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Lepidium sativum Effects on Regulation of Reproduction, Hematological, and Metabolic Indices in Sprague-Dawley Rats

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Lepidium sativum Effects on Regulation of Reproduction, Hematological, and Metabolic Indices in Sprague-Dawley Rats

by

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Abstract

Lepidium sativum (LS) or Garden Cress (GC) is an erect, glabrous, annual herbaceous fast-growing edible plant, belonging to Brassicaceae family grows up to 15-45cm in height. Its easy cultivation and its tolerance to different environmental conditions gave it the ability to spread all around the world. *Lepidium sativum* (LS) seeds are consumed as part of regular diets and used in traditional medicine in many parts of the world to treat different kinds of ailments including hyperglycemia, fracture healing, diarrhea, hypertension, microbial infections, hepatoprotective activity, bronchial asthma, cancer and other disorders. To date, there has been no systematic study done to evaluate the potential effects of LS on reproduction, on GnRH pulsality or on the pulsatile or surge secretions of LH and FSH at brain level. Evidently, the reports from the studies have suggested conflicting results on the effect of LS on reproduction in female and male. LS possess anovulatory and abortifacient properties in females while aphrodisiac effect in males. Furthermore, like many herbs used in folkloric medicine, there is no information as to what constitutes pharmacologic inclusion level. The highest level of inclusion of LS seeds at 50% has shown lethal effects while 10% LS seed inclusion has shown ambiguous toxic and non-toxic effects. Therefore, the objectives of the study were to determine the effect of methanolic LS seed extract on the development and magnitude of pulsatile releases of GnRH, LH and FSH and secondly, to determine the acute and chronic effects of 15% LS seed supplementation on hematological and metabolic indices in the Sprague-Dawley rat.

Twenty mature female rats were ovariectomized and implanted with estradiol -17β (OVEX+ E_2) three weeks before euthanization using standard procedures. After three weeks of ovariectomy, rats were randomly assigned to either of 4 groups: Control (n = 5; 0mg/mL aCSF), Low group (n = 5; 10mg/mL LSE), Mid group (n = 5; 20mg/mL LSE), or High group (n = 5; 40mg/mL LSE). Rats were euthanized using an overdose of Tribromoethanol and both the hypothalamus and pituitary gland of each rat were harvested and perifused separately in artificial cerebrospinal fluid for six hours at a constant temperature of 37°C, and with 95% O_2 and 5% CO_2 aeration. Perifusates were collected at 7.5 minutes for a total of 5 hours consisting of a 120-minute baseline period followed by application of either aCSF or aCSF containing different concentrations of LSE and perifusate collection for 2 hours and finally administration of KCl to the hypothalamic explant or GnRH to the pituitary explant and a final perifusate collection for 60 minutes. LH and FSH concentrations were determined utilizing radioimmunoassay. LSE administration potently stimulated GnRH and gonadotropins secretions. There was no effect of LSE administration on the pulse frequency of GnRH, LH and FSH but LSE significantly increased GnRH pulse amplitude only. The GnRH, LH and FSH hormone secretions were pulsatile both in control and treated groups throughout the sampling period.

Forty-eight male,7 to10 week old Sprague-Dawley rats were utilized for experiment 2 to determine the effect of LSSP on hematological and metabolic indices. Rats were acclimated to housing and feeding conditions for two weeks and then randomly sorted into either of two groups namely Control (0% LS seed inclusion; n = 24) or Treated (15% LS seed inclusion; n = 24) and fed throughout the 8-week duration of this study. Body weights of animals were recorded every other day. Every 2 weeks, 6 rats from each group were sacrificed and trunk blood

samples were collected for hematological and metabolic indices determination. Ingestion of LSSP at 15% had no effect on red blood cells (RBC) count, hematocrit (HCT), hemoglobin concentration (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). No significant effect of LSSP ingestion was observed on plasma glucose concentration or BUN was observed. However, plasma creatinine concentration decreased significantly ($P < 0.0001$) overtime. LSSP ingestion for 8 but not for 6 weeks significantly ($p = 0.045$) reduced total protein and albumin levels but both short- and long-term LSSP ingestion reduced plasma globulin levels. There was no effect of LS supplementation on alanine transaminase (ALT) and aspartate transaminase (AST), although there was an insignificant increase in alkaline phosphatase (ALP) level in LSE-fed rats in the short-term. Overall, irrespective of treatment, ALP levels were significantly higher ($P = 0.005$) after 8 weeks of either normal chow or LSSP feeding.

Overall, results of our studies indicate the stimulatory effect of LSE administration on GnRH and gonadotropins secretion. There was no effect of LSE administration on the pulse frequency of GnRH, LH, and FSH but LSE significantly increased GnRH pulse amplitude only. Ingestion of LS seeds at a 15% level in the diet had no deleterious effects on hematological and metabolic indices in the rat.

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Chapter I: Introduction

Reproduction is a complex series of developmental and neuroendocrine events that is essential for the continuation of species. The hypothalamic-pituitary-gonadal axis (HPG) plays vital role in reproduction. Within this axis, neurons in the hypothalamus synthesize and secrete Gonadotropin Releasing Hormone (GnRH) which controls the secretion of the gonadotropins, Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) from the anterior pituitary gland. These in turn act tropically on the gonads to control gametogenesis and steroidogenesis. Circulating levels of steroidal hormones namely testosterone in males and estrogen and progesterone in females have inhibitory and stimulatory effect on hypothalamo-pituitary gonadal axis through feedback loops in both sexes (Tena-Sempere, 2006). LH acts primarily on Sertoli cells in seminiferous tubules and corpus luteal cells in ovaries to stimulate the secretion of steroid sex hormones. FSH in turn is required to initiate and maintain gametogenesis. The HPG axis is regulated by Kisspeptin-GPR54 signaling pathway leading to depolarization of GnRH containing neurons that are normally found in the medial pre-optic area, with axonal projections to the median eminence, where GnRH is released into hypophyseal circulation (Messager et al., 2005). Both hypothalamic and adenohypophyseal hormone secretion occurs in pulsatile manner and is an essential part of reproduction. In both sexes, this pulsatile secretion is crucial for gametogenesis. In the female specifically, a unique form of hormonal secretion the surge secretion‹ causes ovulation and luteinization. The dynamics of pulsality depends on the stage of the estrous or menstrual cycle in non-primates and primates, respectively. Overall, every pulse of GnRH produces a corresponding pulse of LH and FSH (Tsutsumi & Webster, 2009).

Understanding the physiology of reproduction and the integrated signals that influence it can be important for serving in medicinal purposes in treating reproductive insufficiencies. Medicinal plants represent an almost unexplored reservoir of new substances with potentially useful properties. Compounds from higher plants are of great potential value as medicines, as well as starting points for synthetic analogues and industrial raw materials (Akerele, 1992).

Eighty percent of population in many less developed or developing countries use alternative or complementary medicine to treat illnesses, including reproductive disorders. Because folkloric uses are supported by a long history of human experiences, numerous biologically active plants have been discovered by evaluation of ethnopharmacological data, and these plants may offer local populations immediately accessible therapeutic products. One such plant that is attracting increasing use as food constituents and traditional medications and one that is the major topic of this thesis is *Lepidium sativum* (LS) or Garden Cress (GC).

Garden Cress is an erect, glabrous annual herbaceous, fast growing edible plant belonging to Brassicaceae family. LS seeds have strong antimicrobial activity (Sharma, Vyas, & Manda, 2012), hypoglycemic effect (Eddouks, Maghrani, Zeggwagh, & Michel, 2005), effective in decreasing blood pressure (Maghrani, Zeggwagh, Haloui, & Eddouks, 2005), hepatoprotective effect (Abuelgasim, Nuha, & Mohammed, 2008), and diuretic effect (Patel, Kulkarni, Undale & Bhosale, 2009). Further, the seed have a marked effect on fracture healing (Juma, 2007) and are shown to reduce the symptoms of asthma and improve lung function in asthmatics (Paranjape & Mehta, 2006).

The LS seeds are aperients, tonic, demulcent, aphrodisiac, rubefacient, carminative, galactagogue and emmenagogue (Nadkarni & Nadkarmi, 1954). It is supplemented in the diet of

lactating women to increase the milk secretion during post-natal period (Sahsrabudde & De, 1943) and recommended for diarrhea and dysentery (Kirtikar & Basu, 1933). The roots are bitter, acrid and are useful in treatment of secondary syphilis and tenesmus and used as a condiment (Uphof,1959). With respect to reproduction, LS has also been claimed to have aphrodisiac and sperm concentration increasing effects in males while in females it has anovulatory and abortifacient effects, by mechanisms that are not clearly known. To date, there has been no systematic study done to evaluate the effect of LSE on GnRH pulsality or on the pulsatile or surge secretions of LH and FSH.

Like many herbs used in folkloric medicine, there is no agreement on the ideal level of concentration that is appropriate for physiologic effect or on the safety and efficacy of LS seed usage. Furthermore, there is lack of information on the effect of acute and chronic consumption of LS seeds on hematological parameters and metabolic indices. Although most studies have incorporated LS seeds up to a maximum of 10% supplementation in feeds (Datta, Diwakar, Viswanatha, Murthy, & Naidu, 2011), there is no information as to what constitutes a pharmacologic inclusion level. In addition, the potential deleterious effects of such level of supplementation on hematological profile including liver toxicity is unknown.

Therefore, the purpose of this study was to determine the effect of 15% LS seed supplementation of normal rat chow on hematological and metabolic indices. Specifically, our objectives were to determine the effect of Methanolic Extract of LS seed on the development and magnitude of the pulsatile secretion of GnRH from hypothalamus, LH and FSH from Adenohypophysis that lead to the process of folliculogenesis and steroidogenesis in Sprague-Dawley rats.

Chapter II: Literature Review

Ethnobotany: A Major Source of Food and Medicine

Ethnobotany is the study of the interactive relationships between nonindustrial societies and their floral environment. The ethnobotanical research principally encompasses the utilization of plants in non-western societies (Martin, 1995). Humanity is highly dependent on other organisms such as plants, animal and microbial flora for the continuation of its survival. Plants will remain not only the major source of its food supply but also continue to be valuable source of medicine as they are the producers of unique and diverse chemical compounds (Schultes, 1995). Recognition and development of the medicinal and economic benefits of medicinal plants are on the increase in both developing and industrialized nations (WHO, 1998) but particularly true in developing countries, where herbal medicine in contrast to synthetic drugs has a long and uninterrupted history of use due to the high cost of Western pharmaceuticals and healthcare. In addition, herbal medicines are more acceptable in these countries from their cultural and spiritual points of view (Cunningham,1988).

The plant kingdom represents an extraordinary reservoir of novel molecules. There are estimated 250,000-500,000 plant species around the globe and only a small percentage has been investigated phytochemically. Plant metabolites can also be used to establish taxonomic relationships among plants and may help for a better understanding of certain biological processes. WHO encourages all countries to preserve and use the safe and positive elements of traditional medicine in their national health systems (Akerele,1992). The rapid disappearance of tropical forests and other important areas of vegetation has meant that it is essential to have access to methods which lead to the rapid isolation and identification of bioactive natural

products. The application of plants for medicinal purpose has been known to man since time immemorial. In Africa, as in many parts of the world, medicinal plants still play a pivotal role in treating various ailments. It is estimated that the tropical African flora comprises over 30,000 plant species grouped into about 2500 genera and distinguished by a high degree of endemism. The flora of Africa offers a largely untapped source of potentially useful products and numerous species that are yet to be documented and investigated (Hostettmann,1995).

One plant that is attracting increasing use as food constituents and traditional medications in many parts of Africa and Asia is *Lepidium sativum* (LS) or Garden Cress (GC). The utility of *Lepidium sativum* as a medicinal plant has increased many fold over a period in treating various human systemic disorders but to date, there has been no systematic study done to evaluate the potential effects and mechanistic basis of LS on reproduction. Evidently, the reports existing studies have suggested conflicting results on the effect of LS on reproduction in female and male. LS possesses antiovulatory properties in females (Satyavati, 1984) while it has aphrodisiac effect in males (Nadkarni & Nadkarni, 1954). Furthermore, no study has been done on the effect of *Lepidium sativum* on Gonadotropin releasing hormone (GnRH) pulsality or on the pulsatile or surge secretions of LH and FSH at brain level. Hence the purpose of this study was, therefore to investigate one of traditional medicines, *Lepidium sativum* on the reproductive potentials in female Sprague-Dawley rats.

