Fish oils protects against cecal ligation and puncture-induced septic acute kidney injury via the regulation of inflammation, oxidative stress and apoptosis

ZHAOHENG LIN, JING JIN and XIYUN SHAN

Intensive Care Unit, The People's Hospital of Xishuangbanna Dai Nationality Autonomous Prefecture, Jinghong, Yunnan 666100, P.R. China

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Abstract. Septic acute kidney injury (AKI) is usually caused by sepsis. ω3 fatty acid has been reported to suppress sepsis-induced organ dysfunction to a certain degree. The present study aimed to investigate the effects of ω3 fatty acid in septic renal injury. Sprague Dawley rats were used to establish a cecal ligation and puncture (CLP) model in order to mimic the development of septic injury. The rats were treated with dexamethasone and fish oils (FOs) for 4 days prior to CLP. Alterations in the morphology of the tissues, the renal function and the induction of inflammation, oxidative stress and apoptosis were evaluated. The effects of FOs on nuclear factor-κB (NF-κB), JAK2/STAT3 and p38-MAPK were determined. The rats of the CLP model group exhibited low survival rates and increased expression of serum creatine, blood urea nitrogen, neutrophil gelatinase-associated lipocalin, kidney injury molecule-1 and of proinflammatory cytokines. In addition, the levels of the markers of oxidative injury and apoptosis were increased. The induction of renal injury was notably reversed by administration of dexamethasone and FOs. The expression levels of the protein markers involved in inflammation and apoptosis were measured and the results indicated that FOs inhibited JAK/STAT3 and p-38MAPK signaling, while they concomitantly increased the expression of NF-κB. The present study highlighted that FOs improve CLP-induced mortality and renal injury by inhibiting inflammation, oxidative stress and apoptosis.

Introduction

Sepsis is a life-threatening organ dysfunction, caused by an overwhelming immune response to infection (1). The kidneys are frequently affected by sepsis, which results in the development of acute kidney injury (AKI), inducing unfavorable health outcomes. Previous epidemiological investigations have revealed that patients with AKI (mild or short) tend to have a higher risk of developing chronic- and end-stage kidney disease at later stages of their life (2). Volume resuscitation, antimicrobial therapy and renal replacement therapy remain the main types of treatment for sepsis-induced AKI (3). Recent investigations have focused on the supportive measures that aim to keep the patient alive.

ω3 fatty acids (FAs), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are the main component of fish oils (FOs) (4). Previous studies have shown that enteral nutrition based on FOs affects the induction of sepsis, which is the leading cause of AKI (5,6). Sepsis is a systemic inflammatory response and a severe postoperative infection, which is usually initiated by bacteria and toxins (7). Following the release of bacterial toxins, the immune response is activated, and organ disorders may develop (8). The sepsis-induced kidney inflammatory response involves the increased production of pro‑inflammatory factors, which may contribute to high mortality (9). Concomitantly, the release of TNFα, IL-1β and IL-6 contributes to the inflammatory factors (9). During the development of sepsis, the levels of these pro-inflammatory factors are significantly elevated at the early stage of disease onset affecting the induction of pathophysiological responses (10). ω-3 FAs have shown considerable potential in the prevention of chronic kidney disease in humans (11). The ω-3 fatty acids, especially EPA and DHA, exert anti-inflammatory effects in humans (12). Shih et al (13) demonstrated that FOs could suppress AKI and the inflammatory response in septic mice. The pathophysiology of sepsis remains controversial. In addition to inflammatory reactions, sepsis-induced AKI often involves the induction of oxidative stress and apoptosis (14,15). Previously, the administration of an ω3-FA-rich infusion during parenteral nutrition was shown to alleviate sepsis in patients (16); however, the detailed mechanisms of this process remain unknown.

