Effect of Flaxseed on some hormonal profile and genomic DNA concentration in Karadi lambs

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Abstract. The aims of the current study were to investigate the effect of dietary supplementation of flaxseed powder on some hormonal profile and genomic DNA concentration of Karadi male lambs. Twelve healthy Karadi ram lambs, 6 months old, were randomly divided into 3 equal groups (4 lambs/group); and the treatments were as follow for two months; 0% considered as control group, 4% as a low level (T1), and 8% of flaxseed as a high level (T2) in their feed. Blood samples were collected from each ram lambs every two weeks, serum was separated immediately after sampling, testosterone, estradiol, growth hormone (GH), Thyroid Stimulating Hormone (TSH), and Thyroxine (T4) were measured. Genomic DNA was extracted from each samples. The results showed that testosterone hormone concentration decreased significantly in T2 group (1.575±0.004 n.mol/l) in comparison to control group (6.95±1.992 n.mol/l). Estradiol hormone level significantly increased in T1 (293±4.2 P.mol/l) in comparison to control and T2 (128.5±5.485 and 139±8.083 P.mol/l) respectively. A positive significant effect of flaxseed recorded on growth hormone, which its level increased in T2 group (120±2 ng/ul) in comparison to T1 group (81.5±1 ng/ul). TSH concentration increased significantly in T1 and T2 groups (13.5±2 and 14.5±1 U/ml, respectively) in comparison to control group (7±1.5 U/ml), while T4 hormone was not affected. Flaxseed supplementation caused a significant decrease in Genomic DNA concentration in T2 group (56.2 ng) in comparison to control (28.7 ng). In conclusion, adding flaxseed in lamb rations caused a significant decrease of testosterone, increased estradiol concentration, also each of growth hormone and TSH hormones increased significantly, T4 not affected. This decrease in genomic DNA concentration with flaxseed supplementation may due to one of the mechanisms by which phytoestrogens may influence reproductive physiology that involve in inhibition of enzymes essential for DNA replication.

1. Introduction

Research continues to expand the use of flaxseed for both animal health as well as to develop healthier animal products for humans. The botanical name of flax, *Linum usitatissimum* of the family Linaceae, recognizes its usefulness for a variety of purposes, not only as a food source. The terms “flaxseed” and “linseed” are often used interchangeably, although North Americans use “flaxseed” to describe flax when it is eaten by humans and “linseed” to describe flax when it is used for industrial purposes.
Flaxseed is a very high content of alpha linolenic acid (omega-3 fatty acid) essential for humans; it has a high percentage of dietary fiber, both soluble and insoluble, and finally it is the richest source of plant lignans [1]. Lignans are phytoestrogens. Phytoestrogens are non-steroidal compounds present in plants. These compounds are structurally similar to natural estrogens, such as 17β-estradiol, allowing them to bind with estrogen receptors and thereby to induce biologically detectable effects [2]. Although phytoestrogens can mimic the effects of estradiol (E2), their effects are not necessarily identical, since phytoestrogens can produce estrogenic or anti-estrogenic effects [3]. These compounds compete with endogenous steroids, so the balance between estrogenic and anti-estrogenic activity is determined by the phytoestrogen-estrogen ratio [4]. This might explain why estrogenic effects of phytoestrogens predominate in livestock, whose estradiol plasma concentrations are relatively low (15 pg/ml). In comparison, anti-estrogenic effects are reported mainly in humans, in which estrogen plasma levels are relatively high (50-400 pg/ml) [5].

Flaxseed are the primary source of phytoestrogens (379,012.3 ug/100 ml) [1; 6]. Interest in the effects of phytoestrogens on male fertility has increased in recent years as it has been demonstrated that estrogens play an important role in the male reproductive system [7]. Although there are only few reports, it has been shown that phytoestrogens also cause reproductive disruption in males. In bovines, ingestion of fodder with high quantities of coumestrol causes glandular metaplasia in both prostate and bulbourethral glands [8], gynecomastia and even galactorrhea [9]. In male animal models, phytoestrogens are capable of causing disruptive effects on reproductive parameters. Experimental evidence indicates that long term administration of daidzein to male rats causes a decrease in plasma levels of testosterone as well as erectile dysfunction in a similar way to E2; and these effects are possibly mediated through estrogen receptor (ER) [10]. Similar reduced testosterone levels have been obtained in adult male rats exposed to a high-dose of coumestrol [11]. This decrease in serum testosterone is due to decreased steroidogenesis in testicular Leydig cells caused by isoflavones [12].

