



The impact of long-term consumption of diets enriched with olive, cottonseed or sesame oils on kidney morphology: A stereological study

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Manuscript received on August 27, 2018; accepted for publication on October 29, 2018

How to cite: HASSANZADEH-TAHERI M, HASSANZADEH-TAHERI M, JAHANI F, ERFANIAN Z, MOODI H AND HOSSEINI M. 2019. The impact of long-term consumption of diets enriched with olive, cottonseed or sesame oils on kidney morphology: A stereological study. *An Acad Bras Cienc* 91: e20180855. DOI 10.1590/0001-3765201920180855.

Abstract: To date, most of studies have only focused on metabolic effects of dietary oils while recent evidence proposes that they can influence kidneys structure. Therefore, the aim of this study was to evaluate the impact of long-term consumption of olive, cottonseed and sesame oils on renal morphology in rats. 70 male Wistar rats randomly assigned into seven equal groups and treated with standard diet (control), the standard diet enriched with 10% or 20% (W/W) of either olive oil (OLI10%, OLI20%), cottonseed oil (COT10%, COT20%) or sesame oil (SES10%, SES20%) for 5 months. Quantitative features of the kidney including kidney and cortex volumes and the number of glomeruli were analyzed stereologically. Moreover, kidney sections histologically were evaluated. All of the studied oils in low concentration had no devastating effects on renal morphology and also its pathological features. However, only in SES20% group, kidney volume as well as, cortical volume was higher than the control group. Besides, accumulation of carbohydrate macromolecules and renal fibrosis were markedly increased in SES20% group compared to the control. The results suggest that sesame oil, especially at high concentration, may lead to renal deformities as a result of histopathological changes such as dilatation, fibrosis, and tubular defects.

Key words: cottonseed oil, histopathology, kidney, olive oil, sesame oil, stereology.

INTRODUCTION

A bulk of evidence shows that dietary fats/oils play important roles in health and disease. Over the past 5 decades, the subject of biological effects of dietary oils/fats has been one of the most researched topics among biomedical studies (Ramsden et al. 2016). Subsequently, the landscape of edible oils

extensively has changed. However, to date, most of clinical and experimental research has focused on the effects of edible oils/fats in metabolic and cardiovascular functions. Less is known about the health risks of dietary oils on renal function mainly its structure (Svensson and Carrero 2017).

The kidney is a structurally complex organ that has evolved to subserve a number of important functions: excretion of the waste products of metabolism, regulation of body water and salt, maintenance of appropriate acid balance, and

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secretion of various hormones and autacoids (Kumar et al. 1997). The results of recent studies demonstrated that fatty acids appear to play a pivotal role in kidney diseases. Accordingly, n-3 and n-6 polyunsaturated fatty acids (PUFA) contribute to preserving kidney health (Syren et al. 2018). Taken together, these results put forward the hypothesis that different dietary oils might have different effects on kidneys.

Olive oil because of its prominent role in the Mediterranean diet is one of the most popular edible oils in the world. It is a liquid fat obtained from olives (the fruit of *Olea europaea*; family Oleaceae), a traditional tree crop of the Mediterranean Basin. The oil is produced by pressing whole olives (Syren et al. 2018). Olive oil generally has the highest percentage of monounsaturated fatty acids (MUFA) between edible oils (Assy et al. 2010).

The fifth most produced vegetable oil is cottonseed oil (Wassef et al. 2015). Cottonseed oil is a cooking oil extracted from the seeds of cotton plants of various species, mainly *Gossypium hirsutum* and *Gossypium herbaceum*, that are grown for cotton fiber, animal feed, and oil. Cottonseed has a similar structure to other oilseeds such as sunflower seed, having an oil-bearing kernel surrounded by a hard outer hull; in processing, the oil is extracted from the kernel. Cottonseed oil is used for salad oil, mayonnaise, salad dressing, and similar products because of its flavor stability (Lu et al. 2003).

