental period. During weeks two, four, six and eight the distal tubule degeneration presence was low in the control group at 10.0, 20.0, 10.0 and 15.0%, respectively. During weeks two, four, six and eight the distal tubule degeneration presence was remarkably high at 77.5, 70.0, 85.0 and 87.5%, respectively. Renal proximal tubules for rats that received 0% LS seed inclusion and 15% with tubular degeneration are depicted in Figure 26 for weeks two and four and Figure 27 for weeks

six and eight. Renal distal tubules for rats that received 0% LS seed inclusion and 15% with tubular degeneration are depicted in Figure 28 for weeks two and four and Figure 29 for weeks six and eight.

Testis Histology

The testes displayed no significant change in seminiferous tubule diameter or germinal cell layer thickness throughout the duration of the experiment. There was a significant increase in Leydig cell diameter during week two (Control: 0.0123 mm, Treatment: 0.0134 mm) but throughout the rest of the duration of the experiment the diameters were similar (Table 5).

***In Vitro* Testosterone Synthesis**

The testosterone synthesis of the testis parenchyma was not significant throughout the experiment (Figure 30). Challenging testis parenchyma with hCG did not increase testosterone secretion. During week two there was an increase in testosterone secretion for the control group perifused with 0.01 M PBS 90 minutes into the perifusion at 0.2 ng/mL while the treatment group perifused with 0.01 M PBS, control and treatment group perifused with 0.01 M PBS + hCG remained at 0.1 ng/mL (Figure 30A). There was a trend of 0.1 ng/mL of testosterone secretion. During week four there was a decreasing trend in all groups for the first 120 minutes. The control group perifused with 0.01 M PBS had the lowest testosterone secretions followed by the control group perifused with 0.01 M PBS + hCG. Interestingly, the highest testosterone secretion came from the treatment group perifused with 0.01 M PBS followed by the treatment group perifused with 0.01 M PBS + hCG (Figure 30B). During week eight there was a decreasing tend in testosterone secretion for the first 90 minutes. The control group perifused with 0.01 M PBS had the lowest testosterone secretion at the start of the perifusion. The second

lowest testosterone secretion was the control group perifused with 0.01 M PBS + hCG in the beginning. The highest testosterone secretion in the beginning of the perifusion was the treatment group perifused with 0.01 M PBS + hCG followed by the treatment group perifused with 0.01 M PBS. At 60 minutes the testosterone secretions began to have similar testosterone secretions. After 90 minutes each of the groups secreted approximately 0.1 ng/mL of testosterone (Figure 30C).

Plasma Testosterone

The plasma testosterone level fluctuations were not significant throughout the experiment (Figure 31). The control group increased from week two to week six before decreasing at week eight. The treatment group increased from week two to week four. The plasma testosterone levels then decreased during week six and increased at week eight. At week two the control group plasma testosterone levels were higher at 0.6 ng/mL compared to the treatment group at 0.3 ng/mL. At week four the control group plasma testosterone levels were lower at 0.7 ng/mL compared to the treatment group at 1.1 ng/mL. At week six the control group plasma testosterone levels were higher at 0.9 ng/mL compared to the treatment group at 0.4 ng/mL. At week eight the control group plasma testosterone levels were lower at 0.4 ng/mL compared to the treatment group at 0.5 ng/mL.

Epididymal Sperm Density

Epididymal sperm densities were not significantly different (Figure 32). There was a trending increase in both groups over the duration of the experiment concurrently. During week two the control group had a slightly lower sperm density of 8.2 million sperm/mg Cauda while the treatment group had a sperm density of 9.3 million sperm/mg Cauda. During week four the

control group had a slightly lower sperm density of 12.4 million sperm/mg Cauda while the treatment group had a sperm density of 12.7 million sperm/mg Cauda. During week six the control group had a slightly increased sperm density of 20.2 million sperm/mg Cauda while the treatment group had a sperm density of 19.4 million sperm/mg Cauda. During week eight the control group had an increased sperm density of 32.9 million sperm/mg Cauda while the treatment group had a sperm density of 30.3 million sperm/mg Cauda.

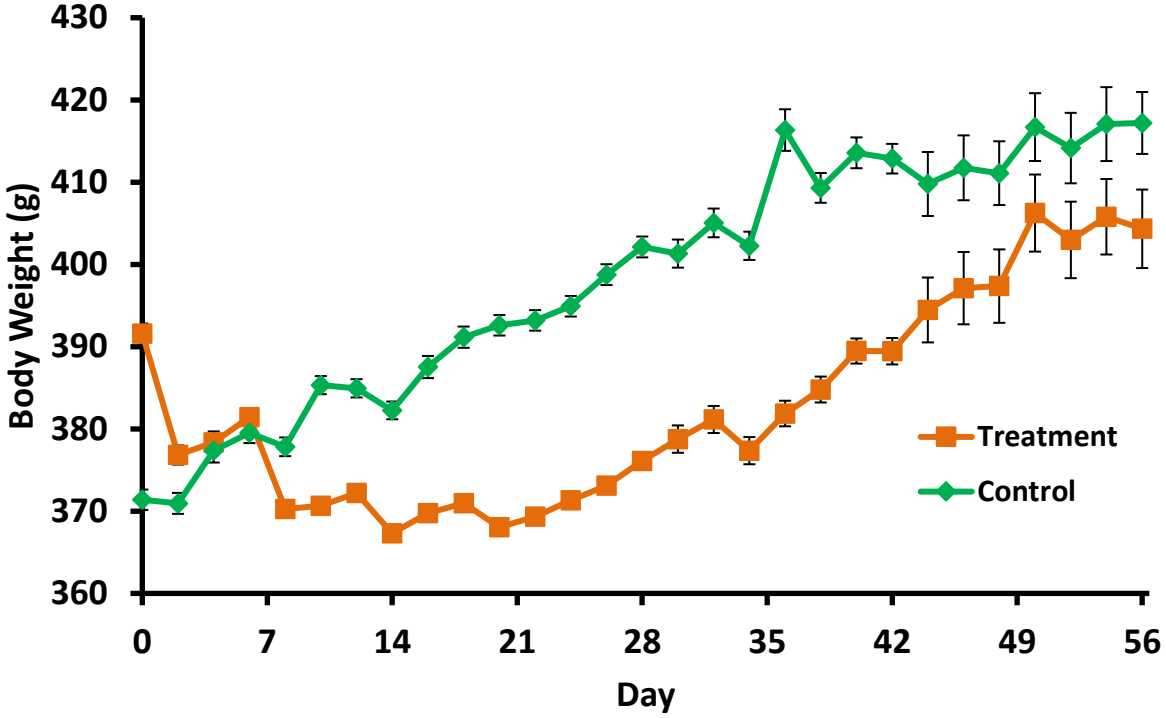

Figure 10. Body weights of Sprague-Dawley rats measured every other day after receiving rat chow with 0% LS seed inclusion (Control; green diamond) and 15% LS seed inclusion (Treatment; orange square).

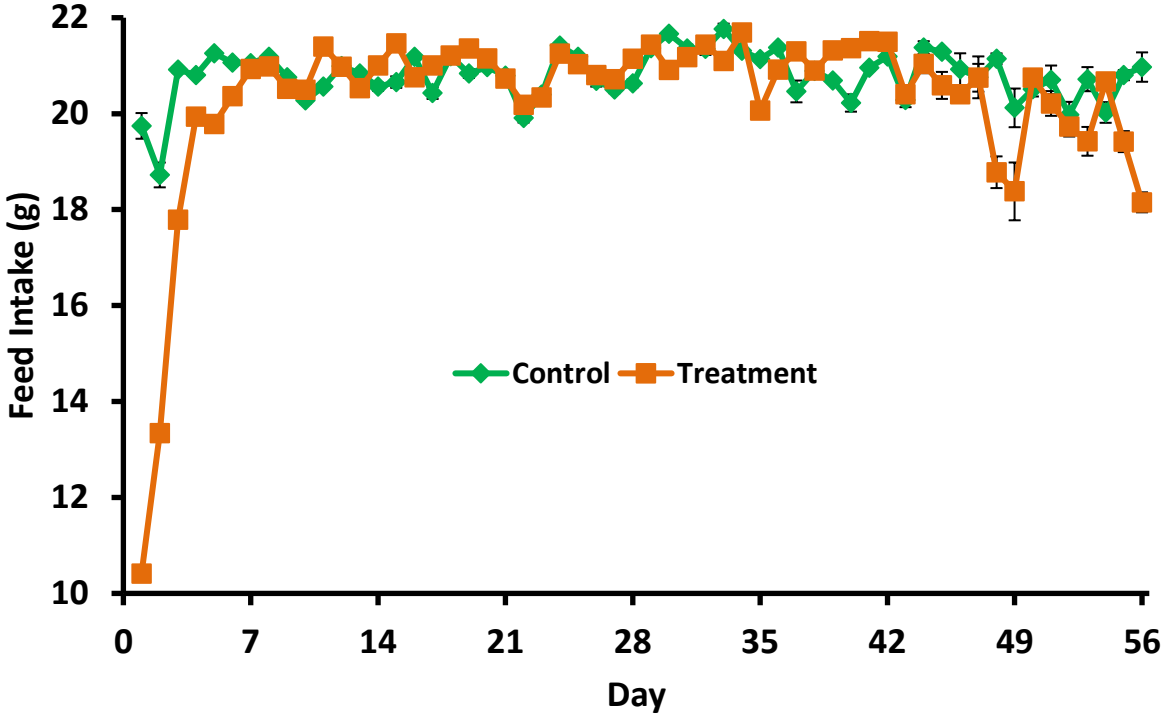

Figure 11. Feed intake of Sprague-Dawley rats measured every day after receiving rat chow with 0% LS seed inclusion (Control; green diamond) and 15% LS seed inclusion (Treatment; orange square).

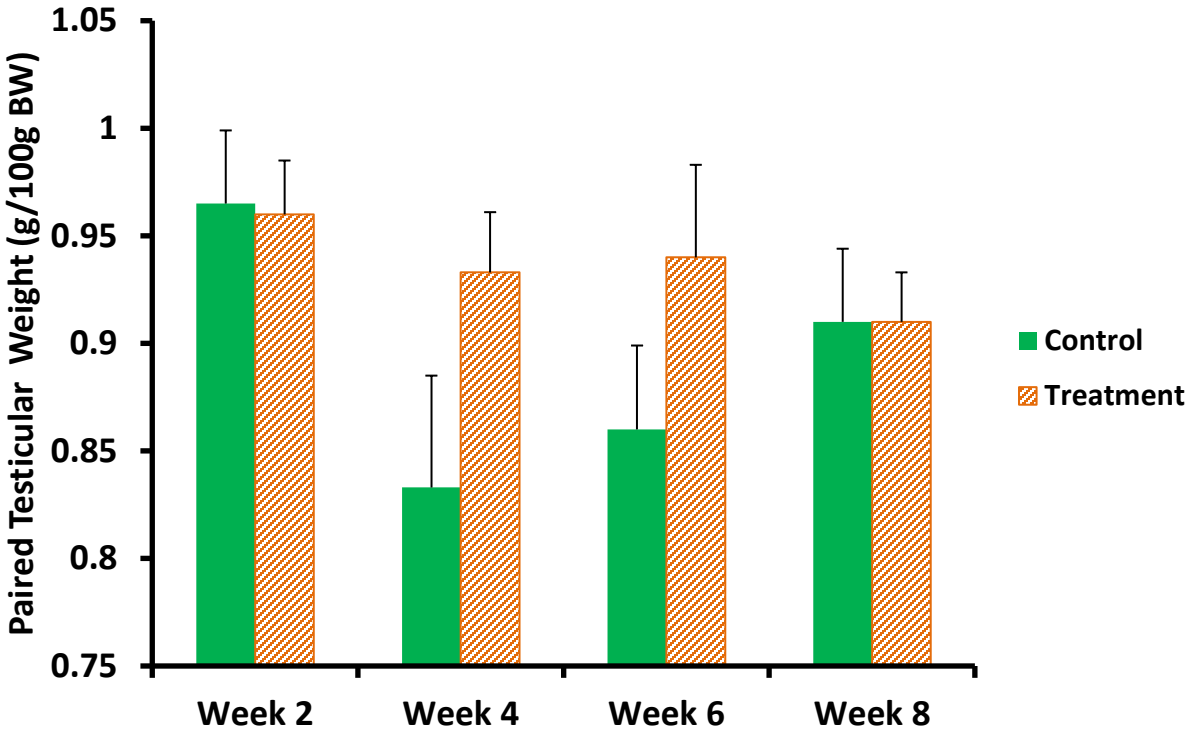

Figure 12. Paired testicular weight (g/100 g BW) measured at each biological time point after receiving rat chow with 0% LS seed inclusion (Control; green solid) and 15% LS seed inclusion (Treatment; orange diagonal stripes).