Distribution, Morphology and Phytochemical Constituents of *Lepidium sativum* (LS)

Lepidium sativum (LS) is an erect, glabrous, annual herbaceous plant, belonging to Brassicaceae family. LS is a polymorphous species and originally cultivated in Ethiopia and Eritrea, Egypt, West Asia and now it is cultivated in the entire world. LS is a fast-growing edible

plant, grows up to 15-45cm in height (Archana, Paramjape, & Mehta, 2006; Datta et al., 2011). It has small white flowers in long racemes. The pods are obovate or broad, eliptic, roduntate, emarginated, notched at apex and winged. It can be grown at all elevations, throughout the year, but the best crop is obtained in the winter season. The plant thrives on any good light soil,but does best on moist loam. LS seed mucilage is used as a substitute for gum Arabic and tragacanthin (Patel, Chauhan, & Patel, 1987). The leaves of the plant are consumed raw in salads, cooked with other vegetables and used to garnish food. Leaves are diuretic and gently stimulant (Archana et al., 2006; Datta et al., 2011; Maghrani et al., 2005). Seeds, leaves and roots of LS are of economic importance. LS is commonly known as garden pepper grass, pepper cress, pepperwort or poor man's pepper (Nadkarni,1976).

LS seeds are brownish red in color and oval-shaped, pointed and triangular at one end smooth, about 3-4mm long,1-2 mm wide and are rich source of proteins (25%), lipids (14-24%), carbohydrates (33-54%) and 8% of crude fiber (Arkroyed, Gopalan, & Balasubramanian, 1966; Mathews, Singhal, & Kulkarni,1993). LS seed contains 20-25% yellowish semidrying oil containing the major fatty acid as alpha linolenic acid (32-34.0 %) (Diwakar, Dutta, Delur, & Naidu, 2010). They also contain natural antioxidants tocopherols and carotenoids (Bhasin, Bansal, Yadav, & Punia, 2011; Yadav, Avijeet, Srivastava, & Anurekha2011; Zia-Ul-Haq et al., 2012) which protect the oil from rancidity (Diwakara et al., 2010). Seven imidazole alkaloids, sinapic acid and sinapin were reported in LS seed (Maier et al., 1998). Sharma et al. (2012) and Yadav et al. (2011) reported the presence of phenolic compounds, alkaloids, cardiac glycosides, anthraquinones glycosides, tannins, steroids, flavonoids in LS seeds. The mineral content of LS seeds includes potassium, phosphorous, Magnesium, Calcium, Sulphur, Sodium, Iron, Copper,

Zinc, Aluminum, Manganese, Boron and Molybdenum. The non-essential amino acid profile for LS seeds includes aspartic acid, glutamic acid, serine, glycine, alanine, tyrosine and proline. The essential amino acid profile for LS seeds include histidine, threonine, arginine, valine, methionine, phenyl alanine, isoleucine, leucine and lysine (Gokavi, Malleshi, & Guo, 2004). The antioxidant properties of LS seeds had a 94.18% DPPH(2,2-diphenyl-1-picrylhydrazyl) inhibition and a reducing power assay absorbance of 0.749 (Panwar & Guha, 2014).

Ethnopharmacological Properties of *Lepidium sativum*

LS seed can be used as a promising multipurpose medicinal source for the treatment of various ailments such as hyperglycemia (Patole, Agte, & Phadnis, 1998) by inhibiting renal glucose reabsorption (Eddouks et al., 2005) and through the alteration of renal glucose transporters (SLGT1) expression in the kidney (Tetsuya et al., 2000) and for fracture healing (Abha, 2007;Yadav et al., 2011) by promoting and accelerating callus formation. The methanolic extract of LS seeds possess significant antidiarrheal activity due to their inhibitory effect both on gastrointestinal propulsion and fluid secretion (Divanji et al., 2009), mediated possibly through dual inhibition of muscarinic receptors and Ca^{2+} channels (Rehman, Khan, Alkharfy, & Gilani, 2012). Due to increasing antibiotic resistance of microorganisms and side effects of synthetic antibiotics, medicinal plants are now gaining popularity in the treatment of bacterial infections. The extract of LS seeds has shown strong antimicrobial activity against the pathogenic organisms *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumonae* and *Proteus vulgaris*. LS acted like standard antibiotics Gentamicin and Ketoconazole when tested for inhibitory effects on *Pseudomonas aeruginosa* and *Candida albicans* due to the presence of tannins in LS seeds which act by inhibition of protein synthesis by forming irreversible complex with proline-

rich proteins (Adam, Salih, & Abdelgadir, 2011). LS is also considered having antihypertensive effects and acts as a diuretic (Patel et al., 2009). The presence of flavonoids, steroids, saponins or organic acids (Maghrani et al., 2005) may produce diuretic effect by stimulation of regional blood flow or initial vasodilation (Stannic & Samarzija,1993) or by inhibiting tubular reabsorption of water and anions (Pantoja, Chiang, Norris, & Concha, 1993). The increased sodium and water excretion activity also provides strong basis for its proved anti-hypertensive action (Jouad, Haloui, Rhiouani, El Hilaly, & Eddouks, 2001). Abuelgasim et al. (2008) reported the hepatoprotective activity of LS by decreased serum activity of alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and bilirubin concentration in carbon tetrachloride CCl_4 - induced liver damage, due to the presence of flavonoids, tannin, alkaloid, coumarin and triterpenes which induced antioxidant effect and a decrease in free radical formation from CCl_4, a main trigger of hepatotoxicity (Sharma & Agarwal, 2011;Wadhal, Panwarl, Agrawall, Saimil, & Patidar, 2012). *Lepidium sativum* seeds have shown anti-cancer activity by inhibiting the growth of human breast cancer cell line MCF-7 (Michigan Cancer Foundation–7). LS shown has been shown to cause significant time- and dose- dependent decrease in cancer cell viability. The effect of LS extract on cancers is generally attributed to the constituent isothiocyanates, specifically benzyl isothiocyanate (Mahassni & Al-Reemi, 2013). LS seeds are shown to reduce the symptoms of asthma and improve lung function in asthmatics (Archana et al.,2006; Paranjape & Mehta 2006) due to the bronchodilator activities. Raval and Ravishankar (2010) reported the antinflammatory, antipyretic and analgesic effect of LS seeds due to the presence of flavonoids, alkaloids, tannins, glucosinolates, sterols and triterpenes (Falana, 2014).

Lepidium sativum and Regulation of Reproduction

Very limited studies have been published in terms of LS role in controlling reproduction in mammals, although LS has been reported to increase the milk secretion (galactagogue) during post-natal period (Sahsrabudde & De, 1943). Al-Yawer, Al-Khateeb, and Al-Khafaji (2006) reported a strong mammotrophic and lactogenic effects of LS seeds on the non-primed mammary gland of adult virgin rats. LS seeds have been shown to act as a emmenagogue (Nadkarni & Nadkarami, 1954), abortifacient (Malhi & Trivedi, 1972), LS has been cited in many articles as an antiovulatory plants (Kamboj & Dhawan ,1982). Kagathara, Shah, and Anand (2015) reported increased proceptive and receptive behaviors of female rats treated with methanolic extract of LS seeds suggesting its possible use to treat various sexual interest and sexual desire disorders in females.

Interestingly, LS seeds in males have been shown to improve sperm parameters including testicular and epididymal caudal sperm concentration due to its antioxidant property. LS constituents with antioxidant properties includes phenols, flavonoids, vitamin E (tocopherol), aromatic compounds and isothiocyanates which maintain viability, membrane integrity and motility of spermatozoa (Naji & Shumran, 2013) and acts as an aphrodisiac (Kagathara et al., 2015). Therefore, contrasting results exist regarding the use of LS seeds as a reproductive medicinal supplement.

The Neuroendocrine Control of Reproduction

Reproduction is important for the survival of all living organisms. Mammalian reproduction is controlled through the interactions among the hypothalamus, pituitary gland and gonads in a complex described as the Hypothalamus–Pituitary–Gonadal axis (HPG). Hormonally

corresponds to the hypothalamus releases gonadotropin-releasing hormone (GnRH) which regulates the synthesis and secretion of gonadotropins (FSH and LH) from gonadotrophs located in the adenohypophysis and these in turn regulate the synthesis and secretion of steroids (estrogen, progesterone, testosterone) from the gonads. 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. This axis also regulates gonadal gametogenesis in both males and females. This axis is exquisitely regulated by feedback systems, mostly of the negative type, although positive feedback exists in female mammals. 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. (Conn & Crowley,1994; Kaiser, Michael, & Chin, 1997 ; Meccariello, Fasno, Pierantoni, & Cobellis, 2014). These two modes of hormonal synthesis and secretion that is pulsatile and surge release involve different subset of neurons in hypothalamic areas (Smith, Cunningham, Rissman, Clifton, & Steiner, 2005).

The hypothalamus is a structure of the diencephalon of the brain located anterior and inferior to 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 (Purnelle, Gérard, Czajkowski, & Bourguignon,1997). The median eminence is part of the inferior boundary of the hypothalamus in the human brain that is attached to the pituitary stalk (infundibulum). It is in the median eminence that the secretions of the hypothalamus (releasing and inhibiting regulatory hormones, known as "hypophysiotropic

hormones") collect before entering the portal system. This structure is part of the anatomical link between the hypothalamus and anterior pituitary gland.

The pituitary gland is cradled within the sella turcica of the sphenoid bone of the skull. It consists of two lobes that arise from distinct parts of embryonic tissue. The posterior pituitary (neurohypophysis) is neural tissue, whereas the anterior pituitary (also known as the adenohypophysis) is glandular tissue. The hypothalamic hormones are secreted by neurons and enter the anterior pituitary through blood vessels called the hypophyseal portal system**,** which allows hypothalamic hormones to be transported to the anterior pituitary without first entering the systemic circulation. The pars nervosa (part of the posterior pituitary gland) is continuous with the median eminence of the hypothalamus via the infundibular stalk. Parvocellular neurons from the hypothalamus terminate in the median eminence of the hypothalamus. The posterior pituitary lobe houses the axon terminals of hypothalamic neurons. It stores and releases into the bloodstream two hypothalamic hormones; oxytocin and antidiuretic hormone (ADH).

Molecular Mechanism of Pulsatile GnRH Regulation

The decapeptide Gonadotropin Releasing Hormone (GnRH) is the key hormone that plays an essential role in neuroendocrine control of reproductive function (Tsutsumi & Webster, 2009). Initially, GnRH is synthesized as a precursor, prepro-GnRH, which is later processed to yield the decapeptide sequence prior to its secretion (Ulloa-Aguirre, 2000). Several central and peripheral signals modulate GnRH neuronal activity. Some of these signals (norepinephrine, kisspeptin and neuropeptide Y) are stimulatory for GnRH release (Ojeda & Skinner, 2005), others are inhibitory (beta-endorphin, progesterone and interleukin-1) while others(estradiol) can be either stimulatory or inhibitory.

In mammals, two independent modes of gonadotropin-releasing hormone (GnRH) regulate the estrous cycle. The Tonic or Pulsatile mode of secretion is caused by GnRH-pulse generator leading to the stimulation of folliculogenesis and steroidogenesis. The LH pulse is produced by a corresponding GnRH pulse from the hypothalamus. Both frequency and amplitude of GnRH pulse are critical for normal gonadotropin release and one reason for the GnRH secretion in pulsatile manner is to avoid the downregulation of the GnRH receptor in the pituitary (Tsutsumi & Webster, 2009).The pulse generator is a bunch of GnRH neurons distributed in the preoptic area and adjacent sites in the rostral region of the hypothalamus, rather than concentrated in a discrete nucleus. These scattered neurons are believed to form a diffuse neural network that functions coordinately as a GnRH pulse generator (Krsmanovic, Hu, Leung, Feng, & Catt, 2009). Westel et al. (1992) reported that intrinsic pulsatile secretory activity was seen in immortalized GnRH neurons indicating that the pulse generator is cell autonomous.

The other mode of secretion is a surge mode and it occurs in female only. GnRH release is triggered by increasing levels of circulating estradiol during the preovulatory period in spontaneous-ovulating species, which is solely responsible for the induction of luteinizing hormone (LH) surges, eventually leading to ovulation. A population of metastin/kisspeptin neurons located in the anteroventral periventricular nucleus (AVPV) mediate GnRH surge as estrogen enhances kiss1 gene expression at the AVPV and thus mediate the estrogen positive feedback action on GnRH release. The other hypothalamic population of metastin/kisspeptin neurons is in the arcuate nucleus (ARC) and could be involved in generating GnRH pulsatile release. Estrogen suppress kiss 1 m RNA levels at ARC nucleus and mediating negative

feedback action of estrogen on GnRH release (Smith et al., 2005). This steroid regulation is nucleus specific.

