Title: Effect of parenteral infusion of fish oil-based lipid emulsion on systemic inflammatory cytokines and lung eicosanoid levels in experimental acute pancreatitis

Authors: Priscila Garla[1], Ricardo Garib[1], Raquel S. Torrinhas[1], Marcel C. C. Machado[1], Philip C. Calder[2], Dan L. Waitzberg[1]

[1]University of Sao Paulo School of Medicine (FMUSP), Department of Gastroenterology, Digestive Surgery Division - LIM 35 and University of Sao Paulo – NAPAN – Food and Nutrition Research Center, São Paulo, Brazil; [2]Human Development and Health Academic Unit, Faculty of Medicine, University of Southampton and National Institute for Health Research Southampton Biomedical Research Centre, University Hospital Southampton NHS Foundation Trust, Southampton, United Kingdom.

Correspondence to Priscila Garla; e-mail: prigarla@gmail.com
Laboratório de Investigação Médica – LIM 35, Faculdade de Medicina da USP
Av. Dr. Arnaldo, 455; Cerqueira César - CEP: 01246903 - São Paulo
Phone: +55 11 30617459

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ABSTRACT: Parenteral fish oil lipid emulsion (FOLE) might mitigate inflammation after injury. Acute pancreatitis (AP) can occur following major surgery and is characterized by tissue and systemic release of inflammatory mediators that contributes to the systemic inflammatory response syndrome and multiple organ failure. **AIM:** We evaluated the effect of short-term FOLE infusion before experimental induction of AP on systemic cytokine and lung eicosanoid profiles. **METHODS:** Lewis rats (n=72) received parenteral infusion of FOLE (FO group) or saline (SS group), or remained without parenteral infusion (CG group) for 48 hr. Thereafter, AP was induced by retrograde injection of sodium taurocholate into the pancreatic duct. Animals were sacrificed after 2, 12 and 24 hr. Blood and lung samples were collected to assess serum inflammatory cytokines (Luminex) and tissue eicosanoids (ELISA), respectively. **RESULTS:** Serum TNF-α increased over time and serum IL-10 decreased from 12 to 24 hr in CG group. In SS group serum TNF-α increased from 12 to 24 hr ($p = 0.039$) and serum IL-10 decreased over time. Both CG and SS groups exhibited increased IL-6/IL-10 ratio ($p=0.040$). From 12 to 24 hr animals from FO group showed decreased serum IL-1 ($p<0.001$), IL-4 ($p<0.002$) and IL-6 ($p=0.050$), and a trend towards increased IL-10 ($p=0.060$). All experimental groups showed a trend towards increased PGE_2 and decreased LTB_4 in the lung at 24 compared with 12 hr**. CONCLUSION:** Parenteral infusion of FOLE for 48 hr before the induction of experimental AP appears to favorably influence the cytokine response without affecting lung eicosanoids at the time points measured. The use of FOLE to prevent and treat AP following major surgery needs to be further explored.

Keywords: omega-3 fatty acids, fish oil, lipid emulsion, systemic inflammatory response, eicosanoids, experimental acute pancreatitis.

INTRODUCTION

Up to 10% of patients who receive traumatic insults, including complex types of gastrointestinal surgery or long periods of pancreatic ischemia during extensive cardiovascular surgery and cardiopulmonary bypass, may develop acute pancreatitis (AP)[1-3]. Procedures involving the handling and infusion of contrast agents in the common bile or pancreatic ducts, such as retrograde cholangiopancreatography, may also cause AP with an incidence ranging from 5 to 40% of patients[4-6]. Once initiated, AP contributes to increased morbidity and mortality, and its severity is related to the degree of injury and the severity of the associated systemic inflammatory response [7].

The inflammatory response plays a key role in development of AP and in the systemic complications of the disease (such as pancreatitis-associated lung injury), which are a main cause of mortality[7]. Clinical and experimental studies suggest that inflammatory cytokines are essential mediators of the pathophysiology of AP. Activation of polymorphonuclear granulocytes and monocytes, associated with release of the pro-inflammatory cytokines interleukin (IL)-1, IL-6 and tumor necrosis factor alpha (TNF-α) in the pancreatic parenchyma, is an early event during the development of AP and has been shown to contribute to the severity of the disease [7].

