RESEARCH ARTICLE

The effects of dietary fish oil on inflammation, fibrosis and oxidative stress associated with obstructive renal injury in rats

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Scope: We examined whether dietary supplementation with fish oil modulates inflammation, fibrosis and oxidative stress following obstructive renal injury.
Methods and results: Three groups of Sprague–Dawley rats ($n = 16$ *per* group) were fed for 4 wk on normal rat chow (oleic acid), chow containing fish oil (33 g eicosapentaenoic acid and 26 g docosahexaenoic acid *per* kg diet), or chow containing safflower oil (60 g linoleic acid *per* kg diet). All diets contained 7% fat. After 4 wk, the rats were further subdivided into four smaller groups ($n = 4$ *per* group). Unilateral ureteral obstruction was induced in three groups (for 4, 7 and 14 days). The fourth group for each diet did not undergo surgery, and was sacrificed as controls at 14 days. When rats were sacrificed, plasma and portions of the kidneys were removed and frozen; other portions of kidney tissue were fixed and prepared for histology. Compared with normal chow and safflower oil, fish oil attenuated collagen deposition, macrophage infiltration, TGF-β expression, apoptosis, and tissue levels of arachidonic acid, MIP-1α, IL-1β, MCP-1 and leukotriene B_4. Compared with normal chow, fish oil increased the expression of HO-1 protein in kidney tissue.
Conclusions: Fish oil intake reduced inflammation, fibrosis and oxidative stress following obstructive renal injury.

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1 Introduction

Chronic renal fibrosis is a common problem in elderly people, and often leads to kidney failure and end stage renal disease [1, 2]. The increasing incidence of renal fibrosis provides impetus for the discovery of prophylactic or therapeutic agents to attenuate such damage. Such agents may include long-chain ω-3 fatty acids, including docosahexaenoic acid (22:6ω-3) and eicosapentaenoic acid (20:5ω-3). Docosahexaenoic acid and eicosapentaenoic acid possess a variety of anti-inflammatory properties. These properties include competition with ω-6 fatty acids such as arachidonic acid (AA) (20:4ω-6) within the phospholipid membrane of cells, together with modulatory effects on leukocyte functions and pro-inflammatory cytokine synthesis [3].

Several studies have investigated the efficacy of docosahexaenoic acid and eicosapentaenoic acid in relation to the function of kidney cells *in vitro*, and also *in vivo* in rats and humans. *In vitro*, docosahexaenoic acid reduces the growth

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Abbreviations: AA, arachidonic acid; **IHC,** immunohistochemistry; **LTB_4,** leukotriene B_4; **MCP,** monocyte chemoattractant protein; **MIP,** macrophage inflammatory protein; **NFκB,** nuclear factor-κB; **UUO,** unilateral ureteral obstruction

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and proliferation of cultured mesangial cells by reducing the activity of platelet-derived growth factor [4]. Eicosapentaenoic acid and docosahexaenoic acid reduce the expression of monocyte chemoattractant protein (MCP)-1 and IL-8 in cultured human kidney cells and endothelial cells by activating peroxisome proliferator-activated receptor-α, which in turn inhibits nuclear factor-κB (NFκB) [5, 6]. Eicosapentaenoic acid inhibits the expression of leukocyte adhesion molecules in glomerular endothelial cells, which in turn prevents leukocytes from binding to glomerular endothelial cells [7]. Docosahexaenoic acid and eicosapentaenoic acid also suppress leukocyte chemotaxis. This anti-inflammatory effect is due in part to the preferential formation of the 5-series eicosanoids derived from eicosapentaenoic acid, which are less potent chemoattractants than the 4-series eicosanoids derived from AA [8–11].

In vivo, eicosapentaenoic acid and docosahexaenoic acid reduce kidney damage and loss of renal function in rats with glomerulonephritis [4]. Eicosapentaenoic acid and docosahexaenoic acid also reduce acute kidney injury following temporary occlusion of the renal artery by attenuating the mRNA expression of TNF-α and inducible nitric oxide synthase [12]. Fish oil also reduces interstitial inflammation, tubulointerstitial proliferative activity, NFκB activation and the protein expression of MCP-1, TGF-β, Smad 2, extracellular signal-regulated protein kinase, cyclo-oxygenase-2 and nicotinamide adenine dinucleotide phosphate oxidase subunits (NOX-4, $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$) in rats with hypertension [13] and renal mass reduction [14]. Dietary supplementation with ω-3 fatty acids contained in canola oil suppresses inflammation and collagen formation in the kidneys of rats with diabetes [15].

The data from these studies indicate that ω-3 fatty acids can potentially reduce inflammation, fibrosis and oxidative stress associated with abnormalities in renal function. Despite the prevalence and importance of obstructive renal injury, no research has examined the efficacy of supplementation of ω-3 fatty acids on the pathophysiology of this condition. We hypothesized that fish oil would reduce inflammation, fibrosis and oxidative stress in renal tissue in rats subjected to unilateral ureteral obstruction (UUO). UUO is an experimental animal model commonly used to investigate fibrogenesis in renal tissue, including the roles of oxidative stress, inflammation and apoptosis [16].

