Oral Omega-3 Fatty Acid for Reduction of Kidney Dysfunction Induced by Reperfusion Injury in Rats

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Introduction. The aim of this study was to examine the effects of oral administration of omega-3 fatty acid on kidney functional disturbances, histological damages, and oxidative stress due to reperfusion injury.

Materials and Methods. Male Sprague Dawley rats received a standard diet for 2 weeks. Through gavage, the rats in acute kidney failure and omega-3 groups received 4 mL normal saline or omega-3 fatty acid (0.4 g/kg) daily. After 2 weeks, the rats underwent surgery and renal ischemia on both sides. During the last 6 hours, the rats were transferred to the metabolic cage for urine sampling. At the end of the period, blood samples were obtained from the aorta and the kidneys were removed for hematoxylin-eosin staining, histological analysis, and oxidative stress measurement. The sham group also received normal saline, but the operation was done without renal ischemia, whereas the control group did not received any substances or operation.

Results. The decrease in glomerular filtration rate induced by reperfusion was relatively improved by omega-3 administration, which resulted in the decrease in plasma urea and creatinine concentrations. In addition, the relative excretion of sodium and potassium, and urine flow rate decreased in the omega-3 group as compared with the acute kidney failure group. The degrees of histologic damages and oxidative stress that had increased following reperfusion injury were also significantly lowered by omega-3 administration.

Conclusions. Preventive oral administration of omega-3 supplement may decrease histological damages, oxidative stress, and kidney dysfunction following reperfusion injury.

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INTRODUCTION

Acute kidney failure (AKF) is a common clinical syndrome. Despite remarkable advances in prevention and treatment of acute disorders, the rate of mortality due to AKF is more than 50%.[1-3] Various factors might cause AKF, among them reperfusion injury is one of most common. Pathophysiologically, damages induced by reperfusion can be divided into arterial endothelium, tubular epithelium, and inflammation.[1,2,4]

In the last past years, the effects of various factors including, omega-3 fatty acids, on reperfusion-induced AKF have been investigated. Fish oil and marine oil are the most important sources of omega-3 fatty acid, which contain eicosapentaenoic acid (EPA) and dexosahexaenoic acid (DHA).[5,6] Clinical

findings have shown that adding EPA and DHA to the diet can have positive effects on inflammatory diseases[7-10] and reduce the risk of mortality and morbidity of severe kidney diseases in patients with glomerulonephritis.[11] Several studies have demonstrated that DHA has anti-inflammatory and immunosuppressive effects.[12,13] In addition, a number of research studies have investigated the effects of omega-3 fatty acid consumption on nephrotoxicity due to cyclosporine, mycotoxin, gentamicin, and formaldehyde,[14-18] as well as renal damages due to diabetes mellitus.[19]

An and colleagues[20] showed that omega-3 fatty acid could reduce oxidative stress, inflammation, and fibrosis in rats with 70% renal mass reduction. It has also been demonstrated that DHA can decrease damages due to reperfusion injury in rats and dogs.[21,22] Hassan and Gronert found that oral administration of omega-3 fatty acid can increase survival, prevent increase of serum creatinine and cytokines, and decrease leucocytes recruitment.[23] However, none of these studies have dealt with the effects of short-term administration of omega-3 on kidney function parameters, histological damages, and oxidative stress in renal tissues altogether following reperfusion injury. Hence, the focus of the present study was to examine these parameters in a rat model.

MATERIALS AND METHODS

Experimental Protocol and Groups

This study was conducted on 32 male Sprague Dawley rats (250 g to 350g) which were kept in 12-hour light and 12-hour darkness with free access to water and standard food in suitable temperature. Throughout the study, all protocols and codes issued by Arak University of Medical Sciences, Arak, Iran, for using laboratory animals were observed. The rats were randomly divided into 4 groups of 8 rats each. In the control group, the rats were kept for 14 days in an ordinary cage and received a normal diet. At the beginning of the last 6 hours of this period, without any injection or operation, the rats were transmitted to a metabolic cage and their urine samples were collected, while at the end of this period, following anesthesia with intraperitoneal administration of pentobarbital sodium (50 mg/kg to 60 mg/kg; Sigma, UK), plasma samples were obtained from the abdominal aorta. Next, the right kidney was removed and after immediate freezing in nitrogen, it was kept in the freezer (-70°C) in order to be measured in terms of oxidative stress parameters (malondialdehyde and ferric reducing antioxidant power [FRAP]). Then, the left kidney was removed and fixed in 10% formaldehyde for histologic study.