Although produced in same gonadotrope cell, concentrations of LH and FSH vary throughout the estrous or the menstrual cycle. Both LH and FSH synthesis is regulated by the frequency of GnRH pulses, with LH favored by fast pulse frequencies (> 1 pulse per h) and FSH favored by slow pulse frequencies (< 1 pulse per 2-3 h). During the follicular phase of the ovulatory cycle, increasing estrogen production activates Kiss-1 neurons in the AVPV overriding the normal tonic repression by ovarian steroids and increasing GnRH pulse frequency and amplitude. This in turn favors LH synthesis leading to the LH surge and triggering ovulation. Following ovulation, luteinization of the granulosa cells increases progesterone production which slows GnRH pulse frequency to decrease LH production and increase FSH production to stimulate the next round of ovulation (Tsutsumi & Webster, 2009). Therefore, the pulsatile and surge release involve different subset of neurons with those in the arcuate nucleus mediating the normal pulsatile release whereas others in the AVPV mediating the surge release (Smith et al., 2005).

The GnRH neurons are activated and stimulated to secrete GnRH through Kisspeptin-GPCR54 signaling pathway. The G protein-coupled receptor 54 (GPR54) and its endogenous ligand, kisspeptin are essential for activation and regulation of the hypothalamic-pituitary-gonadal axis. Kisspeptin (KP) is a peptide product of KiSS-1 gene (Tena-Sempere, 2006), originally considered tumor metastasis suppressors called metastatin and also a potent stimulator of gonadotropic axis. The critical role of Kisspeptin-GPCR54 in reproduction was discovered in late 2003 when two research groups independently reported the presence of loss of function

mutations of GPCR54 gene in patients suffering idiopathic hypogonadotropic hypogonadism (IHH) (De roux et al., 2003; Seminara et al., 2003). Metastin/kisspeptin-GPR54 signaling has been suggested to control ovarian cyclicity through regulating these two modes of GnRH release.

Female Reproduction

The female internal genitalia include the uterus, oviduct and the ovaries. The ovaries are paired organs located on either side of the uterus within the mesovarium portion of the broad ligament below the uterine tubes. The ovaries are oval-shaped, approximately 3-5 cm in length during childbearing years. It has an outer connective tissue layer and an inner connective tissue frame work called stroma. The ovary can be divided into the outer cortex filled with ovarian follicles in various stages of development or degeneration and the inner medulla contains nerve and blood vasculature. The ovary produces both gametes and hormones. Each primary oocyte is enclosed in a primary follicle with a single layer of granulosa cells separated by a basement membrane from an outer layer of cells known as theca. Female humans produce gametes in monthly cycles (average 28 days ; normal range 24-35 days) called menstrual cycles because they are marked by a 3-7 days period of bloody uterine discharge known as the menses or menstruation. The menstrual cycle can be divided into the Ovarian cycle, characterized by the changes that occur in follicles of the ovary and changes in the endometrial lining of the uterus, the Uterine cycle.

The Ovarian cycle consists of follicular phase, ovulation and luteal phase. During follicular phase, under the influence of GnRH from hypothalamus, gonadotropin secretion from the anterior pituitary increases and under the influence of FSH, several follicles in the ovaries

begin to mature. As the follicles grow, their granulosa cells (under the influence of FSH) and their thecal cells (under the influence of LH) start to produce steroid hormones namely Anti-Mullerian Hormone (AMH) from granulosa cells and androgen by theca cells. The androgen is then converted to estrogen by granulosa cells. Gradually increasing estrogen levels in the circulation have several effects. Estrogen exerts negative feedback on pituitary FSH and LH secretion, which prevents the development of additional follicles in the same cycle. At the same time estrogen stimulates additional estrogen production by the granulosa cells. This positive feedback loop allows the follicles to continue estrogen production even though FSH and LH levels decrease. As the follicular phase ends, granulosa cells of the dominant follicle begin to secrete inhibin and progesterone in addition to estrogen. Estrogen which had exerted a negative feedback effect on GnRH earlier in the follicular phase, changes to positive feedback, leading to a preovulatory GnRH surge.

Immediately before ovulation, the persistently high levels of estrogen, aided by rising level of progesterone, enhance pituitary responsiveness to GnRH. As a result, LH secretion increases dramatically, a phenomenon known as LH surge and high levels of estrogen in the late follicular phase prepare the uterus for a possible pregnancy. About 16-24 hours after LH peaks, ovulation occurs. During luteal phase or the post ovulatory, the graafian follicle is transformed into corpus luteum produces steadily increasing amounts of progesterone that continue the preparations for pregnancy. If a pregnancy does not occur, the corpus luteum regresses to corpus albicans, after about two weeks and the ovarian cycle begins again. The luteal cells degenerate, progesterone and estrogen decrease, and this fall removes negative feedback signal to the pituitary and hypothalamus, so secretion of FSH and LH increases.

The uterine cycle is divided into three phases: the menses phase, proliferative phase and secretory phase. Menses, the beginning of the follicular phase in the ovary corresponds to menstrual bleeding from the uterus. Proliferative phase, the latter part of the ovary's follicular phase corresponds to the proliferative phase in the uterus, during which the endometrium adds a new layer of cells in anticipation of pregnancy. During the secretory phase, after ovulation, hormones from the corpus luteum convert the thickened endometrium into a secretory structure meaning that the luteal phase of the ovarian cycle corresponds to the secretory phase of the uterine cycle. If no pregnancy occurs, the superficial layers of the secretory endometrium are lost during menstruation and the uterine cycle begins again.

Polyestrous Cycle in Female Rodents

Female rodents including *Rattus norvegicus* are poliestric, present spontaneous ovulation and show regular and successive estrous cycles. The estrous cycle lasts four to five days. It comprises phases characterized by different cell types observed in vaginal smears namely proestrus, estrus, metestrus and diestrus. The structural changes observed in the vaginal epithelium of female rats during the estrous cycle are induced by estrogen and progesterone. Throughout the estrous cycle, the LH secretion pattern is pulsatile. The first phase proestrus, described as estrus (heat) or the period of sexual receptivity, appears only every 4 or 5 days. Histomorphologically, proestrus phase is characterized by a predominance of nucleated epithelial cells. Estrus phase is characterized by a female animal's readiness to receive a male for coitus characterized by Lordosis Reflex, in which the animal spontaneously elevates her hindquarters (Kagathara et al., 2015). Histomorphologically estrus phase is characterized by clumps of enucleated acidophilic cells. Metestrus or Diestrus phase is characterized by the formation of the

corpus luteum, which produces progesterone. In the absence of pregnancy during the diestrus phase terminates with the regression of the corpus luteum. The lining in the uterus is not shed but is reorganized for the next cycle. Metestrus is characterized by the same proportion of leukocytes, cornified and nucleated epithelial cells. Diestrus is characterized by a predominance of leukocytes.

Estrous versus Menstrual Cycle

There are two differences between estrous and menstrual cycle, one being is that animals that have estrous cycles reabsorb the endometrium or reorganize the endometrium if conception does not occur during that cycle while animals that have menstrual cycles shed-off the endometrium through menstruation. The second difference is in terms of sexual activity, females with estrous cycles are generally only sexually active during the estrus phase of their cycle. This is also referred to as being "in heat". In contrast, females of species with menstrual cycles can be sexually active at any time in their cycle, even when they are not ovulating.

LS Seed Effects on System Functions in Mammals

Renal physiology. The kidneys are two bean-shaped organs found on each side of the body in vertebrates. Anatomically they are located at the back of the abdominal cavity in the retroperitoneal area. Each kidney is attached to ureter, a tube that carries excreted urine to the urinary bladder. The parenchyma of the kidney is divided into the outer renal cortex and the inner renal medulla. Grossly, these structures take the shape of cone-shaped renal lobes, each containing renal cortex surrounding a portion of medulla called a renal pyramid. Between the renal pyramids are projections of cortex called renal columns. Nephron are the urine-producing functional structures of the kidney. The initial filtering portion of a nephron is the renal corpuscle

located in the cortex. This is followed by a renal tubule that passes from the cortex deep into the medullary pyramids. The tip or papilla of each pyramid empties urine into a minor calyx which empty into major calyces, and major calyces empty into the renal pelvis leads to the ureter. At the hilum, renal vein exits the kidney and the renal artery enters. In humans, each adult kidney contains around one million nephrons. The nephron utilizes four processes to alter the blood plasma which flows to it namely filtration, reabsorption, secretion, and excretion.

Elimination of Nitrogenous Waste

The main substances excreted in urine includes metabolic waste products includes urea and creatinine. The liver produces urea as a waste product of the protein catabolism. Blood Urea Nitrogen (BUN) is an indication of renal health that measures the amount of urea nitrogen found in blood. The main causes of an increase in BUN includes increased *protein catabolism*, decrease in glomerular filtration rate (GFR), gastrointestinal hemorrhage.

Creatinine is a breakdown product of creatine phosphate in muscle. Serum creatinine is an important indicator of renal health because it is an easily measured byproduct of muscle metabolism that is excreted unchanged by the kidneys. Creatinine itself is produced via a biological system involving creatine, phosphocreatine and adenosine triphosphate.

LS Seed Effects on Hematological Profile

Ten percent of LS seeds feeding has shown to have no adverse effect on liver and kidney functioning, between control and treated groups in both male and female Sprague-Dawley rats (Datta et.al., 2011). LS seeds are rich source of protein, dietary fibers, minerals, vitamins and essential amino acids. These seeds have shown to improve the hemato-immunological profile,

especially hemoglobin concentration, packed cell volume and Total Leukocyte Count in broiler chicks (Verma, 2016).

The aqueous extract of *Lepidium sativum* has shown antianemic activity in phenylhydrazine- induced anemia on female wistar rats (Sheeba & Sabitha, 2016a). Gupta and Singhal (2011) reported the increased hemoglobin level effect of LS seeds in non-pregnant women ,100 g of Garden Cress seed contains 100 mg of iron. Sheeba and Sabitha (2016b) reported the improved mean hemoglobin level from 9.6 g/dl to 12.1g/dl and RBC level 3.2 million cells/mm3 to 4.0 million cells/mm3 in anemic adolescent girls that were fed with chick peas incorporated with *Lepidium sativum* seeds.LS therefore shown to have antianemic property in combating anemia.

Chapter III: Hypotheses

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

- H_0: *In vitro* female OVEX + E_2 rat treated with LS seed extract (LSE) will have no effect on hypothalamic GnRH and pituitary gonadotropins pulsatile secretion and therefore disrupting folliculogenesis and anovulatory effect.
- H_1: *In vitro* administration of LSE to hypothalamic and pituitary explants will have an effect on GnRH, LH and FSH pulsatile secretion leading to folliculogenesis and steroidogenesis.

Chapter IV: Materials and Methods

The research protocols utilized in this study were approved by the Institutional Animal Care and Usage Committee of SCSU, St. Cloud, MN. 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 SCSU, St. Cloud, MN.

Preparation of Diet

Garden cress seeds were obtained 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 cleaned and rendered free of dust, blended to a fine powder using Waring commercial blender (Waring Products Division, New Hartford, Conn. USA), then stored in polyethylene bags in refrigerator at -20°C until used.

Experiment 1

Effect of methanolic extract of Lepidium sativum on hypothalamic and gonadotropic hormone secretion in ovariectomized, estradiol-implanted (OVEX+E_2) rats.

The main objective of this experiment was to determine the effect of methanolic extract of LS seed on the development and magnitude of the pulsatile secretion of GnRH from hypothalamus, LH and FSH from adenohypophysis that lead to the process of folliculogenesis and steroidogenesis in Sprague-Dawley rats. Twenty mature female rats were ovariectomized and implanted with estradiol -17β (OVX+ E_2) (Tygon flexible plastic tubing R^x50 Silastic - medical grade tubing,1.57 x 3.18mm and Dow Corning 839 Silicone adhesive. Midland, USA) three weeks before euthanization using standard procedures. Ovariectomy is needed to prevent

abrupt variations in the level of estrogen and estradiol replacement is required to ensure that baseline of estrogen is present in the rats.

The animals were housed in the ISELF Vivarium of the Department of Biological Sciences, St. Cloud State University, Minnesota throughout the study. They were maintained under lighting regimen of 12 hours under light and 12 hours in the dark. Rat chow and water was provided *ad libitum*.

Methanolic extraction of LS seeds. Methanolic extraction of *Lepidium* sativum was done by using Sohxelt method. 200g of seeds were powdered and extracted in a Sohxelt apparatus with organic solvent (200g in 500ml of methanol) for 24 hours, The solution was filtered with a vacuum through Whatman qualitative filter paper grade 50 (4.25cm, Whatman Limited, England). The extracts collected were concentrated under reduced pressure using a rotary vacuum evaporator for six hours and stored in sterile containers at 4℃ until used (Patel *et al*., 2009).

