Intravenous omega-3, a technique to prevent an excessive innate immune response to cardiac surgery in a rodent gut ischemia model

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Objectives: Neutrophil infiltration of tissues as part of the inflammatory response to cardiac surgery is one of the major mediators of postoperative multiple-organ dysfunction. Omega-3 fatty acids markedly attenuate endothelial cell inflammatory responses, including upregulation of neutrophil adhesion molecules. The efficacy of a clinically safe form of omega-3 to produce this effect in vivo was examined.

Methods: Rat gut intravital microscopic analysis was used to visualize neutrophil transmigration from the microcirculation into the tissues of the gut. Inflammatory activation was in the form of 30 minutes of ischemia and 90 minutes of reperfusion. Sham, control (0.9% saline infusion over 4 hours), and omega-3 (Omegaven [Fresenius Kabi, Bad Homburg, Germany] infusion over 4 hours) pretreatments were compared.

Results: Ischemia–reperfusion resulted in a 4-fold increase in neutrophil adherence to the endothelium (baseline: 4.3 ± 0.2 vs control group: 19.2 ± 3.5 adherent neutrophils per 100 μm, $P < .01$), which intravenous omega-3 suppressed (7.8 ± 1.7 adherent neutrophils per 100 μm, $P < .01$). Omega-3 pretreatment also reduced neutrophil transmigration into the tissues after reperfusion (sham group: 6.3 ± 0.8 vs control group: 13.2 ± 1.4 vs omega-3 group: 9.4 ± 0.9 neutrophils per field, $P = .037$). Gut tissue levels of the neutrophil-released enzyme myeloperoxidase were similarly markedly reduced with omega-3 pretreatment (sham group: 10.5 ± 1.6 vs control group: 19.0 ± 3.3 vs omega-3 group: 10.1 ± 1.2 U/g, $P = .03$).

Conclusions: Four hours' pretreatment with a relatively safe form of intravenous omega-3 suppressed neutrophil adherence and tissue infiltration, resulting in lower levels of the tissue-damaging enzyme myeloperoxidase. This suggests a possible strategy for diminishing postoperative multiple-organ dysfunction. (J Thorac Cardiovasc Surg 2011;141:803-7)

Increasingly, our patients undergoing cardiac surgery are experiencing multiple-organ dysfunction postoperatively. This is a result of more limited physiological reserve in adult patients with the comorbidities of advanced age, diabetes, hypertension, and diffuse atherosclerosis and also in our pediatric population as a result of more complex procedures at an earlier age, such as the Norwood procedure, truncus arteriosus repair, and arterial switch. A major causative factor of postoperative multiple-organ dysfunction is excessive activation of the innate immune response. The innate immune response is designed to localize a rapid and effective destructive response to where it is needed in the body. It is nonspecific, being the response to infectious agents, trauma, ischemia–reperfusion, and the cardiac surgical process.[1] The response centers around neutrophils entering the tissues at the point of injury, whether that be an infected area or an area that has undergone ischemic injury, and containment of the injury locally through release of factors into the tissues to destroy any infective agent or damaged tissue. Neutrophils are localized to the point of injury by endothelial cells producing adhesion molecules on their surface in the microcirculation at that point to sequester circulating neutrophils as they pass. Unfortunately, the cardiac surgical process produces a systemic inflammatory response, activating this process in the microcirculation of multiple organs. This is inadvertent excessive activation of the innate immune response, which is one of the factors leading to inadvertent multiple-organ injury.[2] This is most easily demonstrated in the lungs, where there is a positive correlation between plasma levels of the neutrophil enzyme elastase and respiratory dysfunction,[3] and inhibition with the experimental drug ulinastatin (which prevents neutrophil degranulation) can attenuate this injury in animal studies but is unapproved for human use.[4] Similar benefits are seen with leukocyte depletion with an arterial filter with bypass[5] but diminish once the filter is removed. In the heart plasma levels of the neutrophil-recruiting cytokine interleukin 6 (IL-6) have been correlated with postoperative myocardial ischemic episodes and echocardiographic wall motion abnormalities in adults,[6] and a recent study in a neonatal population demonstrated that the IL-6 level 4 hours postoperatively was an independent risk factor for myocardial dysfunction.[7]