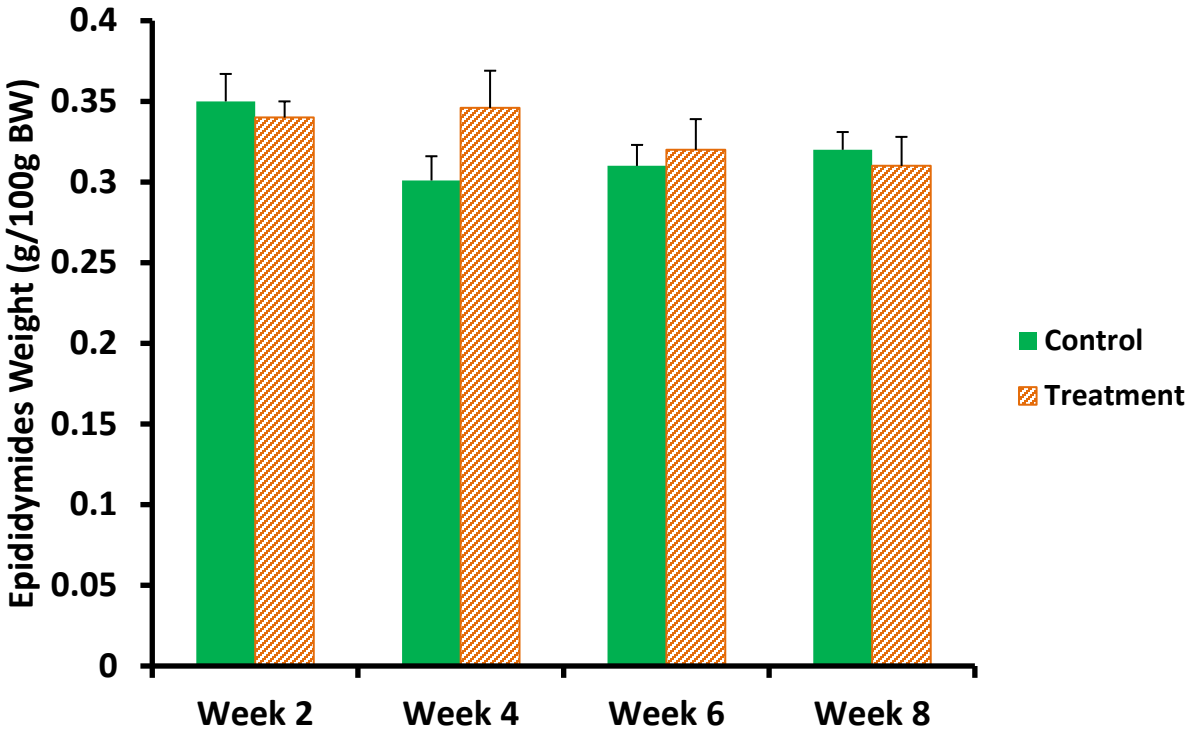

Figure 13. Paired epididymides weight (g/100 g BW) measured at each biological time point after receiving rat chow with 0% LS seed inclusion (Control; green solid) and 15% LS seed inclusion (Treatment; orange diagonal stripes).

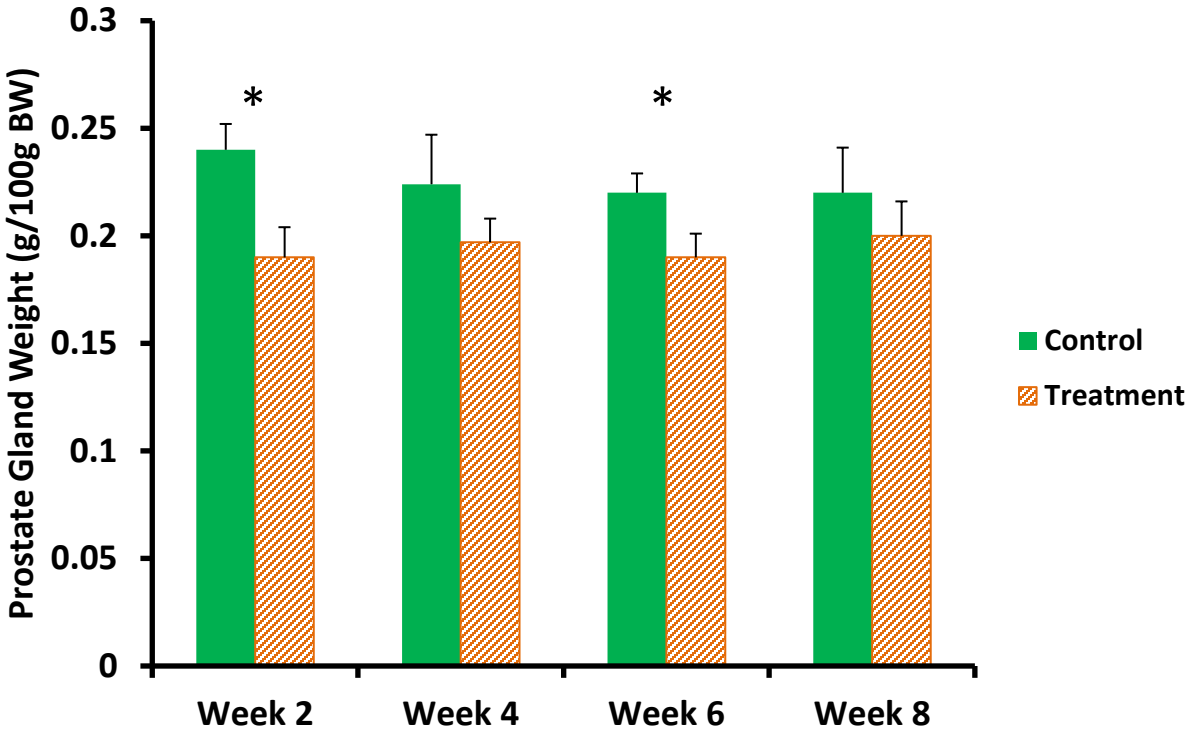

Figure 14. Prostate gland weight (g/100 g BW) measured at each biological time point after receiving rat chow with 0% LS seed inclusion (Control; green solid) and 15% LS seed inclusion (Treatment; orange diagonal stripes). * indicates significantly different at $p<0.05$ compared to the control group.

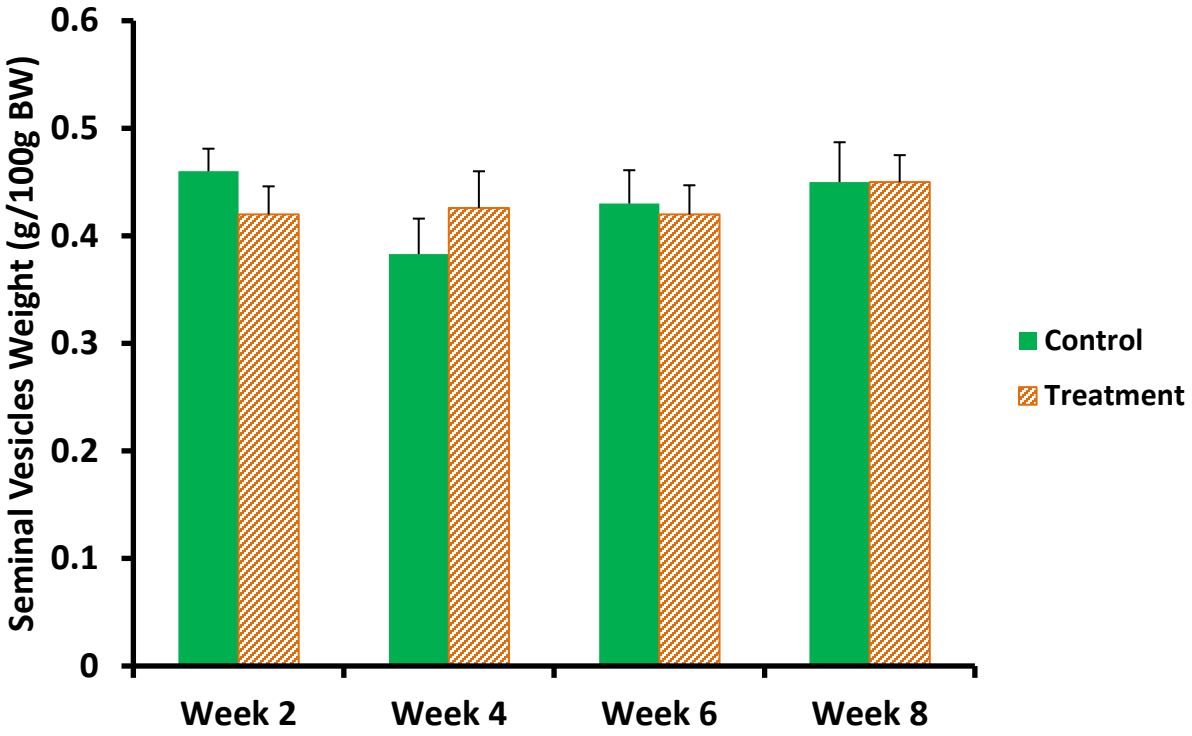

Figure 15. Seminal vesicles weight (g/100 g BW) measured at each biological time point after receiving rat chow with 0% LS seed inclusion (Control; green solid) and 15% LS seed inclusion (Treatment; orange diagonal stripes).

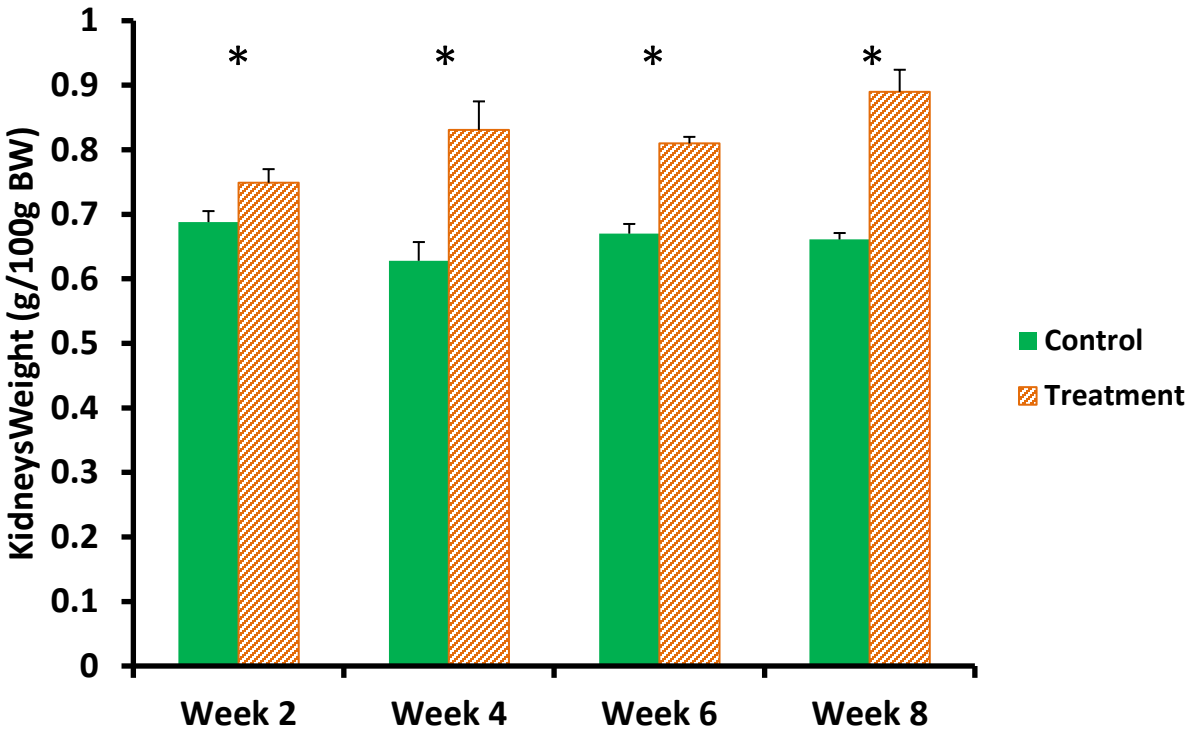

Figure 16. Kidney weights (g/100 g BW) measured at each biological time point after receiving rat chow with 0% LS seed inclusion (Control; green solid) and 15% LS seed inclusion (Treatment; orange diagonal stripes). * indicates significantly different at $p<0.05$ compared to the control group.

