Omega-3 polyunsaturated fatty acids alleviate adenine-induced chronic renal failure via regulating ROS production and TGF-β/SMAD pathway

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Abstract. – OBJECTIVE: To explore the role of omega-3 polyunsaturated fatty acids (ω-3 PUFAs) in adenine-induced rat chronic renal failure and its underlying mechanism. MATERIALS AND METHODS: 30 Sprague Dawley (SD) rats were randomly assigned into three groups, namely sham group, adenine induction group (adenine group) and adenine induction + ω-3 PUFAs treatment group (ω-3 PUFAs group), with 10 rats in each group. Serum and kidney samples were collected after rats were sacrificed. Serum levels of Cr (creatinine) and BUN (urea nitrogen) were detected using commercial kits. HE (hematoxylin and eosin) staining was performed to evaluate the pathological changes of kidneys. Levels of oxidative stress indicators in rat kidney homogenate were detected by relative commercial kits, including SOD (superoxide dismutase), GSH (reduced glutathione), CAT (catalase), and T-AOC (total antioxidant capacity). Reactive oxygen species (ROS) production was also detected by immunofluorescence. Protein expressions of nuclear factor E2 related factor 2 (Nrf2) and transforming growth factor-beta (TGF-β)/SMAD pathway-related genes were detected by Western blot. RESULTS: Serum levels of Cr and BUN in ω-3 PUFAs group were remarkably decreased compared with those of adenine group. Higher contents of SOD, GSH, CAT and T-AOC were observed in ω-3 PUFAs group compared with those of adenine group. Besides, MAD content and ROS production were lower in ω-3 PUFAs group than those of adenine group. Pathological changes of kidneys were alleviated after ω-3 PUFAs treatment. Western blot results demonstrated that ω-3 PUFAs treatment remarkably upregulates Nrf2, HO-1, NQO1, but downregulates relative genes in TGF-β/SMAD pathway. CONCLUSIONS: ω-3 PUFAs alleviated adenine-induced chronic renal failure through enhancing antioxidant stress and inhibiting inflammatory response via regulating Nrf2 and TGF-β/SMAD pathway.

Key Words Omega-3 Polyunsaturated Fatty Acids, Nrf2, TGF-β/SMAD pathway, Adenine, Chronic renal failure.

Introduction

Chronic kidney disease (CKD) is a common disease causing severe economic burden on affected population. The global incidence rate of CKD is about 10%1-3. Early diagnosis and treatment can significantly improve clinical outcomes of CKD. Therefore, precise diagnosis, disease staging and patient management are of great clinical significance3-4. In 2002, Kidney Disease Outcomes Quality Initiative (K/DOQI) of National Kidney Foundation (NKF) published guidelines for the assessment and staging of clinical practice for CKD4. In this guideline, CKD is used to replace the definition of chronic renal failure (CRF) for better understanding of CKD at different stages. A disease staging system based on glomerular filtration rate levels was also proposed4-5. Drug-induced kidney injury is a type of kidney disease caused by exposure to toxins or potentially toxic drugs. The clinical manifestations are abnormal urinalysis, renal pathology, and abnormal renal function6. At present, drug-induced nephrotoxicity mainly includes acute kidney injury, chronic kidney disease, acute interstitial nephropathy and nephrotic syndrome7-9. Adenine is a commonly used drug with nephrotoxicity, which can lead to CKD. Therefore, prevention and treatment for adenine-induced nephrotoxicity have been well recognized10. Secondary damage resulted from CKD should also be urgently prevented, so as to effectively promote CKD treatment3-5.
Active oxygen metabolites and inflammatory reactions are considered as important factors leading to drug-induced CKD. Reactive oxygen species (ROS) are by-products of biological oxidation reactions, including oxygen ions, peroxides, and oxygen-containing free radicals. Restoration of oxygen supply in damaged tissues leads to great consumption of oxygen by activated phagocytic cells, which is called respiratory bursts. Under normal circumstance, ROS production is maintained in a balance via a series of reduced substances. However, excessive production of ROS after external stimuli could be overwhelming. Some certain chemical agents such as free radical scavengers, antioxidants and anti-inflammatory cytokines remarkably alleviate tissue damage in renal toxin injury model. In addition, transforming growth factor-beta (TGF-β)/SMAD signaling pathway is an essential pathway that controls pro-chronic inflammation and fibrosis gene expressions. It is reported that ROS stimulate tissue damage and inflammatory response via activating TGF-β/SMAD pathway. In recent years, the impact of Omega-3 Polynsaturated Fatty Acids (ω-3 PUFAs) on organ damage caused by toxins and ischemia has been well studied. In this study, we aimed to investigate the effect of ω-3 PUFAs on adenine-induced CKD and its underlying mechanism. Our findings may provide important evidence for the clinical application of ω-3 PUFAs in adenine-induced chronic renal failure.