The rats in the sham group received 4 mL of normal saline through gavage for 13 days. Then, the rats were anesthetized and operated, but the kidneys remained intact. During the last 6 hours of reperfusion period, urine sampling was done in the metabolic cage. Finaly, the rats were anesthetized again, plasma samples were obtained, and the kidneys were removed. The same protocol was followed for the AKF group, except for the fact that during the operation, arteries and veins of both kidneys were simultaneously clamped for 30 minutes. The rats in the omega-3 group initially received 0.4 g/kg of omega-3 fatty acid (Alaska deep sea fish oil) for 13 days,[24] and other procedures were done the same as of the AKF group. For providing omega-3, all nature Alaska deep sea fish oil 1-g capsules (Bach No, 50885-2) were used which contained EPA (180 mg) and DHA (120 mg). At the end of experiment, the rats were killed by deep anesthesia.

Measurement of Plasma and Urine Parameters

In order to measure sodium and potassium concentrations of the plasma and urine samples, the Medica EasyLyte apparatus (Instru-Med, Atlanta, USA) was used. Plasma and urine creatinine and urea concentrations and osmolarity were measured by an RA-1000 auto-analyzer (Technicon Instruments New York, USA) and osmometer (Osmomat 010, Gonotec, Berlin, Germany) devices, respectively. Creatinine clearance and relative sodium and potassium excretion values were obtained through the related standard formulas.

Measurement of Oxidative Stress Values

For evaluating the status of oxidative stress, malondialdehyde and FRAP levels in kidney tissue samples were measured. These parameters were measured, as explained in our previous study,[25] through methods introduced by Ohakawa and colleagues and Benzie and Strain, respectively,[26,27] as summarized below.

Measurement of malondialdehyde. The samples

were first weighed and after transfer to test tubes, cold phosphates buffer saline was added to them and was homogenized. Then, 1500 µL of acetic acid (20%), 1500 µL of thiobarbituric acid solution (0.8%), and 1500 µL of sodium dodecyl sulphate (8.1%) were added to all tubes. In the next step, 200 µL of the homogenized sample was added to the test tubes, whereas 200 µL of different concentrations of the standard sample was added to the standard tubes. The tubes containing this suspension were heated in a water bath at 95°C for 60 minutes and were then immediately immersed in ice water. After that, 4 mL of n-butanol was added to each tube and the tubes were centrifuged at 4000 rpm for 10 minutes, while the upper layer was smoothly removed by a sampler as the light absorption of the samples was measured at 532 nm by spectrophotometer (SpectroLab 7500 UV, Spectro, Kleve, Germany). Tetraethoxypropane was used as the external standard.

Measurement of ferric reducing antioxidant power. Initially, a fresh FRAP detector was provided by mixing 25 mL of acetate buffer, 2.5 mL of chloride ferric, and 2.5 mL of tripyridyl triazine solution. In the next stage, standard solution of $FeSO_4$ $7H_2O$ in serial dilutions were provided. Then, 1.5 mL of the FRAP detector was added to each of the tubes and maintained at 37°C for 5 minutes. After that, 50 µL of the tissue extract was added to each of the test tubes and 50 µL of different concentrations of the standard were added to the standard tubes. Finally, the intensity of the obtained stain in 593 nm wavelength was read against the blank. All reagents were purchased from Sigma-Aldrich (St Louis, MO, USA).