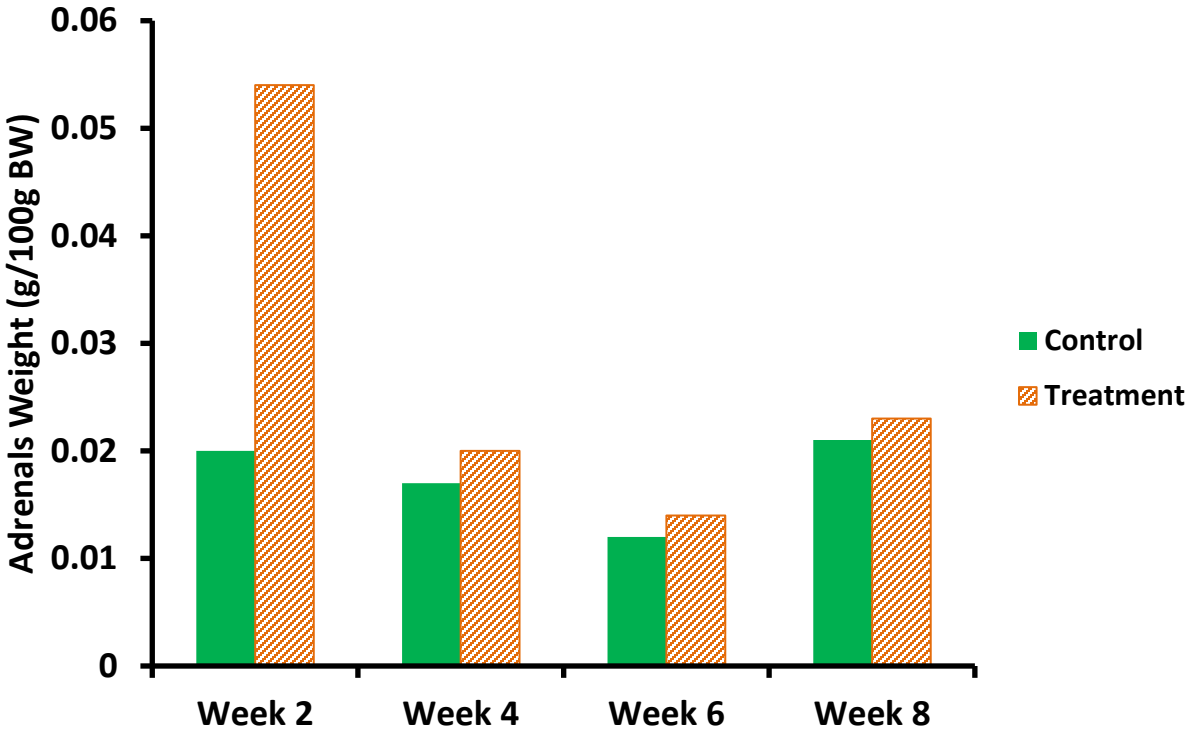

Figure 17. Adrenal gland weights (g/100 g BW) measured at each biological time point after receiving rat chow with 0% LS seed inclusion (Control; green solid) and 15% LS seed inclusion (Treatment; orange diagonal stripes).

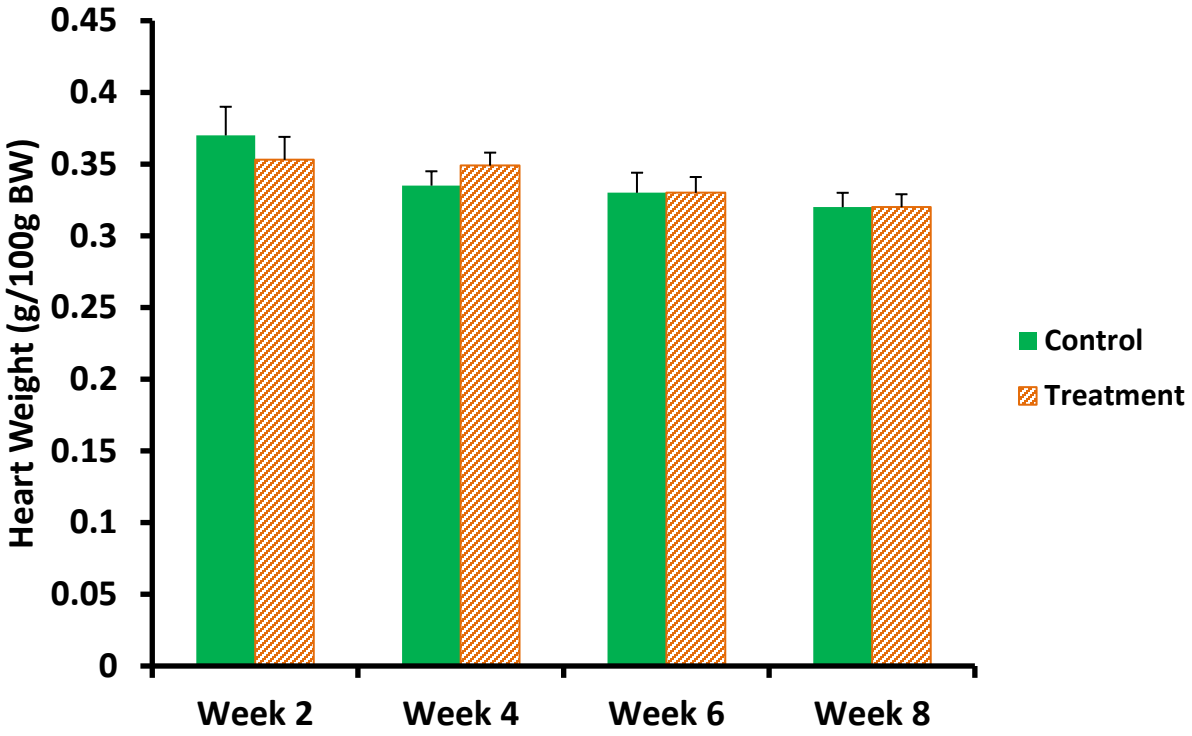

Figure 18. Heart weights (g/100 g BW) measured at each biological time point after receiving rat chow with 0% LS seed inclusion (Control; green solid) and 15% LS seed inclusion (Treatment; orange diagonal stripes).

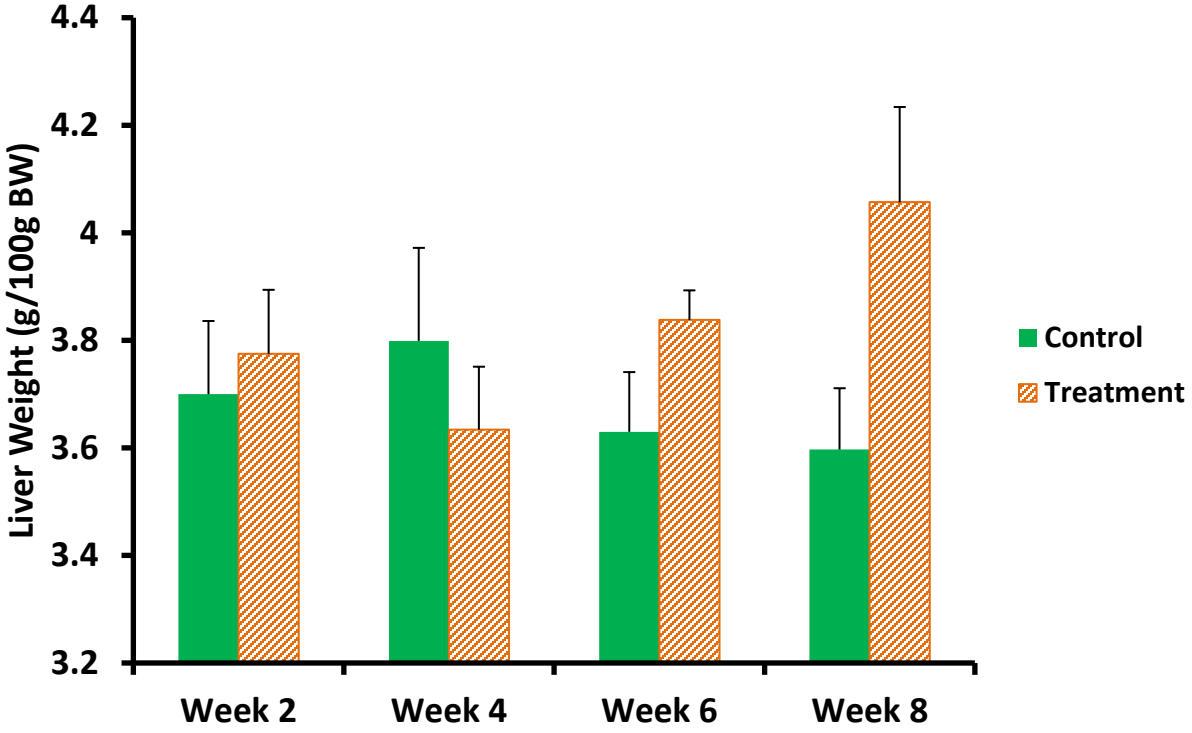

Figure 19. Liver weights (g/100 g BW) measured at each biological time point after receiving rat chow with 0% LS seed inclusion (Control; green solid) and 15% LS seed inclusion (Treatment; orange diagonal stripes).