Sesame oil is an edible vegetable oil derived from sesame seeds. Besides being used as a cooking oil in South India, it is used as a flavor enhancer in Middle Eastern, African, and Southeast Asian cuisines. It has a distinctive nutty aroma and taste (Barnwal and Sharma 2005). Sesame oil is a good mixture of PUFA and MUFA (Orsavova et al. 2015). The oil is popular in Asia and is also one of the earliest-known crop-based oils, but worldwide mass modern production continues to be limited

even today due to the inefficient manual harvesting process required to extract the oil. Nevertheless, a recent review of the literature on biological effects of sesame oil found that it can decrease high levels of cholesterol and inflammation which makes it effective for reducing atherosclerosis and the risk of cardiovascular disease (Hsu and Parthasarathy 2017).

As mentioned before, our knowledge about the effects of dietary vegetable oils on kidney structure is very limited. Consequently, the aim of the research was to evaluate long-term consumption effects of olive oil, cottonseed oil, and sesame oil on the renal structure of healthy rats.

MATERIALS AND METHODS

OILS PREPARATION AND FATTY ACID DETERMINATION

Commercial grades of dietary olive oil (OLI), cottonseed oil (COT) and sesame oil (SES) were purchased from the local market in Birjand, Iran. Fatty acid profiles of the oils, as well as the standard diet (Javaneh-Khorasan, Iran) were determined using a gas chromatograph (YL 6000, Korea) equipped with a CP-Sill 88 capillary column (60m, 25 μm i.d., 0.2 μm film) blindly at the Standard Research Institute, Karaj, Iran. Fatty acid methyl esters were identified on the basis of ISO 5508 (ISIR 4090) and analyzed according to the ISO 5509 (ISIRI 4091) (Hassanzadeh-Taheri et al. 2018a).

ANIMALS AND DIETS

Healthy male Wistar rats (60 days old) were purchased from laboratory animal facility in Birjand University of Medical Sciences, Birjand, Iran. The rats were housed in a temperature-controlled room (22 \pm 2 $^{\circ}\text{C}$) with a 12h light/dark cycle. All animal procedures were conducted and approved in accordance with the guide for the laboratory animals' care and usage of Birjand University

of Medical Sciences, Birjand, Iran (Ethic code: Ir.bums.REC.1396.94). All efforts were made to minimize animal suffering and to reduce the number of animals used. The animals were divided randomly into seven equal groups ($n = 10$) with the mean body weight of 251.41 ± 17.37 g. The groups were fed with the following diets for 5 months:

Group 1 (Control): received a standard diet (containing 3% fat)

Group 2 (OLI10%): received a standard diet 10% (w/w) supplemented with olive oil

Group 3 (OLI20%): received a standard diet 20% (w/w) supplemented with olive oil

Group 4 (COT10%): received a standard diet 10% (w/w) supplemented with cottonseed oil

Group 5 (COT20%): received a standard diet 20% (w/w) supplemented with cottonseed oil

Group 6 (SES10%): received a standard diet 10% (w/w) supplemented with sesame oil

Group 7 (SES20%): received a standard diet 20% (w/w) supplemented with sesame oil.

The concentrations and study period were according to our previous study in which the impact of long term consumption of 6 different dietary fats have been studied (Hassanzadeh-Taheri et al. 2018b). The animals had *ad libitum* access to their respective food and tap water throughout the study.

HISTOLOGICAL EVALUATION

At the end of the study (after 5 months), the rats were anesthetized with ketamine-xylazine (65:10 mg/kg IP) (Hassanzadeh-Taheri et al. 2018c), and fixed by intracardiac perfusion with 4% paraformaldehyde in 0.01M phosphate buffered saline. Both kidneys of each animal were rapidly removed, weighed, and then post-fixed in the same fixative for 48 h at 4 °C. The kidneys were processed by routine histological methods and embedded in paraffin blocks. The tissues were sectioned serially and coronally, using a sliding microtome (Leitz, Italy) at the thickness of 5µm for histopathological

evaluation (left kidney) or 10 µm thickness with 250 µm intervals for stereology (right kidney) (Ulubay et al. 2015).