Histopathological Examinations

At the end of each experiment, the left kidneys of the rats were fixed in 10% formaldehyde (Sigma-Aldrich, St Louis, MO, USA), and stained in hematoxylin-eosin. Then, the severity of renal histopathology was quantified for the degree of Bowman space widening, decrease in the number of erythrocytes in glomerular capillaries, tubular cells necrosis and their exfoliation into lumen, intracellular vacuolization, vascular congestion, and intratubular casts. Scoring the level of histological damages was done by considering the increased Bowman space and the reduced number of erythrocytes in rats that showed the greatest changes in comparison with the sham group as 100% damage. In the rest of the rats, the degree of these damages was measured by comparing them with this group. Other changes such as cell necrosis and exfoliation into the tubular lumens, intracellular vacuolization, vascular congestion, and cast formation were measured as the percentage of the total area under light microscopic study that had been damaged. These percentages were scored as zero with no damage, 1 for 1% to 20% damage, 2 for 21% to 40% damage, 3 for 41% to 60% damage, 4 for 61% to 80% damage, and 5 for 81% to 100% damage.[28,29] Then the total histopathological score was calculated, which was equal to the sum of all scores of different damages.

Statistical Analyses

All data were presented as mean ± standard error, and between groups comparisons of kidney function parameters and oxidative stress values were performed using 1-way analysis of variance followed by Duncan post-hoc test. For determining the exact *P* value, the LSD test was used. The comparison of total histopathologic scores between the groups was made by nonparametric Kruskal-Wallis and Mann-Whitney tests. All statistical analyses were done by using the SPSS software (Statistical Package for the Social Sciences, version 16.5, SPSS Inc, Chicago, Ill, USA). Significance was considered at a *P* value less than .05.

RESULTS

Omega-3 and Kidney Function

Table 1 shows that there were no significant differences between sham and control groups in the measured or calculated parameters. However, ischemia for 30 minutes and reperfusion for 24 hours in the reperfusion group resulted in significant increases in plasma creatinine and urea concentrations, urine flow rate, and relative excretion of sodium and potassium values, and decreases in urine osmolarity and creatinine clearance in comparison with the sham group ($P < .001$ in all).

Omega-3 fatty acid consumption for 2 weeks decreased blood creatinine and urea concentrations, urine flow rate, and relative sodium and potassium excretion values ($P < .001$) and increased urine osmolarity ($P < .01$) and creatinine clearance ($P < .05$) in the omega-3 group in comparison with

Table 1. Laboratory Parameters of Rats With and Without Acute Kidney Failure (AKF) Induced by Reperfusion Injury and Omega-3 Fatty Acid Administration

Parameter	Control	Sham	AKF	Omega-3
Plasma creatinine, mg/dL	0.87 ± 0.02	0.95 ± 0.05	2.75 ± 0.14*	1.09 ± 0.06†
Plasma urea nitrogen, mg/dL	18.91 ± 1.00	18.84 ± 1.20	62.49 ± 1.90*	29.78 ± 2.70*†
Urine osmolality, mOsm/kg/H_2O	1863 ± 116	1913 ± 52	664 ± 63*	1268 ± 196‡§
Urine flow rate, µL/min.BW	25.1 ± 2.9	21.0 ± 1.3	45.9 ± 2.8*	27.8 ± 3.1†
Creatinine clearance, µL/min.BW	6.85 ± 0.80	6.22 ± 0.40	2.57 ± 0.50*	4.28 ± 0.70¶#
Fractional excretion of sodium, %	0.54 ± 0.04	0.47 ± 0.06	3.60 ± 0.70*	0.82 ± 0.19†
Fractional excretion of potassium, %	15.6 ± 2.0	14.2 ± 1.6	69.6 ± 9.0*	27.9 ± 5.6†

*P < .001 in comparison with the sham group.
†P < .001 in comparison with the AKF group.
‡P < .01 in comparison with the sham group.
§P < .01 in comparison with the AKF group.
¶P < .05 in comparison with the sham group.
#P < .05 in comparison with the AKF group.

the AKF group. In the omega-3 group, creatinine concentration, urine flow rate, and relative sodium and potassium excretion values reached the same level as the sham group and there were no significant differences between them. However, blood urea concentration, urine osmolarity, and creatinine clearance still presented significant differences in comparison with their corresponding values in the sham group.