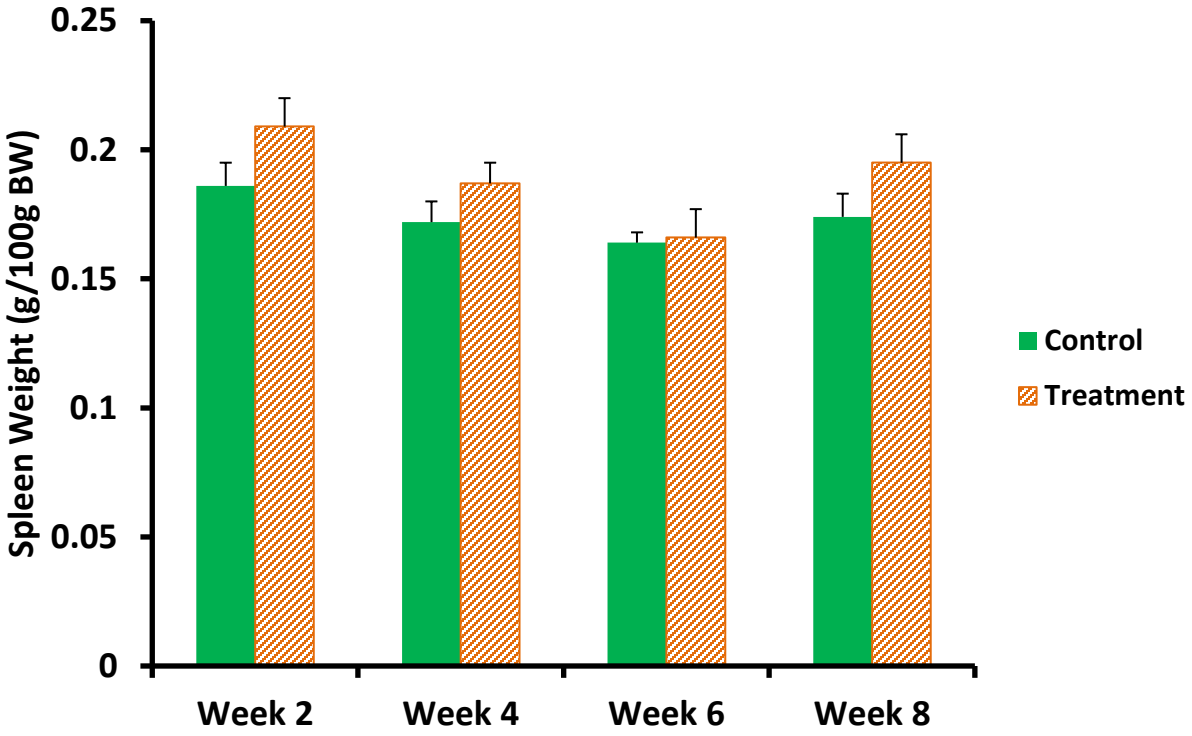

Figure 20. Spleen weights (g/100 g BW) measured at each biological time point after receiving rat chow with 0% LS seed inclusion (Control; green solid) and 15% LS seed inclusion (Treatment; orange diagonal stripes).

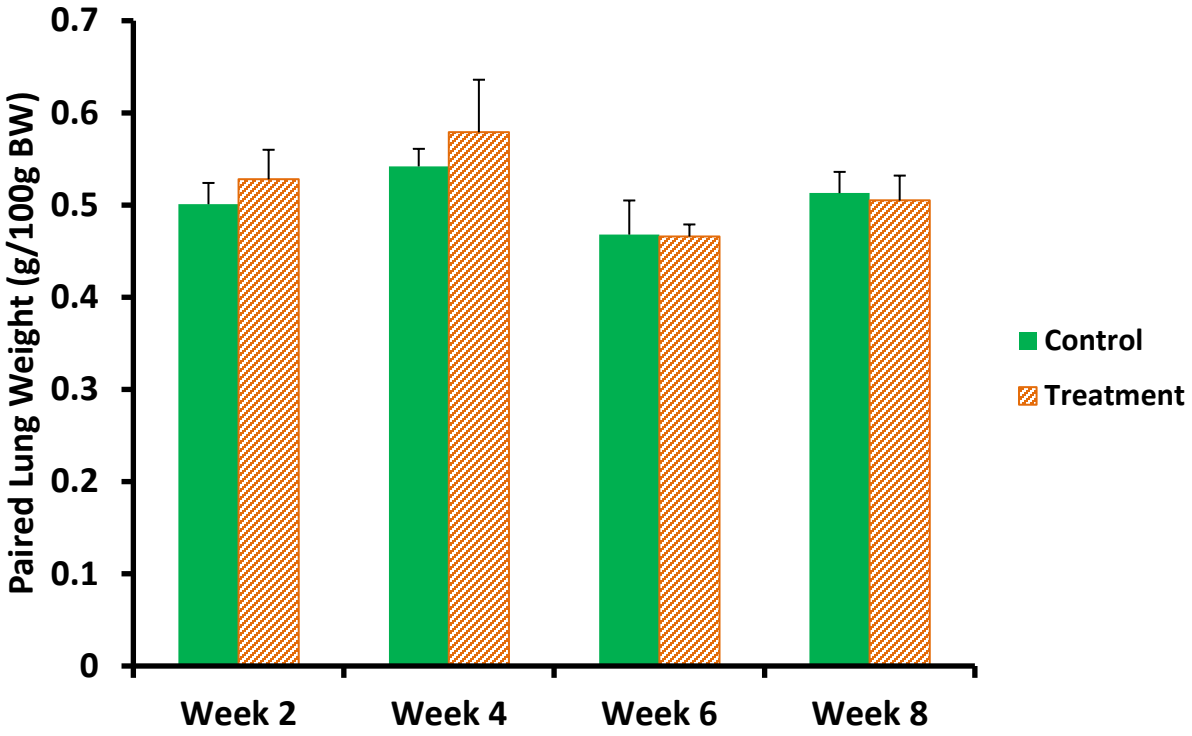

Figure 21. Paired lung weights (g/100 g BW) measured at each biological time point after receiving rat chow with 0% LS seed inclusion (Control; green solid) and 15% LS seed inclusion (Treatment; orange diagonal stripes).

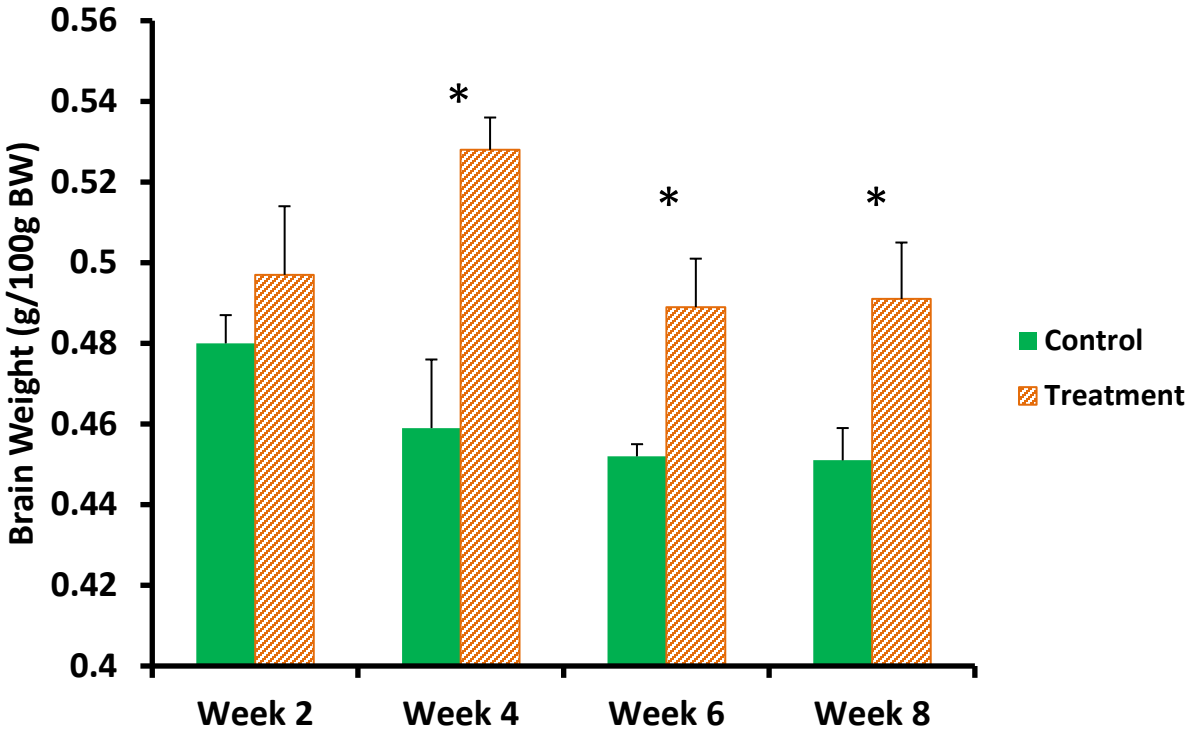

Figure 22. Brain weights (g/100 g BW) measured at each biological time point after receiving rat chow with 0% LS seed inclusion (Control; green solid) and 15% LS seed inclusion (Treatment; orange diagonal stripes). * indicates significantly different at $p<0.05$ compared to the control group.

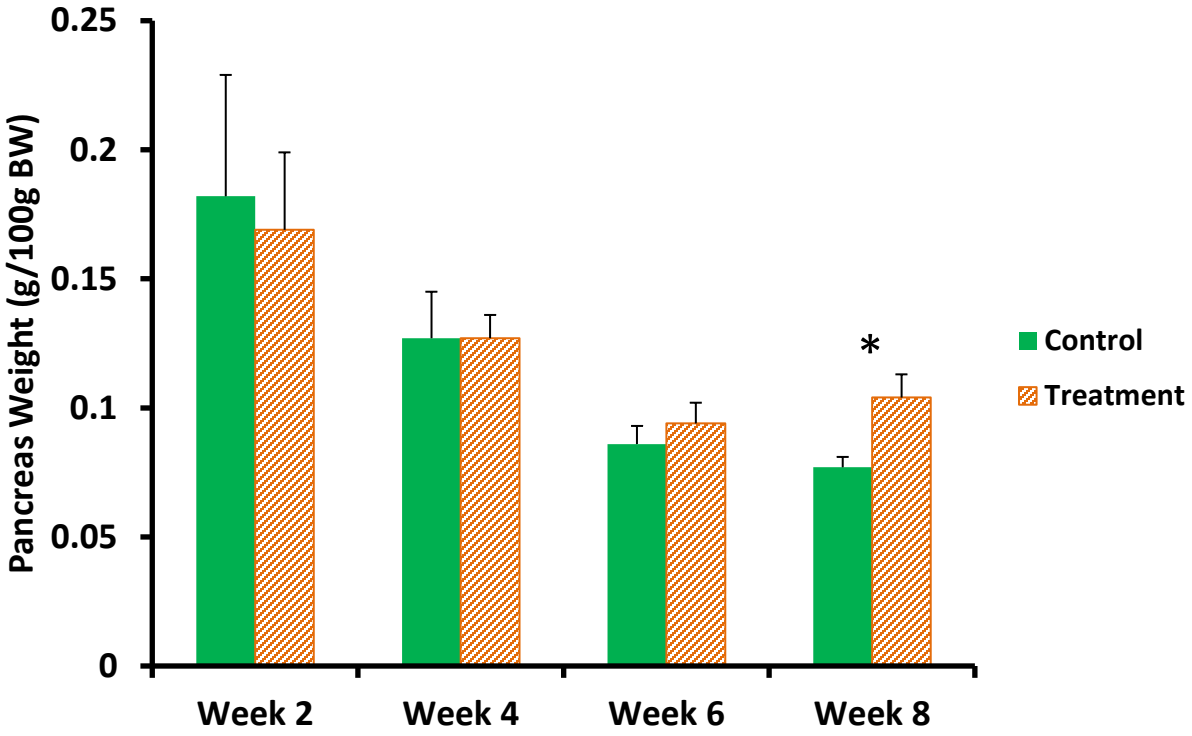

Figure 23. Pancreatic weights (g/100 g BW) measured at each biological time point after receiving rat chow with 0% LS seed inclusion (Control; green solid) and 15% LS seed inclusion (Treatment; orange diagonal stripes). * indicates significantly different at $p<0.05$ compared to the control group.

