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Effects of fish oil-based lipid emulsion on inflammation and kidney injury in mice subjected to unilateral hind limb ischemia/reperfusion

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ABSTRACT

This study investigated the effects of a fish oil-based lipid emulsion (FO) on local skeletal muscle and remote renal damage at 72 h post-reperfusion in a murine model of hind limb ischemia-reperfusion (IR) injury. Mice were assigned to 1 sham group and 3 IR groups. The IR groups were treated daily with either saline or FO from 3 days prior to limb ischemia till 3 days after reperfusion. Limb IR was induced by applying a 4.5-oz orthodontic rubber band above the left greater trochanter for 120 min followed by band-released reperfusion for 72 h. Mice were then sacrificed to harvest blood, muscle, and kidney for analysis. The results showed that IR injury led to upregulation of pro-inflammatory monocytes in blood, infiltration of leukocytes into injured muscle, and over-expression of pro-inflammatory genes in muscle and kidney tissues. Supplementing FO either before or after IR injury alleviated IR-induced inflammatory gene expressions in muscle and kidney tissues. Furthermore, FO given after IR injury reduced circulating pro-inflammatory monocytes, limited muscle leukocytic infiltration, and improved renal histology. These results suggest that FO may protect the muscles from IR injury. FO given after IR injury can better downregulate the inflammation seen in IR-induced remote kidney injury.

1. Introduction

Acute kidney injury (AKI) is frequently encountered during the management of critically ill patients and is usually a consequence of the excessive systemic inflammatory response syndrome (SIRS) from primary injuries such as sepsis, major surgery, cardiogenic shock, and hypovolemic shock that often lead to insidious remote kidney injury as a part of multi-organ failure [1]. Ischemic/reperfusion (IR) injury is among one of the most crucial mechanisms underlying multi-organ dysfunction syndrome caused by surgery, cardiogenic and hypovolemic shock [2–4].

Acute limb ischemia commonly occurs in patients with peripheral artery occlusive disease and in those subjected to vascular trauma or intra-operative tourniquet use [5]. Reperfusion of the ischemic limb results in vital organ injury, which may progress to multiple organ failure with a high mortality rate [6,7]. Events involved in IR injury include the liberation of oxygen free radicals generated locally in ischemic-reperfused limbs, recruitment of neutrophils to remote organs, and endotoxemia caused by bacterial translocation due to altered intestinal permeability [8]. In addition to remote lung and intestinal injury, AKI is also observed following limb IR. Causative association between IR-injury and remote renal injury has been demonstrated in rats following bilateral hind limb IR injuries [9]. Clinical studies have also shown significant renal injuries following supra- or infra-renal abdominal aortic surgery which frequently require intraoperative cessation of blood flow to the lower limbs [10,11]. Furthermore, it has been demonstrated from epidemiological evidence that ischemic rhabdomyolysis of the skeletal muscle may be a major determining factor of

Abbreviations: AKI, acute kidney injury; BUN, blood urea nitrogen; CK, creatinine kinase; CRP, C-reactive protein; Cr, creatinine; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FO, fish oil; HSP72, heat shock protein 72; IL-6, interleukin-6; IR, ischemia-reperfusion; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; MO/MP, monocytes/macrophages; NF-κB, nuclear factor-κB; NGAL, neutrophil gelatinase-associated lipocalin; ORB, orthodontic rubber band SIRS, systemic inflammatory response syndrome; PUFA, polyunsaturated fatty acids; PPAR γ, peroxisome proliferator-activated receptor γ

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renal failure after thoracoabdominal aortic surgery [11].

Fish oil contains high levels of long chain n-3 polyunsaturated fatty acids (PUFAs). These PUFAs, particularly eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6), when consumed in sufficient quantities have been shown to possess many immunomodulatory and anti-inflammatory properties [12]. In addition to the increased competitive inhibition of arachidonic acid-derived pro-inflammatory eicosanoids, other proposed mechanisms involve generation of EPA- and DHA-derived resolvins, decreased activation of nuclear factor (NF)-κB, upregulation of peroxisome proliferator-activated receptor (PPAR)γ, and decreased expression of adhesion molecules and chemoattractants [12]. Studies investigating the effects of fish oil-based lipid emulsion (FO) on hind limb IR-induced remote organ dysfunction are scarce. However, possible benefits of n-3 PUFAs have been demonstrated in other IR animal models. In a murine model of superior mesenteric artery occlusion and reperfusion, intraperitoneal FO pretreatment in rats resulted in the reduction of pulmonary macrophage infiltration and the suppression of intestinal and lung inflammation [13]. A more recent ex vivo study showed that monocytes/macrophages (MO/MP) cultured with EPA and/or DHA-added lymph resulted in a significant reduction in the generation of inflammatory-related proteins in a murine model of intestinal IR [14]. Our previous study also showed that FO administration before ischemia reduced blood inflammatory monocytes, muscle M1/M2 ratio and expression of lung inflammatory mediators 24 h after reperfusion [15]. Since no study has investigated the impact of FO administration on IR-induced kidney injury in limb I/R model, the aim of this study was to explore the anti-inflammatory effects of FO in the context of protecting remote renal injury resulting from the SIRS generated from hind limb IR injury. We hypothesized that FO may improve local skeletal muscle damage, lessen the systemic inflammatory response, and protect against remote kidney injury from limb IR injury by modulating cytokine/chemokine expressions and altering leukocyte recruitment.