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Abbreviation and Acronym
IL-6 = interleukin 6

Pure forms of omega-3 fatty acids have been shown to attenuate inflammatory activation of endothelial cells.[8,9] We have previously shown that exposure of human endothelial cells in vitro to a clinically useful form of omega-3 fatty acids for 4 hours can markedly blunt subsequent inflammatory activation of the endothelium. We have demonstrated that through reducing activation of the inflammatory transcription factor nuclear factor κB, the omega-3 pretreatment reduced endothelial expression of adhesion molecules for neutrophils and release of IL-6.[10] A previous study in mice has shown that intravenous administration of the pure oxidized omega-3 fatty acid eicosapentaenoic acid can reduce neutrophil adherence to the gut endothelium in response to inflammatory stimulation with lipopolysaccharide.[11] The objective of this study was to examine whether a form of intravenous omega-3 fatty acids that is already in safe clinical use could produce similar effects on the innate immune response. We also sought to assess whether these effects extended beyond just inhibiting neutrophil adherence to the endothelium, to migration into the tissues and prevention of release of damaging factors, such as myeloperoxidase, into the tissues.

MATERIALS AND METHODS

We used the technique of intravital microscopy to directly visualize the microcirculation and to characterize the changes in neutrophil adhesion to the endothelium and tissue transmigration of neutrophils after gut ischemia–reperfusion. All experiments were conducted under license from the Department of Health in Ireland in accordance with national guidelines for the care of laboratory animals. All experiments were approved by the ethics committee of the Royal College of Surgeons in Ireland.

Eighteen male Sprague–Dawley rats weighing between 150 and 250 g were used. After achievement of anesthesia with inhalational 2% isoflurane (Forane; Abbott Laboratories, Dublin, Ireland) followed by an intraperitoneal injection of 50 mg/kg sodium pentobarbital (Dolethal; Vétoquinol, Lure, France), a tracheostomy was performed to facilitate breathing, and the left internal jugular vein was cannulated for administration of the control or treatment infusions. A midline laparotomy was performed. The superior mesenteric and coeliac vascular pedicles were isolated at their origin and ensnared. This snare was later tightened to produce ischemia. A segment of gut and its mesentery was exteriorized through the abdominal wall and continuously superfused with bicarbonate buffered saline (containing 131.9 mmol/L NaCl, 4.7 mmol/L KCl, 2.0 mmol/L $CaCl_2$, 1.2 mmol/L $MgSO_4$, and 20 mmol/L $NaHCO_3$ in distilled water, pH 7.4; temperature, 36.5°C–37°C). An inverted microscope with a 40× objective lens (Nikon Diaphot 300, Tokyo, Japan) was used to observe the mesenteric microcirculation, and the images were recorded for later offline analysis.

A single unbranched venule with a diameter of 25 to 35 μm and a length of greater than 100 μm was chosen for observation. After a 10-minute period of baseline observation, the venular diameter was measured online with a video caliper (Microcirculation Research Institute, Texas A&M University, College Station, Tex), and red cell velocity was measured with an optical Doppler velocimeter (Microvessel Optical Doppler RealTime; CircuSoft Instrumentation, Hockessin, Del). The snare was then tightened, and the superior mesenteric and coeliac vascular pedicles were occluded. This was confirmed by occlusion of flow in the observed venule. After 30 minutes of ischemia, the snare was released, and flow recommenced. After re-establishment of flow, the vessel was observed during reperfusion, with measurement of venular diameter and centerline red cell velocity. The following parameters were measured at baseline and 60 minutes of reperfusion: venular diameter in micrometers, red blood cell centerline velocity, mean red blood cell velocity, venular wall shear rate, venular blood flow, leukocyte rolling velocity, leukocyte adherence to the vessel wall, and leukocyte transmigration.