The mechanisms by phytoestrogens may influence reproductive physiology may involve: a) genomic effects on ERα, ERβ and other nuclear receptors (P4, androgen), b) inhibition of enzymes involved in steroidogenesis, such as 3β- and 17β-hydroxysteroid dehydrogenase, aromatase; c) stimulation of sex hormone-binding globulin; d) inhibition of protein tyrosine kinase, important for signal transduction; e) inhibition of DNA topoisomerases I and II, enzymes essential for DNA replication; and f) antioxidant activity [13; 14] and may also work through epigenetic mechanisms involving both hyper and hypomethylation [15; 16].

Finally, the experimental evidence obtained from research in animal models should be considered to evaluate the disrupting effects Flaxseed by phytoestrogens in the reproductive physiology of livestock. It should be considered the possibility that some of the reproductive problems observed in those animals could be caused by the consumption of phytoestrogens and micoestrogens in fodder. In the first step in Karadi lamb, we have designed this project to evaluate the potential effect of flaxseed powder on some hormonal parameters and genomic DNA concentration.

2. Materials and Methods

2.1 Animals, Experimental Design, and Diets:
Twelve healthy Karadi ram lambs, 6 months old, were used in this study at the animal fields, department of Animal Science, College of Agricultural Engineering Sciences, University of Sulaimani, Sulaimani city, Iraq. The lambs were randomly divided into 3 equal groups (4 lambs/group) to receive 3 different levels of flaxseed powder; 0% considered as control group, 4% as a low level (T1), and the last group was received 8% of flaxseed as a high level (T2) in their feed till the end of the experiment (which lasts two months with 2 weeks as adaptation before starting the experiment).
All the lambs were received an equal daily allowance of concentrate ration (3% of the body weight). The Formulation and chemical composition of concentrate diets are presented in (Table 1). The lambs were randomly penned individually indoors on dry earth bedding and the concentrate ration was
supplied once daily (9:00 am). The straw was given *ad libitum*. Daily feed intake and refused were measured and sampled for 8 weeks. The lambs were weighed once weekly from the beginning till the end of the experiment.

2.2 Blood sample collection and DNA extraction:
Blood samples were collected from each ram lamb every two weeks during the experimental period (2 months). The samples were collected before the morning feeding from the jugular veins into 10 ml Vacutainer tubes containing the anticoagulant (EDTA). The serum was separated immediately after sampling by centrifugation at 3,000 rpm for 15 min. Serum samples were analyzed for: Growth Hormone (GH), Thyroid Stimulating Hormone (TSH), Thyroxine (T4), Testosterone Hormone, and Estradiol, were measured by radioimmunoassay (RIA) using commercial kits purchased from local market.

DNA was extracted from each of the blood samples using QIAamp® DNA Blood Mini Kit (QIAGEN GmbH Qiagenstr.1 40724 Hilden Germany) according to the manufacturer’s manual. The quantity of DNA was checked and the quantification was done by Nanodrop spectrophotometer.

2.3 Statistical Data Analysis
Data were analyzed statically by using XLSTAT 2017 software (CRD). Significance was determined at (P<0.05), for the differences between the three levels of Flaxseed (0, 4 and 8 % of basal diet), when a significant interaction identified, Duncan multiple range program was used for the mean comparisons, all the data are presented as means ± SE.