Omega-3 and Indexes of Oxidative Stress

Figure 1 indicates that the malondialdehyde values of kidney tissues in the control and sham groups were not significantly different; however, bilateral occlusion of the renal arteries and veins for 30 minutes and reperfusion for 24 hours increased the level of malondialdehyde in the AKF group in comparison with the sham group (P < .001). Omega-3 fatty acid administration could significantly decrease the malondialdehyde values in the omega-3 group compared with its corresponding values in the AKF group (P < .001), yet it was still significantly different from the sham group (P < .05). The FRAP value of the kidney tissue in the control group was not significantly different from the sham group (Figure 1), but its value in the AKF group was significantly lower than that in the sham group (P < .01). Following oral omega-3 consumption, the level of FRAP in the omega-3 group increased in comparison with the AKF group (P < .001) and reached the same level as that in the sham group.

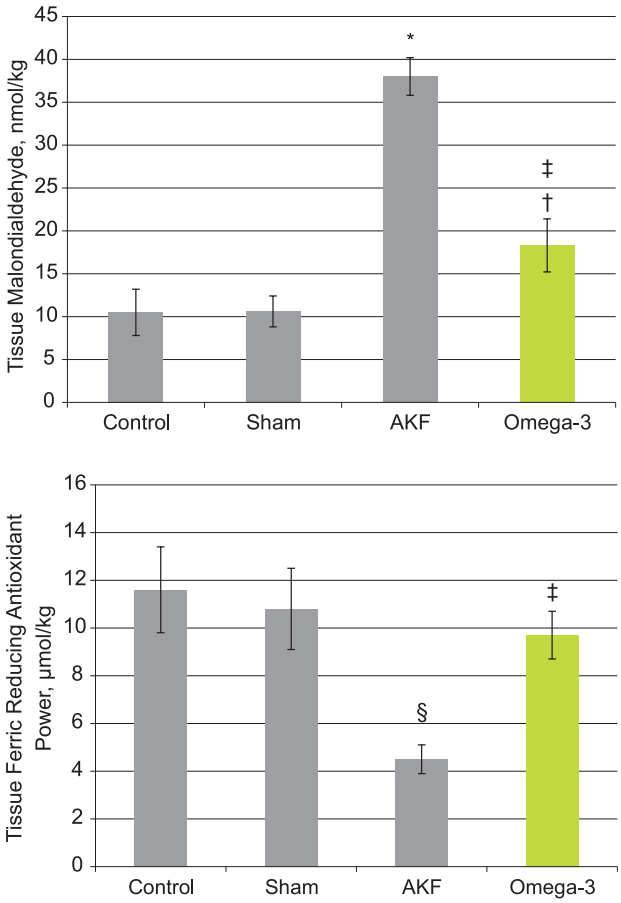

Figure 1. The mean levels of renal tissue malondialdehyde and ferric reducing antioxidant power at the end of reperfusion period in different examined rat groups.
*P < .001 compared with the sham group.
†P < .05 compared with the AKF group.
‡P < .001 compared with the AKF group.
§P < .01 compared with the sham group.

Omega-3 and Renal Histological Damages

No histological damages were observed in the kidneys of the sham group rats (Figures 2A and 2B). Likewise, the same trend was observed in the