2. Materials and methods

2.1. Animals

Thirty-two male C57BL/6 mice (5-week-old, weighing 18–20 g) were purchased from the National Laboratory Animal Center (Taipei, Taiwan). The animals were acclimatized for 4 weeks prior to the experiment. Animals were accommodated in a temperature- and humidity-controlled room with food and water provided *ad libitum*. Care of laboratory animals were in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011), and the experiment protocols were approved by the Institutional Animal Care and Use Committee at Taipei Medical University (LAC-2015-0018).

2.2. Experimental design

Mice were randomly assigned to 1 Sham (n = 8) group and 3 IR groups: a saline-saline group (SS, n = 8), a saline-FO group (SF, n = 8), or a FO-FO group (FF, n = 8). The SS mice served as the positive control and were given daily saline injection from 3 days prior to unilateral left hind limb ischemia till 3 days after the insult. The SF and FF groups were respectively given 3 days of saline or FO-based lipid emulsion prior to ischemic injury followed by 3 days of FO in both groups. In addition to standard chow (Purina No. 5001) provided *ad libitum*, animals were given intraperitoneal (IP) injections of saline or FO (10% Omegaven, Fresenius-Kabi, Hamburg, Germany) at a dose of 10 mL/kg body weight (BW)/day (equivalent to 1 g/kg BW/day of fish oil). This chosen dosage is equivalent to 0.3 g/kg/day in humans [16], which was demonstrated to be therapeutically effective [17]. Previous studies have demonstrated that lipid emulsions given by intraperitoneal injection can be effectively absorbed by the peritoneal cavity with a high rate of absorption and bioavailability [18,19]. All animals, except the sham mice, underwent 120 min of unilateral hind limb ischemia followed by 72 h of reperfusion before sacrifice.

The induction of ischemia was performed as described by Crawford et al [20]. Animals were deeply anesthetized with an IP injection of telitamine/zolazepam (Zoletil, 25 mg/kg) and xylazine (Rumpun, 10 mg/kg), circumferentially shaved over the left upper thigh, and then banded just above the left greater trochanter using a 4.5-oz orthodontic rubber band (ORB; American Orthodontics, Sheboygan, WI). After the procedure, animals were returned to their cages inside the laboratory animal facility. Mice were maintained in a right lateral decubitus position and kept anesthetized throughout the course of the ischemic period. After 120 min of ischemia, reperfusion was initiated by cutting the ORB with scissors. For hydration, all IR mice received subcutaneous injections of 0.5 mL saline upon initial banding with an additional 0.5 mL given at the beginning of the reperfusion period following ORB release. The sham group underwent anesthesia and shaving of the left hind limb but did not undergo the IR procedure. General appearance, physical activity, and body weight of animals were assessed daily by the primary investigator during the experimental course. Those with rapid or progressive body weight loss, open wounds, or persistent recumbency were considered for early euthanization. In this study, all subjects remained healthy throughout the experimental period.

All IR animals were euthanized at 72 h after reperfusion by cardiac puncture. Muscle (gastrocnemius) tissues were taken from the affected limb for cytology and cytokine/chemokine analysis. Whole blood samples were aliquoted into heparinized tubes for the analysis of leukocyte subpopulations. Plasma separated from the remaining blood samples by centrifugation at 1500 × *g* at 4 °C for 10 min was then used for biochemical analysis. For histological analysis, upper half of the right kidney was harvested and fixed with 4% paraformaldehyde in phosphate-buffered saline. All other remaining kidney tissues were snap frozen in liquid nitrogen and stored at -80 °C for further analysis.

2.3. Plasma biochemical markers of renal and muscle injury

Variables measured in plasma included a (1) systemic markers of renal function: blood urea nitrogen (BUN), creatinine (Cr), and neutrophil gelatinase-associated lipocalin (NGAL); (2) biochemical markers of cell injury: creatinine kinase (CK) and lactate dehydrogenase (LDH); and (3) systemic marker of inflammation: C-reactive protein (CRP). Analysis of plasma BUN, CK, LDH were conducted utilizing the VetTest® Chemistry Analyzer (IDEXX Laboratories Inc., Westbrook, MN, USA). Plasma Cr concentrations were analyzed using the Jaffe's reaction and determined by colorimetric method (Cayman Chemical, Ann Arbor, MI, USA). NGAL and CRP levels were measured using commercially-available enzyme-linked immunosorbent assay (ELISA) kits (R & D Systems Inc., Minneapolis, MN, USA).

2.4. Blood leukocyte distribution

The distribution of leukocyte subpopulations was analyzed from samples of heparinized whole blood using flow cytometry. Blood was first separated into 2 aliquots of 100 μL each. For analysis of blood monocytes, one of the aliquots was incubated with PerCP-conjugated anti-CD45 (Biolegend, San Diego, CA, USA), PE-conjugated anti-F4/80 (Biolegend), Pacific blue-conjugated anti-Ly6C (Biolegend), and APC-conjugated anti-CCR2 (R & D Systems, Inc., Minneapolis, MN, USA). For analysis of blood neutrophils, the other was incubated with PerCP-conjugated anti-CD45, FITC-conjugated anti-Ly6G, and APC-conjugated anti-CXCR2 (all Biolegend) antibodies. All antibody concentrations used were compliant with manufacturer recommendations. Samples were incubated at 4 °C in the dark for 30 min. Then, red blood cells in the samples were lysed and leukocytes were stained and suspended in staining buffer (PBS with 0.5% bovine serum albumin). For cytometric analysis, a FACS Canto II flow cytometer (BD Biosciences) was used.