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EXCESSIVE USE OF FLAXSEED MAY POSE A THREAT TO KIDNEY TISSUE: AN EXPERIMENTAL STUDY

KETEN TOHUMUNUN AŞIRI KULLANIMI BÖBREK DOKUSU İÇİN TEHDİT OLUŞTURABİLİR: DENEYSEL BİR ÇALIŞMA

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Öz

Amaç
İyileştirici etkisi nedeniyle yaygın olarak kullanılan keten tohumunun önerilen günlük doz aşıldığında, böbrek dokusu üzerindeki etkilerini araştırmayı amaçladık.

Gereç ve Yöntem
Erkek Wistar Albino cinsi ratlar her grupta 8 adet olacak şekilde 4 gruba ayrıldı. Gruplar: kontrol, OD-1 (1,4 mg/kg/gün), OD-2 (2,8 mg/kg/gün) ve OD-3 (5,6 mg/kg/gün) şeklinde isimlendirildi. Her bir doz aşımı olan gruba, 7 gün boyunca oral gavaj yolu ile öğütülmüş keten tohumu verildi. Deney sonunda kan ve böbrek örnekleri alındı. Kan örnekleri santrifüjlenerek böbrek fonksiyon analizleri için serumlarına ayrıldı. Böbrek örneklerinin yarısı -20 °C'de biyokimyasal analizler yapılana kadar saklandı ve kalan dokular histopatolojik analiz için %10 tamponlu formalin ile fikse edildi.

Bulgular
Serum üre ve BUN seviyeleri, kontrol grubuyla karşılaştırıldığında, doz aşımı olan tüm gruplarda önemli ölçüde arttı (tümü için $p<0.05$). TAS seviyeleri, OD-3 grubunda kontrole göre anlamlı olarak azaldı ($p=0,044$). Tüm aşırı doz gruplarında renal TOS düzeylerinde kontrole göre artış vardı ve ayrıca OSI düzeyi, OD-3 grubunda kontrole göre anlamlı düzeyde arttı ($p=0,016$). OD-1 ve OD-2 gruplarında kontrol grubuna göre histopatolojik değişiklikler gözlenmezken, OD-3 grubunda anlamlı olmayan histopatolojik değişiklikler belirlendi.

Sonuç
Önerilen günlük dozun üzerinde keten tohumu kullanımının böbrek dokusu için tehdit oluşturabileceği kanısına varıldı. Bu nedenle yararı için kullanılırken, toksik etkilerine maruz kalmamak için aşırı ve uzun süreli kullanımdan uzak durulmalıdır.

Anahtar Kelimeler: Böbrek, Doz aşımı, Keten tohumu

Abstract

Objective
We aimed to investigate the effects of flaxseed, which is widely used due to its healing effects, on the kidney tissue when the daily recommended dose is exceeded.

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Material and Methods
We divided the male Wistar Albino rats into four groups of eight, namely the control, OD-1 (1.4 mg/kg/d), OD-2 (2.8 mg/kg/d), and OD-3 (5.6 mg/kg/d) groups. Ground flaxseed was administered to each overdose group by oral gavage for 7 days. At the end of the experiment, blood and kidney samples were collected. Blood samples were centrifuged and serum was separated for renal function analyses. The half of the kidney samples were stored at -20 °C until biochemical analyses and we fixed the remaining tissues with 10% buffered formalin for histopathological analysis.

Results
Serum urea and BUN levels were significantly increased in all overdose groups compared to the control group (for all $p<0.05$). TAS levels significantly decreased in the OD-3 group compared to the control ($p=0.044$). There was an increase renal TOS levels in all overdose groups compared to the control and also, OSI level of the OD-3 group significantly increased compared to the control ($p=0.016$). While histopathological changes were not observed in OD-1 and OD-2 groups compared to the control group, there were non-significant histopathological changes in OD-3 group.

Conclusion
It was concluded that the use of flaxseed above the recommended daily dose can pose a threat to kidney tissue. So, while being used for its benefit, in order not to be exposed to its toxic effects, overuse and long-term use should be avoided.