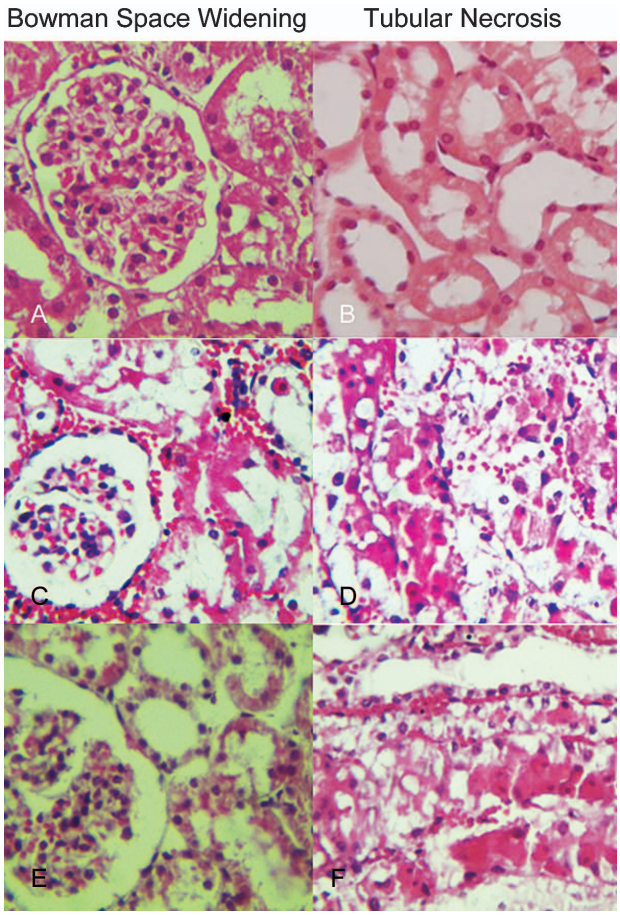

Figure 2. Representative histopathologic alterations in the cortex for Bowman space widening (left) and tubular necrosis (right) in the sham group (A and B), acute kidney failure group (C and D), and the omega-3 group (E and F; hematoxylin-eosin, × 400).

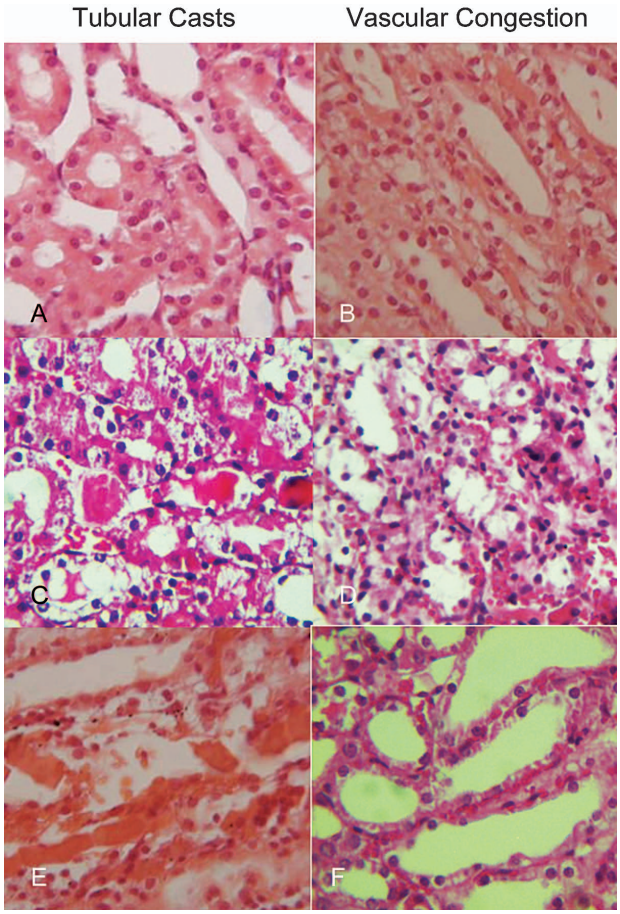

Figure 3. Representative histopathologic alterations in outer medulla for tubular casts (left) and vascular congestion (right) in the sham group (A and B), acute kidney failure group (C and D), and the omega-3 group (E and F; hematoxylin-eosin, × 400).

control group. Hence, data for this group are not shown. In the AKF group, in terms of the cortex, the Bowman space was widened (Figure 2C) and the number of erythrocytes decreased in the glomerular capillaries. In addition, cells in the proximal tubules walls had been severely damaged, vacuolized, and exfoliated into the lumens (Figure 2D). All these damages had a lower score in the omega-3 group compared with the AKF group (Figures 2E and 2F).

In the external medulla of the AKF group, cellular damage to the tubular segments of pars recta (S3) and the thick ascending limb of loop of Henle were more severe than the other segments (Figure 3). The severity of these damages had decreased to some extent in the omega-3 group (Figures 3C and 3D). Also, vascular congestion and intratubular proteinaceous casts in the AKF group were more in comparison with the omega-3 (Figures 3E and 3F). In the internal medulla, the values for vascular congestion and intratubular proteinaceous casts in the omega-3 group had relatively decreased compared with corresponding values in the AKF group (Table 2).