Table 2. Effect of dietary supplementation of LS seeds on relative Bowman's capsule diameter, glomerular diameter and difference in Bowman's capsule and glomerulus diameter

	Bowman's Capsule Diameter (mm)		Glomerulus Diameter (mm)		Δ Bowman's Capsule and Glomerulus (mm)	
Week	Control	Treatment	Control	Treatment	Control	Treatment
2	0.159 ± 0.0003	0.154 ± 0.0003	0.138 ± 0.0002	0.131 ± 0.0002	0.0210 ± 0.0001	0.0225 ± 0.0001
4	0.182 ± 0.0002	0.174 ± 0.0002	0.158 ± 0.0002	0.147 ± 0.0002 *	0.0238 ± 0.0001	0.0264 ± 0.0001
6	0.182 ± 0.0002	0.177 ± 0.0002	0.156 ± 0.0002	0.149 ± 0.0002	0.0260 ± 0.0001	0.0285 ± 0.0001
8	0.164 ± 0.0003	0.186 ± 0.0002 *	0.145 ± 0.0002	0.155 ± 0.0002 *	0.0194 ± 0.0001	0.0311 ± 0.0001 *

Values are mean ± S.E.M. of 48 male Sprague-Dawley rats. * indicates significantly different at $p<0.05$ compared to the control group

Table 3. Effect of dietary supplementation of LS seeds on disease presence of glomerulosclerosis, metaplasia and hyperplasia

	Glomerulosclerosis Presence (%)		Metaplasia Presence (%)		Hyperplasia Presence (%)	
Week	Control	Treatment	Control	Treatment	Control	Treatment
2	27.62 ± 0.428	49.17 ± 0.418 *	29.52 ± 0.437	53.33 ± 0.417 *	44.76 ± 0.476	61.67 ± 0.407
4	43.33 ± 0.415	69.17 ± 0.386 *	38.33 ± 0.407	86.67 ± 0.284 *	46.67 ± 0.417	85.00 ± 0.299 *
6	39.13 ± 0.426	66.67 ± 0.394 *	25.22 ± 0.379	75.83 ± 0.358 *	46.96 ± 0.436	89.17 ± 0.260 *
8	28.33 ± 0.377	75.00 ± 0.362 *	38.33 ± 0.407	82.50 ± 0.318 *	20.83 ± 0.340	81.67 ± 0.324 *

Values are mean ± S.E.M. of 48 male Sprague-Dawley rats. * indicates significantly different at $p<0.05$ compared to the control group

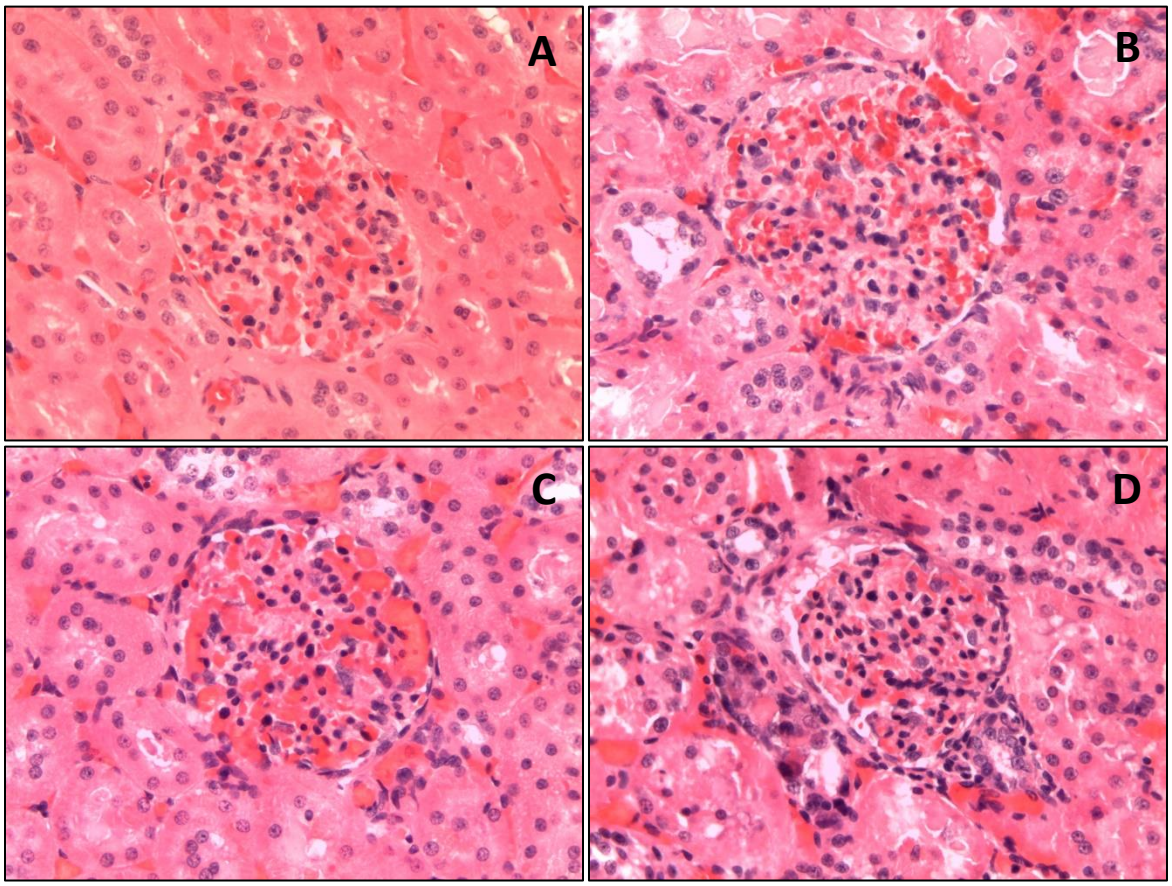

Figure 24. Renal Bowman's capsule and glomerulus from rats receiving A. 0% LS seed inclusion week 2 B. 15% LS seed inclusion week 2 C. 0% LS seed inclusion week 4 D. 15% LS seed inclusion week 4. H&E X 40.

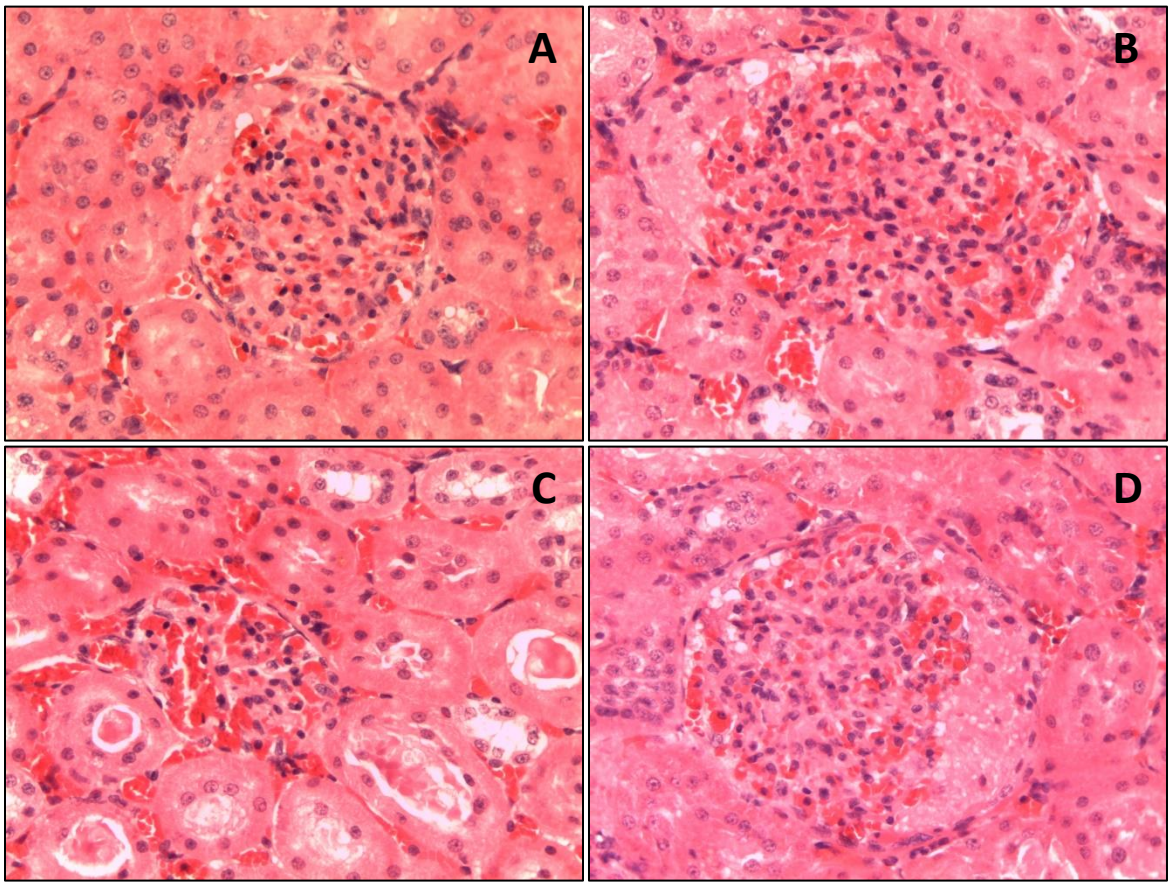

Figure 25. Renal Bowman's capsule and glomerulus from rats receiving A. 0% LS seed inclusion week 6 B. 15% LS seed inclusion week 6 C. 0% LS seed inclusion week 8 D. 15% LS seed inclusion week 8. H&E X 40.

Table 4. Effect of dietary supplementation of LS seeds on proximal tubule diameter, proximal tubule degeneration, distal tubule diameter and distal tubule degeneration

	Proximal Tubule Diameter (mm)		Proximal Tubule Degeneration Presence (%)		Distal Tubule Diameter (mm)		Distal Tubule Degeneration Presence (%)	
Week	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment
2	0.0496 ± 0.000218	0.0441 ± 0.000233	0.00 ± 0.000	35.00 ± 1.21 *	0.0458 ± 0.000193	0.0403 ± 0.000284	10.00 ± 0.760	77.50 ± 1.06 *
4	0.0561 ± 0.000249	0.0559 ± 0.000212	2.50 ± 0.395	60.00 ± 1.24 *	0.0464 ± 0.000267	0.0451 ± 0.000241	20.00 ± 1.01	70.00 ± 1.16 *
6	0.0542 ± 0.000261	0.0509 ± 0.000227	2.50 ± 0.395	47.50 ± 1.26 *	0.0476 ± 0.000287	0.0414 ± 0.000171	10.00 ± 0.760	85.00 ± 0.904 *
8	0.0482 ± 0.000305	0.0561 ± 0.000227 *	0.00 ± 0.000	62.50 ± 1.23 *	0.0396 ± 0.000211	0.0507 ± 0.000309 *	15.00 ± 0.904	87.50 ± 0.837 *

Values are mean ± S.E.M. of 48 male Sprague-Dawley rats. * indicates significantly different at $p<0.05$ compared to the control group