Keywords: Flaxseed, Kidney, Overdose

Introduction

Herbal medicines have been used for therapeutic purposes since ancient times. With the development of the pharmaceutical industry, plant analyses have increased and many herbal medicines have been produced for use in medicine. Some of them are frequently used in modern medicine (such as acetylsalicylic acid, digoxin, and morphine).

Flax is a plant from the Linaceae family and its botanical name is *Linum usitatissimum*. Its seed is also known as flaxseed or linseed. Its spectrum of use in herbal medicine has increased due to a growing body of scientific evidence about it and has suggested that it may be useful in the treatment of some diseases. Flaxseed is a food with high nutritional value due to its content, therefore it is used as a medicinal product (1). Flaxseed contains a high percentage of fat, most of which is composed of alpha-linolenic acid, which is partially converted to docosahexaenoic acid, omega-3 fatty acids, and eicosapentaenoic acid in the body. It also has a variety of bioactive compounds such as fiber, flax protein, lignans, and cyanogenic glycosides (2, 3). The antimicrobial, antioxidant, and anti-inflammatory effects of flaxseed have been proven in many studies, and thanks to these effects, many diseases are healed in herbal medicine (1, 2). It has been shown to have healing effects on burn wounds, skin, mucosal irritation, and even bone healing (3–5). It is used for the treatment of gastrointestinal system diseases such as reflux, gastritis, enteritis, ulcer, dyspepsia, and intestinal diverticulosis. Due to the fiber and mucilage it contains, it is used in constipation so has a laxative effect (2). In addition, it is mentioned that flaxseed has positive effects on diabetes, cardiovascular disease, arthritis, osteoporosis, neurological disorders, cancer, and various chronic diseases (1, 6, 7).

It is generally consumed by people in 4 common forms; whole flaxseed, flaxseed oil, ground flaxseed, and partially defatted flaxseed meal (6). Although it varies according to the indication, it is recommended to use 50 g of flaxseed as an average daily dietary supplement (6, 8–10).

In addition to these positive effects, it is reported by scientific sources that it also contains some toxins. It contains low amounts of cyanogenic glycosides and is nontoxic (8) but can become toxic in long-term use (11). Cyanogenic glycosides can be converted to toxic hydrogen cyanide when the seeds are ground or crushed. Some commercial flaxseeds have been found to contain high levels of cadmium and are not recommended for long-term use. In addition, when flaxseed is not consumed with enough water, it can cause intestinal obstruction.

In light of this information, we predict that the kidney, which is the elimination organ, may be adversely affected as a result of the unawareness consumption of flaxseed who believe that they will get more benefits by consuming more doses. For this reason, we aimed to investigate the effects of flaxseed, which is widely used due to its healing effects, on the kidney tissue when the daily recommended dose is exceeded.

Material and Method

Flaxseed Product and Diets

Ground full-fat flaxseed was used in this study. It was mixed with distilled water and then mashed with a micro cutter, so finally, we prepared aqueous flaxseed mucilage. Each animal was fed with flaxseed at the determined dose by oral gavage except the control group for seven days. Three gradual overdoses were chosen based on the recommended daily dose (10).

Subjects and Experimental Design

A total of thirty-two adult male Wistar rats (250-300 g) were housed in cages with ad libitum food and water. Rats were maintained in a controlled temperature (22 ± 2 °C) environment with a 12 h light/dark cycle and all experiments were conducted during the light cycle. All procedures were arranged in accordance with animal welfare. The study was approved by the Local Ethical Committee for Experimental Animal Ethics of Suleyman Demirel University (Protocol Number: 15.09.2022-06/86) and conformed to the Experimental Animals Research Laboratory and the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines and institutional guideline.

Thirty-two rats were divided into four groups randomly, each of 8 animals:

Group 1: Control (C): Rats were not treated with ground flaxseed.

Group 2: Overdose-1 (OD-1): Rats received 1.4 mg/kg/d ground flaxseed by peroral for 7 days.

Group 3: Overdose-2 (OD-2): Rats received 2.8 mg/kg/d ground flaxseed by peroral for 7 days.

Group 4: Overdose-3 (OD-3): Rats received 5.6 mg/kg/d ground flaxseed by peroral for 7 days (12).