The experiment was terminated at 90 minutes of reperfusion, and the animals were killed with intravenous sodium pentobarbital. Samples of the gut were obtained and stored at –80°C for later determination of tissue myeloperoxidase levels. Three experimental groups were used. A sham group (n = 6) received 5 mL/kg body weight of 0.9% saline (Baxter, Newbury, United Kingdom) as an infusion over 4 hours before intravital microscopy, with no ischemia–reperfusion performed in this group. This group served to demonstrate that the surgical procedure itself did not induce a significant inflammatory response. A control group (n = 6) received the same infusion but underwent 30 minutes of ischemia followed by 90 minutes of reperfusion. An omega-3 group (n = 6) received 5 mL/kg body weight of an omega-3 fatty acid infusion (Omegaven; Fresenius Kabi, Bad Homburg, Germany) over 4 hours before ischemia–reperfusion. The recommended dose for clinical use is 2 mL/kg body weight per day; however, we used 2.5 times this dose because of the higher metabolic rates in small animals. The infusion was administered over 4 hours as per the manufacturer's guidelines and administered as a single preoperative dose based on the observation from our previous in vitro work.[10]

At baseline, there was no significant difference in the mean red cell velocity between the sham, control, and omega-3–pretreated group, which signified that the infusions themselves did not have a significant effect on mean red cell velocity over time. At 60 minutes of reperfusion, there was no difference in red cell velocity between the omega-3 and control groups (P = .78), allowing neutrophil measurements to be directly compared. Venular wall shear rate was also calculated as follows: (Shear rate = (V_{mean}/Venular diameter) × 8(seconds^{-1}).[12] This was because differences between groups might account for differences in neutrophil adherence and velocity. There was no significant difference in the venular wall shear rate at baseline among the sham, control, and omega-3–pretreated groups (P = .9). After ischemia–reperfusion, omega-3 pretreatment did not have a significant effect on shear rate compared with the that seen in control group (P = .971). Again, this rules out any confounding flow-related variables for our neutrophil measurements between groups.

The frozen gut tissue samples were freeze-thawed, homogenized, and centrifuged at 2000 rpm for 10 minutes at 4°C. The supernatants were then assayed photometrically for myeloperoxidase activity. One unit of myeloperoxidase was defined as that degrading 1 micromole of peroxide per minute.[13]

Results are quoted as means ± standard errors of the mean. For multiple comparisons between groups, statistical analysis was performed with 1-way analysis of variance. Where significance was observed, the Tukey test was performed to calculate 95% confidence intervals and identify individual differences. Comparisons between baseline and 60 minutes of reperfusion within a single group were performed with the paired sample t test. All statistical analysis was performed with SPSS software version 15.0 (SPSS, Inc, Chicago, Ill).

RESULTS

At baseline, there was no significant difference in the neutrophil rolling velocity among the sham group (rolling velocity, 65.1 ± 4.3 μm/s), the control group (56.6 ± 3.5 μm/s, P = .2), and the omega-3–pretreated group

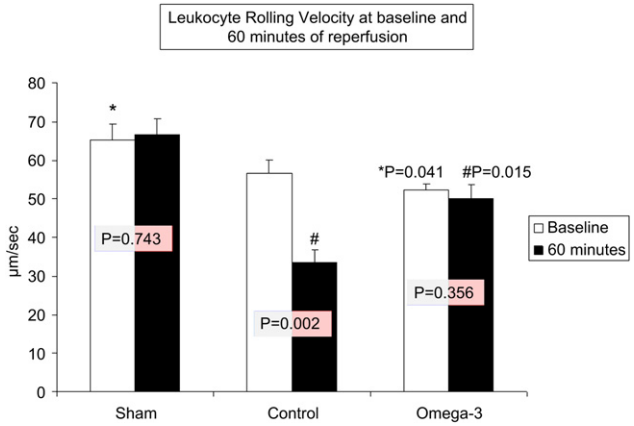

FIGURE 1. Leukocyte rolling velocity was measured at baseline and 60 minutes of reperfusion. The normal reduction with activation of the innate immune response occurred in the control group but was markedly reduced in the omega-3–pretreated group.