Nutrients with anti-inflammatory effects, including n-3 polyunsaturated fatty acids (PUFAs), have been studied in AP with the aim of minimizing the inflammation and improving clinical outcome[8-12]. Fish oil is a source of the n-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). EPA competes

with the n-6 PUFA arachidonic acid (AA) in the cyclooxygenase 2 (COX-2) pathway to produce odd-series eicosanoids with a lower pro-inflammatory potential than those of even-series derived from AA[13,14]. In turn, a change in eicosanoid production can influence other aspects of the inflammatory response including leukocyte infiltration and cytokine production[14]. Furthermore, n-3 PUFAs can reduce inflammatory cytokine production through non-eicosanoid related effects on the pathways that lead to activation of inflammatory cytokine production [14]. Finally, EPA and DHA give rise to mediators that are now recognised to play a central role in resolution of inflammation[15].

The anti-inflammatory effects of n-3 PUFAs rely upon their incorporation into the membrane phospholipids of inflammatory cells[13-15]. Intravenous (i.e. parenteral) administration of fish oil lipid emulsion (FOLE), as source of n-3 PUFAs, results in faster incorporation of these fatty acids into cell membranes compared to when they are administrated enterally[16]. Thus it is possible that the parenteral route may be advantageous in achieving modulation of inflammation by n-3 PUFAs in a short time. Therefore, the present study evaluated the impact of parenteral infusion of FOLE before the induction of experimental AP in rats on systemic and lung inflammatory mediator levels. We hypothesised that animals receiving FOLE would show a lower burden of inflammatory mediators.

METHODS

Animals

Seventy-two adult male Lewis isogenic rats (300-350 g) were purchased from the Multidisciplinary Center for Research in Biological Science Laboratory Animal Area (Campinas, Sao Paulo, Brazil). Prior to the experimental procedures, the animals were adapted for 5 days in metabolic cages at a controlled room temperature (22 ± 2°C) with a 12-h light–dark cycle and with free access to standard rodent chow (Nutrilav, Quimtia, Jundiaí, Brazil) and water. All experimental procedures were approved by the Research Ethical Committee, School of Medicine, University of Sao Paulo, Sao Paulo, Brazil.

Intravenous access

Animals were anesthetized by the intraperitoneal injection of ketamine (100 mg/kg of body weight; Parke-Davis, Ache, São Paulo, Brazil) and xylazine (8 mg/kg of body weight; Bayer, Leverkusen, Germany). Intravenous access was achieved by jugular central venous catheterization (CVC) that was performed according to a standard technique and by using a specific catheter that allows its connection to a gyratory swivel apparatus, which ensured free mobility for the animals[17]. One group of animals did not receive any intravenous infusion for 48 hr (CG group). The delivery of a total daily volume of 6 ml was controlled across all groups with a volumetric infusion pump (Colleague® - Baxter, California, USA). For that purpose, the SS infusion bags were prepared by adding a total of 6 ml of 0.9% saline

125 solution, while the FOLE infusion bags were prepared by adding 0.4 g/kg body
126 weight of FOLE (1 mL - Omegaven® 10%) plus 5 ml of saline solution. All animals
127 had free access to standard oral diet and water *ad libitum* during this period.
128
129 *Experimental acute pancreatitis*
130 After 48 hr of intravenous access, all animals were anesthetized with an
131 intraperitoneal injection of ketamine (100 mg/kg of body weight) and xylazine (8
132 mg/kg of body weight). The pancreas was exteriorized through an abdominal
133 incision and the pancreatic duct catheterized using polyethylene tubing PE-50
134 (Biotechnological). AP was then induced by retrograde injection of 0.1 ml 3%
135 sodium taurocholate solution (Sigma Chemical), according to a standard
136 technique[18]. Following AP induction, animals were sacrificed after 2, 12 and 24 hr
137 by cardiac puncture for blood collection, after being properly anesthetized. Lung
138 samples were collected from the animals sacrificed after 12 and 24 hr.
139
140 *Serum cytokine measurements*
141 Blood samples were centrifuged at 1,000 x g at 4°C for 10 min to isolate serum.
142 Concentrations of IL-1, IL-2, IL-4, IL-6, IL-10, and TNF-α were assessed in 500 µl
143 serum by immunoassay multiplex microspheres, using a commercial kit for rats
144 (07-65K RECYTMAG, Genesis Ltd., Missouri, USA). Plates were read in a
145 Luminex analyzer (Luminex, MiraiBio, Alameda, CA), according to the
146 manufacturer's instructions[19].
147