To evaluate kidney structure and pathological lesions, tissue sections (5 µm) were prepared for staining with hematoxylin and eosin, periodic acid-Schiff (PAS) and Masson's trichrome. To study histological changes in the renal tissue, three random sections from each rat were scanned under a light microscope (UPLAN FI, Japan) and analyzed using Image J software (NIH, Bethesda, USA). Pathological features including degeneration, congestion, infiltration and hemorrhage were assessed and scored for each microscopic field of kidney sections according to a scoring checklist (0= none, 1= mild, 2= moderate, 3= severe) as previously described (Hassanzadeh-Taheri et al. 2018d). The PAS score is defined as 0= no deposits of PAS-positive material, 1= up to one third, 2= one-third to two-thirds, and 3= more than two-thirds of glomerular section positively stain with PAS (Hassanzadeh-Taheri et al. 2016, Wu et al. 2016). Also, interstitial fibrosis in Masson's trichrome stained sections was scored by the following criteria: 0= absent, 1= less than 25% of the area, 2= 25-50% of the area and 3= more than 50% of the area (Wu et al. 2016). The percentage of collagen fibers deposition was measured with the directionality plugin in Fiji- ImageJ (Schindelin et al. 2012).

STREOLOGY

The right kidney of animals was cut in 10 µm thickness with 250 µm intervals for stereological study. The volume of the cortex and whole kidney were estimated by using the Cavalieri's principle and the point counting method (Nyengaard 1999). For this approach 15-20 sections for each rat were used. The point density of the point-counting grid was specified according to a pilot study that included two kidney specimens of adult rats. A grid

of points was laid over the image of the section and the points hitting it were counted and the area sum of the cortex and medulla were calculated separately. The volume of kidney cortex, medulla and glomeruli were determined by applying the following formula:

$$\text{Volume (V)} = t \times a(p) \times \sum P$$

Where 't' is the thickness of the section, 'a/p' is the interpoint area, and '∑p' is the number of points hitting the object of interest in the section. After applying this formula to all samples, the total volume to be estimated from the following formula:

$$\text{Total volume: } V_1 + V_2 + \dots + V_n.$$

To estimate the numerical density of glomeruli and the total number of glomeruli, physical dissector pairs were applied. Serial sections were taken with a systematic unbiased sampling. One of the two sections was selected as a reference and the other as a look-up. Both of the sections were photographed at a magnification of 100X and 400X. An unbiased counting frame was placed on the reference and the look-up sections on the screen of the computer (20*20 cm) to perform the counting according to the dissector method. Afterwards, the numbers of glomeruli seen in the reference section but not in look-up section were counted. The numerical density of glomeruli per volume (cm³) (N_v) was estimated using the following formula:

$$N_v = \sum Q / (t \times A)$$

Where, ∑Q is the total number of glomeruli counted in the reference section, t is the mean section of thickness (10µm), and A is the area of the unbiased counting frame. Finally, the total number of kidney glomeruli (TN) was estimated by the following formula:

$$TN = N_v \times \text{Kidney volume}$$

STATISTICAL ANALYSIS

Results are expressed as mean ± SD in all groups. Variance in data was checked for homogeneity by Kolmogorov–Smirnov test. Statistical differences between groups were detected by one-way ANOVA test followed by Dunnett's test. Furthermore, histopathological grading scores were analyzed between the groups using Kruskal-Wallis test. Statistical significance was inferred at $p < 0.05$. The SPSS software, version 22 was used for all analyses.

RESULTS

FATTY ACID COMPOSITION

The fatty acid composition of all the three oils, as well as standard diet (consist of 3% fat) is presented in Table I. Accordingly, the OLI, COT and SES consisted of 20.7, 33.3, and 20% saturated fatty acids and 67.8, 28.8, and 42% MUFAs respectively. The levels of PUFAs were 10.9, 37.3, and 37.6% of total fatty acids corresponding to OLI, COT, and SES.

TOTAL ENERGY INTAKE AND KIDNEY-BODY WEIGHT RATIO

Based on monitoring of 5-month total food consumption, total energy intake of each rat during the study period was calculated (1g of standard diet: 3.59 kcal; 1g of 10% enriched diet: 3.94 kcal; and 1g of 20% enriched diet: 4.44 kcal). The results of total energy intake, final body and kidney weights, and kidney to body weight ratio are shown in Table II. Accordingly, there was no significant difference in total energy intake between the studied groups. The mean of body weight of the animals treated with all of the experimental diets (except SES10%) was significantly higher than the control group ($p < 0.05$). Compared to the control and other experimental groups, the kidney weight significantly increased only in the SES20% group

TABLE I
Fatty acid composition of the oils and standard diet.