Correspondence to: Dr Zhaoheng Lin, Intensive Care Unit, The People's Hospital of Xishuangbanna Dai Nationality Autonomous Prefecture, 4 Galan South Road, Jinghong, Yunnan 666100, P.R. China
E-mail: linzhaoheng_dz@163.com

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Blood urea nitrogen (BUN) and serum creatine (Scr) are classic biomarkers for renal injury and have been reported as biomarkers for delayed kidney injury (17). Accompanied with increased Scr levels, the glomerular filtration rate (GFR) is reduced (18). The secretion of Scr contributes to 10-40% of total Scr; thus, reductions in GFR may be linked. Additionally, the concentrations of Scr are notably influenced by variable factors, including age, gender, diet and drugs (19). Thus, the levels of BUN and Scr are not sufficient for the diagnosis of early kidney injury. Kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) have been detected in early renal tubular injury (20). The expression of NGAL in both blood and urine have been reported to indicate the presence, severity and development of renal disease, especially in chronic renal disease (21). KIM-1 is a transmembrane protein not found in normal kidney; the expression of KIM-1 and NGAL has been linked to renal ischemia, which usually leads to AKI (22).

In the present study, the cecal ligation and puncture (CLP) model was established to induce sepsis. This model mimics the condition noted in patients with bowel perforation and polymicrobial infection (23). The effects of FOs on sepsis-induced inflammation, apoptosis and oxidative stress were investigated.

**Materials and methods**

**Animals.** Male Sprague Dawley rats (n=32; 7-weeks-old, Qinglongshan Experimental Animal Center, China) weighing 250-330 g were employed. A total of 3 rats were housed per cage and maintained in a 12-h light/dark cycle at 25°C. The experimental protocol was approved by the commission for animal experimentation of the People’s Hospital of the Xishuangbanna Dai Nationality Autonomous Prefecture.

Following acclimation for 1 week, the rats with an initial body weight of 280±25 g were randomly assigned to one of the following groups: Sham-operated (n=8) and CLP (n=24). The former group was used as a control group and the latter as the experimental sepsis group. The CLP model group was randomly assigned to one of the following subgroups: CLP sepsis (n=8) used for model animals, CLP treated with dexamethasone [1 mg/kg, intraperitoneal (i.p.) daily; n=8] used for positive control and CLP treated with FO-containing fat emulsion (2 ml/kg i.p. daily; n=8). The rat model was established by CLP-induced sepsis and intraperitoneally administered with dexamethasone or FOs (Table I) once a day. The induction of sepsis was performed via CLP and the rats were sacrificed following 96 h of treatment. Renal and small intestinal tissues were fixed in 4% formaldehyde at room temperature for ≥24 h for histopathological analysis. Subsequently, the fixed tissues were embedded in paraffin. Sections (5 µm) were sliced. After deparaffinization and rehydration, the sections were stained with hematoxylin (Sigma-Aldrich; Merck KGaA) and measured at 570 nm. BUN was detected with an enzymatic assay kit (MAK006-1KT, Sigma-Aldrich, Merck KGaA) and measured at 570 nm. Blood biochemical analysis was performed according to previous investigations. The blood samples were extracted 24 h following sham or CLP operation and centrifuged at 1400 x g to prepare serum at 4°C for 10 min. Scr levels were evaluated by a creatinine enzymatic assay kit (MAK080-1KT, Sigma-Aldrich, Merck KGaA) and measured at 570 nm. BUN was detected with an enzymatic assay kit (MAK006-1KT, Sigma-Aldrich, Merck KGaA) and measured at 570 nm. The expression levels of NGAL (ab119602, Abcam) and KIM-1 (ab119597, Abcam) were measured using corresponding kits and measured at 450 nm. All measurements were conducted with a multiscan spectrum spectrophotometer (Thermo Fisher Scientific Inc.)