Bilateral ovariectomy and estrogen implantation in the rat. Rats were weighed, and their body weight was recorded. Ketamine-Xylaxine as anesthetic was injected at the rate of 0.07 mL/100gm BW; i.p. The rat was placed in dorsal recumbency position and the abdominal region was shaved and swiped with Iodine-soaked gauze starting from the center and moved outward in a circular motion. Using aseptic procedures, the skin was lifted and horizontally, a nick was made along linea alba. Thereafter using the tip of scissors, a blunt dissection was made in the abdominal muscles to expose the abdominal cavity. The reproductive tract was located by pulling out fatty pad to expose the reproductive tract. Upon locating the uterus, a non-absorbable surgical suture (3-0 (2.0 Metric) perma-hand silk (Ethicon, USA) was tied under the uterine horn

in the area of the junction between the horn and oviduct to cut off blood supply to the horn. The ovary was isolated after two ligations, one superior and the other, inferior. A first cut was made beneath the superior ligation to release the ovary, after which a second cut was made ahead of the inferior ligation to release the ovary. The same procedure was repeated to remove the contralateral ovary. After ensuring that all bleeding had stopped, the muscle layer was approximated using absorbable suture (3.0 Metric Chromic Gut, Ethicon, USA) and the skin layer was also sutured with non-absorbable material. The animal was put in dorsal recumbency and the nape area was shaved and sterilized with alcohol. A 1.5 cm median incision was made and the skin was lifted to permit the subcutaneous placement of the estrogen implant. Thereafter the skin was sutured. Buprenorphine (0.05 mg/Kg BW) and Penicillin (50,000 IU) were injected sub-cutaneously post operatively.

Perifusion of hemi-hypothalamic fragments and pituitary gland. After 3 weeks of ovariectomy, rats used in study were randomly assigned to four groups: Control (n = 5), Low group (n = 5), Mid group (n = 5), High group (n = 5). Rats were euthanized using an overdose (6ml/100gm BW, i.p.) of Tribromoethanol (2.5% TBE) and both the hypothalamus and pituitary gland of each rat were harvested for perifusion as per procedures described later. Each day, five randomly selected rats were sacrificed over the next four days. The use of TBE as a method of euthanasia is necessitated because of the rapidity of its action and the need to obtain both the hypothalamic and pituitary fragments very quickly to ensure they were viable for the study. After the rats were deeply sedated, they were decapitated using small animal guillotine (Wahman Co., Baltimore, MD). Trunk blood was obtained in heparinized EDTA tubes, centrifuged (Eppendorf

Centrifuge 5810 R, Hamburg Germany) at 3000rpm for 5 minutes and stored at -80°C for determination of estradiol-17β in plasma.

The hypothalamus and pituitary were harvested by removing the calvarium to expose the underlying occipital lobe of the brain and the cerebrum using large hemostat from the posterior side. The brain was lifted out of the cranium, giving more visibility for pituitary extraction. The entire pituitary gland was extracted from the surface of the Sella turcica by using the tip of scalpel blade and placed in perifusion unit containing cartridge covered occluded with filter paper at the top and the bottom. Thereafter, the hypothalamus was removed using a scalpel handle fitted with a number 24 blade. First, a cut was made 2 mm anterior to the optic chiasm and another cut was made immediately anterior to the mammillary bodies. Two more cuts were made 2 mm lateral to the hypothalamic sulcus to remove the hypothalamus was isolated and then cut midsagittal to yield two hemi-hypothalamic (HH) fragments. The hemi-hypothalamic fragments were then perifused by placing them in cartridge as described previously for the pituitary gland. Both the pituitary gland and the hemi-hypothalami fragments in the perifusion cartridges were allowed to equilibrate for 60-minutes with artificial cerebrospinal fluid (aCSF) supplied with 95% O_2 and 5% CO_2 at 37°C.

Perifusion of hemi-hypothalamic fragments. The hemi-hypothalamic fragments from OVEX+E_2 Sprague-Dawley female rats were used for the GnRH study. Artificial cerebrospinal fluid (a CSF) buffered with 95% O_2 and 5% CO_2 was used as the perifusion buffer. The hemi-hypothalamic fragments were perifused utilizing a 12-chamber Suprafusion 1000 unit (Brandel Inc., Gaithersburg, MD 20877, USA). The perifusion process consisted of four periods. During the equilibration period, all fragments were perifused with aCSF for 60 minutes. This was

followed by the Baseline period during which all fragments in all treatment groups were perifused with aCSF for 120 minutes with perifusate collection occurring every 7.5 minutes. During the ensuing Treatment period, HH fragments were either perifused with either aCSF only (n = 10; Group = Control) or aCSF containing 10 mg/mL (n = 10; Group = Low), or aCSF containing 20mg/mL LSE (n = 10; Group = High) for 120 minutes with perifusate collection every 7.5 minutes. In the final period, all HH fragments were perifused with aCSF containing 56 mM KCl for 60 minutes with perifusate collection at the same frequency. This was done to validate the viability of the HH fragments during the entire perifusion period that lasted six hours. All perifusates were stored at -20°C until used for GnRH radioimmunoassay analysis.

Perifusion of pituitary gland. Pituitary glands obtained were perifused using a 6-chamber Suprafusion 1000 (Brandel Inc., Gaithersburg, MD 20877, USA) using periods similar to that described above for the HH perifusion, with the following modifications: After the equilibration process, all pituitaries underwent the equilibration period for 60 minutes. This was followed by a Baseline period of 120 minutes during which all pituitary extracts received aCSF for 120 minutes with perifusate collection at 7.5-minute intervals. Thereafter, during the Treatment period that lasted 120 minutes, pituitary extracts received either aCSF (0 mg/mL LSE; n = 5; Group = Control) or aCSF containing 10 mg/mL LSE (n = 5; Group = Low), or aCSF containing 20 mg/mL LSE (n = 5; Group = Mid), or aCSF containing 40 mg/mL LSE (n = 5; Group = High). Perifusates were collected every 7.5 minutes during this period. In the final period, each pituitary fragment was perifused with aCSF containing 10ug of GnRH to validate their functionality and responsiveness throughout the six-hour perifusion process. Samples were

collected every 7.5 minutes and stored at -20°C until perifusates were analyzed for LH and FSH radioimmunoassays.

Radioimmunoassay of GnRH. GnRH concentration was determined using a modification of the technique described by Gazal et al. (1998). Prior to iodination, a carboxymethyl cellulose (CMC) column (Biophoretics-IonSep CM52) was prepared for separation of free ^{125}I from ^{125}I-GnRH. GnRH (Sigma Aldrich chemicals, St. Louis, MO, USA) was iodinated using ^{125}I (Perkin Elmer Laboratories, Boston, MA, USA) by the Chloramine-T method. The GnRH standards used in this assay were 1, 5, 10, 25, 50, 100, 250, 500 and 1,000 pg/mL. The antiserum R1245 (obtained from Colorado State University, Fort Collins, CO, USA) was used 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 into12x75 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 containing 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 except TC tubes as a precipitating agent and then vortexed. The tubes were then centrifuged at 3000 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). The minimum GnRH concentration detected was 1 pg/mL.

Radioimmunoassay of LH. LH concentration was determined using techniques described by Bernard, Valet, Beland, and Lambert (1993). Prior to iodination, Anion exchange

column (BioRad AG 1-X8; 50-100 mesh Chloride form) was prepared. The LH used for making standards and iodination reaction was obtained from Sigma chemicals (Sigma Aldrich chemicals, St. Louis, MO, USA) The LH standards used in this assay were 0.4, 0.8, 1.6, 3.1, 6.2, 12.5,25, 50 ng/ml. LH was iodinated using ^{125}Iodine (Perkin Elmer Laboratories, Waltham, MA, USA) by the chloramine-T method. The antiserum AFP 240580 was used at 1:35,000 dilution as the first antibody and the Goat Anti-Rabbit Gammaglobulin (GARGG) was used as second antibody. Briefly, on day 1, standards, 100ul antibody and tracer (^{125}I-LH; 18,000-20,000 cpm/100ul) were pipetted into 12x75mm borosilicate glass tubes containing 200 µl perifusate and incubated at 4° C for a period of 48 hours. Thereafter, the second antibody, GARGG (200ul of 1:1 dilution of GARGG with PBS-EDTA) was added. This was followed by the addition of 6% polyethylene glycol (500 µl of 6% PEG in 0.01M PBS) to separate the bound and unbound fractions. Thereafter, the tubes were centrifuged at 3000 rpm at 4°C for 15 minutes using the Sorvall RT7 plus centrifuge. The supernatant was then discarded into a waste receptacle and the tubes were loaded on the Packard Cobra II Gamma counter for LH concentration determination. The minimum LH concentration detected by the assay was 0.4 ng/ml.

Radioimmunoassay of FSH. FSH concentration was determined utilizing techniques described by Odell, Parlow, Cargille, and Ross (1968). Prior to iodination, Anion exchange column (Bio Rad AG 1-X8; 50-100 mesh Chloride form) was prepared. FSH was iodinated using ^{125} Iodine (Perkin Elmer Laboratories, Waltham, MA, USA) by the Chloramine-T method. The FSH used for making standards and iodination reaction was obtained from Sigma chemicals. The FSH standards used in this assay were 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

GARGG (Equitech-Bio, Inc, Kerrville, TX, USA) was used 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 (250µL) 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). The minimum FSH concentration detected by the assay was 0.0125ng/ml.

Radioimmunoassay of plasma estradiol-17β. Plasma estradiol-17β concentrations were assayed utilizing a one-day, double-antibody 17β-Estradiol radioimmunoassay kit (MP Biomedicals, Solon, OH 44139, USA). 50 µL of Standards (10-, 30-, 100-, 300-, 1000-, or 3000 pg/mL) or plasma samples in duplicates were pipetted into 12x75 mm borosilicate glass tubes (VWR International, Radnor, PA, USA) and incubated with 500 µL of ^{125}I-estradiol-17β. Then all tubes, except non-specific binding (NSB) and total count (TC) tubes, received 500 µL of estradiol-17β antibody. All tubes were vortexed thoroughly and incubated at 37°C for 90 minutes. Then 500 µL of a precipitant solution was added to all tubes and vortexed thoroughly. All tubes except the TC tubes were centrifuged at 3000 rpm for 20 minutes in 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). The sensitivity of the assay was 10 pg/mL.

Pulse characteristics analysis. Pulse characteristics of GnRH, LH, and FSH were determined using the Pulsefit program. Pulse characteristics included baseline (in pg/ml or ng/ml), total pulse frequency (total pulses over the whole sampling period of 5 hours), pre-treatment pulse frequency (the number of pulses obtained during baseline period over 2 hours), treatment pulse frequency (the number of pulses obtained during applied treatment over 2 hours), post treatment pulse frequency (the pulse frequency obtained in last hour during KCl or GnRH challenge), pulse amplitude (in pg/ml or ng/ml), and finally the interpulse interval (in minutes and corresponds to the time interval between pulses).

Experiment 2

The objective of this experiment was to determine the effect of acute and chronic effect of 15% Lepidium sativum supplementation on hematological parameters, and indices of kidney function and metabolism in male Sprague–Dawley rats.

Preparation of diet. The 15% LS experimental feed was prepared in the form of pellets. LS seeds were ground to produce LS Seed Powder (LSSP) using a laboratory grinder (Waring commercial blender, Waring Products Division, New Hartford, CT 06057, U.S.A) and mixed with regular rat chow that was ground to a fine powder with a mortar and pestle. From our pilot study, we know that rats eat a approximately 20 g of rat chow, on per capita basis, per day. A 15% LS seed mixture in a 20g feed was achieved by mixing 3g of LS seeds with 17g of regular chow and this mixture was then pelleted. Pellets were prepared every week and stored at room temperature.

Animals and study design. Forty-eight mature male rats (Sprague-Dawley) weighing 200-250 g and between 7 and 10 weeks of age were used for this study. Rats were housed in

individual cages under controlled conditions for two weeks prior to the start of the experiment for acclimatization. Rats received *ad-libitum* access to rat chow and water during this period. After the adaptation period, rats were randomly sorted into two groups namely Control (n = 24) and Treated (n = 24).

Feeding and collection of blood and body organs. The animals were fed diet supplemented with ground LS seeds ad libitum. Feed intake and feed refusal were weighed every day and feed refusals were discarded. Body weight of animals were recorded every other day. Rats were fed either normal rat chow (Group = Control; n = 24) or rat chow with 15% LSSP (Group = Treated; n = 24) throughout the 8-week duration of this study.

Specimen collection and processing. Every 2 weeks, 6 rats from each experimental group were sacrificed and trunk blood samples were collected into centrifuge tube containing 100 µl of 5% EDTA for plasma collection. Plasma was stored at -80°C until hematological, liver and kidney functional endpoints analyses.

Chemical and hematological analyses. Plasma samples were analyzed for aspartate transaminase (AST) and alanine transaminase (ALT), alkaline phosphatase (ALP) and for the concentrations of total protein, albumin, globulin, Blood Urea Nitrogen (BUN), creatinine, hemoglobin (Hb) concentration, packed cell volume (PCV), red blood cells(RBC), white blood cells and other hematological indices using the IDEXX Vet Connect Plus Reference Laboratories (www.idexx.com).