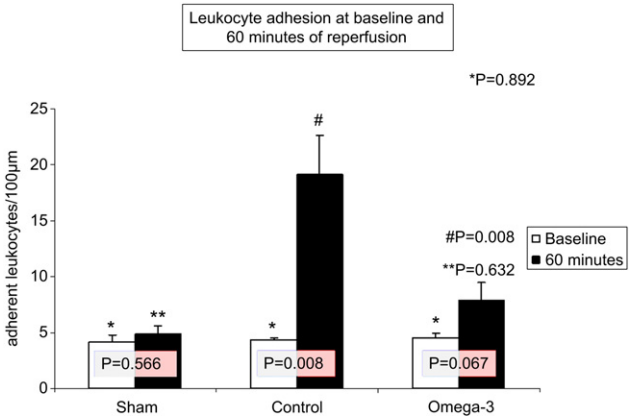

FIGURE 2. The number of leukocytes adherent to the endothelium was measured at baseline and 60 minutes of reperfusion. It was increased in the control group as part of the normal innate immune response, but very little adhesion occurred in the omega-3–pretreated group.

(52.4 ± 1.5 μm/s, $P = .7$). In the sham group neutrophil rolling velocity at 60 minutes of sham reperfusion (66.7 ± 4.0 μm/s) was not significantly different from the baseline value (65 ± 4.3 μm/s, $P = .7$); that is, the surgical procedure alone had no effect on neutrophil rolling velocity over time. At 60 minutes of reperfusion, neutrophil rolling velocity was significantly reduced in the control group (33.6 ± 3.2 μm/s) compared with the baseline value (56.6 ± 3.5 μm/s, $P = .002$). In the omega-3–pretreated group neutrophil rolling velocity at 60 minutes of reperfusion (50.1 ± 3.6 μm/s) was not significantly different from the baseline value (52.4 ± 1.5 μm/s, $P = .4$); that is, omega-3 pretreatment prevented neutrophil rolling on the endothelium (Figure 1).

With neutrophil adhesion to the endothelium, at baseline there was no significant difference among the sham, control, and omega-3–pretreated groups ($P = .9$). At 60 minutes of reperfusion, neutrophil adhesion in the control group (19.2 ± 3.5 adherent neutrophils per 100 μm) was significantly increased compared with the baseline value (4.3 ± 0.2 adherent neutrophils per 100 μm, $P = .008$). However, there was much less adherence in the omega-3–pretreated group (7.83 ± 1.66 adherent neutrophils per 100 μm) compared with that seen in the control group ($P = .008$). In addition, the number of adherent neutrophils at 60 minutes in the omega-3–pretreated group was not significantly different from that seen in the sham group ($P = .632$, Figures 2 and 3).

Finally, neutrophil transmigration across the endothelium into the tissues was assessed. At baseline, there were no differences in the number of transmigrated neutrophils between the control and omega-3–pretreated groups (4.7 ± 0.3 vs 4.7 ± 0.3 neutrophils per field, respectively; $P = 1.0$). At 60 minutes of reperfusion, the number of transmigrated neutrophils in the control group (13.2 ± 1.4 neutrophils per field) was significantly increased compared with the baseline value (4.7 ± 0.3 neutrophils per field, $P = .002$), as was the case in the omega-3–pretreated group (9.4 ± 0.9 neutrophils per field, $P = .018$). However, omega-3 pretreatment significantly attenuated the transmigration of neutrophils compared with that seen in control animals ($P = .037$, Figure 4). Gut myeloperoxidase levels were measured to confirm the intravital findings of reduced tissue neutrophil infiltration because myeloperoxidase is released by neutrophils into the tissues after they transmigrate. The control group had increased levels of tissue myeloperoxidase after ischemia–reperfusion compared with those seen in the sham group (19.0 ± 3.3 U/g in the control group vs 10.5 ± 1.6 U/g, $P = .04$). Omega-3 pretreatment prevented the increase in tissue myeloperoxidase levels (10.1 ± 1.2 U/g in the omega-3 group, $P = .031$, Figure 5).

DISCUSSION

Neutrophil–endothelial interactions are early and critical events in patients with acute inflammation.[14] Neutrophils are normally targeted to sites of tissue damage and anchored through the expression of cell adhesion molecules. Dysregulation of this process results in excessive and inappropriate extravasation of leukocytes. These activated neutrophils release reactive oxygen species and other toxic enzymes, such as elastase, myeloperoxidase, hydrolase, and collagenases. Excessive production of these results in organ injury and is found in hyperinflammatory states associated with critical illness and multiple-organ dysfunction syndrome.[15]