Sample Collection and Tissue Preparation

After the experiment, rats were euthanized under anesthesia with ketamine (90 mg/kg) and xylazine (10 mg/kg) intraperitoneally. Blood samples were taken and kidney tissues were removed. The blood samples were taken into the serum separated tubes and centrifuged at 10.000 rpm for 5 min. to separate serum for renal function marker analysis. The part of kidney tissue was placed in 10% neutral formalin for the histological procedure and the part of the tissue was collected for biochemical analyses. Renal tissues belonging to each group stored at -20ºC were weighed separately and diluted 10 times with 50 mM phosphate buffer (pH 7.4) after bringing them to room temperature. Homogenization was completed by treatment with the tissue shredder (Janke&Kunkel, Ultraturrax T-25, Germany) and then with the sonicator (UW-2070, Bandeun Electronic, Germany). Tissue samples were centrifuged at 10.000 rpm for 10 min. with a refrigerated centrifuge, and the supernatant was taken and transferred to eppendorf tubes. TAS and TOS parameters were studied by spectrophotometric method using ELISA kits (Rel Assay Diagnostics kit, Mega Tip, Gaziantep/Türkiye) and microplate reader (Biotek® Instruments, USA) in the supernatants obtained.

Identification of Serum Biochemical Markers

The renal biochemical parameters of urea, uric acid, BUN, and creatinine in serum were measured in a clinical chemistry autoanalyzer (Gesan production Chem 200, Italy) at the MAKU-VET Animal Hospital.

Identification Of Tissue Total Antioxidant Status (TAS)

It was carried out by applying the spectrophotometric protocol with commercially purchased TAS kits in the renal homogenates acquired from all groups. To indicate the total antioxidant status, the antioxidants present in the specimen induce the reduction of ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) radicals in the kit, causing the navy blue-green color of ABTS to disappear. The total antioxidant capacity is determined by measuring the wavelength of 660 nm in the microplate reader. This assay is set with Vitamin E analog (Trolox), a stable antioxidant solution, and defined as Trolox equivalent (mmol Trolox Equiv/L) (13).

Identification of Tissue Total Oxidant Status (TOS)

It was performed by applying spectrophotometric protocol with commercially purchased TOS kits in renal homogenates acquired from all groups. This assay is a colorimetric method evaluated spectrophotometrically at 530 nm. The oxidants in the specimen oxidize the iron ion complex. The ferric ion creates a chromogenic compound complex in an acidic medium. The density of the color is commensurate to the quantity of oxidant in the sample. The assay is adjusted with hydrogen peroxide and results are indicated as H_2O_2 equivalent (µmol H_2O_2 Equiv/L) (14).

Identification of Tissue Oxidative Stress Index (OSI)

OSI, which is a marker of the rate of the oxidative stress, was calculated according to the following formula: OSI (arbitrary unit) = [TOS (µmol H2O2 equiv/l) /TAS (mmol Trolox equiv/l) x 100] (15).

Histopathological Procedure

At the end of the experiment, rats were weighed and sacrificed and their right kidney was removed. Kidneys were placed in 10% neutral buffered formalin at room temperature. Later, they were processed with alcohol, blocked with paraffin, and sectioned. Slides, formalin-fixed, paraffin-embedded, stained with H&E technique. On slices with 3-4 µm thickness, the histopathological changes assessment of kidney tissue was performed using light microscopy. Cortex and medulla were evaluated at x200 or x400 magnifications separately in ten different areas of each slide and used a modified semi-quantitative scale for evaluation; [(0): no damage, (1): mild damage, (2): moderate damage, (3): severe damage] (16). Finally, the analyses were carried out on images taken using NIKON digital camera ((ECLIPSE Ni-U, Nikon, Tokyo, Japan).) and the scale bar was added to the images

Statistical Analyses

The statistical analyses were made by using a Windows® compatible SPSS® 20.0 program.

Biochemical parameters and histopathological scores were compared between the groups with One-way ANOVA (post hoc Tukey) tests. Variables are shown as mean ± standard deviations. P < 0.05 was set as the significance value.