Statistical data analyses. Data in Experiment 1 were analyzed using the PROC MIXED procedure of SAS (SAS Institute Inc., NC, USA.) for repeated measures. The main effect of LSE treatment dose, period and dose and period interactions on GnRH, LH and FSH secretions

were determined using the PDIFF analyses. Further, the effect of LSE treatment on GnRH, LH, and FSH pulse characteristics were determined. In Experiment 2, a MANOVA analysis was used to determine the effect of LSE dose and duration of treatment on growth and organ development, and hematological. Liver and kidney function. Means comparisons were done using Tukey's test. Differences were considered significant at $P < 0.05$. Data are reported as Mean ± Standard Error.

Chapter V: Results

Effect of LSE on the Hypothalamic GnRH Secretion

Hypothalamic GnRH secretory profiles in OVEX+E_2-implanted explants obtained from Control, 10mg/mL LSE treated–and 20mg/mL LSE-treated rats are shown in Figure 1. Pooled profiles from all treatment groups are shown in Figure 2. Baseline GnRH was pulsatile in Control and all treatment groups. Mean baseline GnRH concentrations was significantly greater ($P < 0.0001$) in hypothalamic explants that were later exposed to high LSE treatment. LSE administration at the high dose (20mg/mL LSE) significantly increased GnRH secretion over baseline levels. No effect of low LSE administration (10mg/mL LSE) on GnRH secretion was observed (Table 1). The administration of KCl had no significant effect on the release of GnRH in the last hour of experiment. Averaged over the entire sampling period, explants in the high group had significantly higher ($P < 0.0001$) mean GnRH secretion than either those in the Control or Low LSE groups (Table 2).

Baseline GnRH pulse frequencies were similar among Control, Low- and High-treatment groups ranging from 1.4 to 1.9 pulses/2 hours. Overall, no effect of LSE treatment was observed on total pulse frequency. However, LSE administration at the high dose significantly increased ($P = 0.01$) GnRH pulse amplitude (Table 3).

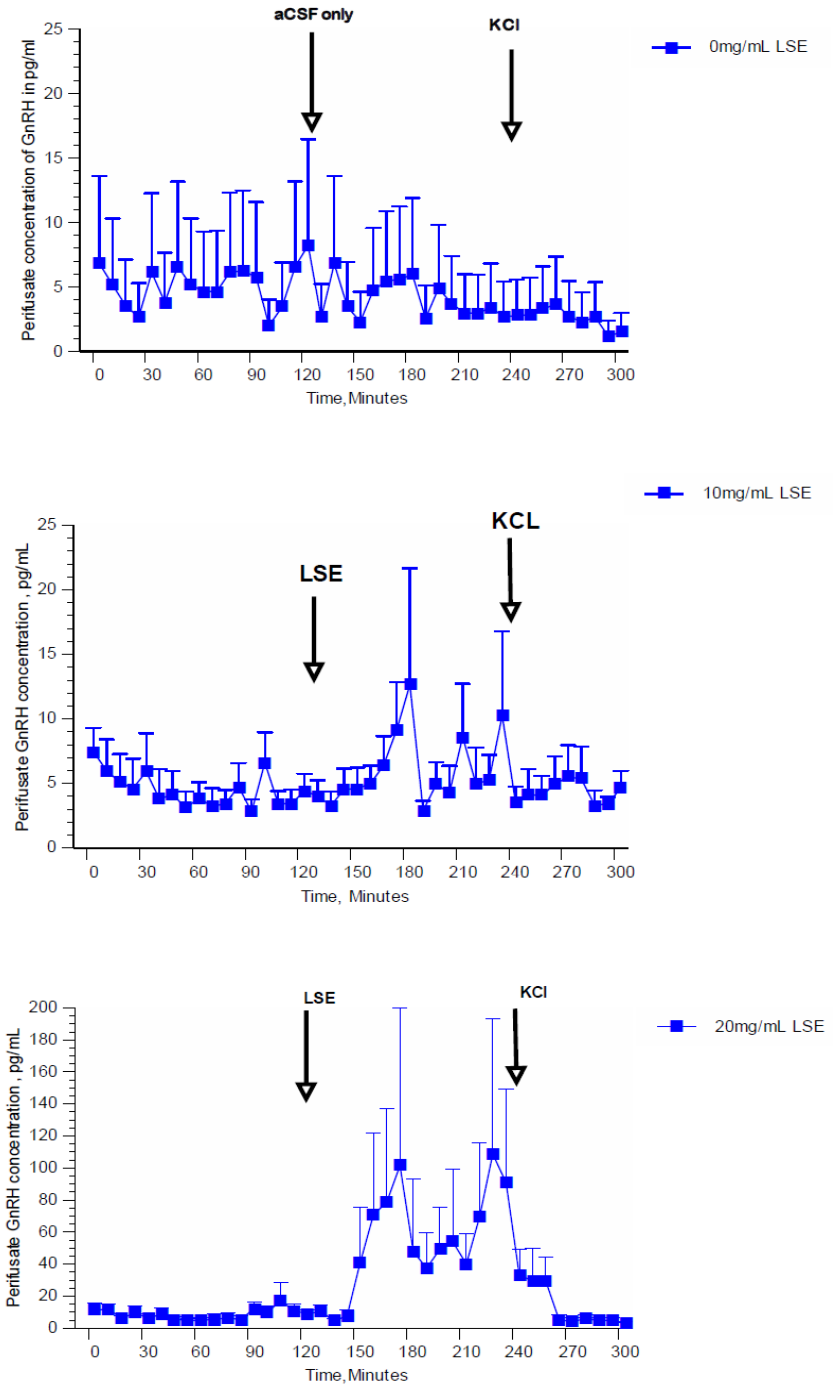

Figure 1. *In vitro* GnRH secretion patterns from hemi-hypothalamic explants obtained from OVEX+E_2 Sprague-Dawley rats treated with aCSF only (n = 10; top panel), low LSE treatment, 10mg/mL LSE (n = 10; middle panel), high LSE treatment, 20mg/mL LSE (n = 10; bottom panel).

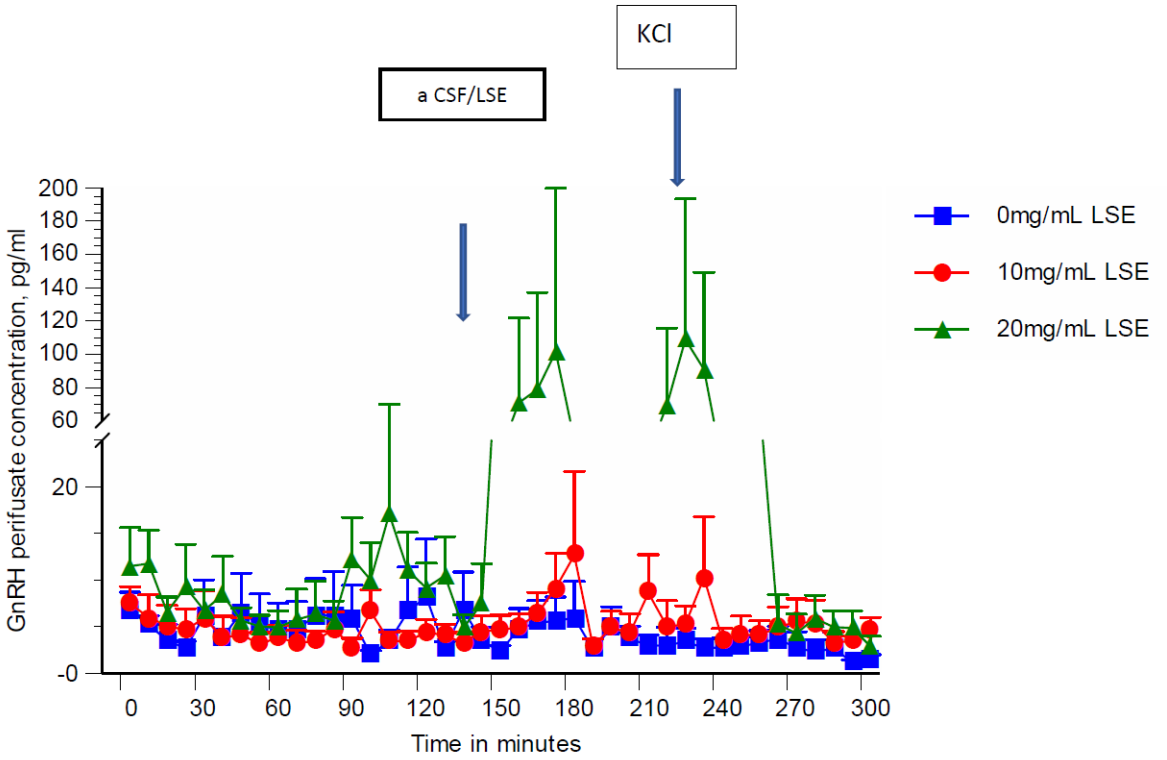

Figure 2. Effect of LSE on GnRH secretion in hemi-hypothalamic explants obtained from OVEX+E_2 Sprague-Dawley rats. Hemi-hypothalami in Control-, Low- and High-groups were treated with 0 mg/mL (n = 10), 10 mg/mL (n = 10), or 20mg/mL (n = 10) LSE, respectively.

Table 1. LSE effect on GnRH secretion periodic means. Comparison of LSMean GnRH concentration secreted by Hemi- hypothalamic explants obtained from OVEX+E_2 Sprague-Dawley rats during the different experimental periods. Values are LSMeans ± SE.

Treatment	**Period I** (Baseline)	**Period II** (aCSF / LSE)	**Period III** (KCl)
Control, aCSF	$5.1 \pm 3.36^{a+}$	$4.6 \pm 3.36^{a+}$	$2.5 \pm 4.47^{a+}$
10 mg/mL LSE	$4.5 \pm 3.24^{a+}$	$6.2 \pm 3.27^{a+}$	$4.32 \pm 4.31^{a+}$
20 mg/mL LSE	$21.5 \pm 3.51^{a++}$	$59.2 \pm 3.46^{b++}$	$29.6 \pm 4.93^{a++}$

^{a,b}LSMeans within a row with different superscripts are significantly different, P < 0.0001.
$^{+, ++}$LSMeans within a column with different superscripts are significantly different, P = 0.0008.

Table 2. LSE effect on overall GnRH secretion. GnRH values were averaged overall sampling periods. LSMeans within the same column with different superscripts are different, P <0.0001.

Treatment	**Overall Mean GnRH, pg/mL**
Control	4.1 ± 2.18[a]
10 mg/mL LSE	5.0 ± 2.11[a]
20 mg/mL LSE	36.7 ± 2.46[b]

Table 3. Effect of LSE treatment on GnRH pulse characteristics.

Pulse Characteristics	**Control (0 mg/ml LSE)**	**Low Dose (10 mg/ml LSE)**	**High Dose (20 mg/ml LSE)**
Baseline (ng/mL)	1.6 ± 0.61	1.6 ± 0.30	3.4 ± 1.92
Total Pulse Frequency (Total pulses/5 hours)	4.6 ± 0.88	4.4 ± 0.75	4.2 ± 0.47
Pre-Treatment - Pulse Frequency (No. of pulses/two hours)	1.6 ± 0.52	1.9 ± 0.66	1.4 ± 0.43
Treatment – Pulse Frequency (No. of pulses/ two hours)	2.3 ± 0.72	1.7± 0.47	2.3± 0.52
Post Treatment – Pulse Frequency (Pulses/hour)	2.8 ± 0.78	2.7± 0.66	2.8 ± 0.42
Pulse Amplitude (pg/ml)	6.5 ± 2.75[a]	5.6 ± 1.55[a]	97.7 ±41.51[b]
Interpulse Interval (minutes)	5.8 ± 2.55	6.1 ± 1.50	8.8 ± 2.00

LSMeans within the same row with different superscripts are significantly different, P = 0.01.

Effect of LSE on the Secretion of LH from the Pituitary

The profile of LH secretion in pituitary fragments obtained from all treatment groups is shown in Figure 3. Baseline LH secretion was pulsatile in all groups. Baseline LH secretion in pituitary explants obtained from Control rats, much to our chagrin, was significantly greater ($P < 0.0001$) that observed in all other treatment groups.

LSE administration at all doses significantly increased ($P < 0.0001$) pituitary LH secretion. GnRH challenge of pituitary explants post aCSF or LSE administration had no further effect ($P > 0.05$) on pituitary LH secretion. Mean pituitary LH secretion, averaged over 5 hours of perifusion was similar across all treatment groups (Table 5).

Overall, no effect of LSE administration was observed on either LH pulse frequency, pulse amplitude or interpulse intervals (Table 6).