Eicosanoid measurements

Lungs were washed in PBS and frozen in liquid nitrogen before storage at -80ºC for later analysis. Lungs were homogenized in phosphate buffered saline (PBS, 100 mg tissue/mL) and the concentrations of leukotriene B_4 (LTB_4), prostaglandin E_2 (PGE_2), thromboxane B_2 (TB_2) and lipoxin A_4 (LXA_4) were determined by using commercially available ELISA kits, according to the manufacturer's protocols [20].

Fatty acid measurements

Lipids were extracted from plasma and homogenized lungs with chloroform: methanol (2:1) and phospholipids were isolated by thin layer chromatography using a mixture of hexane: ethyl ether: acetic acid (90: 30: 1), according to the method of Folch et al [21]. Fatty acid methyl esters were prepared by incubation with 140 g/L of methanol and boron trifluoride at 80°C for 60 min. Subsequent to the extraction process, the fatty acid methyl esters were dried and separated by gas chromatography (Shimadzu Model GC-2010) with flame ionization detection, and an Omegawax 250 (Supelco) column. The operating conditions of the column corresponded to an initial temperature of 180°C (1 min) and then 270°C (5 min), with a total run time of 36 min. Fatty acid methyl esters were identified and quantified by comparison with external standards.

Statistical analysis

Data were analysed using SPSS 18.0 for Windows software (SPSS, Chicago, IL, USA). They were analysed by Kruskal-Wallis test and multiple comparisons between the groups were carried out with the Behrens-Fisher test. Lipid mediator concentrations were analysed with the statistical software R 3.1.0 (Core Team, 2014). The nime package was used to adjust the statistical models. In all cases a value for $P < 0.05$ was taken to indicate a significant didfference.

RESULTS

Serum cytokine concentrations

Kinetics of serum cytokines for SS, FOLE and CG groups are shown in Figure 1. Both SS and FOLE groups exhibited an acute increase of IL-1 (FOLE; 12 versus 2 hr $p = 0.010$), but IL-1 was then significantly decreased only in FOLE group at 24 hr (24 versus 12 hr $p < 0.001$). However, IL-1 concentrations did not differ between experimental groups at any time point. All groups presented increased serum IL-2 concentrations over time with no difference between them. In FOLE group, serum IL-4 concentrations were lower at 24 hr than at 12 hr ($p < 0.002$), and serum IL-6 was higher at 12 hr than at 2 hr ($p = 0.006$) and then decreased from 12 to 24 hr ($p = 0.050$). Serum TNF-α concentrations increased over time in all groups, but the increase was smallest in FOLE group. Serum concentrations of IL-10 decreased from 2 to 12 hr in both SS ($p = 0.002$) and FOLE ($p = 0.010$) groups, and from 12 to 24 hr in both SS ($p = 0.008$) and CG ($p = 0.007$) groups. FOLE group presented a trend towards increased concentrations of IL-10 at 24 compared with 12 hr ($p = 0.060$). Finally, the IL-6/IL-10 ratio increased with time in all three groups (Figure

2). This ratio was higher at 24 hr than at 2 hr in SS and CG groups (p=0.04), but not in FOLE group (figure 2).

Lung eicosanoid concentrations

Lung eicosanoid concentrations are shown in Figure 3. All groups presented similar decreased concentrations of LTB_4 ($p = 0.010$) and increased concentrations of PGE_2 ($p < 0.010$) from 12 to 24 hr. There were no differences between groups. Concentrations of TXB_2 and LXA_4 did not change from 12 to 24 hr, although FOLE group showed a weak trend towards lower TXB_2 concentration at 24 compared with 12 hr ($p = 0.30$).

Serum and lung fatty acids

FOLE and SS groups presented higher serum DHA levels at 2 hr (FOLE; $p = 0.02$; SS $p = 0.01$) and higher lung EPA levels at 12 hr (FOLE; $p = 0.020$; SS $p = 0.010$), compared to CG group. There were no differences between groups for serum EPA or lung DHA ($p > 0.05$). The ratio EPA/AA in lung at 12 hr after AP was higher in FOLE ($p= 0.036$) and SS ($p=0.001$) compared to CG group. The ratio DHA/AA did not demonstrate statistical differences between groups (figure 4).