**Evaluation of inflammatory factors**

**ELISA assays.** The serum samples were extracted 24 h following sham or CLP operation. The expression levels of tumor necrosis factor (TNF)-α (ER006-96), interleukin (IL)-1β (ER008-96), and IL-6 (ER003-96) in the serum were determined by ELISA kits (Shanghai ExCell Biology, Inc.) following the manufacturer’s protocol. The samples were centrifuged at 1,500 x g for 15 min at 4°C. Subsequently, the supernatant was collected, and the measurements were conducted at 450 nm using a multiscan spectrum spectrophotometer (Thermo Fisher Scientific Inc.).
Immunofluorescence staining. The expression levels of TNF-α, IL-1β, and IL-6 were detected in renal and intestinal tissues by immunofluorescence. Sections (5 µm) of renal and intestinal tissues were obtained. The samples were incubated with blocking solution (5% bovine serum albumin in PBS) and permeabilized (0.1% Triton X-100 in PBS). Primary antibodies against TNF-α (1:100, ab6671, Abcam), IL-1β (1:100, ab9722, Abcam), and IL-6 (1:200, YB-0782R, Yubo Biological Technology Ltd.) were used for sequential double immunofluorescence staining for 2 h at room temperature in the dark. The goat anti-rabbit IgG H&L (Alexa Fluor® 488) secondary antibody (1:500, ab1500, Abcam) were conjugated with fluorescein isothiocyanate. The sections were mounted in an anti-fading agent (DAPI; Invitrogen; Thermo Fisher Scientific, Inc.) for 5 min at room temperature, and subsequently imaged and analyzed with a fluorescence microscope (Olympus Corporation) at x100 magnification.

Assessment of antioxidant activity and oxidative stress markers in renal tissues. The levels of oxidative stress biomarkers were determined according to a previous investigation (26). The renal tissues were homogenized in 50 mmol/l phosphate buffer, and subsequently centrifuged at 12,000 x g for 20 min at 4°C. The supernatant was collected from the samples for the detection of the markers of oxidative stress. The concentration of malondialdehyde (MDA) was detected with an MDA assay kit (Beijing Solarbio Life Sciences). The activity level of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were evaluated by commercial kits (Jiancheng Bioengineering Institute). All detection was conducted according to manufacturer's protocols.

Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labelling (TUNEL) staining. The TUNEL assay was performed as described previously (27). The paraffin-embedded renal tissues were sliced into 5 µm sections at room temperature. After deparaffinization, the samples were rehydrated with a gradient concentration (90, 80, 70%) of ethanol and immersed in 4% formaldehyde in PBS for 9 min. Subsequently, the sections were treated with proteinase K (20 µg/ml) and incubated in a nucleotide mixture containing fluorescein-12-dUTP and TdT for 1 h at 37°C. The cell nuclei were stained with 4,6-diamidino-2-phenylindole for 5 min at room temperature, and the sections were imaged with a fluorescence microscope (Olympus Corporation).

Western blotting. Frozen renal tissues were thawed and total protein extraction was performed by RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific). The proteins were analyzed by immunoblotting using SDS-PAGE (10%) and transferred to polyvinylidene fluoride membranes. The blots were probed with antibodies against the following proteins: Bcl-2-associated X protein (Bax; 1:1,000, cat. no. 2772), Bcl-2, cleaved-caspase3 (1:1,000, cat. no. 9664), caspase 3 (1:1,000, cat. no. 9662), phosphorylated (p)-JAK2 (1:1,000, cat. no. 5209), JAK2 (1:1,000, cat. no. 3230), p-STAT3 (1:2,000, cat. no. 9145), STAT3 (1:1,000, cat. no. 12640), p-p38MAPK (1:1,000, cat. no. 4511), p-1xBα (1:1,000, cat. no. 5209), 1xBα (1:1,000, cat. no. 4812), p-p65 (1:1,000, cat. no. 3031), p65 (1:1,000, cat. no. 8242), and GAPDH (1:1,000, cat. no. 5174; all Cell Signaling Technology, Inc.). Incubation with the primary antibodies was performed overnight at 4°C. The following morning, the membranes were washed and incubated with horseradish peroxidase-conjugated secondary anti-rabbit IgG (1:2,000, cat. no. 7074, Cell Signaling Technology, Inc.) for 2 h. The expression levels of each protein were determined using enhanced chemiluminescence reagents (Chemidoc™XRS, Bio-Rad Laboratories, Inc.). The images were collected and quantified using Quantity One Software v4.6.6 (Bio-Rad Laboratories, Inc.).