Neutrophil activation occurs after cardiopulmonary bypass because of many factors, including contact with the extracorporeal circuit and activated platelets, although activated complement components formed after contact activation are probably the main activators of neutrophils.[16] The technique of intravital microscopy has been used widely to characterize the sequence of events that leads to the eventual transmigration of leukocytes into the tissue spaces as a result

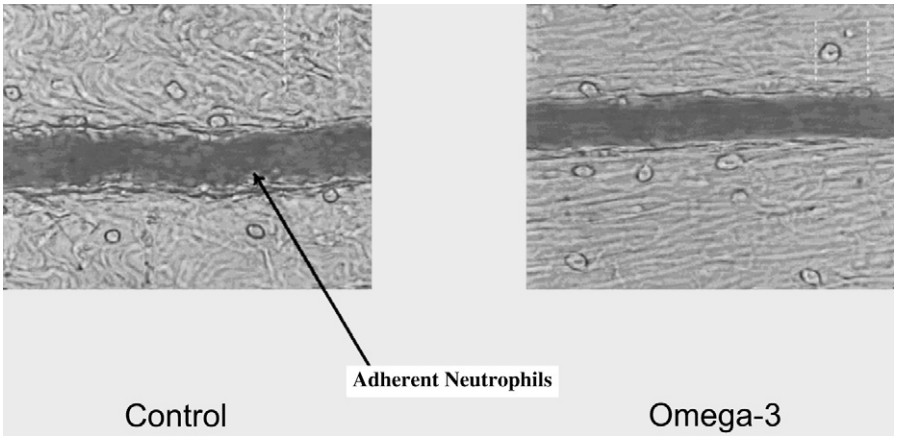

FIGURE 3. Images from intravital microscopy at 60 minutes of reperfusion. A mesenteric venule is shown in the center with multiple adherent leukocytes in the control group and very few in the omega-3–pretreated group.

of a multitude of inflammatory insults. In the postcapillary venule margination of leukocytes to the periphery occurs as a result of hemodynamic forces. This is followed by rolling of the leukocyte along the endothelium, firm adhesion, and then transmigration or diapedesis of the leukocyte into the tissue spaces.[17] In this study we observed that gut ischemia–reperfusion injury resulted in a significant reduction in mean red cell velocity, reduced mesenteric blood flow, and reduced venular wall shear rate. This was associated with a reduction in the velocity of leukocytes rolling along the endothelium, increased adhesion of leukocytes to the endothelium, and increased transmigration of leukocytes into the tissue spaces. This indicates upregulation of cell adhesion molecules, causing the increased leukocyte–endothelial interactions we observed.[12] Myeloperoxidase is a neutrophil enzyme, and measurements of increased tissue levels of myeloperoxidase reflect leukocyte infiltration into the tissues.[18] Pretreatment with a clinically relevant omega-3 infusion did not have a significant effect on baseline mean red cell velocity, blood flow, or venular wall shear rate. There was no effect on baseline adhesion or transmigration of leukocytes. After gut ischemia–reperfusion, mean red cell velocity, blood flow, and venular wall shear were significantly reduced in the omega-3–pretreated group, which is similar to what was seen in the control group. Omega-3 did not have a significant effect on the reduction in these parameters because of ischemia–reperfusion compared with the control group. We know that vessel wall shear affects leukocyte adherence to the endothelium.[12] Thus our observation of equal hemodynamic changes in the control and omega-3–pretreated groups after ischemia–reperfusion is important. We can safely infer that any observed beneficial

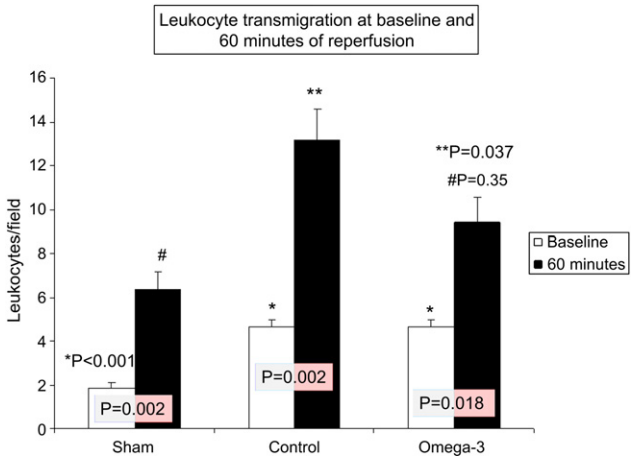

FIGURE 4. The number of leukocytes present in the extravascular tissues having traversed the endothelium of the venule were counted at baseline and 60 minutes of reperfusion. Omega-3 pretreatment attenuated the normal response of leukosequestration in the tissues.