2 Materials and methods

2.1 Animals, diet and experimental design

All experimental procedures were conducted with approval from the University of Queensland Animal Ethics Committee (Approval Number MED/RBWH/310/07/UQ). Male Sprague–Dawley rats (body mass range 180—220 g) were used in this study. The rats were allowed free access to water, and were housed four rats *per* cage in an air-conditioned room on a 12/12 h light/dark cycle. The rats were fed *ad libitum* with approximately 15 g *per* day *per* animal of one of the following diets for 4 wk: standard rat chow, rat chow supplemented with linoleic acid (18:2ω-6) (70 g safflower oil/kg diet, containing 53.8 g linoleic acid/kg) or rat chow supplemented with eicosapentaenoic acid and docosahexaenoic acid (70 g fish oil/kg diet, containing 18.7 g eicosapentaenoic acid/kg and 14.4 g docosahexaenoic acid/kg). The ratio of eicosapentaenoic acid:docosahexaenoic acid in the present study was 1.3:1, which is the same as that used in other animal studies [4] and human studies [17–19]. This intake is equivalent to a dietary intake of 0.5 g eicosapentaenoic acid and docosahexaenoic acid for a 75-kg adult. This intake is lower than that used in most clinical trials; however, to prepare the fish oil chow, it was not possible to incorporate more than 70 g fat *per* 1 kg chow. The specialized diets were modified AIN93G diets (Specialty Feeds, Glen Forrest, WA, Australia), and were identical to the AIN93G diet with regard to energy (16.1 MJ/kg), protein (19.4%) and total fat (7%). The fatty acid composition of the three diets is described in Table 1.

After 4 wk, the rats in each dietary group were further subdivided into four smaller groups ($n = 4$ animals *per* group) resulting in 12 groups in total. Animals in nine of these 12 groups underwent surgery to induce UUO after the initial 4-wk supplementation period. UUO was maintained for a further 4, 7 or 14 days in each of the dietary groups before the rats were sacrificed. Rats were fed with their respective diets during the period of UUO. Animals in the remaining three groups ($n = 4$ *per* group) were fed normal chow, safflower oil or fish oil for six wk, and did not undergo surgery.

Table 1. Fatty acid composition of diets

	Normal chow	Safflower diet	Fish oil diet
Linoleic acid (18:2-ω-6) (g)	13.0	54.0	1.1
Oleic acid (18:1) (g)	42.0	9.0	5.0
Eicosapentaenoic acid (20:5-ω-3) (g)	0.0	0.0	18.7
Docosahexaenoic acid (22:6-ω-3)	0.0	0.0	14.4
Total ω-6 (g)	13.3	54.0	2.0
Total ω-3 (g)	7.8	0.1	34.0
Other fatty acids (g)	48.9	6.9	34.0

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2.2 Surgery

The rats were anesthetized with pentobarbital sodium (60 mg/kg, intraperitoneal injection). Normal body temperature was maintained throughout surgery. For UUO surgery, the left kidney was exposed with a mid-abdominal incision and a double ligature was placed on the left ureter approximately 1 cm from the renal hilum. The abdominal incision was then closed with 4–0 silk and Michel clips. For the control operation, the rats were anesthetized and then left to recover without any sham surgery. Rats were monitored daily for signs of wound or systemic infections, anorexia, weight loss, acute toxicity and average food consumption. No differences were seen amongst groups.

2.3 Preparation of tissue

At the times of sacrifice, rats were heavily anesthetized with pentobarbital sodium. Body mass was then recorded, blood was collected *via* a retrograde catheter in the aorta into sterile heparinized tubes, and the obstructed kidney was removed prior to death. Fatty tissue surrounding the kidney was removed, the kidney was blotted and weighed and it was quickly sliced transversely (2–3 mm slices) to the length of the kidney in the equatorial plane. The slices closest to the center were fixed in 10% buffered formalin for histology and immunohistochemistry (IHC), and the adjacent slices of kidney tissue were snap frozen in liquid nitrogen and stored at −80°C in two portions in RNAse-free cryovials for protein, fatty acid or oxidative stress analyses. Blood was centrifuged for 10 min at 450 × *g* at room temperature to obtain plasma. Plasma was stored at −80°C for the analysis of fatty acids, creatinine and C-reactive protein.

2.4 Fatty acid analysis

The full details of the methods used to analyze fatty acids are presented as Supporting Information. The fatty acid content of plasma and kidney tissue was analyzed according to previously published methods [20, 21]. Kidney tissue was thawed at room temperature, and 200 mg were homogenized in physiological saline using a Polytron. Plasma was thawed at room temperature, and then centrifuged at 12 250 × *g* for 2 min. Lipids were extracted from homogenized tissue and plasma samples using chloroform:methanol. The total lipid extract from the plasma and cellular fractions was fractionated by thin-layer chromatography and the phospholipid fractions retained. The cellular and plasma fractions were then trans-esterified by methanolysis (1% H_2SO_4 in methanol at 70°C for 3 h). Fatty acid methylester extracts were separated and measured on a Hewlett-Packard 6890 gas chromatograph equipped with a 50 mm capillary column (0.32 mm internal diameter SGE, Victoria, Australia) coated with 70% cyanoproply polysilphenylene-siloxane (BPX-70) (0.25 μm film thickness), which was fitted with a flame ionization detector. Helium was the carrier gas and the split-ratio was 20:1. The injector temperature was set at 250°C and the detector temperature at 300°C. The initial oven temperature was 140°C and programmed to rise to 220°C at 5°C *per* minute and upon reaching that temperature, was maintained for 3 min. Fatty acid methylester extracts were identified based on the retention time of standards obtained from Nucheck Prep (Elysian, MN, USA) using the Chemstation software.

2.5 Leukotriene B_4 and isoprostanes analysis

The full details of the methods used to analyze leukotriene B_4 and isoprostanes are presented as Supporting Information. Isoprostanes (8-iso-$PGF_{2\alpha}$) and AA were extracted from kidney homogenate supernatant using a modified method from [22]. Leukotriene B_4 (LTB_4) was extracted from kidney homogenate supernatant using solid-phase extraction [23]. Lipids were derivatized using previously reported methods [24]. Samples were analysed for AA, isoprostanes and LTB_4 concentration using a Varian 320 MS/MS, with a Varian 450 gas chromatograph equipped with a CP8400 auto sampler. LTB_4, AA and 8-iso-$PGF_{2\alpha}$ isoprostane concentrations were divided by the protein concentration in the original homogenized supernatant. Because LTB_4 and 8-iso-$PGF_{2\alpha}$ isoprostane are derivatives of AA, data for LTB_4 and 8-iso-$PGF_{2\alpha}$ isoprostanes are expressed as a ratio of AA.