Statistical analysis. Experiments were performed in triplicate, and data are presented as mean ± standard deviation. GraphPad Prism 6 (GraphPad Software, Inc.) software was used to analyze the results. Kaplan-Meier analysis was performed to derive survival curves and survival differences was analyzed by log-rank test. Multiple comparisons were assessed by analysis of variance followed by a Bonferroni post-hoc test. P<0.05 was considered statistically significant.

Results

Effects of FOs in sepsis on the survival of septic rats. To investigate the effects of FOs on the survival rate of septic rats, the survival rate between the CLP group and FOs-treated group was compared. A total of 8 rats did not survive 48 h of sepsis induction in the CLP group. The survival rate was reduced to 25% at 48 h and reached 0% at 72 h. Following treatment of the animal with dexamethasone, the survival rate was increased to 69% within 48 h. The survival rate was estimated to 71% at 48 h following FOs intervention. The average survival rate of the FOs and dexamethasone groups was significantly higher than that of the CLP group (Fig. 1A).

| Table I. Content of fish oil. |
|-------------------------------|-------------------|
| Main components               | Contents (g/100 ml)|
| Fish oil                      | 10                |
| EPA                           | 1.25-2.82         |
| DHA                           | 1.44-3.09         |
| Myristic acid                 | 0.1-0.6           |
| Palmitic acid                 | 0.25-1            |
| Palmitoleic acid              | 0.3-0.9           |
| Stearic acid                  | 0.05-0.2          |
| Oleic acid                    | 0.6-1.3           |
| Linoleic acid                 | 0.1-0.7           |
| ALA                           | ≤0.2              |
| SDA                           | 0.05-0.4          |
| Eicosanoic acid               | 0.05-0.3          |
| ARA                           | 0.1-0.4           |
| Docosanoic acid               | ≤0.15             |
| docosapentaenoic acid         | 0.15-0.45         |
| Vitamin E                     | 0.015-0.0296      |

EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ALA, α-linoleic acid; SDA, stearidonic acid; ARA, arachidonic acid.
Effects of FOs on renal injury induced by CLP. H&E staining was performed for histopathological evaluation (Fig. 1B). Histopathological alterations are direct indications of renal injury. In contrast to the control group, the model group presented glomerular atrophy, dilatation of the renal capsule cavity, destruction of tubular structures, and local focal epithelial cell necrosis. However, pretreatment with FOs or dexamethasone alleviated glomerular atrophy and epithelial necrosis.

Effects of FOs on CLP-induced renal dysfunction. Renal injury was assessed in rats subjected to CLP for 24 h. The levels of Scr, and BUN, NGAL and KIM-1 in the serum were significantly elevated in the model group at 24 h post-CLP compared with the control group. However, treatment with dexamethasone and FOs significantly diminished the levels of Scr, BUN, NGAL and KIM-1 compared with those of the model group (Fig. 2).

Effects of FOs on CLP-induced inflammatory cytokines expression in the serum. The expression levels of TNFα, IL-1β and IL-6 were determined by ELISA and by immunofluorescence assays in serum samples from CLP-rats. The results indicated that the expression levels were significantly increased in the model group compared with the control group, whereas administration of FOs or dexamethasone significantly
suppressed the induction of TNF-α, IL-1β and IL-6 expression in the CLP group (Fig. 3A). Immunofluorescence analysis of the renal tissue samples verified the aforementioned results (Fig. 3B). The data indicated that FOs could reduce the induction of inflammation in the CLP group.

**Effects of FOs on CLP-induced oxidative stress and apoptosis.** The induction of inflammation is usually accompanied with the induction of oxidative stress and apoptosis (28). The effects of FOs on CLP-induced renal oxidation injury were presented in Fig. 4A. The results indicated that CLP induced a significant increase in the MDA levels compared with the control group. In addition, the activity of the antioxidant enzymes SOD and GSH-Px was significantly decreased in the CLP model compared with the control group. The MDA levels of the CLP rats that received treatment with dexamethasone and FOs were reduced by 60.4 and 57.7% compared with those of the CLP model group. Moreover, the activity levels of SOD and GSH-Px in animals treated with dexamethasone were increased by 135.8 and 134.5%, whereas FOs treatment resulted in increases of 104.0 and 104.3%, respectively.