Results

Biochemical Findings

Serum urea and BUN levels were significantly increased in all overdose groups compared to the control group (for all p=0.018, p=0.033, and p=0.000, respectively). There was no significant change in creatinine levels compared to the control. The uric acid level increased in the OD-2 group compared to the control, but this increase was not statistically significant (Table 1). A statistically significant decrease in renal TAS levels was observed in the OD-3 group compared to the control (p=0.044). Although there was an increase in

Table 1 Effect of flaxseed on serum levels of renal function markers

	Urea (mg/dL)	Uric Acid (mg/dL)	BUN (mg/dL)	Creatinin (mg/dL)
Control	52.0 ± 6.52	1.37 ± 0.69	24.29 ± 3.04	0.71 ± 0.10
OD-1	61.00 ± 4.72*	1.31 ± 0.40	28.50 ± 2.20*	0.71 ± 0.03
OD-2	60.25 ± 7.04*	1.85 ± 0.98	28.15 ± 3.29*	0.71 ± 0.07
OD-3	66.87 ± 3.64*	1.37 ± 1.03	31.25 ± 1.70*	0.72 ± 0.13

Values are expressed in mean ± SD. n = 8 per group. One way ANOVA (post hoc Tukey test) was used for comparison between groups. *p < 0.05 was considered statistically significant when compared to the control group. BUN: Blood urea nitrogen; OD-1: Overdose-1; OD-2: Overdose-2; OD-3: Overdose-3.

Table 2 Effect of flaxseed on total antioxidant/oxidant status in renal tissues

	TOS (µmol H_2O_2 eq/L)	TAS (mmol Trolox eq/L)	OSI (AU)
Control	4.90 ± 0.97	1.28 ± 0.20	0.38 ± 0.07
OD-1	5.50 ± 1.42	1.31 ± 0.22	0.42 ± 0.12
OD-2	5.08 ± 1.62	1.12 ± 0.19	0.46 ± 0.17
OD-3	6.49 ± 2.23	0.94 ± 0.33*	0.79 ± 0.45**

Values are expressed in mean ± SD. n = 8 per group. One way ANOVA (post hoc Tukey test) was used for comparison between groups. p < 0.05 was considered statistically significant when compared to the control group. *: p=0.044; **: p=0.016; TAS: Total antioxidant status; TOS: Total oxidant status; OSI: oxidative stress index; OD-1: Overdose-1; OD-2: Overdose-2; OD-3: Overdose-3.

renal TOS levels all overdose treatment compared to the control but not statistically significant. According to the calculations, the OSI level of the OD-3 group showed a statistically significant increase compared to the control (p=0.016) (Table 2, Figure 1).

Histopathological Findings

The control group showed normal tissue architecture based on the kidney tissues' histopathological evaluation. Both OD-1 and OD-2 groups showed normal structuring and no noticeable pathological changes were observed. But mild/moderate histopathological changes were observed in the OD-3 group. When the Malpighi corpuscle was evaluated, glomerular degeneration and Bowman's capsule dilatation were observed in some of them. In addition, mild/moderate capillary hemorrhage and vasocongestion were observed in some intertubular areas. The eosinophilic changes, a marker of degeneration, were observed in some tubular epithelium (Fig. 2). Minimal mononuclear cell infiltration was observed in some areas. The histopathological scores were shown in Table 3.

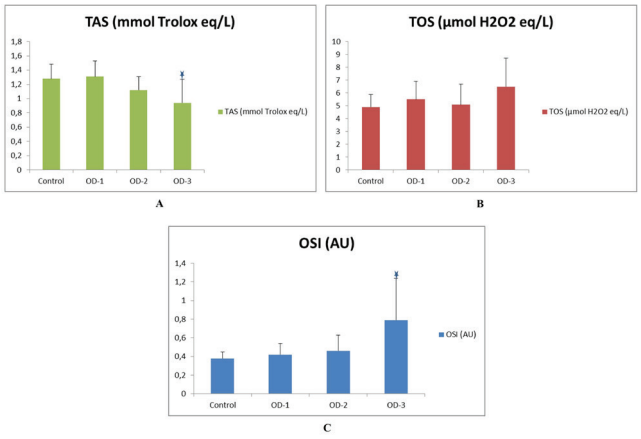

Figure 1:
Comparison of TAS, TOS, and OSI levels of kidney tissues in experimental groups.
A: Total Antioxidant Status (TAS) values; B: Total Oxidant Status (TOS) values; C: Oxidative Stress Index (OSI) values; *comparison with the control group (p<0.05).