Table 1. Formulation and chemical composition of concentrate diets

| Ingredients (%) | Control | T1 | T2 |
|-----------------|---------|----|----|
| Flaxseed        | 0       | 4  | 8  |
| Barley          | 41      | 37 | 33 |
| Wheat           | 30      | 30 | 30 |
| Yellow Corn     | 15      | 15 | 15 |
| Soybean meal    | 12      | 12 | 12 |
| Salt            | 1       | 1  | 1  |
| Minerals and vitamins | 1 | 1 | 1 |

**Chemical Composition**

| Crude protein (CP) % | 15.72 | 16.19 | 16.66 |
| Metabolized Energy (ME) | 12.47 | 12.42 | 12.37 |

*Calculated metabolized energy ME (MJ/ kg DM) and CP representing two components of the feeds from the tables of chemical analysis of the Iraqi feed materials [17], except ME of Flaxseed calculated as stated by [18].

3. Results and Discussion:
Flaxseed has high levels of energy and protein and promotes feed intake and weight gain [19], the results of the current study showed adding flaxseed powder to the lambs rations doesn’t have a significant effect (P<0.05) on the live body weight under the condition of the current experiment, although the lambs increased in their weights but the statistical analysis showed that doesn’t belong to the effect of the treatments (low and high levels of ground flaxseed) data is not shown.

3.1. Testosterone: is secreted primarily by the testicles of males and, to a lesser extent, the ovaries of females. On average, in adult males, levels of testosterone are about 7 to 8 times as great as in adult females [20] As the metabolism of testosterone in males is more pronounced, the daily production is about 20 times greater in men [21], [22] Females are also more sensitive to the hormone [23]. Testosterone is the primary male sex hormone and an anabolic steroid. In male humans,
testosterone plays a key role in the development of male reproductive tissues such as testes and prostate, as well as promoting secondary sexual characteristics such as increased muscle and bone mass, and the growth of body hair [24]. The result of the current study showed adding flaxseed powder supplementation caused gradually decrease of the concentration of testosterone hormone with increasing the time of exposure and the amount of flaxseed, at the 8 weeks of exposure, testosterone concentration recorded its significant lowest level (P<0.05) in T2 (1.575±0.004 n.mol/l) in comparison to control group (6.95±1.992 n.mol/l) table (2), same results have recorded by [10], they found that long term administration of daidzein to male rats causes a decrease in plasma levels of testosterone as well as erectile dysfunction in a similar way to E2; and these effects are possibly mediated through ER. Similar reduced testosterone levels have been obtained in adult male rats exposed to a high-dose of coumestrol [11]. This decrease in serum testosterone is due to decreased steroidogenesis in testicular Leydig cells caused by isoflavones [12]. Long term dietary administration of genistein in rats, suppresses the steroidogenic response of Leydig cells to hCG and cAMP by down regulating the expression of the cytochrome P45017α-hydroxylase/C17-20 lyase enzyme (P450scc), which initiates steroidogenesis in these cells [25] reducing serum levels of testosterone and androstenedione [26]. A high phytoestrogen diet in male rats can also block spermatogenesis, induce germ cell apoptosis [27], and decrease the expression of ERs and AR in the cauda epididymis as well as increase lipoperoxidation in epidydimal sperm. These effects are possibly mediated by disruption of the steroid regulation of the epididymis, resulting in decreased quality of sperm, and thereby reducing fecundity [28]. On the other hand, chronic dietary exposure to genistein in combination with vinclozoline (a fungicide considered an endocrine disruptor) reduces sperm count and motility, litter size and increased post-implantation loss [29]. In vitro studies show that mouse and human spermatozoa exposed to genistein and daidzein in different doses, alone or in combination, accelerate capacitation [30] and acrosome loss, which may possibly impair fertility [31]. Perinatal exposure to phytoestrogen diets can also decrease steroidogenesis and androgen secretion by testicular Leydig cells in the adult rat [12].