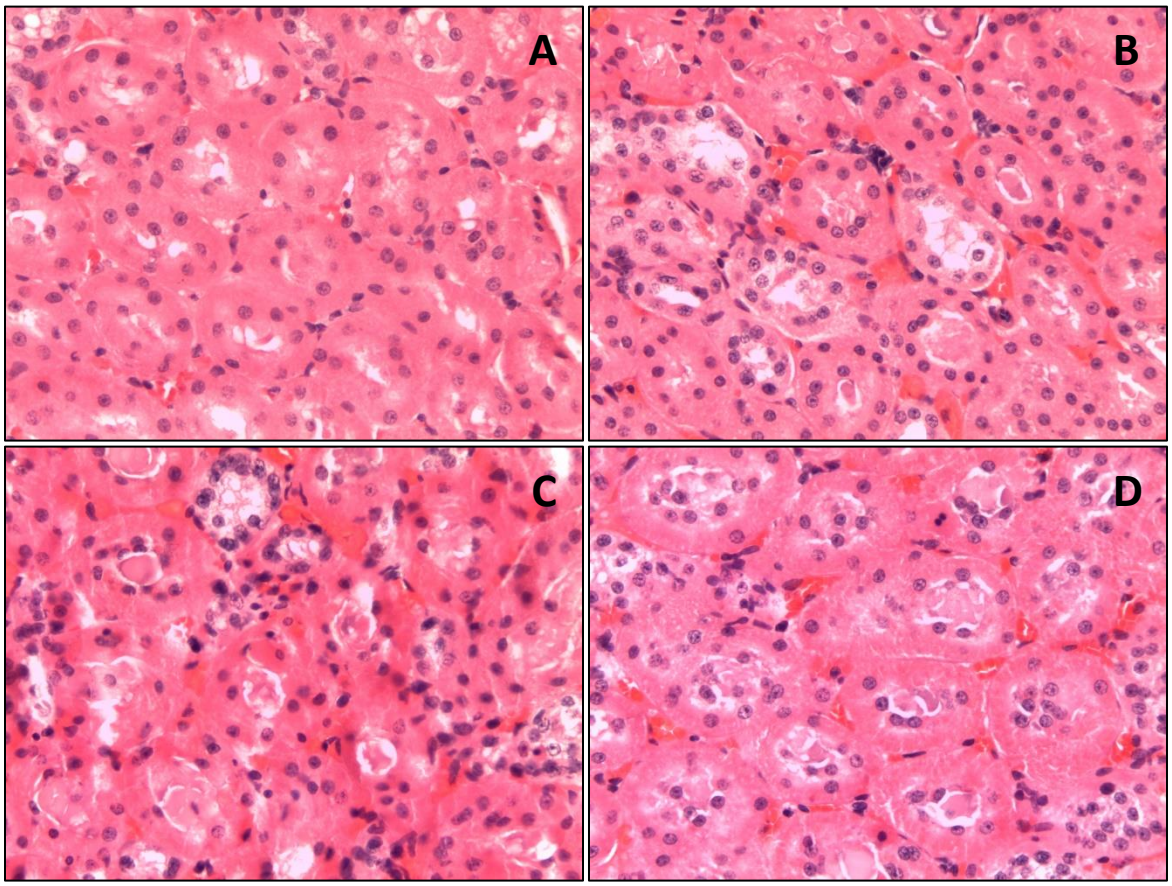

Figure 26. Renal proximal tubules rats receiving A. 0% LS seed inclusion week 2 B. 15% LS seed inclusion week 2 C. 0% LS seed inclusion week 4 D. 15% LS seed inclusion week 4. H&E X 40.

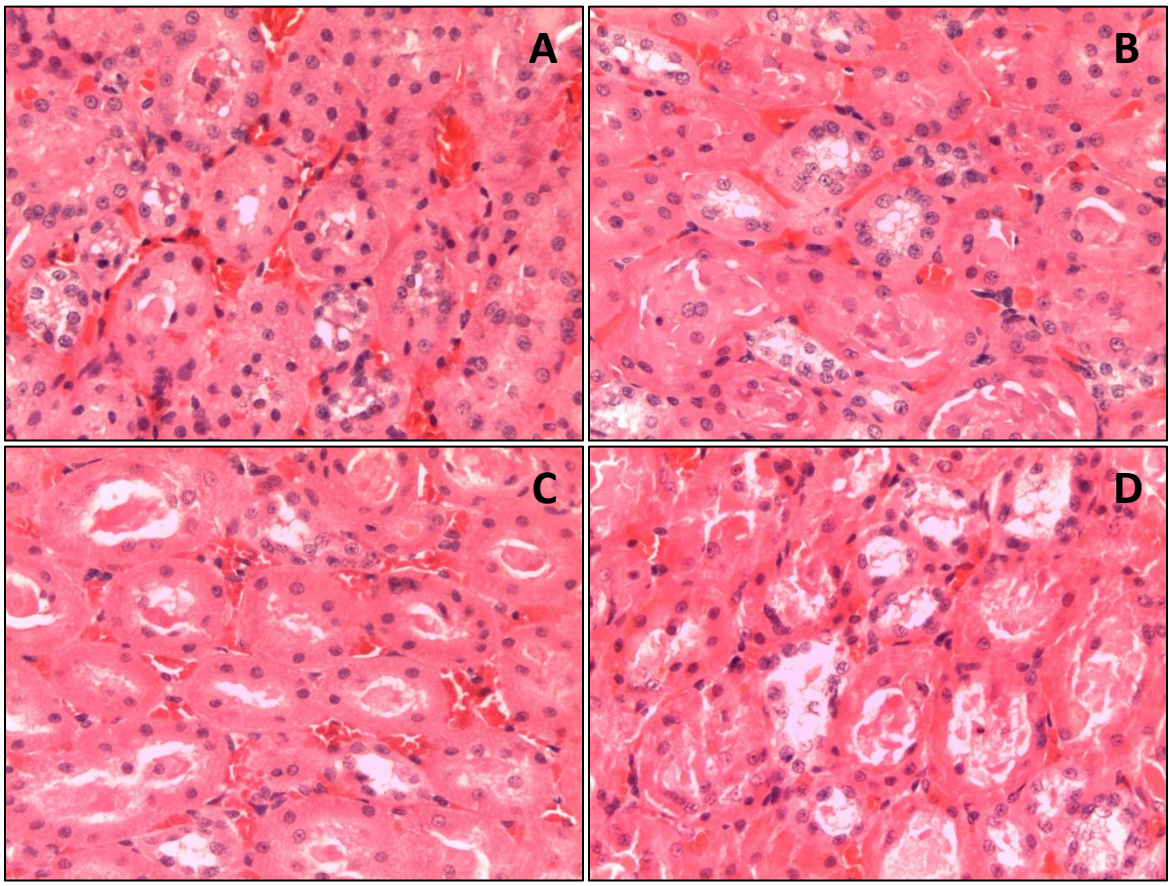

Figure 27. Renal proximal tubules rats receiving A. 0% LS seed inclusion week 6 B. 15% LS seed inclusion week 6 C. 0% LS seed inclusion week 8 D. 15% LS seed inclusion week 8. H&E X 40.

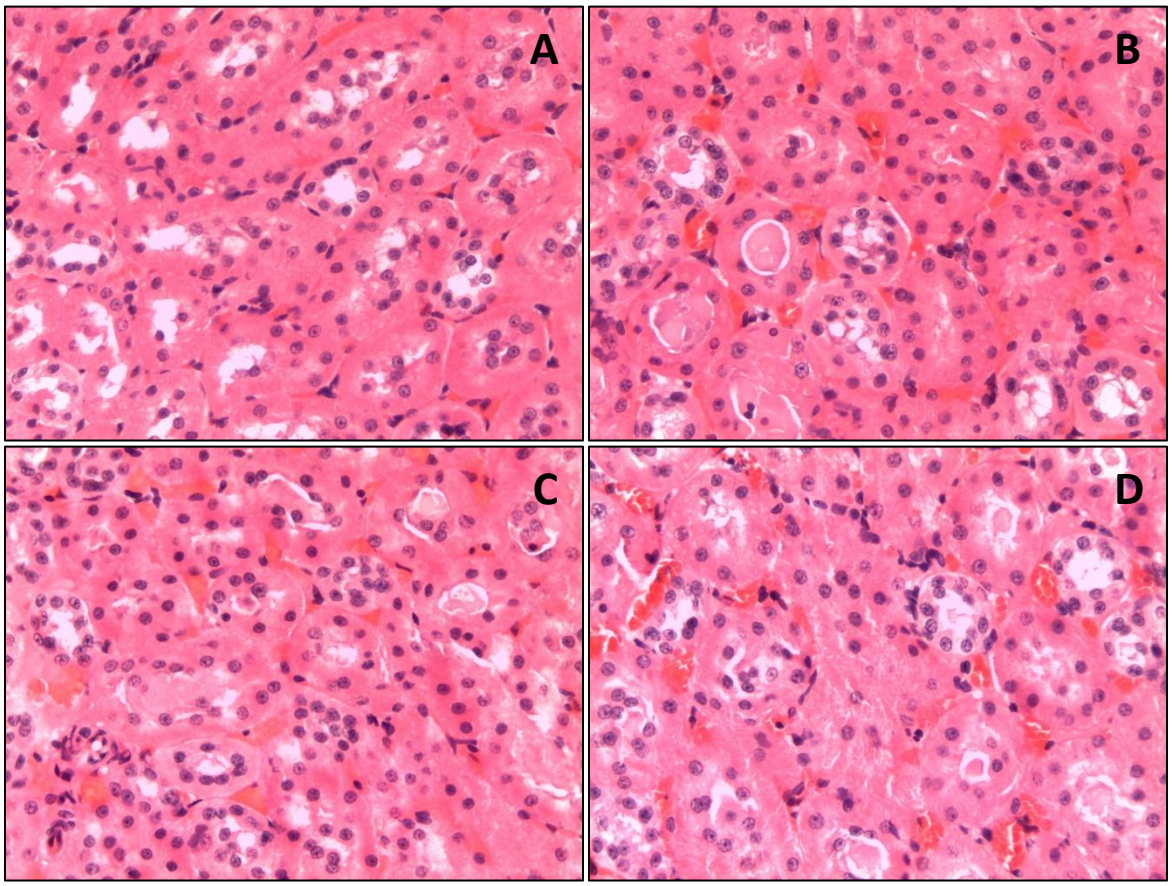

Figure 28. Renal distal tubules rats receiving A. 0% LS seed inclusion week 2 B. 15% LS seed inclusion week 2 C. 0% LS seed inclusion week 4 D. 15% LS seed inclusion week 4. H&E X 40.

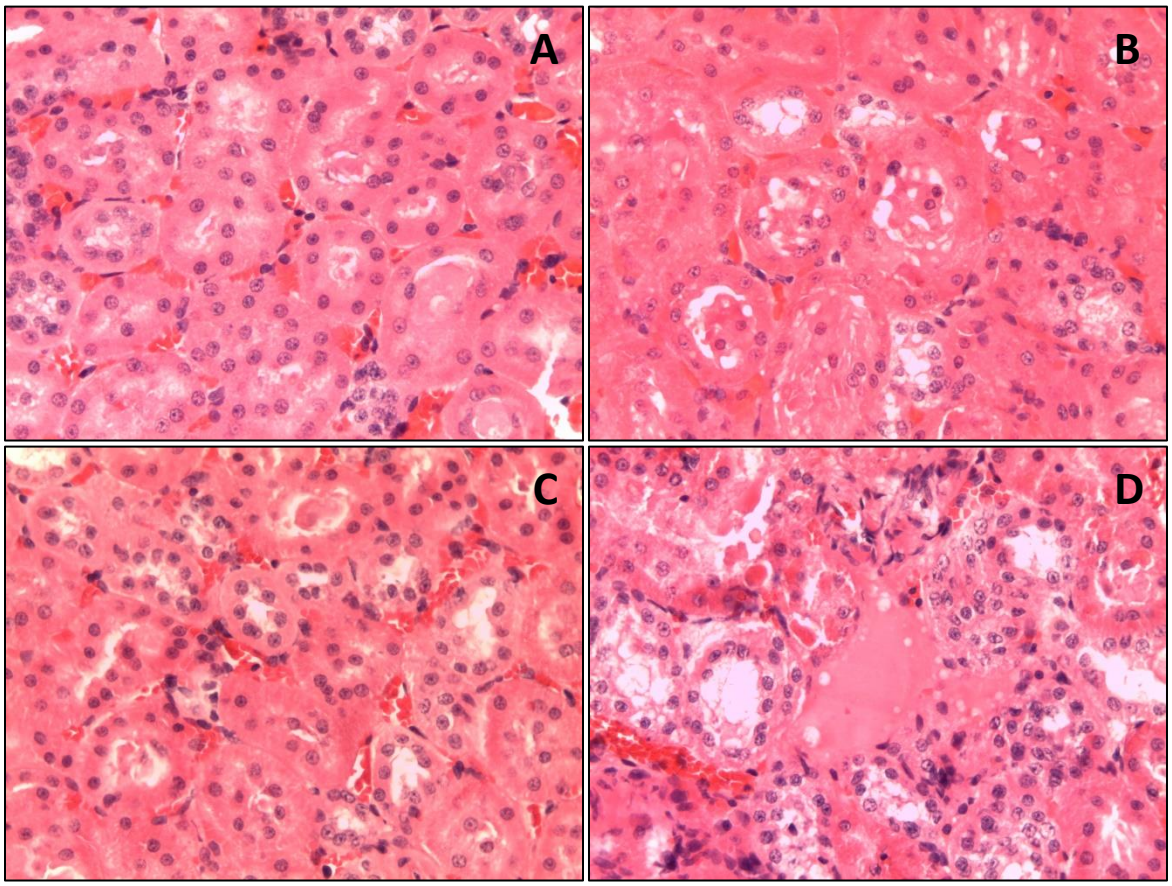

Figure 29. Renal distal tubules rats receiving A. 0% LS seed inclusion week 6 B. 15% LS seed inclusion week 6 C. 0% LS seed inclusion week 8 D. 15% LS seed inclusion week 8. H&E X 40.