DISCUSSION

This study investigated the effect of intravenous FOLE in an experimental model of AP with the underlying hypothesis being that isolated infusion of FOLE would reduce the burden of inflammatory mediators. The work is of clinical

relevance because AP is a serious medical condition in which poor outcome is linked with an excessive inflammatory response[1-3,7]. The experimental design allowed an evaluation of the effect of prior parenteral infusion of FOLE on the kinetics (2, 12, 24 hr) of systemic cytokines after the induction of AP in rats as the main marker of the inflammatory intensity of disease.

An experimental model of AP induced by retrograde injection of sodium taurocholate in the biliopancreatic duct in rats has been important for understanding the modulation of inflammation resulting from acute pancreatic injury [18]. From these experiments, it has become evident that an initial release of pro-inflammatory cytokines occurs two hours after trauma induction [22,23]. In rats with AP induced by 4% sodium taurocholate, an increase of pro-inflammatory cytokines is also observed later (e.g. 12 and 24 hours after the insult of pancreatic injury) [24]. Our findings suggest that administration of FOLE prior to induction of AP may favorably modulate systemic cytokine levels from a period beyond 12 hr after the induction.

Current evidence supports that n-3 PUFAs, particularly EPA and DHA found in fish oil, can attenuate inflammation and can therefore prevent or treat diseases with an inflammatory component [14,15]. Moreover, experimental studies and clinical trials have shown that parenteral infusion of lipid emulsions (LEs) containing fish oil can affect the synthesis of immune mediators in an inflammatory environment, including eicosanoids and inflammatory cytokines [8-12].

FOLE used as a supplement to parenteral nutrition has been shown to exert a favorable effect on the systemic inflammatory response in experimental acute

pancreatitis, particularly when compared to parenteral infusion of soybean oil lipid emulsion rich in omega-6 fatty acids[13].

Recently, a LE containing only fish oil has been infused alone in experimental studies, but also in some clinical trials, as a pharmacological agent to modulate the immune response. In preliminary clinical studies, the isolated infusion of LE containing only fish oil was safe and well tolerated and also effective in decreasing clinical severity and/or inflammatory markers in surgical and septic patients[25,26]. Despite omega-3 fatty acids showing a promising role as a pharmaconutrient, few studies have explored the impact of pure infusion of FOLE on the acute inflammatory response.

Thus n-3 PUFAs may be able to lower the risk of developing AP in susceptible patients or may be able to treat already established AP. In order to use n-3 PUFAs effectively in such an acute setting, rapid incorporation of such fatty acids would be desirable. Parenteral infusion of fish oil allows faster incorporation of n-3 PUFAs into plasma and blood cells than seen with enteral supplementation[16]. Thus intravenous administration would be preferred over oral/enteral.

After 2 hours of AP induction, there was an increase in DHA but not in EPA in serum and, after 12 hours an increase in EPA but not DHA in lung tissue. Unexpectedly, we detected the presence of the n-3 fatty acids EPA and DHA in the other two studied groups, particularly in the SS group. It is likely that the presence of n-3 fatty acids in the standard oral diet enriched with omega-3 fatty acids (0.19% per kg) may partially explain these findings. Serum represents fatty acids being transported to cells and tissues and being made available for use, while lung

represents the composition of the cells present which will include both lung and infiltrating inflammatory cells. Thus the different pattern of n-3 PUFA incorporation seen may reflect the differences in composition of transport and functional pools and may also relate to use of fatty acids from these pools as part of the inflammatory process itself. In our study, the ratio of EPA/AA was more favorable in the FOLE and SS groups.

Immediately after onset of pancreatic tissue injury, the release of pro-inflammatory mediators such as IL-1, -2, -6, -8 and TNF-α occurs. The increase of these inflammatory cytokines is followed by the release of anti-inflammatory mediators such as IL-10. Our data illustrate these changes with a generalised increase in serum concentrations of IL-1 and -2 and TNF-α and a decrease in serum IL-10 and an overall increase in inflammatory burden as demonstrated by the increasing ratio of IL-6 to IL-10. There was also a time-dependent increase in the AA-derived lipid mediator PGE_2 in lung tissue.