Total histopathologic score in the AKF group had significantly increased in comparison with the sham group. Although this score had decreased in omega-3 group, its amount was still significantly different from that in the sham group (Table 2).

DISCUSSION

This study was conducted to investigate the effects of oral omega-3 supplementation on changes in plasma parameters, excretory function, histological damages, and oxidative stress in kidneys following 30 minutes bilateral renal ischemia and 24 hours of reperfusion. The absence of significant differences in the values of all parameters of control and sham groups was indicative of the fact that the

Table 2. Histopathological Scores of Rats With and Without Acute Kidney Failure (AKF) Induced by Reperfusion Injury and Omega-3 Fatty Acid Administration

Histopathology Groups	Sham	AKF	Omega-3
Cortex			
Bowman space enlargement	0	V	III
Proximal tubule injury	0	III	I
Thick ascending limb injury	0	III	I
Reduced number of erythrocytes in glomerular capillaries	0	V	II
Intracellular vacuolization	0	IV	II
Outer medulla			
Pars recta (S3) injury	0	V	I
Thick ascending limb injury	0	V	II
Vascular congestion	0	IV	II
Intratubular proteinaceous casts	0	IV	II
Inner medulla			
Vascular congestion	0	III	I
Intratubular proteinaceous casts	0	IV	II
Total histopathological score*	0	45	19

*P < .01 for comparison of AKF and omega-3 groups, and P < .001 for comparisons of the AKF and omega-3 groups with the sham group.

stress due to operation has not been able to cause changes in the sham group. Hence, all changes in the parameters of the AKF group are related to reperfusion injury.

The comparison between the findings for AKF and sham groups indicates that following renal reperfusion injury, the level of creatinine clearance, as an index for glomerular filtration rate, decreased which resulted in the great increase in plasma creatinine and urea concentrations in this group. The decrease in the amount of glomerular filtration rate can be due to the decrease in renal blood flow, increase in the hydrostatic pressure within the Bowman space, back leak of glomerular filtration rate, or a combination of them following renal ischemia which has been reported by other studies.[1,30]

The great increase in the relative sodium excretion in the AKF group is indicative of sharp decrease in sodium reabsorption due to tubular damages which is verified by histological examinations in this study. Regarding the increase in the relative excretion of potassium in the AKF group, it can be stated that in addition to decreased tubular reabsorption of potassium, its increased secretion by the cheaf cells located in the distal tubules and cortical collecting ducts probably play roles that is due to high plasma potassium and aldosterone hormone concentrations.[31]

The increase in the amount of urine flow rate in the AKF group indicates the decrease in the amount of tubular reabsorption of water. In the case of renal failure, various factors can lead to decreased water reabsorption, among which interruption in countercurrent multiplier,[32] weakened antidiuretic hormone function, and the decrease in the number of aquaporin water channels in the collecting ducts can be mentioned.[33,34] Tubular cells damage, especially in the thick ascending limb of loop of Henle, and their decreased ability in reabsorption of minerals following ischemic damage lead to a decrease in osmotic gradient of medulla and, as a result, the decrease in reabsorption of water. The decreased urine osmolarity in this group is also indicative of disturbance in medulla hyperosmolarity that is due to the damage in the countercurrent multiplier.

The examination of cortical and medullary regions showed that renal reperfusion injury has resulted in evident tubular necrosis in renal tubules, increased Bowman space, cell vacuolization, decreased number of erythrocytes in glomerular capillaries, formation of intra-tubular casts, and medullar congestion in the AKF group. The main cause of these histological lethal and sub-lethal damages during ischemia is the great decrease in adenosine triphosphate due to poor oxygenation in cells. Cellular adenosine triphosphate depletion following ischemia leads to disturbance in mitochondria function, inflammatory processes activation, and decreased sodium/calcium exchange. Interruptions in mitochondria function through production of reactive oxygen species, and consequently, DNA damage can bring about necrosis. The increase in the amount of malondialdehyde and decrease in the amount of kidney tissues FRAP in Figure 1 proves this point.