**Materials and Methods**

**Chemicals and Reagents**

ω-3 PUFAs was purchased from Sinopharm Chemical Reagent (Shanghai, China). Adenine injection was obtained from QiluPharma (Jinan, China). Commercial kits were purchased from Jiajicheng Bioengineering Institute (Nanjing, China), including MDA (malondialdehyde), T-AOC (total antioxidant capacity), CAT (catalase), GSH (reduced glutathione), SOD (superoxide dismutase), Cr (creatinine) and BUN (urea nitrogen) determination kits. Coarse balance, electronic thermometer and 721-type spectrophotometer were obtained from Inesa Analytical Instrument (Shanghai, China).

**Animals and Experimental Protocol**

30 adult Sprague Dawley (SD) rats weighing from 180-220 g were obtained from Vital River Laboratory Animal Technology (Beijing, China). Rats were housed in the environment with a 12 h light/dark cycle and free access to food and water. Rats in sham group were intragastrically administrated with 0.01 mL/g distilled water for 28 consecutive days. Rats in adenine group were also intragastrically administrated with 0.01 mL/g distilled water for 28 consecutive days. Meanwhile, intragastrical administration of 150 mg/kg·d adenine was performed on the 7th day 2 h after distilled water administration. Rats in ω-3 PUFAs group were intragastrically administrated with 0.01 mL/g ω-3 PUFAs for a total of 28 days. Intragastrical administration of 150 mg/kg·d adenine was also performed on the 7th day. Body weight and daily activities were recorded during the administration period. This study was approved by the Animal Ethics Committee of Sichuan University Animal Center.

**Assessment of Renal Function**

Body weight of rats was daily recorded before intragastrical administration. Bilateral kidney tissues were harvested and weighed immediately after the rats were sacrificed. Kidney index = kidney mass / body mass. 2 mL of blood sample were centrifuged at 3500 g/min for 30 min. Serum levels of Cr and BUN were measured by sarcosine oxidase method and urease method, respectively.

**Histological Examination**

Coronal sections of kidney tissues were prepared for histological examination. Kidney sections were fixed with 10% formaldehyde and paraffin-embedded. Tissues were then stained with hematoxylin and eosin (HE) (Boster, Wuhan, China). Histological changes were assessed by semi-quantitative examination of renal tubular necrosis. Evaluation criteria were applied as 0 (no damage), 1 score (<10%), 2 scores (11-25%), 3 scores (26-45%), 4 scores (46-75%) and 5 scores (>76%). Five randomly selected fields of each sample were observed.