Table 5. Effect of dietary supplementation of LS seeds on seminiferous tubule diameter, germinal cell layer thickness and Leydig cell diameter

	Seminiferous Tubules (mm)		Germinal Cell Layer Thickness (mm)		Leydig Cell Diameter (mm)	
Week	Control	Treatment	Control	Treatment	Control	Treatment
2	0.300 ± 0.0004	0.306 ± 0.0004	0.0573 ± 0.0001	0.0548 ± 0.0001	0.0123 ± 0.00001	0.0134 ± 0.00001 *
4	0.362 ± 0.0003	0.363 ± 0.0003	0.0638 ± 0.0001	0.0596 ± 0.0001	0.0139 ± 0.00001	0.0139 ± 0.00001
6	0.343 ± 0.0003	0.355 ± 0.0003	0.0629 ± 0.0001	0.0600 ± 0.0001	0.0126 ± 0.00001	0.0130 ± 0.00001
8	0.322 ± 0.0004	0.329 ± 0.0003	0.0562 ± 0.0001	0.0581 ± 0.0001	0.0125 ± 0.00001	0.0124 ± 0.00001

Values are mean ± S.E.M. of 48 male Sprague-Dawley rats. * indicates significantly different at $p<0.05$ compared to the control group

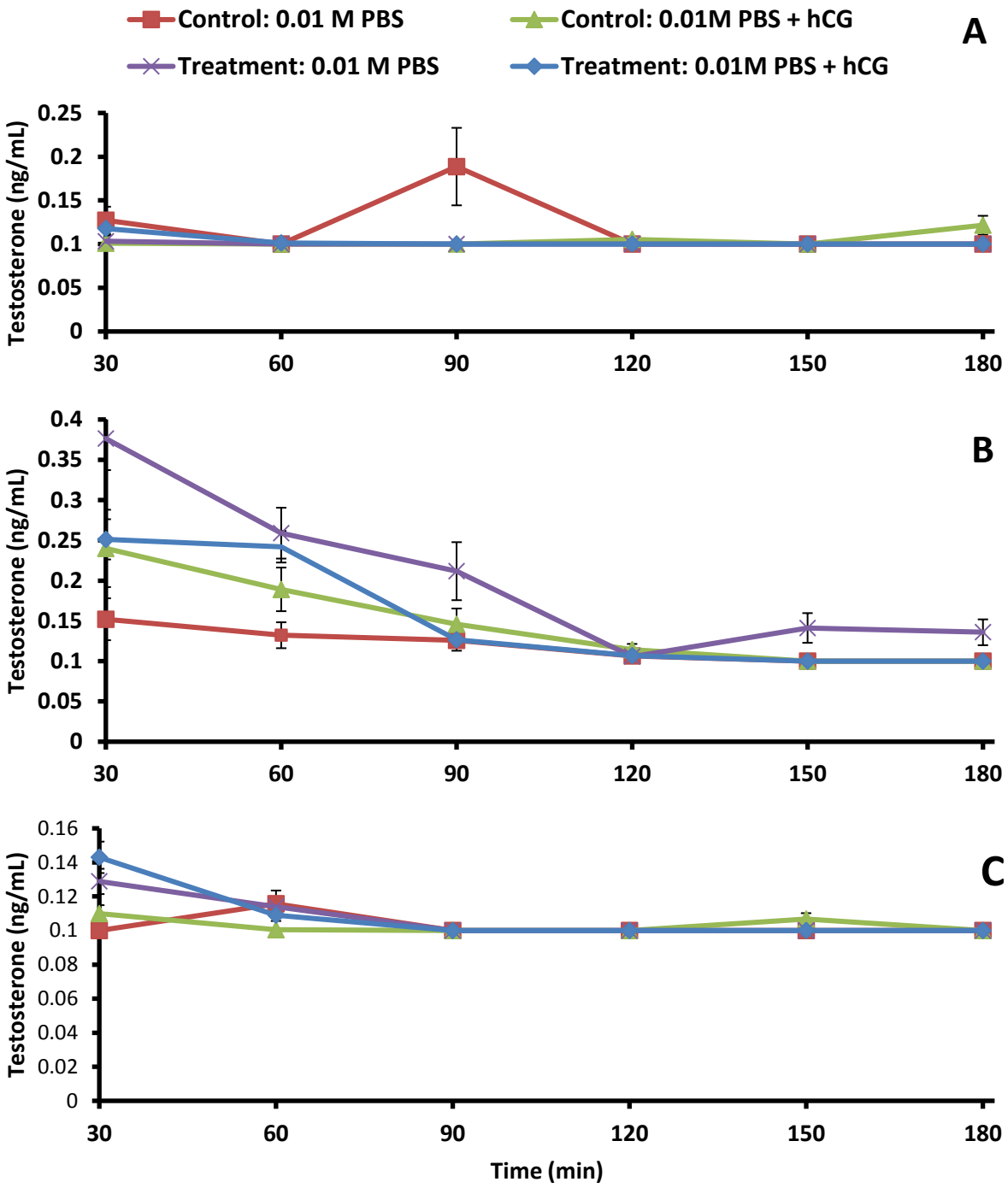

Figure 30. Perifusion of testis parenchyma from male Sprague-Dawley rats for testosterone. Control groups perifused with 0.01 M PBS (red square) and 0.01 M PBS + hCG (green triangle) while treatment groups were perifused with 0.01 M PBS (purple x) and 0.01 M PBS + hCG (blue diamond) at each biological time point: A.) Week 2, B.) Week 4 and C.) Week 8.

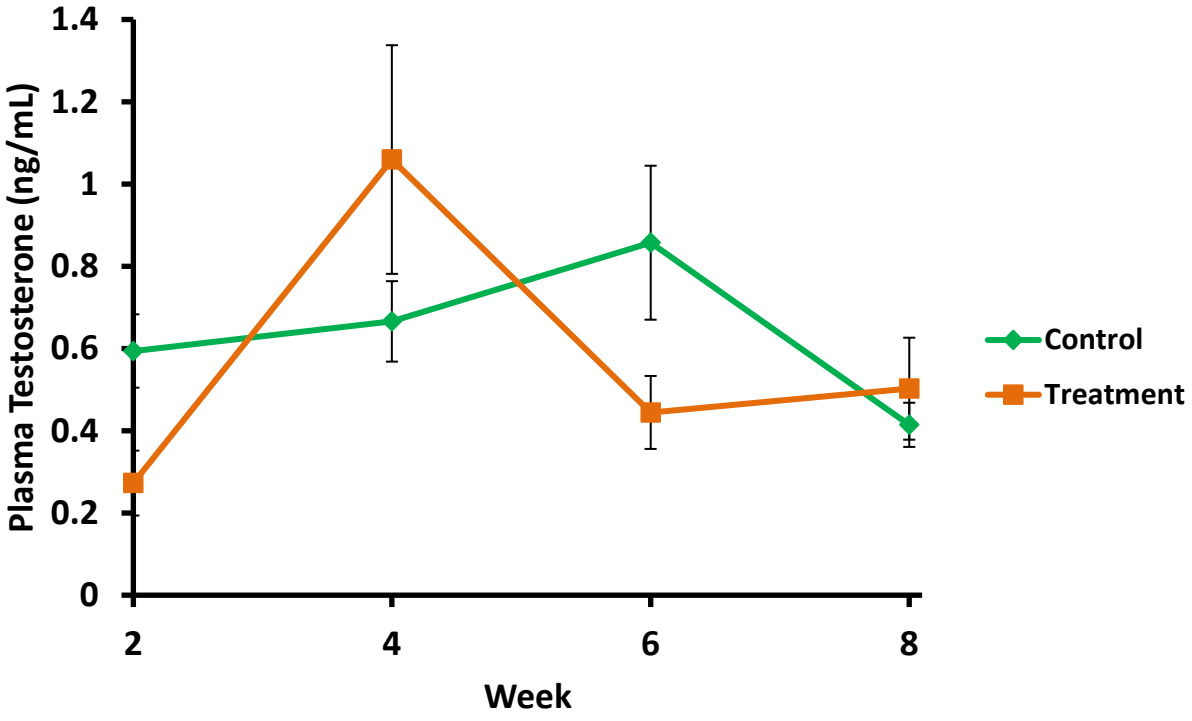

Figure 31. Plasma testosterone from male Sprague-Dawley rats measured at each biological time point after receiving rat chow at 0% LS seed inclusion (Control; green diamond) and 15% LS seed inclusion (Treatment; orange square).

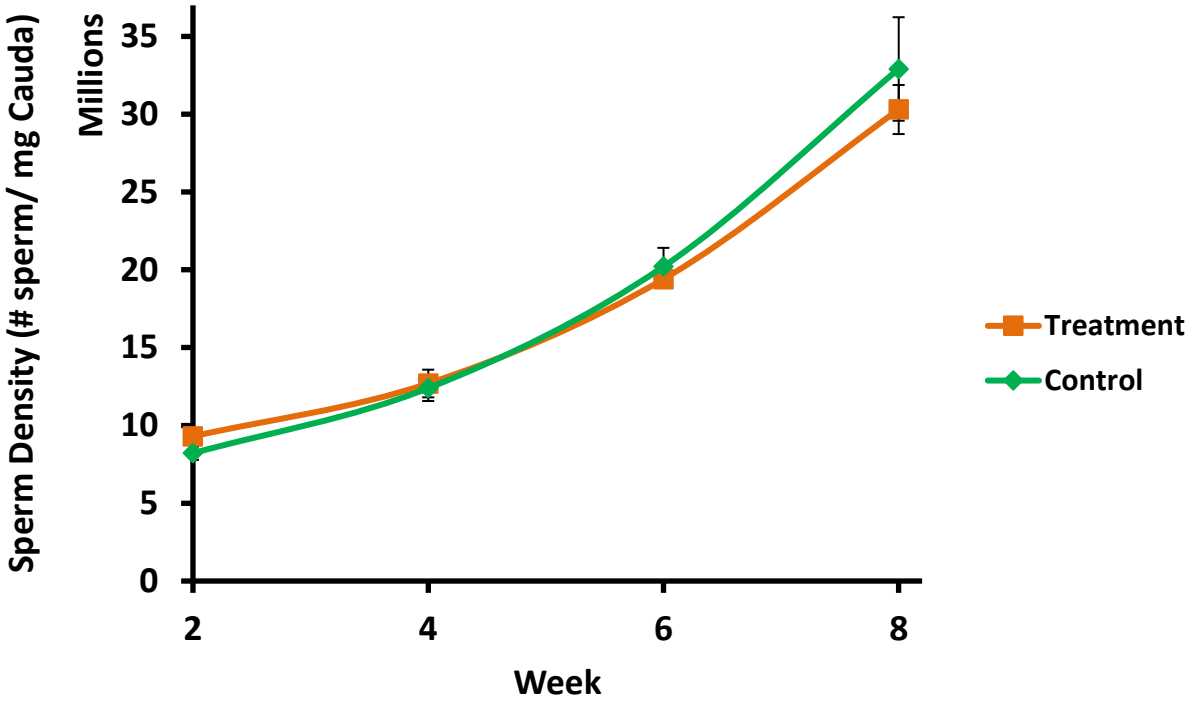

Figure 32. Sperm density from male Sprague-Dawley rats measured at each biological time point after receiving rat chow at 0% LS seed inclusion (Control; green diamond) and 15% LS seed inclusion (Treatment; orange square).