Fatty acid	Olive oil (%)	Cottonseed oil (%)	Sesame oil (%)	Standard diet (%)
<i>Saturated</i>				
C4	-	-	-	-
C6	1.1	1.7	1.4	19.3
C8	-	-	-	0.9
C10	1.2	0.9	0.8	6.3
C12	0.9	0.4	0.4	4.6
C13	-	-	-	-
C14	0.2	0.6	0.2	3.8
C15	-	0.5	-	-
C16	12.9	22	10.3	18.8
C17	0.2	0.4	-	0
C18	3.7	5.9	6.2	4.3
C20	0.5	0.9	0.7	0
C22	-	-	-	-
<i>Un-saturated</i>				
C8:1	-	-	-	-
C10:1 <i>trans</i>	-	-	-	-
C10:1 <i>Cis</i>	-	-	-	-
C12:1 <i>trans</i>	-	0.1	-	-
C12:1 <i>Cis</i>	-	0.2	-	-
C14:1 <i>trans</i>	-	0.1	-	-
C14:1 <i>Cis</i>	0.1	0.3	-	-
C16:1 <i>trans</i>	-	-	-	-
C16:1 <i>Cis</i>	1.3	0.5	0.2	0.5
C17:1 <i>trans</i>	-	-	-	-
C17:1 <i>cis</i>	0.3	-	-	-
C18:1n-9 <i>trans</i>	-	-	-	-
C18:1n-9 <i>Cis</i>	66.1	27.6	41.7	19.6
C18:2n-6	9.8	33.2	37.1	19.7
C18:3n-3	1.1	4.1	0.5	1.8
C20:1	-	-	-	-
CLA	0.2	0.5	-	0

CLA: conjugated linoleic acid. Total fats of standard diet were 3%. Values are expressed as percentage by weights.

($p < 0.001$). Likewise, the ratio of kidney weight to body weight only was greater in SES20% group than control, significantly ($p < 0.001$).

HISTOLOGICAL EVALUATION

The results of the semi-quantitative histopathological evaluation are presented in Table III. Kruskal-Wallis global comparison revealed a significant difference between the groups in the mean rank of congestion as well as degeneration ($p < 0.001$ each). Kidney sections of SES20% treated group revealed higher mean rank

of congestion and degeneration compared to the control group ($p < 0.001$ each). Other experimental groups showed normal kidney architecture without any significant inflammations, hemorrhage, and infiltration or degenerations (Figure 1).

PAS-positive staining can be used to stain structures containing a high proportion of carbohydrate macromolecules, such as glycoproteins, glycogen, and proteoglycans. The PAS score comparison revealed that SES20% treated animals had significantly increased ($p < 0.001$) carbohydrate content compared to the

TABLE II
Total energy intake, body and kidney weights, and kidney to body weight ratio.

Groups	Total energy intake (kcal/rat/5months)	Body weight (g)	Kidney weight (g)	Kidney to body weight ratio (*1000)
	Control	10356.03±305.78	304.62±19.87	1.11±0.07
OLI 10%	10673.46±335.56	379.71±29.37*	1.22±0.12	3.29±0.29
OLI 20%	10614.35±382.15	360.00±15.80*	1.07±0.06	3.00±0.207
COT 10%	10232.77±529.26	371.00±18.46*	1.17±0.14	3.15±0.39
COT 20%	9667.30±348.85	355.04±18.12*	1.17±0.12	3.31±0.38
SES 10%	10106.10±510.15	338.62±40.23	1.06±0.104	3.11±0.18
SES 20%	10191.84±1054.32	373.12±23.78*	1.75±0.10*	4.72±0.38*

Values are expressed as mean± SD ($n=10$). OLI, olive oil; COT, cottonseed oil; SES, sesame oil. *Significant difference from the control.

TABLE III
Score of histopathological changes in the kidney section.