The increase in cytosolic calcium, in addition to having a vasoconstrictor effect, by activating phospholipases, endonucleases, and proteases, breaking the cellular skeleton and intervening in energy metabolism, results in tubular epithelium cells necrosis.[35] Furthermore, during reperfusion following ischemia, with increases in the production of vasoconstrictor substances and capillary congestion, the amount of renal blood flow undergoes a steady decrease which contributes to the incidence of acute tubular necrosis.

In the rats that received the omega-3 dietary supplement, the level of creatinine clearance had

increased in comparison with its corresponding values in the AKF group which led to the reduction in plasma urea and creatinine concentrations in this group (Table 1). In addition, relative potassium and sodium excretion values as well as urine flow rate had all decreased while urine osmolarity had increased which were indicative of improvements in reabsorption in renal tubules in the omega-3 group. Therefore, omega-3 could decrease renal function disturbances following reperfusion injury which is in agreement with the findings of other studies.[21,22] Hassan and Gronert,[23] in a study on mice, showed that omega-3 dietary supplement by increasing the level of protectin D1 (PD1), and resolvins, as well as increasing the hemoxygenase expression plays its protective role against 30 minutes of ischemia and reperfusion for 24 hours. Moreover, other studies had already demonstrated the protective effects of resolvins on kidneys.[36]

It has been shown that PD1 can preserve glomerular filtration rate, suppress recruitment of polymorphonuclears and inflammation, and decrease tubulointerestitial fibrosis in kidneys following reperfusion injury.[36,37] Thus, omega-3, probably by increasing PD1 and resolvins levels and increasing the expression of hemoxygenase, protects the kidneys against reperfusion injury. On the other hand, it has been shown that lipopolysaccharide induces tumor necrosis factor-α expression and exposure to omega-3 can prevent the expression increase through inhibiting nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB).[38]

In most cells, NF-κB is kept inactive through inhibitor of kappa B inhibitory subunit,[39] while inhibitor of kappa B phosphorylation can lead to its destruction, and as a result, NF-κB activation. However, the EPA component of omega-3 prevents reductions in IκB level to prevent NF-κB activation. Also, the amount of reactive oxygen species production increases in renal tissues following reperfusion injury.[1] This point is demonstrated by the increase in malondialdehyde and the decrease in FRAP levels in reperfusion group of this study. The present study and other studies[40] have shown that omega-3 can restore the antioxidant defense mechanism of tissues. Several studies have shown that NF-κB is a redox-sensitive transcription factor,[41,42] which is activated by low concentrations of hydrogen peroxide. Therefore, omega-3 is likely to be capable of preventing NF-κB activation and activation of tumor necrosis factor-α by reducing reactive oxygen species production. On the other hand, omega-3 supplement decreased oxidative stress, inflammation, and tubulointerestitial fibrosis in the rats that nearly 84% of their kidneys had been removed.[20] Arachidonic acid plays an important role in signal induction routes involved in inflammation, reactive oxygen species generation, cell proliferation, and extracellular matrix production.[43,44] By cyclooxygenase, lipoxygenase, or cytochrome P450, arachidonic acid is converted to a number of strong eicosanoides, such as thromboxane A2 and leukotriene A4 which are highly proinflammatory, prooxidant, and prothrombotic. However, omega-3 reaction with these enzymes decreases the level of inflammatory products generation or increases production of anti-inflammatory agents.[45,46] Hence, part of the anti-inflammatory and antioxidant effects of omega-3 is accomplished through limiting the access to arachidonic acid for participation in signal induction and enzyme pathways.

CONCLUSIONS

We can conclude that omega-3 dietary supplement can result in the relative improvement of functional disturbances, oxidative stress, and histological damages following renal reperfusion injury. These changes are probably brought about by reducing the level of pre-inflammatory factors, increasing the level of anti-inflammatory and protective factors of kidney and decreasing the access to arachidonic acid.

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CONFLICT OF INTEREST

None declared.

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