2.6 Creatinine and C-reactive protein analysis

Plasma creatinine concentration was analyzed using the Jaffe method (QuantiChromTM Creatinine Assay Kit DICT 500, Bioxys, Brussels, Belgium). C-reactive protein was measured using a high-sensitivity turbidimetric assay (Kamiya Biomedical, Seattle, WA, USA). Both assays were conducted using an automated analyzer (Cobas MIRA, Roche Diagnostics, Basel, Switzerland).

2.7 Cytokine analysis

Kidney tissue was homogenized using a Bio-Plex cell lysis kit (BioRad Laboratories, Hercules, CA, USA) specific for multiplex assays. Briefly, kidney tissue was washed with a wash buffer in a glass tube, then 0.5 mL cell lysis buffer were added before the kidney tissue was homogenized for 20 s. The kidney homogenate was then transferred to a polypropylene tube, frozen at −80°C, thawed and centrifuged at 1450 × *g* to collect the supernatant. The supernatant was then frozen until the day of analysis. The supernatant was added undiluted to a multiplex assay (Rat Cytokine/Chemokine Panel, Lincoplex, Millipore, Billerica, MA, USA) to analyze the concentrations of the cytokine IL-1β and the

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chemokines MCP-1 and macrophage inflammatory protein (MIP)-1α. The assay was conducted according to the manufacturer's guidelines using the Luminex® 100 IS system (Millipore, Billerica, MA, USA). All samples were analyzed in duplicate. The intra-assay coefficient of variation was 8.0% for IL-1β, 15.4% for MCP-1 and 9.5% for MIP-1α. After comparison with a standard curve, cytokine/chemokine concentrations were adjusted for the protein concentration of each sample. Protein concentration was measured using the BCA protein assay (Thermo Fisher Scientific, Rockford, IL, USA).

2.8 Histological studies

Kidney tissue was fixed in 10% buffered formalin. The fixed tissue was embedded in paraffin using routine histological techniques, sectioned at 4 μm, and mounted on SuperFrost Plus glass slides (Menzel Glaeser, Braunschweig, Germany). A complete set of sections was batch stained with hematoxylin and eosin (H&E) for morphological assessment of apoptosis, and with picosirius red and Masson's trichrome for the assessment of fibrillar collagen as a marker of fibrosis. Remaining sets of sections were used for IHC using ED-1 for macrophages, α-smooth muscle actin (α-SMA) for activated myofibroblasts, TGF-β1 as a profibrotic cytokine and TNF-α as a marker of inflammation. Apoptosis was counted using × 400 magnification microscopy in the tubular epithelium of 10 random corticomedullary fields *per* section. Morphological characteristics counted were: (i) shrunken eosinophilic cells with condensed, marginated nuclear chromatin and intact cell membranes; (ii) discrete apoptotic bodies comprising large, dense, pyknotic, nuclear fragments usually surrounded by a narrow eosinophilic cytoplasmic rim and (iii) clusters of small apoptotic bodies (assessed as a single apoptotic occurrence). The presence of apoptosis was verified enzymatically using the ApopTag® Peroxidase *In Situ* Apoptosis Detection Kit (Millipore). Morphological assessment of apoptosis in tissue sections is an accepted method for quantification [25, 26]. These data were used for graphical representation of the incidence of apoptosis.

2.9 Immunohistochemistry (IHC)

The following antibodies were used as primary antibodies for IHC: mouse monoclonal anti-α-SMA clone 1-A4 (Sigma, St. Louis, MO, USA; 1:400) for myofibroblasts; rabbit polyclonal anti-TGF-β1 and anti-TNF-α, (Cell Signaling; Beverly, MA, USA; 1:50); and mouse monoclonal anti-ED-1 (Serotec; Oxford, UK; 1:150) for macrophages. Kidney sections for IHC were dried overnight at 37°C, dewaxed in xylene and rehydrated through graded ethanol to PBS. Endogenous peroxidase activity was blocked by immersion in 3% hydrogen peroxide and 0.1% sodium azide in PBS for 10 min at ambient temperature. Sections were washed three times in PBS for 5 min, before they were incubated for 20 min with normal goat serum diluted 1:10 with PBS, pH 7.4. Sections were then incubated with the primary antibody in PBS overnight (18 h) at 4°C. They were then thoroughly washed in PBS (3 × 5 min changes), and incubated with the appropriate secondary antibody (DAKO Envision, DAKO Cytomation, Glostrup, Denmark; 1:2000) for 30 min. Visualization of antibody localization was facilitated with diaminobenzidine hydrochloride. Sections were rinsed three times with PBS for 5 min followed by counterstaining with Mayer's hematoxylin, blued in Scott's tap water, dehydrated in ethanol, cleared in xylene and mounted using Depex mounting medium and glass coverslips. Negative controls were prepared without primary antibody and were consistently negative.

2.10 Morphometry

Quantification of picosirius red (red stain for collagen) Masson's trichrome (blue stain for collagen) and brown diaminobenzidine hydrochloride (α-SMA for myofibroblasts, ED1 for macrophages, TGF-β1 and TNF-α proteins) was achieved by scanning the stained slides using the Aperio system (Spectrum, Aperio, CA, USA), and selecting 10 fields at × 200 magnification *per* section. Image morphometry was carried out using either the Aperio ImageScope software (% of field, for picrosirius red), or ImagePro Plus image analysis software (Version 4.1.29, Media Cybernetics, Silver Spring, MD, USA) (area of stained color in square microns in each of the selected view). Fields for picrosirius red, Masson's trichrome and α-SMA were not considered if they contained large blood vessels which also stain positive for these stains. For quantification of ED1 positive cells (macrophages) in the kidney, ten fields from the cortex and outer medulla of each kidney section were randomly chosen and ED1 positive cells counted at × 400 magnification. All slides were assessed by the same investigator (GG), who was blinded to the treatment procedure.