The stress response of trauma is characterized by the initial release of high amounts of the pro-inflammatory cytokines IL-1 and TNF-α, which are primarily responsible for the production of other cytokines such as IL-6, the main cytokine responsible for the acute phase response[7,14,15]. In the current study, the TNF-α response was blunted in the group receiving FOLE. In addition, 24 hr after AP the animals treated with FOLE showed decreased serum IL-1 and IL-6 concentrations compared to the earlier timepoint (12 hr) and showed a strong trend towards an increase in concentration of the anti-inflammatory IL-10.

These findings are in concordance with previous clinical studies evaluating the effect of parenteral regimens containing FOLE on inflammatory markers. In these studies patients with severe pancreatitis receiving LEs containing FO showed decreased levels of C-reactive protein (CRP), an acute phase response protein mainly stimulated by IL-6 [9-11]. In addition, patients with severe AP receiving a fish oil containing lipid emulsion exhibited higher plasma IL-10/TNF-α ratio in comparison to the control group[12].

It is important to highlight that the beneficial effects of FOLE in modulating the inflammatory response were seen during the period of 12 to 24 hr and that earlier in the response (2 to 12 hr) the changes in the cytokine profile were similar to those seen in the SS group. Thus, FOLE was advantageous later in the inflammatory response, perhaps reflecting an active role of n-3 PUFAs in resolution of inflammation.

In experimental studies[22,23], intravenous volume replacement with saline solution, especially in hypertonic concentration, suggests the favorable modulation of local and systemic inflammatory responses. In rats subjected to AP, the infusion of saline solution, at two concentrations of 0.9% and 7.5% reduced the serum concentrations of IL-6 and TNF-α 2 hours after induction of AP[24]. In the present study, the concentrations of pro-inflammatory cytokines in the SS group did not change significantly at 2 and 12 hours after the induction of AP. However, we found an increase of serum TNF-α and a reduction of the anti-inflammatory IL-10 in the SS group when compared to the later periods of 12 to 24 hours.

The strong trend seen towards an increase in concentration of IL-10 seen at 24 hr compared with 12 hr in the FOLE group may be important because of the

anti-inflammatory role of this cytokine. Interestingly this effect mirrors a finding from an earlier clinical trial of a LE including FO in patients with AP[11]. The authors found benefits in the FOLE group with increased plasma levels of EPA, and a significant increase IL-10 in relation to the control group who received an n-6 rich LE.

Some cases of AP are associated with systemic organ disease. In particular, pulmonary injury is a potentially devastating complication of AP and COX-2 plays a role in its development [27]. Consistent with this, we observed an increase in the COX-2 metabolite PGE_2 in lungs at 24 compared with 12 hr. However, another COX-2 metabolite TXB_2 was not altered between these time points while the 5-lipoxygenase metabolite LTB_4 decreased and the pro-resolving 15-lipoxygensase metabolite LXA_4 was unchanged. These different patterns may in part reflect that we did not assess eicosanoid concentrations at the 2 hr time point. The increase in PGE_2 and decrease in LTB_4 seen from 12 to 24 hr may reflect a partitioning of arachidonic acid towards the COX-2 rather than the 5-lipoxygenase pathway and this could be due to greater induction of COX-2 than lipoxygenase in this model.

Presently, FOLE did not affect arachidonic acid-derived eicosanoid concentrations in the current study. This finding is similar to the findings of Killian et al. who reported no differences in the concentrations of prostaglandins and leukotrienes in pancreatic tissue between rats receiving infusions of saline or LEs rich in n-6 or n-9 fatty acids [8]. Actually, the potential anti-inflammatory effects drove by changes in eicosanoids levels after parenteral infusion of fish oil seem more related to a decrease in AA-derived / EPA-derived eicosanoids ratios induced by an increase of

eicosanoids derived from EPA, than a decrease of the absolute amount of those derived from AA [28].

Clinical trials have reported that peri- or post-operative infusion of parenteral regimens containing LEs providing FO compared to standard LE rich in omega-6 FA in surgical patients can decrease post-operative levels of inflammatory cytokines (IL-1, IL-6 and TNF-α) and arachidonic acid derived eicosanoids [25,26,29,30]. These effects have been linked to clinical benefits. The current study also suggests a single role for FOLE in controlling inflammation in AP. Considering that serum levels of inflammatory mediators can be used as a prognostic factor in AP[7], our findings suggest a potential benefit in using the isolated infusion of FOLE to modulate the systemic cytokine profile and potentially control the course of AP intensity. However a limitation of the current study is that the FOLE was provided prior to initiation of AP. It will be important to evaluate the effect of FOLE in modulating inflammation when administration is started soon after initiation of AP. Another limitation of the current study is that no metabolic or behavioural changes in the animals were assessed.