**Terminal Deoxynucleotidyl Transferase dUTP Nick-end Labeling (TUNEL) Assay**

Apoptosis in kidney sections was detected according to the instructions of in situ DNA terminal transferase (TUNEL) assay (ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit; Chemicon, Millipore, Billerica, MA, USA). Kidney tissues were sliced into 5-μm thick sections and counterstained with methyl green. The number of TUNEL-positive cells in 10 random fields was counted using a high power microscope.
Biochemical Measurements
Abdominal cavity was exposed by midline abdominal incision. The abdominal aorta was cannulated under the branch of the renal artery, followed by ligation of the proximal segment above the branch of renal artery. The left renal vein was cut open. After the color of kidney tissue changed from red to white, the kidney was quickly removed and placed in liquid nitrogen. Tissues were homogenated for detecting levels of MDA, T-AOC, CAT, GSH and SOD.

For evaluating production of intracellular reactive oxygen species (ROS), intracellular superoxide level assay was detected by a fluorescent microscope (Eclipse Ti-SR, Nikon Co., Tokyo, Japan). The density of the images was detected with a laser scanning confocal microscope (Zeiss Ltd., Göttingen, Germany) in arbitrary units per millimeter square field.

Western blot
Kidney tissues were added with lysis buffer and shaken on ice for 30 min. The total protein was separated after the centrifugation at 14,000 g/min for 15 min at 4°C. Protein concentration was calculated by bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). The extracted proteins were separated on a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Western blot analysis was performed according to standard procedures.

Statistical Analysis
The t-test was used for comparing continuous variables. Categorical variables were analyzed using χ²-test or Fisher’s exact probability method. Kaplan-Meier method was performed to evaluate the survival time of patients and Log-rank test was used to compare the differences between different curves. SPSS 22.0 (Statistical Product and Service Solutions) was used for data analysis (IBM, Armonk, NY, USA). The data were expressed as mean ± standard deviation (x±s). p<0.05 was considered statistically significant.

Results
ω-3 PUFAs Pretreatment Improved Renal Function in Adenine-Induced Rats
Body weight and ratio of renal weight/body weight of rats in adenine group were remarkably decreased compared with those of sham group (p<0.05), indicating the successful construction of adenine-induced chronic renal failure model in rats. Significant improvements of body weight and ratio of renal weight/body weight were found in ω-3 PUFAs group compared with those of adenine group, suggesting that 20 mg/kg ω-3 PUFAs remarkably elevated renal function recovery (Figure 1A and 1B).

Subsequently, we detected serum levels of Cr and BUN in rats of each group. Serum level of Cr was remarkably elevated in adenine group and ω-3 PUFAs group compared with sham group (p<0.05). In particular, ω-3 PUFAs pretreatment could decrease serum level of Cr. However, serum level of Cr in ω-3 PUFAs group was still higher than that of sham group (Figure 1C). Similar results were observed in serum level of BUN (Figure 1D).

ω-3 PUFAs Preserved Renal Histologic Structure and Mitigated Neutrophil Infiltration
No significant pathological changes of renal microstructure were found in sham group. Enlarged tubular lumen and flat tubular epithelium were observed in adenine group. Besides, disordered cells with granular denaturation and karyopyknosis were shown in renal tissues of adenine group. Significant glomerular contraction, interstitial proliferation and inflammatory cell infiltration were also found. Renal injury in adenine group was less than that of ω-3 PUFAs group (Figure 2A). Similar results were obtained from Masson staining (Figure 2B). Kidney tubules injury score in adenine group and ω-3 PUFAs group was higher than that of sham group (p<0.05).

ω-3 PUFAs Decreased Renal Tubular Cells Apoptosis after Adenine-Induced Renal Injury
We next detected adenine-induced apoptosis in kidney tissues by TUNEL assay. The amount of TUNEL-positive cells in adenine group was remarkably larger than that of sham group. However, ω-3 PUFAs group presented a lower amount of TUNEL-positive cells compared with that of adenine group (Figure 2C and 2D, p<0.05).