3.2. Estradiol: is an estrogen sex hormone and the major female sex hormone, it is responsible for the development of female secondary sexual characteristics, its levels in men are much lower compared to those in women, estradiol has important roles in men as well. Estradiol is produced especially within the follicles of the ovaries, but also in other tissues including the testicles, the adrenal glands, fat, liver, the breasts, and the brain. It is produced in the body from cholesterol through a series of reactions and intermediates [32]. The effect of estradiol (and estrogens in general) upon male reproduction is complex. Estradiol is produced by action of aromatase mainly in the Leydig cells of the mammalian testis, but also by some germ cells and the Sertoli cells of immature mammals [33] It functions (in vitro) to prevent apoptosis of male sperm cells [34] Suppression of estradiol production in a subpopulation of sub-fertile men may improve the semen analysis [35]. As it is illustrated in table (2) estradiol concentration reached its highest significant level (P<0.05) by adding 4% (T1) flaxseed as a source of phytoestrogen to the lambs diet, that is mean this amount is enough to raise estradiol level in lambs body, This result is in agreement with [36] which recorded increased oestrogen level with diet containing flaxseed oil.

Phytoestrogens have lower affinity for estrogen receptors (ER) than estradiol (E2), and most of them exhibit a higher affinity for ERβ than for ERα [37; 38] by approximately 30 fold. Other phytoestrogens, such as resveratrol, bind to ERβ and ERα with comparable affinity, but with a 7,000-fold lower activity than E2 [39]. In comparison, E2 recruits the co-regulators of both types of receptors in a nonselective way [40]. The ligand-receptor complex generated is capable of inducing transcriptional activity [41]. However, the concentration required for isoflavones (genistein, daidzein, glicetin and equol) to induce transcriptional activity is 104 higher than E2, and their activity is lower than that of the steroid. This lower transcriptional activity of phytoestrogens is offset by their higher bioavailability, since the free circulating fraction is more than 50%, compared with the 4.5% for E2. Furthermore, phytoestrogen circulating levels are one order of magnitude higher than those of E2 (ng/ml versus pg/ml). This greater accessibility to ERs explains why in the presence of endogenous estrogens, isoflavones behave as
estrogenic antagonists, while in the absence of estrogens they behave as weak agonists [2]. In addition to the interaction with ERs, phytoestrogens may also modulate the concentration of endogenous estrogens by binding or inactivating some enzymes, such as P450 aromatase, 5α-reductase, 17β-hydroxyysteroid dehydrogenase (17β-OHDH), topoisomerases, and tyrosine kinases.

3.3 Growth hormone: is a protein hormone that is synthesized and secreted by somatotrophs in the anterior pituitary. It participates in growth and metabolism, growth hormone binds to its receptor on target cells, Fat cells (adipocytes), it stimulates them to break down triglyceride and suppresses their ability to take up and accumulate circulating lipids. Also a majority of the growth promoting effects of growth hormone is actually due to insulin-like growth factor-I (IGF-I), a hormone that is secreted from the liver and other tissues in response to GH, acting on its target cells. We have previously recorded that flaxseed supplementation caused significantly (P<0.05) decrease in fat percentages and improvement of carcass traits in Karadi ram lamb meat [42], so the reason of that result may due to the effect of flaxseed supplementation on increasing the secretion of GH, as shown in the result of the current study table (3), which GH secretion in T1 and T2 groups stayed at a high level during the whole experimental periods in comparison to control group, even the differences were not significant at the three first periods (2, 4, 6 weeks) but it reached a significant (p<0.05) higher level in T2 group (120±2 ng/ul) at the end (8 weeks) of the experiment in comparison to T1 group (81.5±1 ng/ul) table (3), which means with the increasing of the ratio flaxseed level and time of exposure, GH secretion has increased also, this may give an evidence that flaxseed supplementation causes increasing of GH secretion. It was reported that isoflavones in flaxseed could affect the growth and metabolism in animals. [43] Found Da (3 mg kg-1 BD) could promote the growth of chest and femoral muscle and significantly increase the RNA to DNA ratio in male broilers. [44] Demonstrated that Da or ipriflavone (3 mg kg-1 BD) could facilitate the skeletal muscle growth. The positive physiological effect of SDG on the growth of skeletal muscles could be similar to that of isoflavones. The myofibrillar formation included following steps [45; 46] satellite cells differentiated to sarcoblast, magnanimous sarcoblasts conjugated to form a myotubule, myoneme transformed to myofilament, and the muscle fiber formed.