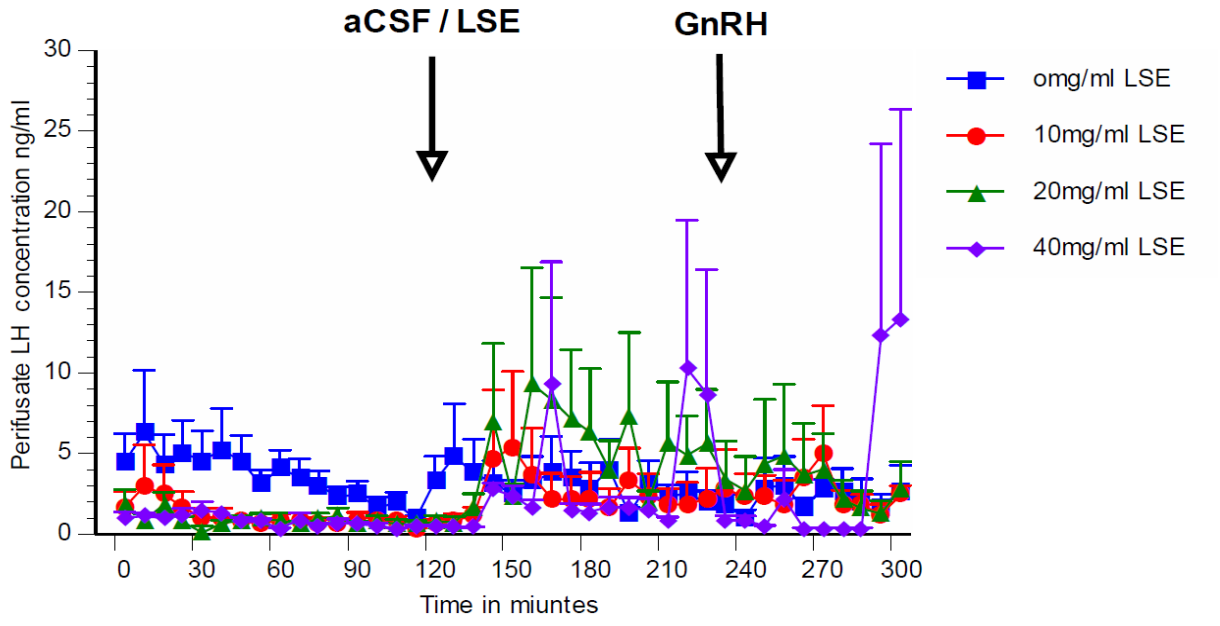

Figure 3. *In vitro* LH secretion patterns from pituitary glands explants obtained from OVEX+E_2 Sprague-Dawley rats treated with aCSF only (control n = 5); low LSE treatment, 10mg/ml LSE (n = 5); medium LSE treatment, 20mg/ml LSE (n = 5); high LSE treatment, 40mg/ml LSE (n = 5).

Table 4. LSE effect on LH secretion–periodic means. Comparison of LSMean LH concentration secreted by Pituitary explants obtained from OVEX+E2 Sprague-Dawley rats during the different experimental periods. Values are LSMeans ± SE.

Treatment	**Baseline LH (P I)**	**Treatment Period (PII)**	**GnRH Treatment (PIII)**
Control 0mg/mL LSE	$3.6 \pm 0.44^{a+}$	$3 \pm 0.45^{a+}$	$1.9 \pm 0.68^{b+}$
10 mg/mL LSE	$1.1 \pm 0.43^{a++}$	$2.5 \pm 0.44^{b+}$	$2.5 \pm 0.60^{a+}$
20 mg/mL LSE	$0.9 \pm 0.43^{a++}$	$4.9 \pm 0.45^{b++}$	$3.1 \pm 0.58^{b+}$
40 mg/mL LSE	$0.7 \pm 0.46^{a++}$	$3.0 \pm 0.46^{b+}$	$4.5 \pm 0.83^{b++}$

^{a,b} LSMeans within a row with different superscripts are significantly different, P = 0.004, P = <0.0001.

$^{+, ++}$ LSMeans within a column with different superscripts are significantly different, P <0.0001.

Table 5. LSE effect on overall LH secretion. LH values were averaged overall sampling periods. Overall nonsignificant effect.

Treatment	**Overall Mean LH, ng/mL**
0 mg/mL LSE	2.8 ± 0.31^{a}
10 mg/mL LSE	2.0 ± 0.29^{a}
20 mg/mL LSE	2.9 ± 0.28^{a}
40 mg/mL LSE	2.8 ± 0.35^{a}

Table 6. Effect of LSE administration on LH pulse characteristics in OVEX + E_2 rats treated with either 0 mg/mL LSE. Control (n = 5), 10mg/mL LSE, Low (n = 5), 20 mg/mL LSE, Mid (n = 5) or 40 mg/mL LSE, High (n = 5).

Pulse Characteristics	**Control (0 mg/ml LSE)**	**Low Dose (10 mg/ml LSE)**	**Medium Dose (20 mg/ml LSE)**	**High Dose (40 mg/ml LSE)**
Baseline (ng/mL)	0.5 ± 0.32	0.3± 0.08	0.5 ± 0.28	0.4 ± 0.06
Total Pulse Frequency (Total pulses/5 hours)	6.0 ± 1.1	4.4 ± 1.21	4.6 ± 1.50	2.2 ± 0.73
Pre-Treatment - Pulse Frequency (No. of pulses/two hours)	2.4 ± 0.60	1.2 ± 0.58	0.6 ± 0.40	0.6 ± 0.60
Treatment – Pulse Frequency (No. of pulses/two hours)	2.2 ± 0.58	1.8 ± 0.58	3.4 ± 1.66	1.6 ± 0.24
Post Treatment – Pulse Frequency (Pulses/hour)	3.4 ± 0.98	3.2 ± 1.02	4.0 ± . 1.22	1.8 ± 0.37
Pulse Amplitude (pg/ml)	2.3 ± 0.73	3.4 ± 1.20	6.6 ± 4.53	26.8 ± 24.88
Inter pulse Interval (minutes)	5.3 ± 0.76	8.0 ± 3.95	3.9± 1.15	3.7 ± 1.65

Effect of LSE on the Pituitary FSH Secretion

The pituitary FSH secretory profiles in OVEX+E_2-implanted explants obtained from Control, 10mg/mL LSE treated, 20mg/mL LSE and 40mg/mL LSE treated rats are shown in Figure 4. Perifusate FSH secretion was pulsatile during the baseline period and there was a tendency for mean FSH levels to be greater in Control rats relative to other treatment groups.

LSE administration at all doses significantly increased ($P < 0.0001$) pituitary FSH secretion with the most stimulatory effect observed in the explants that were perifused with the highest dose of LSE (40 mg/ml LSE). Further, administration of GnRH had no significant effect on the release of FSH in the last hour of experiment (Table 7). Averaged over the entire sampling

period, explants in the medium- and high-LSE group had significantly higher ($P < 0.0001$) mean FSH secretion than either those in the Control or Low LSE groups (Table 8).

Administration of Low-dose but not Medium-or High-dose LSE significantly increased FSH pulse frequency. GnRH administration significantly increased FSH pulse frequency in the Low-LSE-dose-treated but not the medium-LSE- or the high LSE-treated explants (Table 9). There was no effect of LSE treatment on perifusate FSH pulse amplitude.

Plasma Gonadotropins and Estradiol-17β Levels

Plasma LH and FSH levels in trunk blood obtained from OVEX+E_2 Sprague-Dawley rats pre-perifusion are shown in Figure 5. Plasma estradiol-17β levels averaged 601 ± 121pg/mL.

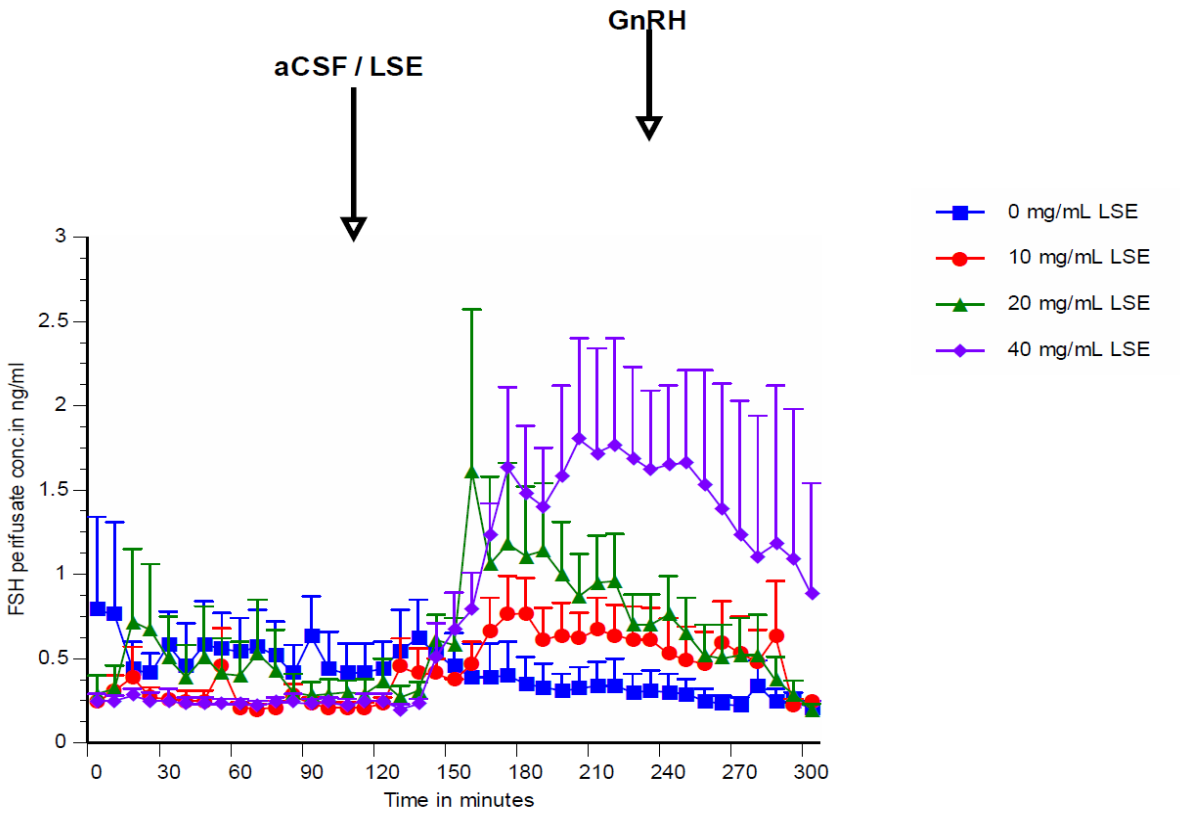

Figure 4. *In vitro* FSH secretion patterns from pituitary glands explants obtained from OVEX+E_2 Sprague-Dawley rats treated with aCSF only (Control, 0 mg/mL LSE, n = 5); Low LSE treatment (10 mg/ml LSE, n = 5); Medium LSE treatment (20mg/ml LSE, n = 5); High LSE Treatment (40mg/ml LSE, n = 5).

Table 7. LSE effect on FSH secretion–periodic means. Comparison of LS Mean FSH concentration secreted by Pituitary explants obtained from OVEX+E_2 Sprague-Dawley rats during the different experimental periods. Values are LS Means ± SE.

Treatment	**Baseline FSH (P I) ***	**Treatment Period (P II)**	**GnRH Treatment (P III)**
0 mg/mL LSE	$0.5 \pm 0.06^{a+}$	$0.4 \pm 0.06^{a+}$	$0.3 \pm 0.09^{a+}$
10 mg/mL LSE	$0.3 \pm 0.06^{a++}$	$0.5 \pm 0.06^{b++}$	$0.5 \pm 0.08^{b+}$
20 mg/mL LSE	$0.4 \pm 0.06^{a+}$	$0.8 \pm 0.06^{b++}$	$0.5 \pm 0.08^{a+}$
40 mg/mL LSE	$0.2 \pm 0.06^{a++}$	$1.2 \pm 0.06^{b++}$	$1.3 \pm 0.08^{b++}$

^{a,b}LSMeans within a row with different superscripts are significantly different, P = <0.0001.
$^{+, ++}$LSMeans within a column with different superscripts are significantly different, P <0.0001.

Table 8. LSE effect on overall FSH secretion. FSH values were averaged over all sampling periods. LSMeans within the same column with different superscripts are different, P <0.0001.

Treatment	**Overall Mean FSH, ng/mL**
0 mg/mL LSE	0.4 ± 0.04^{a}
10 mg/mL LSE	0.4 ± 0.04^{a}
20 mg/mL LSE	0.6 ± 0.04^{b}
40 mg/mL LSE	0.9 ± 0.04^{c}

Table 9. Effect of LSE administration on FSH pulse characteristics in OVEX + E_2 rats treated with either 0 mg/mL LSE. Control (n = 5), 10mg/mL LSE, Low (n = 5), 20 mg/mL LSE, Mid (n = 5) or 40 mg/mL LSE, High (n = 5). Means within the same row with different superscripts are different.