ω-3 PUFAs Decreased ROS Production and Tissue Impairment by Enhancing Antioxidant Capacity
It is reported that adenine severely damages antioxidant capacity of kidney and stimulates ROS production. In the present study, we detect-
Omega-3 polyunsaturated fatty acids and chronic renal failure

ed antioxidants levels in renal homogenate using relative commercial kits. The data demonstrated that levels of T-AOC, ACT and SOD were higher in ω-3 PUFAs group than those of adenine group (Figure 3B-3D). ROS production was also detected using immunofluorescence assay. ω-3 PUFAs pretreatment remarkably alleviated ROS accumulation (Figure 3E and 3F). Besides, MDA level was also decreased in ω-3 PUFAs than that of adenine group (Figure 3A).

**ω-3 PUFAs Upregulated Nrf2 and Nrf2 Downstream Genes by Increasing Nrf2 Nuclear Translocation**

To further explore the mechanism of ω-3 PUFAs in protecting adenine-induced chronic renal failure, we collected cytoplasm and nucleus of adenine-induced kidney tissues, respectively. Expression of nuclear Nrf2 was higher in ω-3 PUFAs group than that of sham group and adenine group (Figure 4A). Western blot results also demonstrated stronger nuclear translocation of Nrf2 in ω-3 PUFAs group compared with that of sham group and adenine group. Similarly, downstream genes of Nrf2 were also upregulated in ω-3 PUFAs group than those of adenine group, including HO-1 and NQO1 (p<0.05). Furthermore, TGF-β/SMAD pathway-related genes were detected by Western blot. The data elucidated that ω-3 PUFAs pretreatment results in downregulated TGF-β, α-SMA, SMAD and FN, as well as upregulated E-cad (Figure 4B), indicating that ω-3 PUFAs regulates adenine-induced chronic renal failure via TGF-β/SMAD pathway.

**Discussion**

Chronic kidney disease (CKD) is a type of kidney disease in which there is gradual loss of kidney function over a period of months or years. Drug-induced renal failure is a crucial cause of acute kidney diseases. A great number of ROS produced after cardiac macrovascular surgery, kidney transplantation and shock could lead to CKD. Studies have shown that Nrf2 is a significant nuclear transcription factor. Nrf2
is capable of defending against oxidative stress. After binding to anti-oxidation response elements (ARE) in the nucleus, Nrf2 regulates expression levels of multiple downstream antioxidant genes. Recent studies have demonstrated that ω-3 PUFAs is a potent Nrf2 inducer. Functionally, ω-3 PUFAs possess anti-oxidative and anti-apoptotic abilities, which exert a protective effect on drug-induced CKD. At present, CKD poses a great burden on the medical resources. In-depth studies are urgently needed to improve the clinical outcomes of CKD.

**Figure 2.** ω-3 PUFAs prevents adenine-induced renal injury in renal morphology. Renal sections were stained with hematoxylin and eosin and examined using a light microscopy (200×).

**A.** HE staining of renal tissues in rats of sham group (n=10), adenine group (n=10) and ω-3 PUFAs group (n=10).

**B.** Masson staining of renal tissues was assessed the tubulointerstitial fibrosis.

**C.** Representative images (magnification ×100, scale bar=50 μm) of TUNEL immunostaining in the adenine-induced renal injury.

**D.** TUNEL-positive cells per 103 germ cells of testes. Data were expressed as mean ±SD. *Significant difference vs. sham group (p<0.05); #significant difference vs. adenine group (p<0.05).
Oxidative stress is an adaptive reaction caused by the imbalance between the active oxygen components and antioxidant system. Generally, excessive production of ROS exceeds the removal abilities of antioxidant enzymes and antioxidants. Oxidative stress is mainly manifested as inflammatory cell infiltration, increased secretion of proteases, and abundance of oxidation intermediates. ROS accumulation exerts an important role in ischemia-reperfusion injury. Hypoxia-induced reduction of ATP production and dysfunction of calcium ion channels activate calcium-dependent proteases. Xanthine dehydrogenase is, thereafter, hydrolyzed to xanthine oxidase and accumulated in the lesioned tissues. After oxygen supply is restored in the ischemic tissue, xanthine oxidase is activated to xanthine. Subsequently, superoxide ion radicals are generated and disproportionate to hydrogen peroxide and hydroxyl radicals. The large number of oxygen free radicals damages the function and structure of cells, eventually resulting in cell damage.