2.11 Western blotting of HO-1

Renal tissue was disrupted in ice-cold cell RIPA buffer (0.15 M NaCl, 0.025 M NaF, 0.5 M EDTA, 0.1% SDS, 1.0% Igepal CA-630 in 50 mM Tris-Cl, pH7.5) containing protease and phosphatase inhibitors (10 μg/mL leupeptin, 10 μg/mL aprotinin, 100 μg/mL PMSF, 1 mM sodium orthovanadate) (Sigma-Aldrich, Botany, Australia) using a tissue homogenizer. Cell debris was removed by centrifugation at 16 000 × *g* for 15 min at 4°C. Protein concentration was determined in each tissue extract by a Bradford protein assay (Bio-Rad Pty, Sydney, Australia) and spectroscopy at 595 nm. Forty microgram of total protein was electrophoresed on SDS-polyacrylamide gels using a Bio-Rad mini

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Protean unit, transferred to a polyvinylidene difluoride membrane and blotted with HO-1 (1:1000) (Stressgen, BC, Canada). Horseradish peroxidase-conjugated secondary antibody diluted at 1:2000 in 5% blotto was used. Protein bands were visualized using enhanced chemiluminescence, X-ray film was scanned using a Hewlett Packard ScanJet 3200 C at 300 dpi and Scion Image (β4.0.2) software was used to quantify the density of the bands in arbitrary densitometry units. Membranes were routinely stained with Coomassie blue (Sigma-Aldrich), or pan-actin immunoblots were used to verify equal protein loading of lanes. Levels of expression for HO-1 were normalized against actin for graphs of densitometry.

2.12 Statistical analysis

The main comparisons of interest were (1) fish oil *versus* safflower oil and (2) fish oil *versus* normal chow. Furthermore, pooled data for each of the three UUO groups (4, 7 and 14 days) were compared individually with the control group (no UUO) using independent *t*-tests to assess the impact of ureteral obstruction on kidney function, inflammation, fibrosis and apoptosis. Within the UUO groups and the control group, one-way analysis of variance and independent *t*-tests were used to compare differences in each dependent variable between the normal chow, safflower oil and fish oil diets. The false discovery rate was used for these multiple comparisons [27]. Data are presented as means ± SD, and statistical significance was accepted at $p < 0.05$.

3 Results

3.1 Effects on renal function

Consistent with previous research [28], plasma creatinine concentration was higher ($p < 0.05$) in the 4-day UUO group fed with normal chow, and all three 14-day UUO groups compared with the respective control groups that did not undergo UUO (Supporting Information Fig. 1). Diet did not alter plasma creatinine concentration in the UUO groups ($p \geq 0.10$) or the control group ($p \geq 0.57$), despite substantial improvements in tissue injury and fibrosis in response to fish oil, as described in the following results sections.

3.2 Effects on fatty acids

The docosahexaenoic acid and eicosapentaenoic acid content, and the ratio of ω-3:ω-6 was higher ($p < 0.05$) in kidney tissue and plasma in the rats fed with fish oil compared with normal chow and safflower oil (Supporting Information Figs. 2 and 3). Conversely, AA content was lower ($p < 0.05$) in kidney tissue and plasma in the rats fed with fish oil compared with normal chow and safflower oil.

Independent of any dietary influence, UUO itself altered the fatty acid content of plasma and kidney tissue. Docosahexaenoic acid and eicosapentaenoic acid content, and the ratio of ω-3:ω-6 were lower in the plasma and kidney tissue of all three UUO groups fed with fish oil compared with control group fed with fish oil ($p < 0.05$). Conversely, AA content was higher in the plasma and kidney tissue of the 4-day UUO and 7-day UUO rats fed fish oil compared with the control rats fed fish oil ($p < 0.05$). Docosahexaenoic acid content and the ratio of ω-3:ω-6 were higher in the plasma and kidney tissue of the 4-day UUO and 7-day UUO rats fed safflower oil compared with the control group fed safflower oil ($p < 0.05$). The AA content was lower in the kidney tissue of the 4-day UUO and 14-day UUO rats fed safflower oil compared with the control rats fed safflower oil ($p < 0.05$).

3.3 Effects on fibrotic and inflammatory markers

Diet did not alter plasma C-reactive protein concentration in any of the UUO groups or the control group (Supporting Information Fig. 4). Using tissue extracts, the kidney content of inflammatory markers IL-1β, MCP-1 and MIP-1α was lower in the 4-day UUO rats fed with fish oil compared with safflower oil ($p < 0.05$) (Figure 1). Using IHC, renal TGF-β protein was increased in UUO animals and was mostly interstitial. Fish oil treatment decreased the expression of TGF-β1 in the 7- and 14-day UUO rats compared with normal chow and safflower oil ($p < 0.05$) (Figure 2). The epithelial changes in UUO animals with and without fish oil were subtle, yet the photomicrographs represent those differences, and significant differences were found using morphometry. TNF-α expression increased in the UUO rat kidneys and was lower in the 4- and 7-day UUO rats fed with fish oil compared with normal chow ($p < 0.05$) (Figure 3). TNF-α was expressed mainly in the cytoplasm of the tubular epithelium, and occasionally in macrophages. Macrophage numbers increased in UUO kidneys, and were lower in the 4- and 14-day UUO rats fed with fish oil compared with safflower oil ($p < 0.05$) (Figure 2). The LTB_4 content of kidney tissue was lower in the 14-day UUO rats fed with fish oil compared with safflower oil ($p < 0.05$) (Figure 4).