Pulse Characteristics	**Control (0 mg/ml LSE)**	**Low Dose (10 mg/ml LSE)**	**Medium Dose (20 mg/ml LSE)**	**High Dose (40 mg/ml LSE)**
Baseline (ng/mL)	0.2 ± 0.05	0.2 ± 0.03	0.1 ± 0.03	0.2 ± 0.03
Total Pulse Frequency (Total pulses/5 hours)	2.8 ± 1.02	4.8± 1.11	3.4 ± 0.75	4.0 ± 1.30
Pre-Treatment - Pulse Frequency (No. of pulses/two hours)	2.0 ± 0.71	0.6 ± 0.40	0.2 ± 0.2	0.4 ± 0.40
Treatment – Pulse Frequency (No. of pulses/two hours)	0.6 ± 0.24^{a}	3.4 ± 0.51^{b}	3.2 ± 0.86^{ab}	3.0 ± 0.83^{ab}
Post Treatment – Pulse Frequency(Pulses/hour)	0.8 ± 0.37^{a}	4.2 ± 0.80^{b}	3.2 ± 0.86^{ab}	3.6 ± 1.03^{ab}
Pulse Amplitude (pg/ml)	0.5 ± 0.28	0.4 ± 0.10	0.9 ± 0.46	1.0 ± 0.22
Inter pulse Interval (minutes)	6.8 ± 2.23	4.9 ± 1.03	7.4 ± 3.12	3.7 ± 1.12

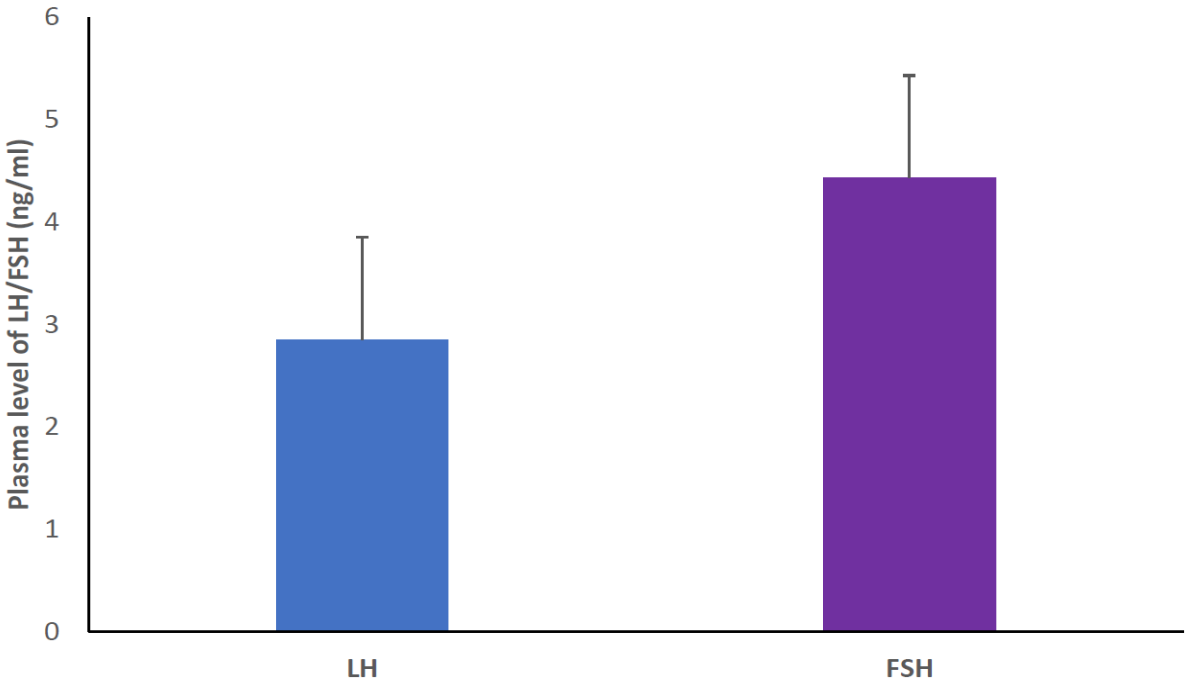

Figure 5. Plasma LH and FSH levels in OVEX + E_2 Sprague-Dawley rats (n = 12). Plasma was harvested from trunk blood obtained 21-days post-ovariectomy and estradiol implantation.

Effects of LSE on Hematological and Metabolic Indices

Mean red blood cell (RBC) count, hematocrit (HCT) and hemoglobin concentration ranged between 9.6 ± 0.15 (M/µL), 52.4 ± 0.7 % and 16.3 ± 0.2 (g/dL), respectively in Control rats between week 6 and week 8 after LSE administration. Ingestion of LSE at 15% for 6 or 8 weeks had no effect on HCT, RBC count and Hemoglobin concentration (Figure 6). Further, no significant effect of LSE ingestion on plasma glucose concentration (Figure 7) or BUN (Figure 8) was observed. However, plasma creatinine concentration decreased significantly ($P < 0.0001$) overtime, although there was an insignificant treatment by time interaction (Figure 8).

There was no effect of short-or long-term LSE ingestion on MCV, MCH, and MCHC (Table 10). LSE ingestion for 8 but not for 6 weeks significantly ($p = 0.045$) reduced total

protein and albumin levels but both short- and long-term LSE ingestion reduced plasma globulin levels (Table 12).

There was no effect of LSE supplementation on alanine transaminase (ALT) and aspartate transaminase (AST). Although there was an insignificant increase in alkaline phosphatase (ALP) level in LSE-fed rats in the short-term, overall, irrespective of treatment, ALP levels were significantly higher ((P = 0.005) after 8 weeks of either normal chow or LSSP feeding (Table 11).

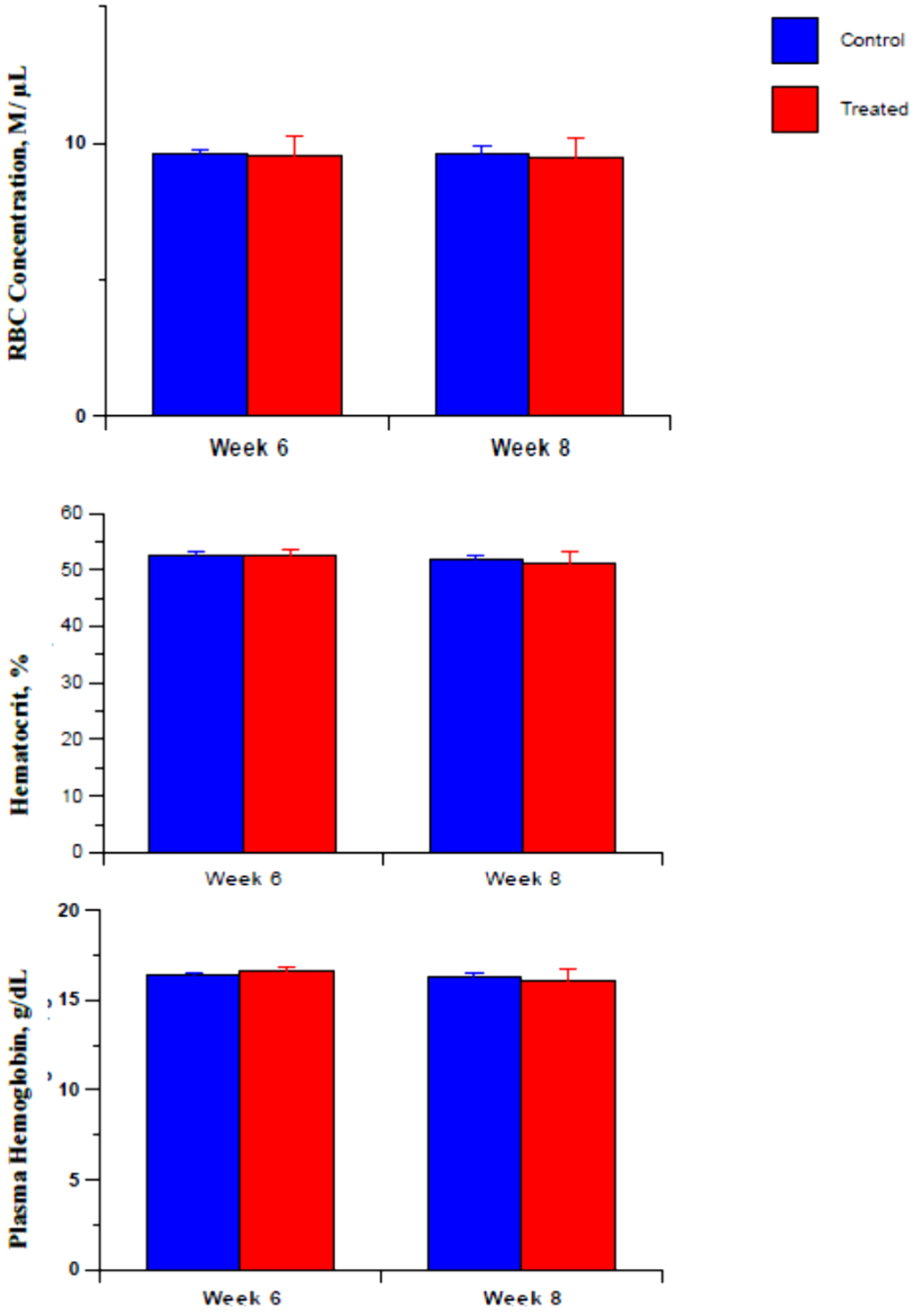

Figure 6. Effect of 15% dietary feeding of LSSP for week 6 and 8 on RBC (top panel), hematocrit (middle panel), and hemoglobin (bottom panel) levels in male rats.

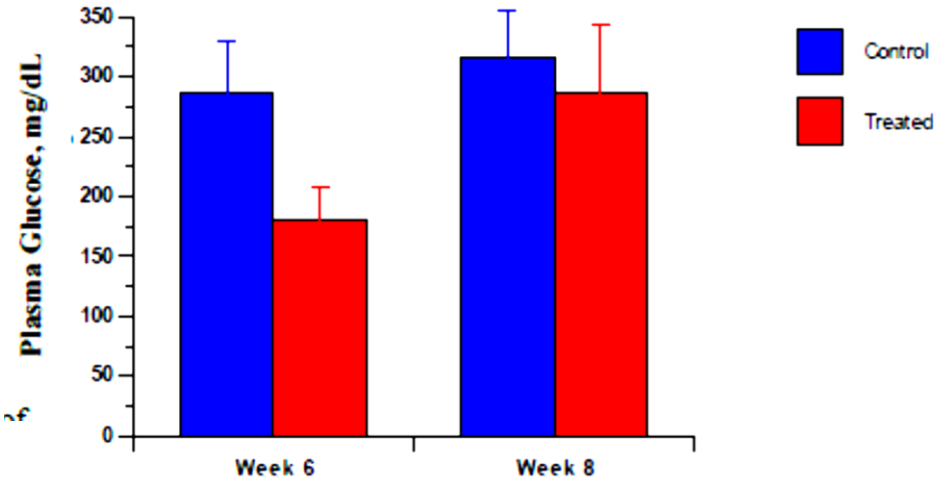

Figure 7. Effect of LSSP supplementation for 6- or 8-weeks on plasma glucose concentration in male Sprague-Dawley rats. Values are Mean ± Standard error.

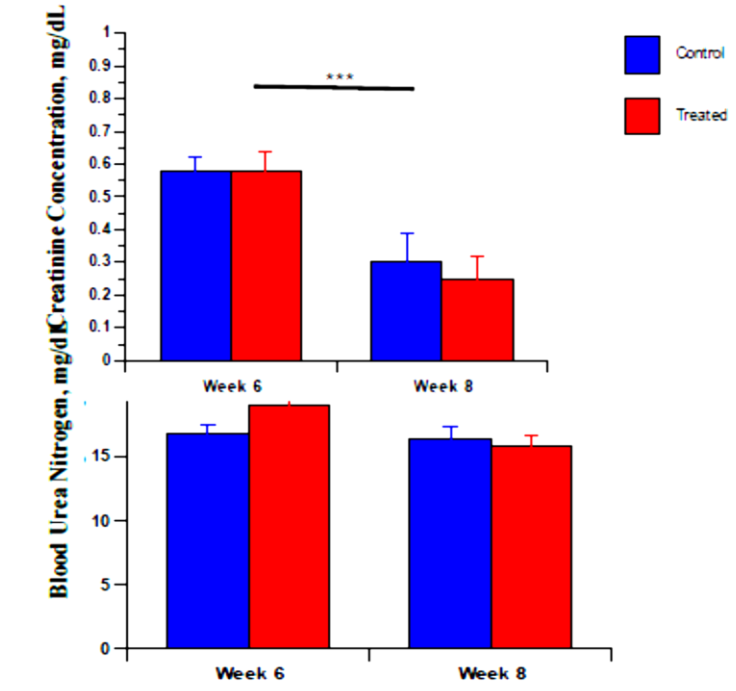

Figure 8. LSSP supplementation and kidney function**.** Mean plasma Creatinine (Top) & BUN (Bottom) concentration in male Sprague-Dawley Control (n = 6) and Treatment (n = 6) rats. ***Significant week effect on creatinine levels, P = 0.0001

Table 10. Effect of 15% LSSP ingestion for 6 (n = 6) and 8 (n = 6) weeks on hematologic profile in male Sprague-Dawley rats. Values are Mean ± S.E.M. No significant difference was observed between groups and duration of feeding.