**TUNEL staining.** The induction of apoptosis in the present animal model was stimulated by CLP treatment (Fig. 4B). The percentage of TUNEL-positive cells was increased in the CLP model group compared with the control group. Treatment of the rats with dexamethasone and FOs markedly decreased the number of apoptotic cells. Moreover, the expression levels of the apoptosis-related proteins were detected (Fig. 4C). Bcl-2 expression levels were significantly reduced, whereas the expression levels of Bax and cleaved-caspase 3 were significantly increased in the CLP model compared with those of the control (Fig. 4C). The ratio of Bax/Bcl-2 was significantly increased in the model group, while dexamethasone and
FOs treatment significantly reduced the ratio (Fig. 4C). This indicated the effects of FOs on cell apoptosis. Therefore, administration of dexamethasone and FOs caused a notable decline in the induction of apoptosis by CLP.

**FOs negatively regulates the activation of JAK/STAT3, p38-MAPK, and nuclear factor-κB (NF-κB) signaling pathways.** FOs were determined to affect the activation of JAK2/STAT3, p38-MAPK and NF-κB proteins in the renal tissues of CLP rats. Our results indicated that the phosphorylation levels of JAK2, STAT3, p38MAPK, IκBα and p65 were significantly reduced in renal tissues of CLP rats treated with FOs compared with the model group (Fig. 5).

**Histology.** H&E staining of intestinal tissues derived from the CLP model group revealed renal injury as demonstrated by...
tissue destruction and inflammation. This was reversed by dexamethasone and FOs treatment (Fig. 6A). Immunofluorescence revealed that FO treatment suppressed the production of the pro-inflammatory factors, IL-1β, IL-6 and TNFα in the small intestine of cLP rats (Fig. 6B).

**Discussion**

In the present study, the survival rates of rats subjected to CLP-induced sepsis were prolonged following treatment with FOs and dexamethasone. A recent study has shown that in a similar in vivo model, the administration of FOs alone led to ~70% survival at 48 h following CLP induction (29). Dexamethasone alleviated the induction of inflammation caused by sepsis and reduced the extent of lipid peroxidation (30). In the present study, dexamethasone was used as a positive control. The current investigation evaluated the therapeutic effects of FOs as a pharmaconutrient in the absence of standard administration of food and water. The levels of Scr, BUN, KIM-1 and NGAL were measured to evaluate kidney injury as described previously (20).

Previous investigations have shown that ω3 FAs can reduce inflammation, fibrosis and oxidative stress, which are associated with renal function abnormalities (31-33). The protective effects of FOs on AKI and their associated mechanism of action were examined. In the CLP model, kidney injury was evident with distinct changes in the levels of biochemical markers histopathological markers of renal function and injury. The results indicated that treatment of the GLP rats with FOs alleviated the changes in the expression of the histopathological markers, increased the animal survival duration, and decreased the levels of Scr, BUN, KIM-1 and NGAL.
NGAL is a reliable marker for GFR function (34,35). The reduced NGAL levels after pretreatment of FOs suggests that FOs may alleviate renal injury by improving GFR. The downregulation of SCr and BUN further confirmed this assumption. AKI patients with the existence of KIM-1 expression usually have poor prognosis (36). FOs reduce KIM-1 expression in cLP-induced rats, suggesting the potential effects of FOs on prognosis of sepsis-induced renal injury; however, further investigation is required.

Excessive inflammation is a critical step during septic shock (37). In sepsis, multiple mediators of inflammation participate in organ injury (37). The proinflammatory cytokines IL-1β, IL-6 and TNFα are produced by damaged renal tubule cells or extrarenal cells, serving as potential critical contributors to renal injury (38). The current study confirmed that the anti-inflammatory effects of FOs were mediated by regulation of pro-inflammatory cytokine secretions as reported previously (39). In addition, the data demonstrated that CLP-induced septic intestinal injury could be reduced by FOs treatment. H&E staining of intestinal tissues derived from the CLP model group confirmed the induction of renal injury as demonstrated by tissue destruction and inflammation. This was reversed by dexamethasone and FOs treatment. In addition, immunofluorescence staining indicated that FOs inhibited the CLP-induced production of the pro-inflammatory factors, IL-1β, IL-6 and TNFα in the small intestine. These results supported the aforementioned findings regarding the effects of external FOs on sepsis-induced organ dysfunction. The inhibitory effects of FOs on inflammation may suppress the progression of AKI.