3.4 Effects on tubulointerstitial fibrosis

Tubulointerstitial fibrosis was assessed using α-SMA for activated myofibroblasts, picrosirius red and Masson's trichrome staining for fibrillar collagen and tubular epithelial apoptosis as a measure of tubular atrophy. The number of cells expressing α-SMA increased at all times in the UUO animals, and fish oil reduced the number of α-SMA+cells in the 4- and 14-day UUO rats compared with safflower oil

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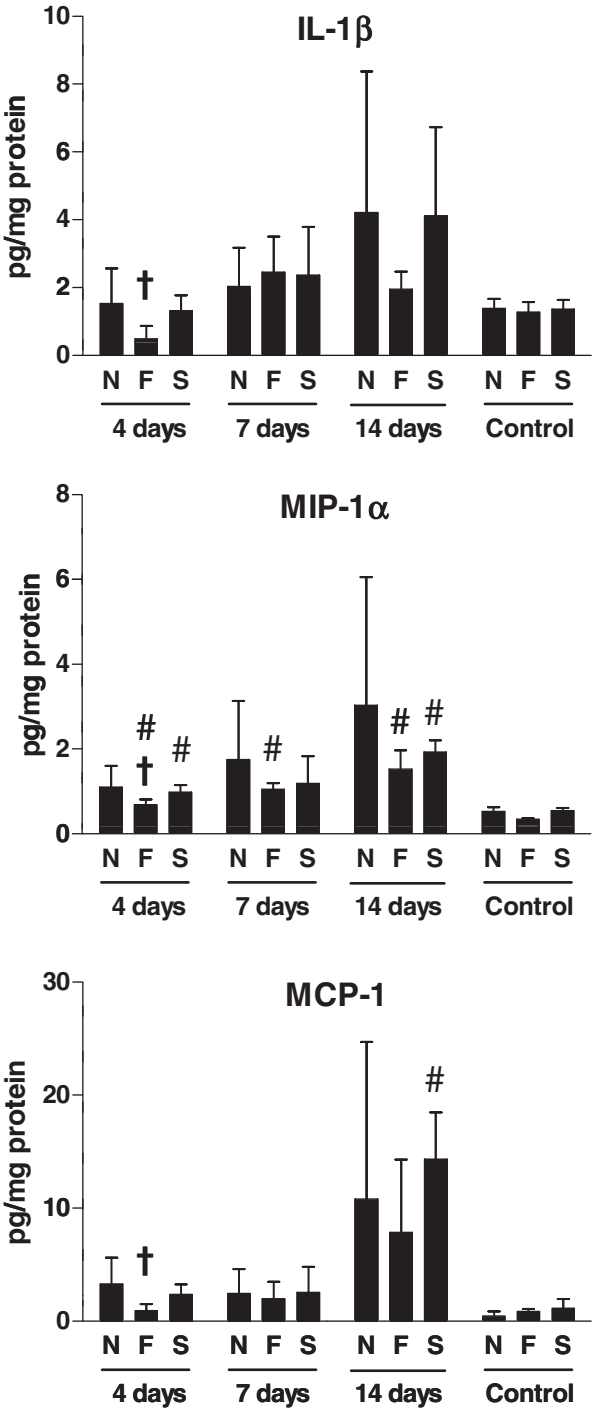

Figure 1. Cytokine content in kidney tissue. Data are means ± SD. N, normal chow; F, fish oil supplemented chow; S, safflower oil supplemented chow. $^{\dagger}p<0.05$ *versus* respective safflower oil group. $^{\#}p<0.05$ *versus* respective control group.

($p<0.05$) (Figure 5). Data for picrosirius red and Masson's trichrome morphometric quantification were in general agreement; therefore, only the data for picrosirius red are presented. Collagen deposition increased in the UUO animals and was lower in the 7- and 14-day UUO rats fed fish oil compared with safflower oil ($p<0.05$) (Figure 5). Apoptosis was lower in the 7- and 14-day UUO rats fed fish oil compared with normal chow and safflower oil ($p<0.05$) (Supporting Information Fig. 5).

3.5 Effects on oxidative stress

The expression of HO-1 was higher in the 7-day UUO rats treated with fish oil compared with normal chow ($p<0.05$) (Figure 6). Fish oil supplementation did not alter the 8-iso-$PGF_{2\alpha}$ isoprostanes content of kidney tissue in 14-day UUO rats (one-way analysis of variance $p=0.16$) (Figure 4).

4 Discussion

We investigated whether dietary feeding with fish oil would reduce inflammation, fibrosis and oxidative stress in renal tissue in rats subjected to ureteral obstruction. Compared with the control diet (composed mainly of oleic acid), the fish oil diet, containing docosahexaenoic acid and eicosapentaenoic acid, attenuated macrophage infiltration (at 14 days UUO), collagen deposition, TGF-β1 expression and apoptosis (at 7- and 14 days UUO). Compared with the safflower oil diet (composed mainly of linoleic acid), the fish oil diet attenuated MIP-1α, IL-1β and MCP-1 expression (at 4 days UUO), LTB_4 content (at 14 days UUO), collagen deposition (at 4 days UUO), fibrosis and macrophage infiltration (at 4 and 14 days UUO), and TGF-β1 expression (at 4, 7 and 14 days UUO). These findings provide support for consuming oily fish as part of the regular diet, particularly for older individuals who are vulnerable to obstructive disorders of the upper urinary tract.

The lack of improvement in plasma creatinine concentration in response to dietary modification likely relates to the model of UUO, in which the ureter remains completely and permanently blocked. Accordingly, structural repair was evident, without any corresponding change in excretory function and associated change in plasma creatinine concentration.