Week	**MCV(fL)**		**MCH(pg)**		**MCHC(g/dL)**	
	Control	Treated	Control	Treated	Control	Treated
6	54.8 ± 1.33	55.3 ± 1.09	17.0 ± 0.27	17.4 ± 0.22	31.2 ± 0.36	31.6 ± 0.40
8	55.0 ± 1.84	54.0 ±0.86	17.0± 0.32	16.9 ± 0.16	31.1 ± 0.51	31.4 ± 0.61

Table 11. Effect of 15% LSSP ingestion for 6 (n = 6) and 8 (n = 6) weeks on liver enzymes in male Sprague-Dawley rats. Values are Mean ± S.E.M.

Week	**ALP(U/L)**		**ALT(U/L)**		**AST(U/L)**	
	Control	Treated	Control	Treated	Control	Treated
6	1.7 ± 0.42*	59.7 ± 37.15*	59.7 ± 4.90	59.7 ± 7.23	137.8 ± 4.46	151.5 ± 25.1
8	157.2 ± 18.19**	144.8 ± 13.97**	104.8 ± 38.46	88.5 ± 45.89	137.8 ± 22.4	99.7 ± 17.45

*,**Numbers within same column row with different superscripts are significantly different,
** = P = 0.005

Table 12. Effect of 15% LSSP ingestion for 6 (n = 6) and 8 (n = 6) weeks on plasma total protein, albumin and globulin levels in male Sprague-Dawley rats. Values are Mean ± S.E.M. Numbers within a row with different superscripts are significantly different.

Week	**Total Protein (g/d L)**		**Albumin (g/d L)**		**Globulin (g/d L)**	
	Control	Treated	Control	Treated	Control	Treated
6	6.63 ± 0.10^{a}	6.58 ± 0.05^{a}	3.33 ± 0.06^{a}	3.43 ± 0.03^{a}	3.30 ± 0.07^{a}	3.15 ± 0.04^{b}
8	6.64 ± 0.45^{a}	5.57 ± 0.32^{b}	3.12 ± 0.24^{a}	2.53 ± 0.20^{b}	3.52 ± 0.23^{a}	3.03 ± 0.13^{b}

^{a,b}Trt effect, $P < 0.05$

Chapter VI: Discussion

The aim of this study was to determine the effect of LSE on hypothalamic and pituitary hormone secretion of GnRH and gonadotropins as well as the effect of ingestion of LSSP at 15% on hematological and metabolic indices in Sprague-Dawley rats. We have demonstrated that pulsatile secretion of GnRH and gonadotropins is inherent in hypothalamic and adenohypophyseal explants obtained from ovariectomized, estrogen implanted female rats. Our results further indicate that LSE administration potently stimulated GnRH and gonadotropins secretion. There was no effect of LSE administration on the pulse frequency of GnRH, LH and FSH but LSE significantly increased GnRH pulse amplitude only. The observed pulsatile secretion of GnRH and gonadotropins in hypothalamic and pituitary explants is in line with the result of Tsutsumi and Webster (2009) and Messager et al. (2005) in-vivo and Purnelle et al. (1997) in-vitro.

To our knowledge, no previous work has evaluated the effect of LS extracts on the pulsatility of GnRH and gonadotropins secretion. Limited studies have reported the effect of herbal extracts on the secretion of gonadotropins.

Phytochemical screening of the methanolic extracts of LS seed revealed the presence of sterols (Chatoui et al., 2016). Similarly, Sharma et al. (2012) and Yadav et al. (2011) reported the presence of phytoestrogen in LS seeds. Phytosterol in *Lepidium sativum* has been reported to stimulate gonadotropins secretion in ovariectomized, estradiol-implanted Chinchilla rabbits (Imade et al., 2018) presumably by mimicking the effects of endogenous estrogen which then trigger ERs, thereby resulting in gonadotropins secretion. Similarly, Jashni, Jahromi, Ranjbary, Jahromi, and Kherameh, 2016 reported significant increased levels of GnRH and gonadotropins

in female Wistar rats fed aqueous extract of Asparagus root. Steroid saponins are most likely the estrogenic component extracted from asparagus roots known to stimulate hypothalamic pituitary-gonadal axis hormones secretion.

Our findings are consistent with previous studies showing that increased secretory response of LH and FSH may be ascribed to phytosterol constituent of LS extracts which mimic estrogen structurally and functionally (WocBawek-Potocka et al., 2013). We speculate that phytosterol content of LS extract bind either with ERs and exerts stimulatory effects on GnRH secretion by acting directly on the GnRH neurons, which express functional estrogen receptors or indirectly via estrogen-sensitive interneurons such as GPCR–KiSS peptin neuronal signaling pathway (Krsmanovic et al., 2009; Messager et al., 2005; Tena-Sempere, 2006; Tsutsumi & Webster, 2009).

The primary effects of estrogen on GnRH secretion is mediated by estrogen receptor alpha (ERα) through KNDy neurons which release neurokinin B (NKB), dynorphin and kisspeptin (Navarro, 2012). Kisspeptin neurons in the hypothalamus are thought to relay information about estrogen status directly to GnRH neurons. Kisspeptin, which is coded by the *Kiss1* gene, is expressed in the arcuate (Arc), a nodal point in negative feedback regulation of GnRH and gonadotropins secretion (Gottsch et al., 2009).

NKB and dynorphin regulate the secretion of kisspeptin. NKB is the stimulating peptide which initiates the pulsatile release of GnRH by activating TACR3 NKB receptors, on mutually connected KNDy neurons to release kisspeptin in an autocrine signaling pathway. Kisspeptin then activates the GPR54 receptors on GnRH neurons inducing the pulsatile release of GnRH. Eventually the pulse is terminated by dynorphin which acts on κ-opioid receptors (KOR) in

KNDy neurons to inhibit NKB and kisspeptin secretion and inhibits GnRH secretion acting directly on GnRH neuron receptors.

Estrogen damages hypothalamic beta-endorphin containing neurons (Desjardins, Brawer, & Beaudet, 1993) by the conversion of estradiol to catechol estrogen and subsequent oxidation to o-semiquinone free radicals. Therefore, the high level of estrogen observed in the current study, together with the phytoestrogen activity of the LSE may have suppressed β-endorphin levels and therefore precipitated an increase in GnRH synthesis and secretions through the direct stimulation of metabotropic glutamate receptors (mGluRs), and initiation of second messenger signaling (Gu & Moss, 1996) involving the activation of adenylyl cyclase. Increased concentrations of cyclic AMP activated PKA, which catalyzes phosphorylation of cyclic AMP response element binding (CREB) protein ultimately lead to increased GnRH production (Boulware et al., 2005; Mermelstein, 2009).

The results of current study indicate a stimulatory effect of LSE on hypothalamic and pituitary reproductive hormone secretion. Since the hypothalamic and pituitary fragments were perifused separately in vitro, our results suggest that the effect of LSE on gonadotropins secretion from the pituitary is independent of the secretion of GnRH from the hypothalamus. Therefore, LSE effect is manifested directly at the level of both the hypothalamus and pituitary. This rhythmic pattern of GnRH release seems to be Ca^{2+} dependent and mediated by increase in cytosolic calcium levels. During cell activation, mitochondria play an important role in Ca^{2+} homoeostasis due to the presence of a fast and specific Ca^{2+} channel in its inner membrane, the mitochondrial Ca^{2+} uniporter. This channel allows mitochondria to buffer local cytosolic Ca^{2+} changes and controls the intramitochondrial Ca^{2+} levels. A local rise in intracellular calcium is

associated with neurotransmitter and neurohormonal secretions. Natural plant flavonoid has been reported to directly activate the mitochondrial calcium uniporters (Montero et al.,2004). High Ca^{2+} has been shown to stimulate the activity of key metabolic enzymes resulting in raised energy production for the stimulated cell.

The other possibility of increased GnRH secretion is that GnRH neurons themselves do not express ER alpha (Hrabovszky et al., 2000). Therefore, they are not able to properly respond to changes in the sex steroid milieu, which constitute a major regulatory drive to the gonadotropic axis. Indeed, in the absence of negative feedback of sex steroids after ovariectomy, GnRH pulses are significantly increased (Gore. Windsor-Engnell, & Terasawa, 2004).

The result gathered indicate that the hypothalamus and pituitary explants are capable of secreting GnRH and gonadotropins in pulsatile manner as observed during the baseline period in Control and all treatment groups and further LSE administration at all doses significantly increased the secretion of all three hormones. Further, the administration of KCl had no significant effect on the release of GnRH in the last hour of experiment. Potassium chloride (KCl) depolarizes cell membranes by changing the ion concentration and creating an electrical gradient, and in that way is able to open Ca^{2+} channels, ultimately inducing massive release of GnRH. In our hands however, KCl administration did not induce any further release of GnRH. This may be due to depletion of the GnRH pool or an ineffective dose of KCl. Likewise, GnRH challenge of pituitary explants post aCSF or LSE administration had no further effect on pituitary gonadotropins secretion. Future studies should investigate the use of a higher concentration of KCl and GnRH to stimulate the further release of GnRH and gonadotropins.

The presented data demonstrate that the effect of LS extract on the secretion of these hormones are believed to be mimicking that of estrogen by acting directly or indirectly on the hypothalamus and the pituitary. LS extract containing phytosterol, may effectively modulate GnRH and gonadotropins secretion in OVEX+E_2 implanted rats by acting directly on CNS. In future research it is crucial to fractionate the LS extract to identify specific types of phytosterols present in LS to better understand the mechanism by which LS exert its effect on reproduction. There are very few reports in the literature regarding the safety of LS seeds as to what constitutes a pharmacologic inclusion level and the potential deleterious effects of LSSP on hematological profile and metabolic indices. At two percent level LS effect was non-toxic, but at ten percent effect was toxic and non-fatal while lethal at 50% (Adam, 1999). In the current study, we determined the effect of 15% LS seed supplementation of normal rat chow on the hematological and serobiochemical parameters in male Sprague–Dawley rats.

Our research result revealed that the values of hematological indices fall within safety limit (Datta et al., 2011) who evaluated the effect of different levels of LS seeds feedings and found absence of significant treatment effect between control and LS seeds fed groups.

Significant increase in Alkaline phosphatase (ALP) levels over the duration of feeding of either regular chow or LSSP may indicate the positive effect of the presence of isothiocyanates present in LS seeds. Isothiocyanates in LS seeds (Virtanen & Saarivirta, 1962; Burow & Bergner, 2007) could be responsible for high ALP levels in rats. Therefore, the changes observed in serum chemistry may not be of toxicological significance as isothiocyanates are beneficial compounds present in Cruciferae family which are shown to be potent inducers of phase II detoxification enzymes involved in metabolism of xenobiotics (Munday & Munday, 2004).

Similar results are reported by Datta et al. (2011) of elevated serum ALP at a 10% level of LS and Adam (1999). Similarly, Bafeel and Ali (2009) observed significant increase in serum ALP levels. Likewise, in broilers fed LS seed inclusion (Shawle, Urge, & Animut, 2016) showed higher ALP activity.

In our study, plasma creatinine concentration decreased significantly overtime but LSE ingestion for 8 but not for 6 weeks significantly reduced total protein and albumin levels and both short- and long-term LSE ingestion reduced plasma globulin levels. This is in agreement with the results of Adam (1999) who reported that both the short and long-term LS feeding significantly lowered total protein content and albumin among the 10% and 50% treatment groups. Differences in our study and other previous studies with respect to the LSSP feeding on hematological characteristics and liver enzymes could be due to the difference in the route of LSSP administration, suspension of seed extract and duration of feeding.

In conclusion, our results indicate a direct effect of LSE on the stimulation of gonadotropin secretion directly at the level of the pituitary. The exact mechanism of action of LSE in this regard is not clear, although a possibility remains a direct effect on the flavonoid in the LSE on the release of Calcium from the mitochondria (Montero et al., 2004).

We did not observe any effect of LSE administration on the pulse frequency of GnRH or the gonadotropins (Misztal, Gorski, & Romanowicz, 2008) but we observed that GnRH pulse amplitude was enhanced by LSE administration.

Ingestion of LS seeds at a 15% level in the diet was well tolerated and had no deleterious effects on hematological and metabolic indices in the rat.

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