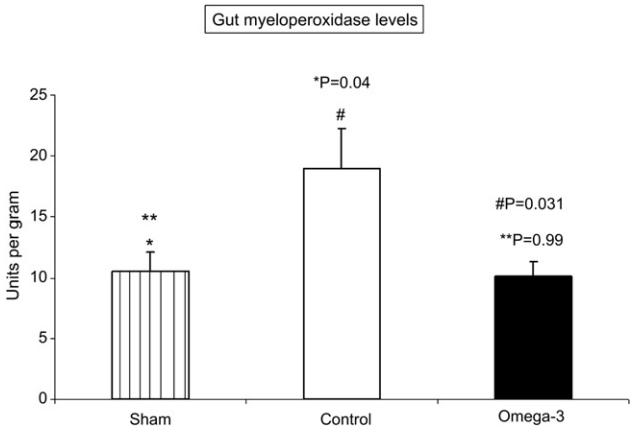

FIGURE 5. Gut myeloperoxidase levels were measured from homogenized tissue after 90 minutes of reperfusion. Myeloperoxidase is released from activated neutrophils in the tissues and was found to be reduced in the omega-3–pretreated group compared with the control group, confirming our findings of reduced transmigration on intravital microscopy.

effect of omega-3 pretreatment to attenuate the upregulation of leukocyte–endothelial interactions caused by ischemia–reperfusion is due to a difference in cell adhesion molecule expression and not due to effects on microcirculatory hemodynamics.

Pretreatment with omega-3 significantly attenuated the reduction in leukocyte rolling velocity, reduced the number of leukocytes firmly adherent to the endothelium, and reduced the number of transmigrated leukocytes occurring as a result of gut ischemia–reperfusion injury compared with the control group. The reduced transmigration of leukocytes was directly observed by using intravital microscopy and also demonstrated by the reduced gut myeloperoxidase levels seen in animals pretreated with omega-3.

These findings are consistent with previous in vitro work assessing the effect of pure forms of omega-3 on endothelial cell adhesion molecule expression[8] and monocyte adhesion to endothelial cells in culture[8,9] and in a murine intravital microscopic model in which leukocyte rolling and adhesion to the venular endothelium in response to systemic lipopolysaccharide was reduced.[11] However, these pure forms of omega-3 could never be administered safely in their current forms to human subjects. We have worked with a clinically safe form of omega-3 and have demonstrated that it has similar anti-inflammatory effects in vitro on endothelial cells to pure forms.[10] We have also shown that it mediates its effects through inhibition of the acute inflammatory transcription factor nuclear factor κB and has minimal effects on the neutrophil and that its effects on the endothelium are reversible within 48 hours.[10] This omega-3 formulation was originally designed as a lipid component of parenteral nutrition and has an established safety profile.[9,19] In this study we used a higher dose of the omega-3 infusion that is used currently clinically. This was because we believed that with the rat's higher metabolic rate, they would need a higher dose to have a bioequivalent effect to that seen in human subjects. We believe that in human subjects the normal maximum recommended dose would be sufficient to induce the anti-inflammatory effect because a previous study in healthy volunteers using the omega-3 infusion for 4 hours on 2 days resulted in reduced activated monocyte cytokine production.[9] However, if the infusion was to be used at higher doses, then the potential adverse effects to look for would be hyperglycemia, metabolic acidosis, and bleeding tendency caused by abnormal platelet function.

It appears from this study that a single dose over 4 hours just before the inflammatory insult has potent effects to minimize neutrophil–endothelial interactions (ie, the innate immune response). We plan to conduct further studies in a cardiopulmonary bypass circulatory arrest model to assess its efficacy in attenuating multiple-organ dysfunction. A single preoperative intravenous dose of omega-3 might be a simple addition to our current strategies to optimize recovery after cardiac surgery.

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