Our data, indicating that fish-oil reduced cytokine content and macrophage counts in kidney tissue following short-term UUO (*i.e.* 4 days), are consistent with data from *in vitro* studies [5, 6, 29]. Diaz Encarnacion *et al.* [13] reported that fish oil reduces macrophage infiltration and fibrosis in kidney tissue from salt-sensitive rats with hypertension. The fish oil diet used in their study contained almost four times the amount of fish oil used in the present study (260 g/kg *versus* 70 g/kg). Our findings indicate that smaller doses of fish oil are also effective for reducing inflammation and fibrosis in the kidney following ureteral obstruction.

An *et al.* [14] examined the therapeutic effects of 12 wk of 0.3 g/kg/day gastric gavage with eicosapentaenoic acid and docosahexaenoic acid (ratio 7:1) in rats, after induction

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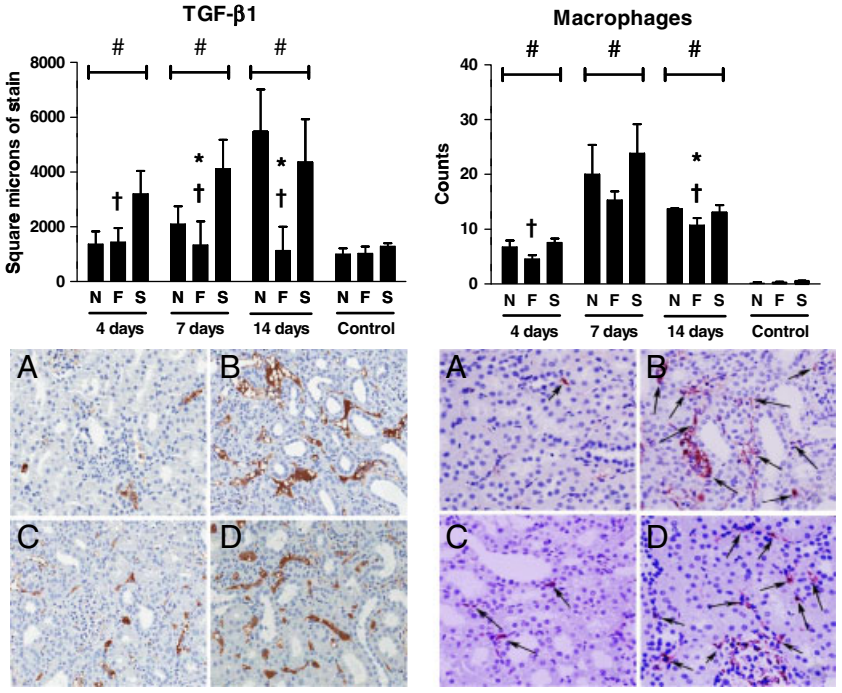

Figure 2. Morphometry and representative images for TGF-β IHC (left), ED1 counts and ED1 IHC (right). Data are means ± SD. N, normal chow; F, fish oil supplemented chow; S, safflower oil supplemented chow. $^{*}p<0.05$ *versus* respective normal chow group. $^{\dagger}p<0.05$ *versus* respective safflower oil group. (A) Control group (14 day no UUO) on normal chow. (B) 14 day UUO group on normal chow. (C) 14 day UUO group on fish oil diet. (D) 14 day UUO group on safflower oil diet. Brown coloring indicates TGF-β localization. Brown-stained cells indicate ED1^{+}cells. $^{\#}p<0.05$ *versus* respective control group.

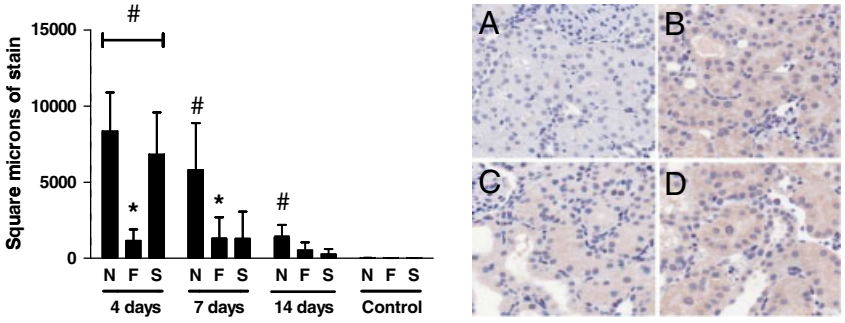

Figure 3. Morphometry and representative images for TNF-α IHC. Data are means ± SD. N, normal chow; F, fish oil supplemented chow; S, safflower oil supplemented chow. $^{*}p<0.05$ *versus* respective normal chow group. (A) Control group (14 day no UUO) on normal chow. (B) 4 day UUO group on normal chow. (C) 4 day UUO group on fish oil diet. (D) 4 day UUO group on safflower oil diet. Brown coloring indicates TNF-α localization.

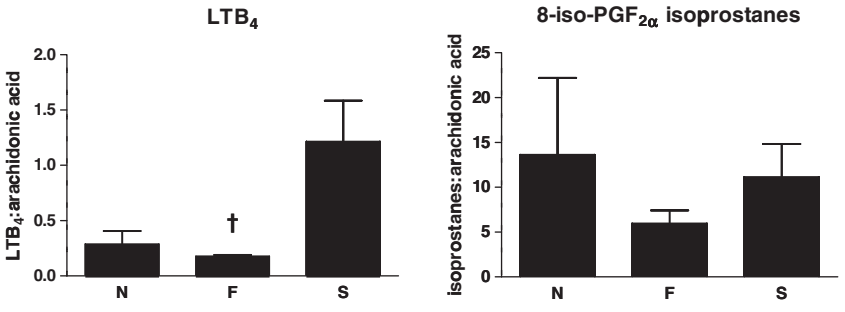

Figure 4. The leukotriene B_4 and 8-iso-$PGF_{2\alpha}$ isoprostanes content of kidney tissue. Data are means ± SD. N, normal chow 14 day control rats (*i.e.* no UUO). F, fish oil 14 day UUO rats. S, safflower oil 14 day UUO rats. $^{\dagger}p<0.05$ *versus* safflower oil group.