Groups	Alterations			
	Congestion (Mean rank)	Haemorrhage (Mean rank)	Degeneration (Mean rank)	Infiltration (Mean rank)
	Control	32.30	40.00	29.25
OLI 10%	32.40	36.50	29.25	30.50
OLI 20%	32.40	33.00	29.25	34.00
COT 10%	32.40	36.50	29.25	34.00
COT 20%	32.40	36.50	35.75	30.50
SES 10%	32.40	33.00	36.70	37.50
SES 20%	53.20*	33.00	59.05*	48.00

Scoring was done as follows for each microscopic field: none (0), low (1), mild (2) and severe (3). OLI, olive oil; COT, cottonseed oil; SES, sesame oil. *Significant difference ($p<0.05$) from the control.

control group (Figure 2). The structure of kidneys of the studied groups was depicted in Figure 3.

Masson's trichrome is a three-color staining protocol used in the histology, which produced red fibers, blue collagen, pink cytoplasm, and dark brown nuclei. The amount of blue collagen staining was significantly increased ($p<0.001$) in SES20% treated animal when compared to the control group (Figures 4 and 5).

STEREOLOGICAL RESULTS

All stereological results are summarized in Table IV. Mean volume of the kidney was significantly increased ($p<0.001$) in SES20% group in comparison with control group. There was no

statistical difference between the other studied groups. The mean cortex volume of SES20% group also were significantly higher ($p<0.001$) than control as well as other studied groups. There was no significant difference in the total number of glomeruli between the groups ($p=0.14$).

DISCUSSION

The results of the present study revealed that all of the studied oils in low concentration (10% added to the diet) had no devastating effects on renal morphology and also its pathological features. However, in the 20% concentration, kidney of animals treated with sesame oil showed markedly

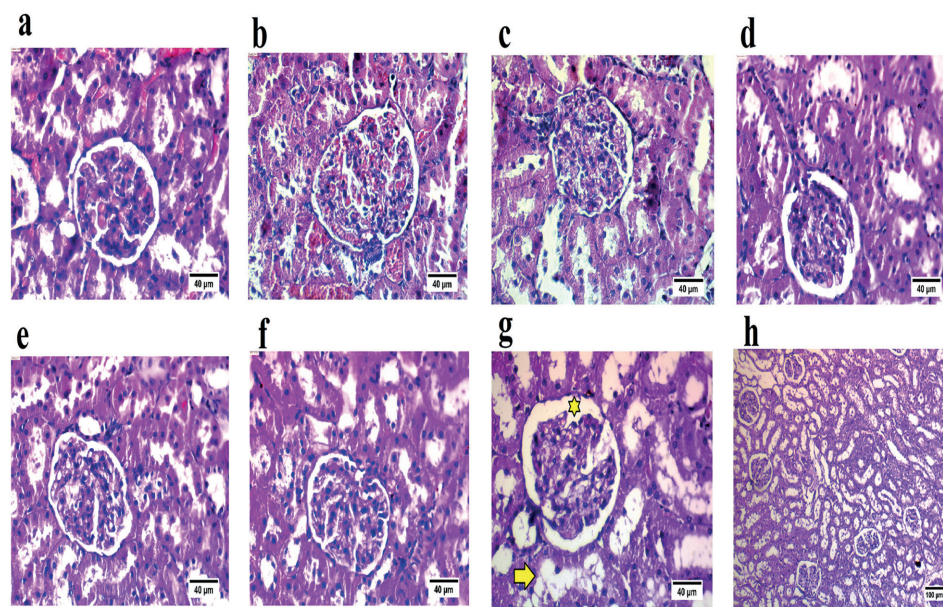


Figure 1 - Histopathological assessment of the kidney sections of rats in the control (a), Olive 10% (b), Olive 20% (c), Cottonseed 10% (d), Cottonseed 20% (e), Sesame oil 10% (f) and Sesame oil 20% (g, h). The sections were stained with hematoxylin and eosin dyes. The star represents urinary space dilatation and arrow shows a proximal tubule with lysosome accumulation.