**Table 2.** Effect of flaxseed powder on testosterone and estradiol hormones of Karadi ram lambs.

| Hormones        | Periods (8 weeks) | 2     | 4     | 6     | 8     | 2     | 4     | 6     | 8     |
|-----------------|-------------------|-------|-------|-------|-------|-------|-------|-------|-------|
|                 | C     | T1    | T2    | C     | T1    | T2    | C     | T1    | T2    |
| Testosterone    | 0.925 | 4.625 | 3.6   | 2.515 | 4.2   | 2.946 | 4     | 3.4   | 1.748 |
| (n.mol / L)     | ± ±    | ± ±    | ± ±    | ± ±    | ± ±    | ± ±    | ± ±    | ± ±    | ± ±    |
|                 | a a    | a a    | a a    | a a    | a a    | a a    | a a    | a a    | a a    |
| Estradiol       | 151   | 113.5 | 151   | 147.4 | 148   | 145   | 140   | 220   | 134.8 |
| (P.mol / L)     | ± ±    | ± ±    | ± ±    | ± ±    | ± ±    | ± ±    | ± ±    | ± ±    | ± ±    |
|                 | a a    | a a    | a a    | a a    | a a    | a a    | a a    | a a    | a a    |
| Results are represented as mean ± MSE, values in rows not sharing the same letter are different at the (P<0.05) level of significance, based on the Duncan Multiple Range.
Table 3. Effect of flaxseed powder on GH, TSH and T4 hormones of Karadi ram lambs.

| Hormones | Periods (8 weeks) | 2 | 4 | 6 | 8 |
|----------|-------------------|---|---|---|---|
| GH (ng/ul) | C | T1 | T2 | C | T1 | T2 | C | T1 | T2 | C | T1 | T2 |
|           | 30 ±1.9 a | 56.5 ±1 a | 59 ±1.7 a | 50 ±1 a | 61.5 ±2 a | 74 ±1.4 a | 75±3 a | 71.5 ±2 a | 85 ±9 a | 100 ±6 ab | 81.5 ±1b | 120 ±2a |
| TSH (u/ml) | 13.5 ±1a | 28 ±1a | 28 ±2.9 a | 12.5 ±1 a | 22.5 ±0.8 a | 25.5±0.8 a | 12 ±1 a | 19 ±1 a | 19 ±4 a | 7 ±1.5b | 13.5 ±2 a | 14.5 ±1 a |
| T4 (n.Mol/L) | 123.2 ±10.392 a | 132.9 ±0.13 a | 112.7 ±7.910 a | 126.5 ±6.640 a | 131.5 ±0.68 a | 117 ±4.041 a | 138 ±7.217 a | 123.75 ±6.34 a | 117.5 ±4.330 a | 146.55 ±15.906 a | 124.4 ±6.8 a | 118.5 ±11.662 a |

Results are represented as mean ± MSE, values in rows not sharing the same letter are different at the (P<0.05) level of significance, based on the Duncan Multiple Range.

3.4. Thyroid Stimulating hormone (TSH): (with a half-life of about an hour) stimulates the thyroid gland to secrete the hormone thyroxine (T4), which has only a slight effect on metabolism. T4 is converted to triiodothyronine (T3), which is the active hormone that stimulates metabolism. About 80% of this conversion is in the liver and other organs, and 20% in the thyroid itself [47].

TSH is secreted throughout life but particularly reaches high levels during the periods of rapid growth and development, as well as in response to stress. The hypothalamus, in the base of the brain, produces thyrotropin-releasing hormone (TRH). TRH stimulates the anterior pituitary gland to produce TSH. Somatostatin is also produced by the hypothalamus, and has an opposite effect on the pituitary production of TSH, decreasing or inhibiting its release.