of 5/6 nephrectomy. They discovered that this treatment regimen reduced inflammation (cyclo-oxygenase-2, NFκB p65, MCP-1, PA-1), fibrosis (TGF-β, α-SMA fibroblasts, Smad 2, extracellular signal-regulated protein kinase 1/2) and oxidative stress (nicotinamide adenine dinucleotide phosphate oxidase). Some of our findings are similar to those of An *et al.* [14]. In the present study, however, we used a different model of kidney disease, used a different ratio of eicosapentaenoic acid and docosahexaenoic acid, and treated rats prophylactically (versus

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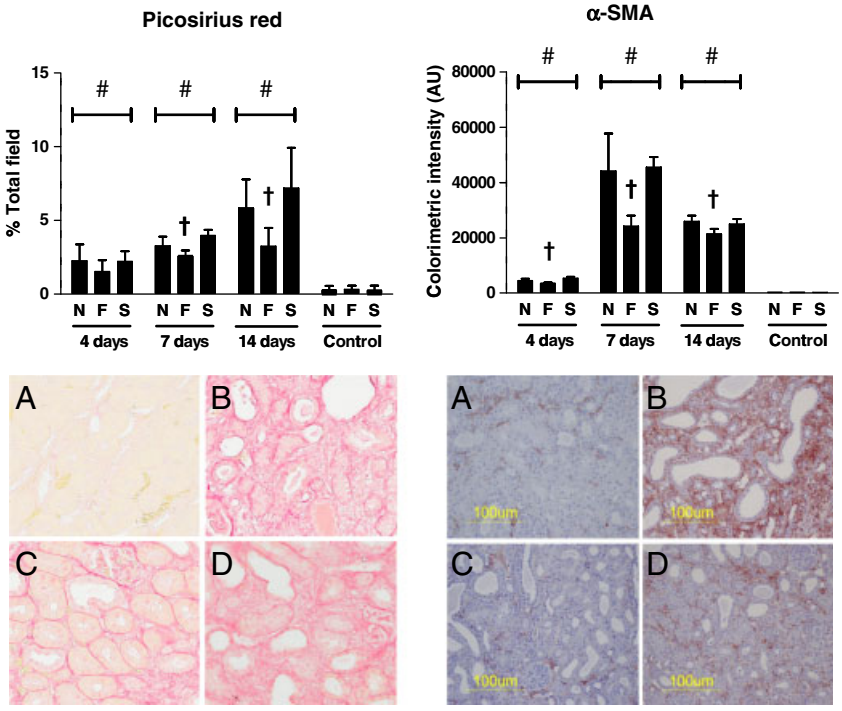

Figure 5. Tubulointerstitial fibrosis. Morphometry and representative images for α-SMA expression are demonstrated on the left. Brown staining indicates α-SMA-positive myofibroblasts. The figures on the right indicate morphometry and representative images for picosirius red histochemical stain for collagen. Red staining indicates fibrillar collagen. (A) Control group (14 day no UUO) on normal chow. (B) 14-day UUO group on normal chow. (C) 14-day UUO group on fish oil diet. (D) 14-day UUO group on safflower oil diet. Data in graphs are means ± SD. F, fish oil supplemented chow; S, safflower oil supplemented chow. $^{*}p<0.05$ *versus* respective normal chow group. $^{\dagger}p<0.05$ *versus* respective safflower oil group.

therapeutically) for a shorter period of time. We also observed some separate effects of ω-3 fatty acids, including reduced apoptosis, AA and LTB_4, together with increased HO-1 expression. Our findings therefore complement those of An *et al.* [14].

The lower expression of the chemokines MCP-1 and MIP-1α following the fish oil diet may have reduced the number of macrophages that entered the kidney – at least following 4 days of ureteral obstruction. Fish oil also reduced macrophage infiltration in kidney tissue at 14 days of ureteral obstruction. We observed that fish oil significantly reduced the LTB_4 content of kidney tissue at 14 days UUO. We measured the LTB_4 content of kidney tissue at 14 days UUO because TGF-β1 expression, collagen deposition and apoptosis were greatest at this time. The decrease in LTB_4 in response to ω-3 fatty acids could account for the lower number of macrophages in rats that were fed with fish oil. ω-3 fatty acids target eicosanoids derived from AA (*e.g.* PGE_2, LTB_4, thromboxane B_2, 5-hydroxyeicosatetraenoic acid). In contrast with AA, eicosapentaenoic acid promotes the production of eicosanoids derived from cyclo-oxygenase and 5-lipoxygenase (*e.g.* LTB_5, LTE_5) [8, 30]. We did not measure LTB_5, but the fish oil diet in our study may have increased LTB_5 content in kidney tissue. Compared with LTB_4, however, LTB_5 is a weaker chemoattractant, and it does not actually reduce leukocyte chemotaxis [31]. We therefore suggest that the lower number of macrophages in the kidney tissue of rats that were fed fish oil resulted mainly from reduced synthesis of LTB_4, rather than increased formation of LTB_5. LTB_4 most likely acts as an intermediate factor that regulates macrophage infiltration, rather than an end-product that directly influences fibrosis in obstructed kidney tissue.

We present novel data that ω-3 fatty acids increased the expression of HO-1 in kidney tissue following ureteral obstruction. This increase in HO-1 expression could account for the lower degree of apoptosis that we observed in UUO rats fed fish oil, because HO-1 plays an important anti-apoptotic role following ureteral obstruction [32]. The lower incidence of apoptosis was also likely linked with lower macrophage infiltration of kidney tissue in response to fish oil supplementation. Our finding that fish oil did not alter the 8-iso-$PGF_{2\alpha}$ isoprostanes content of kidney tissue suggests that ω-3 fatty acids do not modulate the pathway downstream of AA that leads to the formation of 8-iso-$PGF_{2\alpha}$ isoprostanes. HO-1 has many reported associations. In some cases an increase in HO-1 expression is seen as a marker of increased oxidative stress, but there are also many papers that link increased HO-1 expression with improved cell survival during oxidative stress in kidney, heart and liver tissue [33–35]. We propose that our observation of greater HO-1 expression in rats fed fish oil is also evidence of improved cell survival.