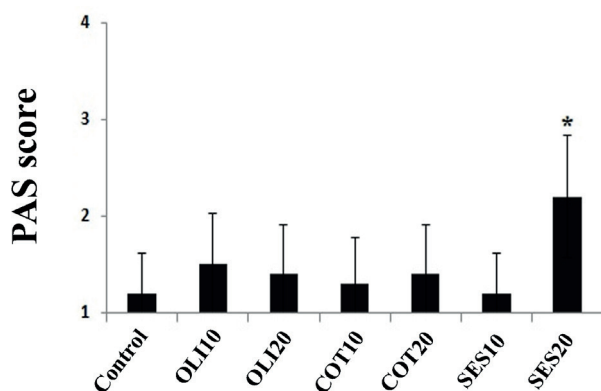


Figure 2 - Quantitative analysis of PAS-positive deposits. Control, Olive 10% (OLI10), Olive 20% (OLI20), Cottonseed 10% (COT10), Cottonseed 20% (COT20), Sesame oil 10% (SES10) and Sesame oil 20% (SES20). Values were presented as mean± SD. * $p < 0.05$ compared with the control group.

morphological changes and histological alterations. In line with increases in kidney weight, kidney total volume and cortical thickness significantly increased in SES20% treated rats. Moreover, histological evaluation revealed that tubular and glomerular congestion, as well as urinary space

dilatation were the most evident alterations in kidney tissues belong to SES20% treated animals. Consistent with histopathological changes, special staining techniques showed that PAS-positive reaction of glomeruli and interstitium -representing carbohydrate accumulation- significantly increased in SES20% group. Moreover, renal collagen deposition assay using Masson’s trichrome staining technique showed that fibrosis score significantly increased in SES20% groups compared to the control and other experimental groups. Our findings reveal that the results of histopathological examinations and stereological quantification confirm each other.

Several experimental studies demonstrated that rodent diet enriched in fat (more than 30% of energy) considered as a high-fat diet (Yang et al. 2017). In the current study 10 and 20% enriched diets with the oils providing about 22.5 and 40.1% calories, respectively. Moreover, toxicological assessments in experimental animals usually were categorized into four classes: acute, sub-acute, sub-

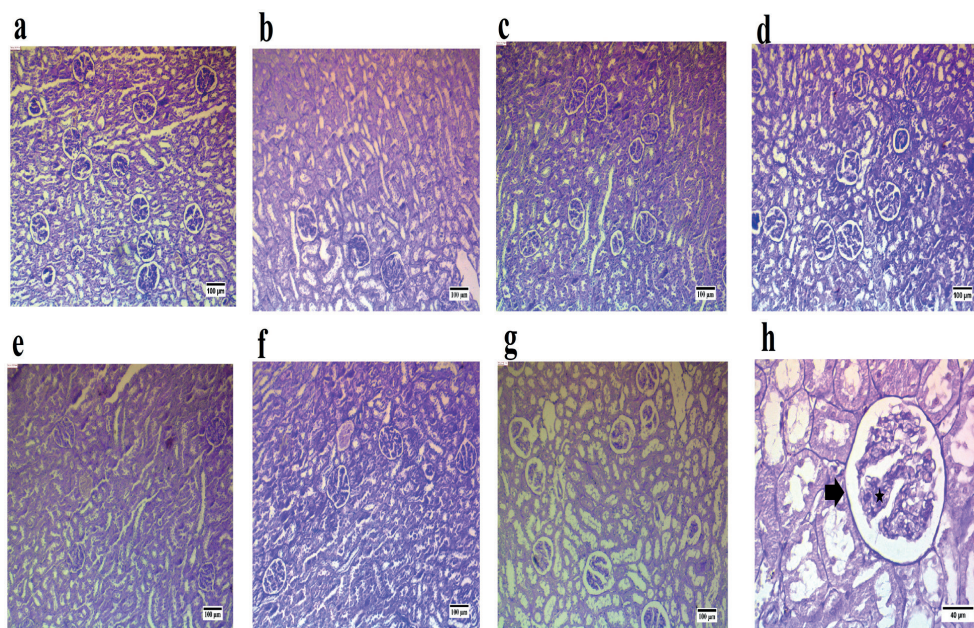


Figure 3 - Evaluation of carbohydrate content in the kidney of rats using PAS staining. Control (a), Olive 10% (b), Olive 20% (c), Cottonseed 10% (d), Cottonseed 20% (e), Sesame oil 10% (f) and Sesame oil 20% (g, h). The arrow shows thickening of Bowman’s capsule and the star represents carbohydrate accumulation (PAS-positive) in the glomerulus. Generally, the sections of SES20% group have purple appearance while those from the other groups have blue appearance.