In summary, parenteral infusion of FOLE for 48 hr before the induction of experimental AP appears to favorably influence the cytokine response without affecting lung eicosanoids at the time points measured. The use of FOLE to prevent and treat acute pancreatitis following major surgery needs to be further explored.

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Conflicts of interest

PCC has received speaking honoraria from Fresenius-Kabi, B.Braun and Baxter Healthcare. DLW has received speaking honoraria from Fresenius-Kabi and B.Braun.

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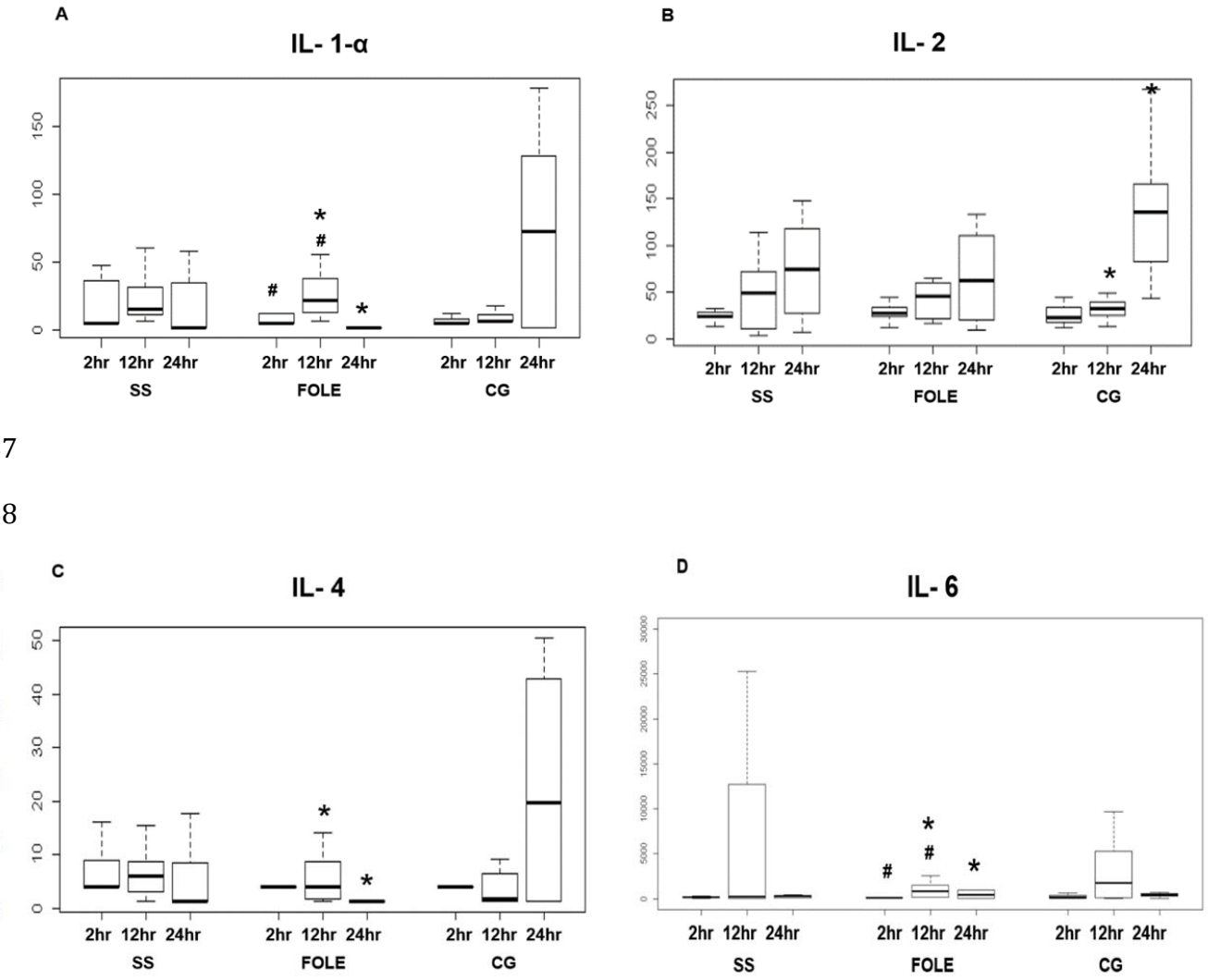
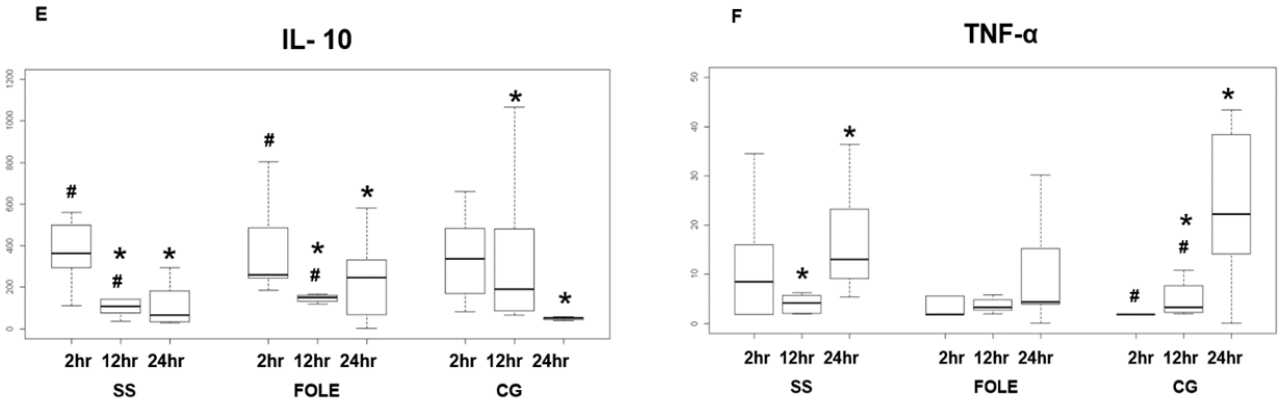
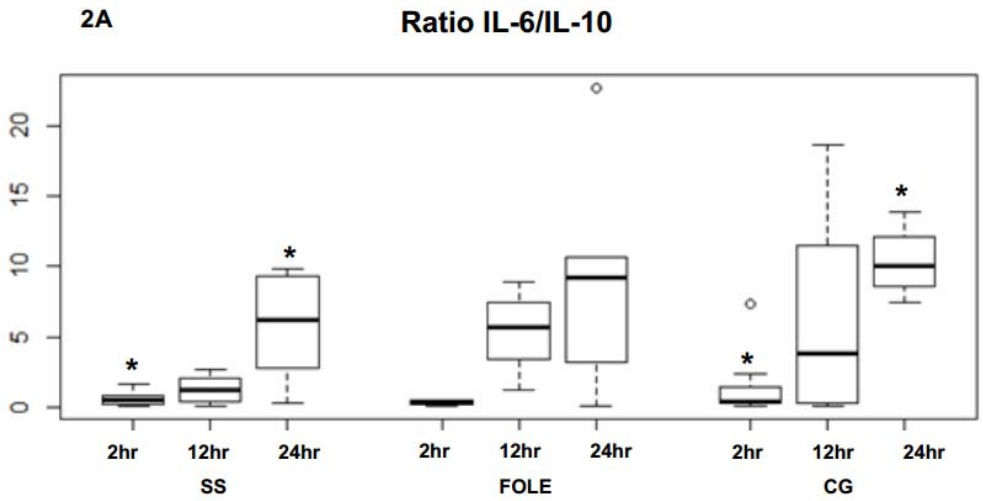
Figure 1: Serum cytokine concentrations (pg/ml) in rats treated with different intravenous infusions prior to the induction of acute pancreatitis.