The concentration of thyroid hormones (T3 and T4) in the blood regulates the pituitary release of TSH; when T3 and T4 concentrations are low, the production of TSH is increased, and, conversely, when T3 and T4 concentrations are high, TSH production is decreased. This is an example of a negative feedback loop [48]. In the present study TSH levels were non significantly decreased gradually in all three groups during the first three time points (2, 4, 6 weeks) when it reached the end of the study (8 weeks) TSH level was significantly (P<0.05) increased in T1 and T2 groups (13.5±2 and 14.5±1 U/ml) respectively, in comparison to control group (7±1.5) as shown in table (3).

3.5. Thyroxine (T4) hormone: recorded no significant (P<0.05) differences among all three groups studied in this experiment, but as it is mentioned above when T3 and T4 concentrations are low, the production of TSH is increased, and, conversely, when T3 and T4 concentrations are high, TSH production is decreased as illustrated in table (3). This result is not agree with [49] which they found ewes fed linseed meal for 14 days have increased concentrations of T4 compared to all other days (0, 1, and 7), which did not differ.

3.6. Genomic DNA concentration: As shown in Figure 1, T2 group (28.7 ng) showed significant (P<0.05) less concentration by %51 in comparison to Control group (56.2 ng), while T1 (45.6 ng) showed %19 less genomic DNA concentration, this reduce in genomic DNA concentration with flaxseed supplementation as a phytoestrogen source may due to one of the mechanisms by phytoestrogens may influence reproductive physiology may involve inhibition of DNA topoisomerases I and II, enzymes essential for DNA replication [13; 14]. To our best knowledge, this study is the first to report the effect of flaxseed powder on genomic DNA concentration, this result is not agree with the results obtained by [50], they found that the DNA content in phytoestrogen (SDG- or Da-) treated rats seemed to be higher than that of the control group, and the RNA concentrations were significantly increased by adding phytoestrogens. Another study [49] showed feeding flaxseed meal and/or
implanting ewes with E2 did not alter concentrations of DNA, RNA, or protein, or the RNA: DNA or protein:DNA ratios.

It is now evident that fatty acids, either directly or via its metabolites, act via a great variety of signaling pathways to influence numerous metabolic, inflammatory, and other biological processes. In the past decade, nutrigenomics has provided the ideal conceptual framework and the necessary technological tools to address the global effects of dietary fatty acids and has greatly contributed to a major advancement in our understanding of the molecular action of dietary fatty acids. So far, the focus has been on the molecular characterization of specific signaling routes, coupled with the description of the whole genome effects of dietary fatty acids. In the future, greater emphasis will have to be placed on the functional consequences of specific target gene regulation to fully understand the functional impact of dietary fatty acids and their potentially preventive effect in specific disease conditions. It can be foreseen that nutrigenomics will continue to make a push toward a more mechanistic and genomics-driven approach within the domain of nutritional sciences and further promote the implementation of high-throughput technologies [51].

Health benefits of flax lignans reside in their antioxidant capacity as sequestrators of hydroxyl radicals, and as estrogenic compounds due to their structural similarity to 17-β-estradiol. The antioxidant capacity of SDG is related to the suppression of the oxidant conditions due to oxygen species. SDG diglycoside and its aglycone, secoisolaricresinol display a very high antioxidant capacity and act as protectors against damage to DNA and liposomes – especially in the epithelial cells of the colon exposed to these compounds – during the metabolism of colon bacteria which transform them into mammal lignans [52; 53].

Figure 1. showing effect of flaxseed powder on genomic DNA concentration in Karadi male lambs, different letters show significant differences (P<0.05).

4. Conclusions

The present study shows that dietary supplementation of flaxseed powder resulted in significant (P<0.05) decrease of testosterone hormone, while estradiol had increased by adding 4% flaxseed powder to the diet. Growth hormone and TSH hormones increased significantly (P<0.05), but T4 hormone didn’t change under the condition of this experiment. In addition, genomic DNA decreased significantly (P<0.05), this decrease in genomic DNA concentration with flaxseed supplementation as a phytoestrogen source may be due to one of the mechanisms by which phytoestrogens may influence reproductive physiology that involve in inhibition of DNA enzymes essential for DNA replication.
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