**Figure 3.** ω-3 PUFAs attenuated oxidative stress injury by the assessment of biochemical parameters. A, MDA content in kidney tissues. B, T-AOC content in kidney tissues. C, CAT content in kidney tissues. D, SOD content in kidney tissues. E, DHE staining of kidney tissues in sham group, adenine group and ω-3 PUFAs group. ROS exhibited red fluorescence under fluorescent microscope. F, Density of ROS was reported as arbitrary units per millimeter square field. Data were expressed as mean ±SD. *Significant difference vs. sham group (p<0.05); †significant difference vs. adenine group (p<0.05).
Nrf2 is a crucial transcriptional factor involved in oxidative stress. Under normal conditions, cytoplasmic Nrf2 is inactive and easily degraded. However, Nrf2 is dissociated from Keap1 and translocated in the nucleus stimulated by oxidative stress. Nuclear Nrf2 can bind to ARE, a DNA promoter binding sequence of phase II detoxification enzyme genes and antioxidant enzyme genes. HO-1 and NQO1 reduce oxidative stress damage by synergistically scavenging ROS or nitrogen species. In addition, activated Nrf2 also upregulates GSH, GST and SOD, further strengthening the antioxidant function.

Cytokine network regulation is greatly involved in drug-induced CKD. Among them, transforming growth factor-β1 (TGF-β1) is a crucial cytokine. TGF-β1 is secreted by Kupffer cells, which activates the synthesis of type I procollagen and type III collagen in adjacent hepatic stellate cells. Scholars have confirmed that TGF-β1 can induce collagen production in renal tissue through TGF-β1/SMAD and ERK signaling pathways. SMAD protein family is the most important intracellular effector molecule in TGF-β1/SMAD pathway. SMAD2 and SMAD3 are phosphorylated by TGF-β1 in renal injury to form hetero-oligomers with SMAD4, thereafter promoting nuclear translocation. SMAD negatively regulates TGF-β1/SMAD signaling pathway via inhibiting phosphorylation of SMAD2 and SMAD3.

ω-3 PUFAs are important components of biological cell membranes. Studies have shown that ω-3 PUFAs exert a variety of physiological functions, such as anti-inflammatory, immune regulation, anti-oxidation, development promotion of the nervous system and retina. Animal researches confirmed that ω-3 PUFAs have a protective effect on heart, intestine, liver, brain and other tissues during ischemia-reperfusion injury. However, the effect of ω-3 PUFAs on drug-induced renal failure has not been reported. Our data showed that adenine treatment results in significant histopathological changes, higher levels of oxidative stress and lower antioxidant capacity compared with those of sham group. Levels of MDA and ROS in kidney tissue of ω-3 PUFAs group were decreased, while levels of T-AOC, CAT, GSH, GSH/GSSG and SOD were increased than those of adenine group, indicating that ω-3 PUFAs intervention can reduce oxidative stress.
stress and enhance antioxidant activity. Relative investigations have shown that ω-3 PUFAs can significantly reduce the oxidative stress products and increase the anti-oxidative substances in the lesioned tissues, thereby reducing poison-induced tissue damage. In this study, Nrf2 was downregulated in adenine group than that of sham group. Besides, expressions of TGF-β/β3 with its promoter methylation during the pathogenesis of endometriosis. Eur Rev Med Pharmacol Sci 2017; 21: 4509-4515.

Conclusions
We found that ω-3 PUFAs alleviated adenine-induced chronic renal failure through enhancing antioxidant stress and inhibiting inflammatory response via activating Nrf2 and inhibiting TGF-β/β3/SMAD pathway.

Conflict of Interest
The authors declared no conflict of interest.

References