Chapter VI: Discussion

Our overall goals in these studies were first to determine the effect of LS seed consumption, both in the acute and chronic states, on the endocrinology of ovulation, secondly on the development of visceral organs and finally on the indices of spermatogenesis in the male rat model. We have demonstrated that a preovulatory surge-like GnRH secretion is inducible *in vitro* in the ovariectomized, estrogen-primed, progesterone treated rats. Our results indicated that LS supplementation tended to cause an earlier, attenuated preovulatory surge-like GnRH secretion.

Temporally, LH secretion from the pituitary gland in all treatment groups decreased over time. Perifusate LH levels were high varying from 15 to 36 ng/mL pre-GnRH administration. We observed no stimulatory effect of 10 ng/mL GnRH administration. Consequently, no surge-like LH secretion was observed. Future studies should investigate the use of a higher concentration of GnRH to stimulate the surge of LH. There was however a suppressive effect of progesterone in progesterone treated rats on average LH secretion.

Overall, FSH decreased from time 0 to about 180 minutes before stabilizing. Average FSH levels were significantly higher in ovariectomized, estrogen-primed, progesterone-treated rats compared to ovariectomized, estrogen-primed rats. LSE administration significantly increased average FSH secretion in ovariectomized, estrogen-primed rats but had no effect in the ovariectomized, estrogen-primed, progesterone-treated rats. Administration of GnRH at 10 ng/mL had no effect of FSH secretion. Future studies should investigate the use of a higher concentration of GnRH to stimulate the surge of FSH. There may be differential regulation of

FSH secretion because of the increased average FSH secretion observed in the ovariectomized, estrogen-primed, LSE treated rats.

The observed earlier and attenuated surge-like secretion of GnRH is in line with Sharief et al. (2004) who observed LS seeds to have contraceptive properties in 100% of mice treated with one g/day administration of LS seeds orally. LS seeds utilized as an aqueous extract have also been shown to act as an abortifacient with unknown mechanisms (Nath, et al., 1992). Interestingly, 200 mg/Kg of ethanolic extract of LS seeds showed 0% inhibition of ovulation in copper acetate-induced ovulation (Vohora, et al., 1973). LS seeds have been shown to contain 3.7 mg/g dry weight of lepidine with the highest content of lepidine in the plantlets at 10.4 mg/g during the vegetative state (Pande, et al., 2002). Lepidine could be a possible alkaloid that lends anti-ovulatory properties (Kumar & Sachin, 2013). To date, there have been no systematic studies completed on the effects of lepidine on reproductive function. Further research is needed to investigate if a higher concentration of LS seed aqueous extract is needed to yield anti-ovulatory effects. Interestingly, female mice that were cyclooxygenase 2 (COX-2) deficient appeared to be infertile (Dinchuk, et al., 1995). LS seeds have been shown to have anti-inflammatory properties (Raval, et al., 2009). Subsequently, LS seeds could have another pathway yielding anti-ovulatory properties by inhibiting COX-2. The anti-inflammatory mechanism for COX-1 or COX-2 inhibition specificity has not yet been elucidated for LS seeds.

Our results indicate that there was a strong feed aversion for rats being fed 15% LS seed inclusion. This yielded a sharp decline in body weight for the LS-supplemented rats. The aversion lessened after the rats became convinced the LS supplemented feed was safe as evident in previous studies (Forbes, 2007). Importantly, after this feed aversion, feed intake became

similar between control and LS included groups. Weight changes increased over time concurrently between the two groups. These results were similar in broilers fed up to forty-two days at 0, 0.75, 1.50 and 2.25% LS seed inclusion (Shawle, et al., 2016).

There was no effect of LS seed inclusion on adrenal gland, heart, liver, spleen or lungs. This is in agreement with previous results obtained in Wistar rats fed 10% LS seed inclusion (Datta et al., 2011). Similarly, LS supplementation at 2.25 % of broilers had no effect on heart and liver weights (Shawle et al., 2016). We observed however a significant increase in brain weight in rats fed 15% LS seed inclusion during weeks four, six and eight. It is unknown why the brain weight increased. We speculate this may be due to water accretion in the brain. The exact mechanism by which this might have occurred is not clear. Future research is needed to delineate the molecular mechanisms surrounding the increased cerebral weight. There also was a significant increase in pancreatic weight during week eight. We speculate this was due to pancreatitis. Eddouks and Maghrani (2008) determined LS seeds to have hypoglycemic activity with no plasma insulin changes while collecting blood samples during a four-hour period of aqueous LS extract infusion. The authors attributed the hypoglycemic effects of LS seeds to be extra-pancreatic occurring possibly by inhibiting renal glucose reabsorption. The hypoglycemic state possibly caused a decreased release of pancreatic insulin secretion and therefore an increase in pancreatic weight. Further research is needed to investigate the effects of chronic consumption of LS seeds on pancreatic function.

In our hands, chronic LS feeding had no significant effect on paired testicular, epididymides or seminal vesicle weight. However, the prostate gland showed a significant decrease during weeks two and six for rats fed 15% LS seed and a nonsignificant decrease during

weeks four and eight for the treatment group. It is of interest to note that these are testosterone dependent organs displaying opposite weight changes. It is unclear why these differential weight changes have occurred.

Histologically, there were no significant effect of LS supplementation on seminiferous tubule diameters or germinal cell layer thickness. There was however, an effect of acute but not chronic LS feeding on Leydig cell diameter. No significant Leydig cell diameter differences was observed in week four, six and eight. *In vitro* testosterone production did not differ significantly. There were also no significant changes in plasma testosterone. Therefore, our results provide no support for the aphrodisiac claims of LS seed ingestion (Mali, et al., 2007) at 15% inclusion. Future research should investigate the effect of different LS seed extracts on testosterone production and plasma levels. The increase in fertility for males may be a testosterone-independent function.

Chronic LS supplementation had neither stimulatory nor inhibitory effect on epididymal sperm density. This is different from results obtained by Naji et al. (2013a, 2013b) that investigated the effects of phenol and tocopherol extracts of LS seeds in male white rabbits. The authors determined the extracts to increase sperm concentration in the cauda epididymis and testis. The authors proposed the increased in sperm concentration was from the isothiocyanate or phenolic compounds with antioxidant activity similar to results from Gonzales et al. (2006) using *Lepidium meyenii* roots. The reason our results differed may be because Naji et al. (2013a, 2013b) utilized phenol and tocopherol extractions that would yield a more concentrated extract of isothiocyante and phenolic compounds than our 15% inclusion of ground LS seeds. Christie (2010) found that tocopherol may have a protective function towards sperm serving as an

antioxidant. The extraordinary effects of antioxidants like vitamin E have been shown to protect rabbit testes against lipid peroxidation and testosterone-induced lipid peroxidation (Aydilek, Aksakal, & Karakilçik, 2004). Vitamin supplementation in boars increased semen production and percentage of motile sperm cells but did not affect sperm morphology or libido (Audet, Laforest, Martineau, & Matte, 2004). This yields evidence that the antioxidants within LS seeds can increase fertility in higher concentrations via different extracts.

Both acute and chronic LS feeding significantly increased kidney weight in our study. Adam (1999) observed no renal abnormalities in Wistar rats fed 2% LS seed inclusion but at 50% LS seed inclusion there were significant deleterious effects on the kidneys. The deleterious effects were less marked in rats fed 10% LS seed inclusion. In addition to the renal weight increase, histological analysis showed the LS supplementation induced significant increase in glomerulosclerosis and tubular degeneration throughout the duration of the study. Similar results were observed in Wistar rats fed 50% LS seed inclusion (Adam, 1999). At 2.25% LS seed inclusion in broilers, there was no kidney weight change (Shawle, et al., 2016). Glomerulosclerosis presents with sclerosis of the glomerulus and an increase in matrix (Sugahara, Hosaka, Mineshige, Kamiie, & Shirota, 2015). The increased matrix led to significantly-expanded Bowman's space. Ingestion of LS seeds is a concern because nearly 20% of glomerular diseases are from glomerulosclerosis and glomerulosclerosis is a major cause of end-stage renal disease (Chen, et al., 2016). This therefore suggests that, in our hands, LS supplementation at 15% increases the susceptibility to glomerulosclerosis and represents a major health concern.

The weight increase can also be explained by significant presence of increased glomerular hyperplasia and metaplasia of the Bowman's capsule. There was also significant increase in proximal and distal convoluted tubule diameter with significant tubular degeneration. Similar tubular degeneration was seen in Wistar rat's that were fed 50% LS seeds and less marked in rats fed 10% LS seeds for three and six weeks and absent in rats fed 2% LS seeds (Adam, 1999).

Interestingly, one study showed that ethanolic LS seed extract administration ameliorated renal tubular dilation (Yadav & Srivastava, 2012) caused by the anticancer drug cisplatin (Molitoris, Dahl, & Geerdes, 1992). Yadav et al. (2012) hypothesized the cisplatin induced oxidative stress nephrotoxicity was decreased by the antioxidant properties of the ethanolic extract of LS seeds.

Transforming growth factor-β1 (TGF-β1) is a cytokine involved with glomerular extracellular matrix production in streptozotocin-induced diabetic rats (Schaan, et al., 2001). TGF-β1 is responsible for the generation of glomerulosclerosis in diabetic rats (Bertoluci, Schmid, Coimbra, & Lachat, 1996). In adult male Wistar rats, Eddouks et al. (2008) induced diabetes via streptozotocin injection and lowered plasma glucose levels concurrent with increased urinary glucose excretion over the course of fifteen days by administration of aqueous LS seed extract. The author's hypothesized the aqueous LS seed extract inhibits renal glucose reabsorption. The diabetic rats treated with aqueous LS seed extract had similar urinary TGF-β1 levels to diabetic control rats. Both diabetic rat TGF-β1 levels did decrease after fifteen days. Therefore, the TGF-β1 levels from the streptozotocin induced diabetic rats receiving aqueous LS

seed extract may be masking the toxic effects of LS. In our study, there was a significant presence of glomerulosclerosis and renal tubular degeneration after fourteen days.

Glomerulosclerosis and tubular atrophy are clinical manifestations of immunoglobulin A nephropathy (IgAN) (Mizerska-Wasiak, et al., 2014). N-acetylgalactosamine exposure is crucial to the pathogenesis of IgAN. Increased exposure to N-acetylgalactosamine is correlated with an increased risk factor for glomerulosclerosis (Xu, et al., 2014). N-acetylgalactosamine is located bound to the IgA1 hinge region on immunoglobulin A (Kerr, 1990). IgAN is characterized by the deposition of polymeric IgA1 within the renal mesangial cells (Donadio & Grande, 1997). Allen et al. (2001) and Hiki et al. (2001) demonstrated IgA1 molecules that had an aberrant O-glycosylation deposited within the renal mesangial cells. With this evidence LS seed ingestion could result in the aforementioned renal toxicity by a(n) unknown phytochemical(s) in LS seeds by cleaving N-acetylgalactosamine from IgA1. Future research is needed to investigate the effects of LS seeds and IgA1 molecules.

Chapter VII: Conclusion

Aqueous LS seed extract had no significant effect on GnRH, LH or FSH secretions during preovulatory hormonal surge secretion. Aqueous LS seed supplementation demonstrated a tendency to advance and attenuate a preovulatory GnRH surge. Although limited studies have suggested an anti-ovulatory effect of LS seed ingestion, further research is needed to confirm this. Further, whereas chronic LS seed supplementation lead to no deleterious effects on testes, epididymides, seminal vesicles, adrenal glands, heart, liver, spleen and lungs, gross histopathology was observed in the kidney indicating nephrotoxicity. Deleterious effects in the weight of the prostate gland, brain and pancreas were also observed by chronic consumption of LS seeds. Overall, these results indicate a cautionary approach to the consumption of LS seeds for medicinal purposes because of a possible narrow therapeutic index.

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