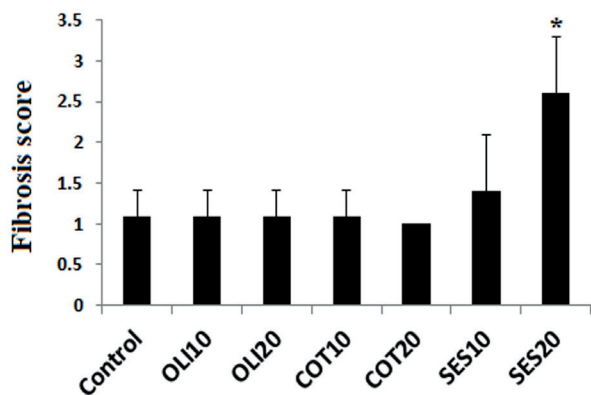


Figure 4 - The extent of renal fibrosis is represented semi-quantitatively by the fibrosis score. Control, Olive10% (OLI10), Olive 20% (OLI20), Cottonseed 10% (COT10), Cottonseed 20% (COT20), Sesame oil 10% (SES10) and Sesame oil 20% (SES20). Values were presented as mean± SD. * $p < 0.05$ compared to the control group.

chronic and chronic. Acute toxicity test is defined as a single exposure for less than 24 h, sub-acute toxicity refers to repeated exposures for 1 month or less, sub-chronic toxicity refers to repeated exposures for 1 to 3 months and chronic toxicity

assay refers to repeated exposures for more than 3 months (Hassanzadeh-Taheri et al. 2018d). Therefore, in the present study two concentrations of the oils added to normal diets because they are more reassembling to normal and high-fat diets and also we have chosen 5-month investigation to simulate long-term consumption pattern like what happens in real life.

Recently, research demonstrates that high-fat diet may cause renal alterations (Syren et al. 2018). Previous studies demonstrated that chronic administration of dietetic lipids can cause abdominal obesity and significantly alters the renal cortical structure in rats (Altunkaynak et al. 2008). There is evidence showing that in chronic kidney diseases, kidney weight increases (Levey and Coresh 2012). Similar to our findings, Abdel-Rahman and colleagues reported that 12 weeks treatment with a high-fat diet (consist of 20% fat) markedly increased kidney weight in rats

TABLE IV
Mean volumes of the kidney and cortex and total glomeruli number.

Groups	Estimations		
	Kidney Mean Volume (mm ³)	Cortex Mean Volume (mm ³)	Total number of glomeruli (Mean rank)
Control	37± 0.14	27±0.06	286,324±6430
OLI 10%	36± 0.09	27±0.19	294,302±6493
OLI 20%	37±0.43	28±0.07	281,509± 5700
COT 10%	37± 0.21	27±0.10	213,645±14762
COT 20%	38±0.11	28± 0.2	224,525±9471
SES 10%	36±0.21	25±0.12	304,736±11923
SES 20%	80±0.13*	47.3±0.12*	213,714±9462

OLI: olive oil; COT, cottonseed oil; SES, sesame oil. *Significant difference ($p<0.05$) from the control.