Ureteral obstruction increases the synthesis of TNF-α and TGF-β1, both of which regulate fibrosis and collagen deposition [36]. TGF-β is integral in mediating fibrosis and collagen formation [2]. Fish oil – specifically eicosapentaenoic acid – may therefore have restricted fibrosis in our study by reducing TGF-β synthesis. Other research has also

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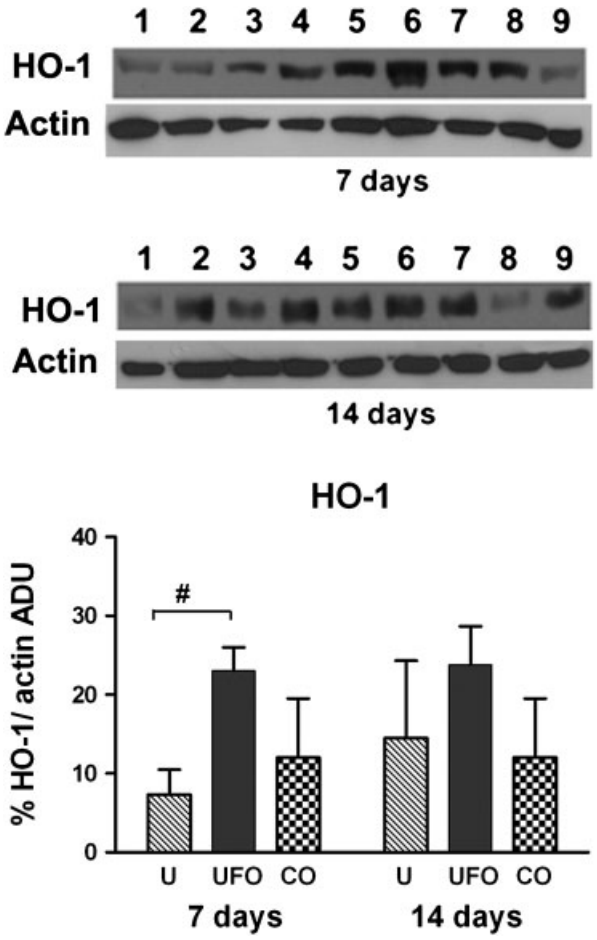

Figure 6. A summary of the Western immunoblot results for HO-1 is presented. The representative blots for 7- and 14-day animals for HO-1 and actin loading controls are as follows: lanes 1–3 are UUO rats fed with normal chow, lanes 4–7 are UUO rats fed with fish oil (FO) and lanes 8–9 are control rats fed with normal chow. The lower figure represents the mean densitometry ± SE for each group with each lane normalised against actin loading (% HO-1/actin as arbitrary densitometry units or ADU). U,UUO; UFO,UUO plus FO; CO,controls. HO-1 was significantly elevated at 7 days in the UUO plus FO animals versus UUO alone ($^{\#}p < 0.05$). No other comparisons within the 7 or 14 day sets were significant.

reported that fish oil attenuates TGF-β secretion from mesangial cells [37] and cardiomyocytes treated with endothelin-1 [38].

The time course for the effects of fish oil varied over time. Compared with the safflower oil diet, the fish oil diet reduced the expression of IL-1β, MIP-1α and MCP-1 after 4 days UUO, but not after 7 days or 14 days UUO. The reason for this difference is not clear. The types of cells producing these cytokines in kidney tissue may change as ureteral obstruction proceeds, for example, from pro-inflammatory cells that assist in breaking down and removing damaged tissue, to anti-inflammatory cells that promote tissue regeneration. These cells may respond differently to ω-3 fatty acids. Compared with the normal chow, the fish oil diet reduced HO-1 expression after 7 days UUO, but not after 14 days. Again, the reason for this difference is not clear. HO-1 expression appeared to increase after between 7 days and 14 days UUO in rats fed normal chow, whereas it did not change over this period in rats fed the fish oil diet. The amount of ω-3 fatty acids in kidney tissue also remained unchanged over this period. Together, these findings suggest that the ω-3 fatty content of kidney tissue was saturated after 7 days UUO, and consequently, HO-1 did not increase beyond this time point.

An unexpected finding in this study was that the ureteral obstruction itself altered the fatty acid content of plasma and tissue from the damaged kidney. This effect varied depending on whether rats were fed with fish oil or safflower oil. The mechanisms by which obstructive renal injury reduced the ω-3 fatty acid content in the circulation and kidney tissue are unclear, but warrant further investigation. This response may have implications for the efficacy of dietary fish oil supplementation in patients with poor kidney function.

In summary, we have provided strong evidence that a short period of prophylactic treatment with moderate doses of fish oil attenuated the pathophysiology associated with obstructive renal injury. These findings suggest that individuals at higher risk of urinary tract obstruction, such as the elderly population, may benefit from maintaining a diet high in fish oil. Clinical trials have investigated the effects of fish oil supplementation in patients with IgA nephropathy, chronic glomerular diseases, polycystic kidney disease, systemic lupus erythematosis, and type 2 diabetes mellitus treated for hypertension. The findings from these clinical trials have generally been limited because of weaknesses in the study design (*e.g.* open-label trials, small sample size) [39]. Further clinical trials are therefore warranted to examine the prophylactic and therapeutic effects of fish oil supplementation in individuals with obstructive renal injury and other kidney injuries.

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