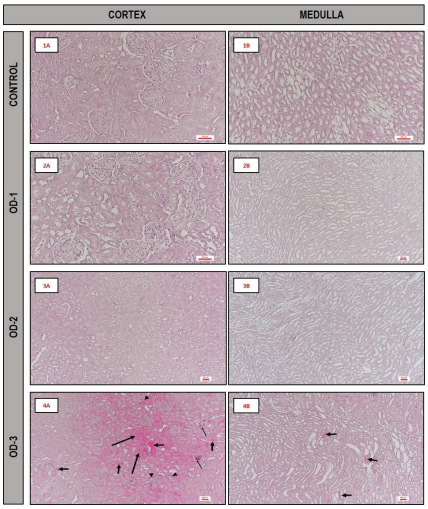

Figure 2:
Representative histopathological changes of kidney tissue between the groups.
(1A, 1B) Control rats, normal tissue architecture. (2A, 2B) Rats treated with 1.4 mg/kg/d, showing the cortex and medulla. Normal tissue architecture, no obvious pathological changes. (3A, 3B) Rats treated with 2.8 mg/kg/d, had normal tissue architecture, and no obvious pathological changes. (4A, 4B) Rats treated with 5.6 mg/kg/d, showing mild/moderate glomerular degeneration with a thick arrow, eosinophilic tubular epithelium with a long thin arrow, and intertubular hemorrhage/congestion with a short thin arrow. Mononuclear cell infiltration with an arrowhead. H-E, scale bars = 50 µm

Table 3 Effect of flaxseed on total antioxidant/oxidant status in renal tissues

	Control	OD-1	OD-2	OD-3
Glomerular degeneration	0.13 ± 0.35	0.25 ± 0.46	0.25 ± 0.46	0.38 ± 0.51
Tubular degeneration	0.25 ± 0.46	0.25 ± 0.46	0.13 ± 0.35	0.50 ± 0.53
Hemorrhage/congestion	0.13 ± 0.35	0.13 ± 0.35	0.25 ± 0.46	0.38 ± 0.51
Mononuclear cell infiltration	0.13 ± 0.35	0.13 ± 0.35	0.13 ± 0.35	0.25 ± 0.46

Values are expressed in mean ± SD. n = 8 per group. One way ANOVA (post hoc Tukey test) was used for comparison between groups. P < 0.05 was set as the significance value. No significant difference between the groups. OD-1: Overdose-1; OD-2: Overdose-2; OD-3: Overdose-3.

Discussion

Flaxseed has been used and is still being used in the health field, as its many healing effects have been discovered. Although it varies according to the purpose of use, 50 g/day of flaxseed is stated to be a safe dose by many sources (6, 8–10). Until now, many studies have been conducted on the curative effects and indications for use of flaxseed, and the toxic content of flaxseed has also been mentioned in the literature, however, the modeled-overdose experimental studies are limited.

Therefore, we aimed to see the biochemical and pathological changes on the tissue if the recommended daily dose is exceeded by using flaxseed overdose models in our experiment. We conducted this study using three different overdose experimental models. At the end of the experiment, the overdose groups were compared between each other and also to the control group in terms of oxidative stress and histopathological changes.