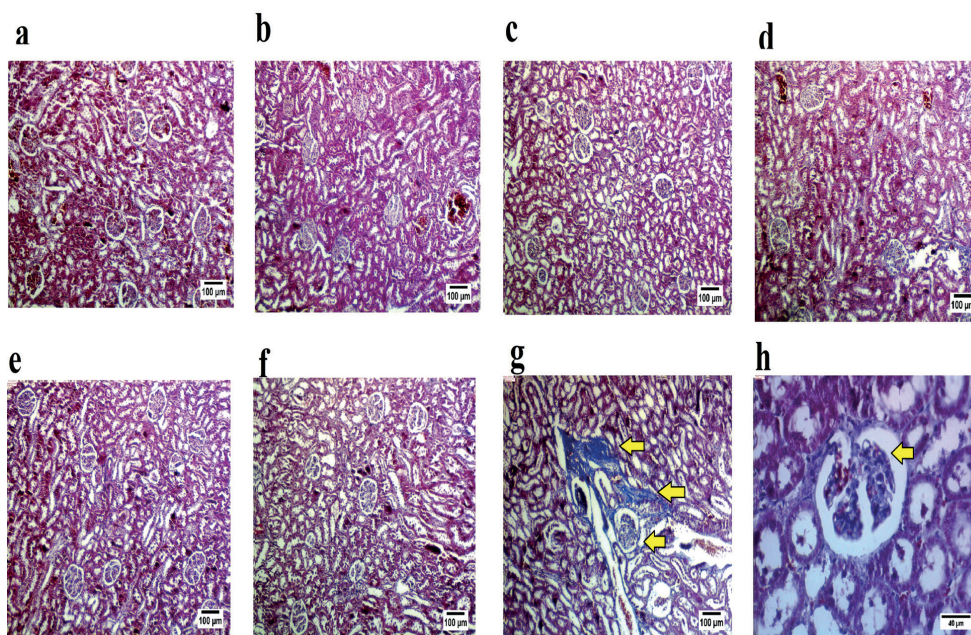


Figure 5 - Evaluation of renal collagen deposition using Masson's Trichrome staining. Control (a), Olive 10% (b), Olive 20% (c), Cottonseed 10% (d), Cottonseed 20% (e), Sesame oil 10% (f) and Sesame oil 20% (g, h). The arrows clearly show collagen deposition areas (blue fibers) both in glomeruli and interstitium.

(Abdel-Rahman 2010). Evidence also shows that high-fat diet can cause morphological alterations in kidney such as increasing glomerular volume, dilatation in urinary space and increasing in cortical thickness (V Mathew et al. 2011). In the recent study in which nephrectomized rats were evaluated by MRI (Magnetic Resonance Imaging) technique, scientists reported that kidney weight in response to increasing glomeruli volume elevation

is increased (Bennett et al. 2013). According to evidence cortical thickness in acute progressive kidney diseases is decreased. On the other hand, in diabetic patients, the cortical thickness is increasing during the disease development (Golalipour et al. 2007).

Histopathological evaluations revealed that in SES20% group, PAS-positive staining in both glomerular and tubular structures significantly

increased. Most sugars include at least one glycol formations at positions 2 and 3. The periodate ion (in periodic acid) selectively oxidizes glycols, yielding two aldehyde groups which firmly anchored to the tissue. The aldehydes can be detected with one of several chromogenic reagents like Schiff's reagent. Schiff's reagent covalently combines with aldehydes to form a red-purple compound. Thus, PAS-positive tissues indicating the high content of carbohydrate that usually observes in diabetic nephropathy and some other renal diseases (Kiernan 2001). This is in good agreement with Wu et al. study in which 12-week high-fat diet consumption (45% calorie from fat) caused a significant increase in the amount of PAS-positive staining in both glomerular and tubular structures of mice kidneys (Wu et al. 2016).

Similar to carbohydrate macromolecules accumulation, assessments of renal fibrosis with Masson's trichrome staining techniques revealed that in SES20% group renal fibrosis markedly increased compared to the control and other studied groups. It is previously reported that imbalance between renal lipogenesis and lipolysis in high-fat treated mice could induce renal fibrosis (Kume et al. 2007).

The evidence from this study suggests that sesame oil in long-term and high intake may cause structural and histopathological changes in kidneys. Taken together, our findings confirm that different types of fat regardless their proportion in a diet could influence renal structure differently. We propose that further research using molecular methods and immunohistochemical techniques to put more light on mechanisms behind sesame oil induced kidney damage.

ACKNOWLEDGMENTS

This study was financially supported by Birjand University of Medical Sciences (Grant number: 455109). We gratefully acknowledge the help

provided by Ms. Sara Nanvazadeh in fatty acids determination.

AUTHOR CONTRIBUTIONS

Mohammadmehdi Hassanzadeh-Taheri designed the study, Mahsa Hassanzadeh-Taheri, Farnaz Jahani and Zahra Erfanian performed the experiment, Hesam Moodi analyzed the data and co-wrote the paper draft. Mehran Hosseini discussed the data and wrote the manuscript. All authors read and approved the final version of the manuscript.

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