Oxidative stress is a critical step required for the pathogenesis of AKI (40). Oxidative stress, as well as inflammation, can promote the severity of sepsis (41). Excessive oxidative stress is one of the main reasons inflammation is induced (41). Early in 1994, FOs have been found to promote antioxidant enzyme expression in murine lupus nephritis (39). Free radicals increased the levels of lipid peroxidation and led to the generation of reactive aldehyde metabolites, such as MDA (42). The antioxidant defense system includes the enzymes (SOD and GSH-Px) that exert protective effects on lipid peroxidation (43). In the present study, FOs caused a significant inhibition in the levels of MDA, and promoted the activity of SOD and GSH-Px in the tissues of septic rats. This suggested that they could suppress CLP-induced oxidative stress by increasing the activity levels of the antioxidant enzymes. Therefore, FOs may attenuate renal injury in the rat CLP model by inhibiting oxidative stress.

Furthermore, the present study reported the suppressive effects of FOs on the induction of apoptosis caused by CLP. The data showed that increased induction of apoptosis was noted in renal tissues from the CLP group, while pretreatment of the rats with FOs could effectively alleviate renal injury by reducing the levels of apoptosis. The expression levels of...
specific apoptotic proteins were determined. The Bcl-2 family of proteins exerts key regulatory functions with regard to apoptosis induction. The Bax to Bcl-2 ratio has been used as a marker for the activation of caspase 3 (44,45). The present study indicated that the expression levels of cleaved caspase-3 and Bax were increased, whereas the expression levels of Bcl-2 were decreased in the CLP model group. Moreover, pretreatment of the rats with FOs could reverse the changes noted in the expression levels of these proteins. The results indicated that FOs exhibited a strong protective effect against CLP-induced apoptosis.

It has been reported that FOs inhibit the NF-κB signaling pathway in sepsis-induced liver injury (46). The activation of p38 has been shown to increase the expression levels of the inflammatory cytokines, such as TNF-α, IL-1β and IL-6 in microglial cells (47). Furthermore, TNF-α serves a critical role in the endothelial dysfunction of patients with sepsis via activating the p38-MAPK and the NF-κB pathways (48). The suppression of the TNFα/p38-MAPK/caspase-3 signaling pathway is also considered a key mechanism in the prevention of sepsis-induced myocardial injury in rats (49). In the present study, the results indicated that FOs inhibited p38-MAPK and NF-κB signaling activation by CLP. In addition, the JAK/STAT signaling pathway is involved in immune and inflammatory responses, by regulating the expression of several target genes (50,51). JAK is an upstream regulator of STAT activation, which has been rarely investigated in sepsis-induced organ dysfunction. In the current study, the activation of JAK2/STAT3 signaling pathway was inhibited by FOs. This was noted in the renal tissues from CLP rats. The results indicated that FOs could inhibit the CLP-induced activation of the NF-κB, p38-MAPK, and JAK2/STAT3 signaling pathways. Whether the effects of FOs on renal function and induction of oxidative stress, apoptosis and inflammation were mediated by these signaling pathways remains unclear. Thus, further investigation is required.

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Availability of data and materials
The datasets generated and/or analyzed during the current study are not publicly available due to data confidentiality in our hospital but are available from the corresponding author on reasonable request.

Authors’ contributions
ZL drafted the manuscript, data analysis, and made substantial contributions to the design of the present study and resolved the problems during investigation. JJ prepared experimental samples and detection. XS prepared the manuscript, and was involved in western blotting and ELISA. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The animal experiment protocol was approved by the Commission for Animal Experimentation of the people's hospital of Xishuangbanna Dai Nationality Autonomous Prefecture.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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