Oomah and Mazza reported in their study that long-term use of flaxseed would lead to some toxic effects (17). Histopathologically, there was no difference between OD-1 and OD-2 groups compared to the control group. It does not mean that long-term consumption of these doses is safe since the current experiment has an acute process. Pathological changes were observed in the OD-3 group, although not significant compared to the control group. According to these results, the OD-3 level signals danger for the kidney tissue and seems to be out of the safe range. Many studies mention many compounds in flaxseed that have a damaging effect on the human and animal body. Wiesenfeld et al. reported that the high amount of unsaturated fatty acids in its content oxidizes in vivo and causes the formation of free fatty acids. Therefore, long-term use of flaxseed will reduce antioxidant compounds while increasing oxidative stress (18). In the present study, especially in the OD-3 group, the increase in BUN and urea (for all $p=0.018$, $p=0.033$, and $p=0.000$, respectively), and the increase in TOS and OSI ($p=0.016$) in the tissue support the findings. While some literature mentions the antioxidant activity of flaxseed (19), paradoxically others mention that flaxseed triggers the formation of oxidative stress (18). Some studies have mentioned that oxidative stress results in kidney tissue damage (20, 21). In our study, it was seen that excess flaxseed disrupted the oxidative balance and tissue damage could be explained by this.When the literature is examined, it is revealed that the efficacy is dose-dependent or related to long-term exposure. Since kidney tissue is a tissue with a high blood supply, it will be easily affected by oxidative stress, which has systemic activity. The presence of pathological changes in the kidney tissue of the OD-3 group and the shift of the balance in the oxidative system in favor of TOS indicate that the kidney tissue is affected.

In addition, linatine, a compound in flaxseed, is a pyridoxine antagonist. Pyridoxine deficiency is associated with homocysteinemia and leads to renal failure (22). This clinical condition develops as a result of long-term use. Although it was used for a short time in the current experiment, it may generate the pathological basis of future kidney failure due to the high dose. Elmowafy A., showed an increase in plasma levels of uric acid, urea, and creatinine in rats treated with 10% flaxseed diet compared to the control (23). Ahmed S. M. et al. found that uric acid, urea, and creatinine levels increased in the flaxseed oil treatment compared to the control group (24).

Another study has mentioned that cyanogenic glycosides (linamarin, linustatin, neolinustatin) in flaxseed are toxic if consumed without cooking (10). As a result of chewing and digesting flaxseed, hydrogen cyanide, a strong toxic compound, is produced (25). Consumption of flaxseed by grinding is one of the traditional methods and in the current experiment, rats were fed using this method. And the resulting oxidative stress is triggered probably by the toxic products, so the resulting reactive oxygen species (ROS) may damage tissue and cause pathological changes As a matter of fact, oxidative stress-related kidney tissue damage studies have been reported in the literature until now (26–28). A study reported that if flaxseed consumption increases, urinary excretion of thiocyanate, a metabolite of cyanogenic glycosides, also increases (8). In other words, high-dose use or long-term use of flaxseed is a burden on the kidney. This means that the acute period of high-dose flaxseed consumption may adversely affect kidney functions.

Like many plants, flaxseed has a load of cadmium, a toxic heavy metal absorbed from the soil. Cadmium has a long half-life in the human body, thus high-dose exposure or long-term exposure causes accumulation in tissues. One of the organs primarily affected by cadmium toxicity is the kidneys, manifested by decreased glomerular function (29). Although pathological findings were observed in the highest dose group of the current study, it suggests that it may be a sign of toxicity, but further determination analyses are still required to confirm this toxicity.

Although no toxicity has ever been reported in clinical studies of adding flaxseed to the diet, it has

been reported that flaxseed contains potentially toxic compounds such as cyanogenic glycosides, linatine, and cadmium. In conclusion, it is important to recognize that conclusive scientific data is needed to support the concept of toxicity from any of these compounds. However, although there is no short-term striking effect of using flaxseed above the recommended daily dose, it is recommended to avoid at least long-term use to avoid its toxic effects.

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Conflict of Interest Statement
The authors have no conflicts of interest to declare.

Ethical Approval
This study was approved by the Local Ethical Committee on Animal Research of Suleyman Demirel University, Isparta and was performed according to ethical rules (Protocol number: 15.09.2022-06/86).

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Availability of Data and Materials
The datasets analyzed in this study are available through the corresponding author upon acceptable request.

Author Contributions
İ.A: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation, Visualization; Writing-original draft.

Ş.Y.: Data curation; Formal analysis; Investigation; Methodology; Visualization; Validation; Writing-review & editing.

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