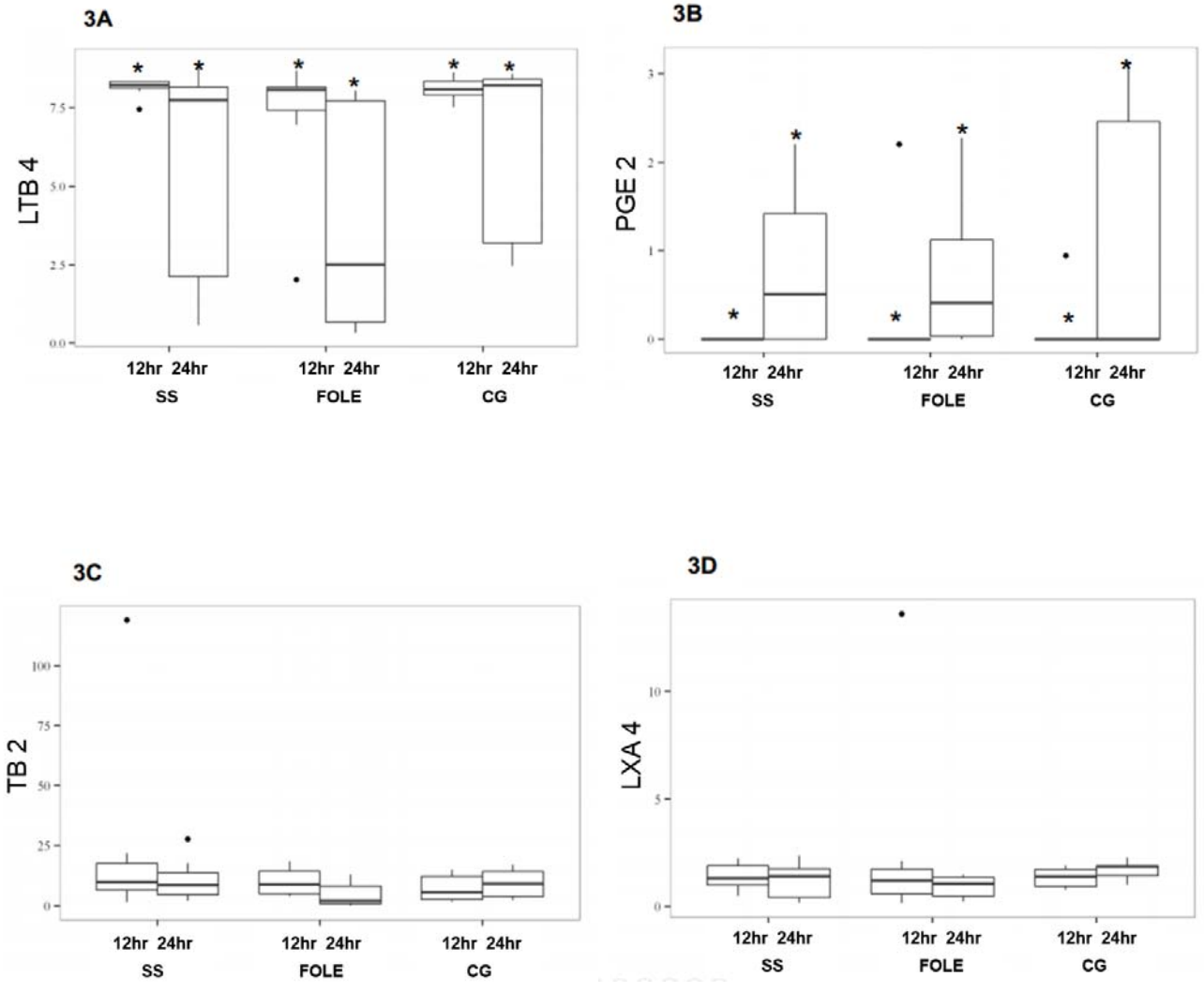
Legend - SS: saline solution; FOLE: fish oil lipid emulsion; CG: control group. A: IL-1 expression ($^{\#}$FOLE; p= 0.01) and (*****FOLE; p= 0.0006); B: IL-2 expression (*****CG; p= 0.0017); C: IL-4 expression (*****FOLE; p= 0.0019); D: IL-6 expression ($^{\#}$FOLE; p= 0.005) and (*****FOLE; p= 0.05); E: IL-10 expression ($^{\#}$FOLE; p= 0.01); ($^{\#}$SS; p= 0.002) and (*****FOLE; p= 0.06); (*****SS; p= 0.008); (*****CG; p= 0.007); F: TNF-α expression ($^{\#}$CG; p= 0.001) and (*****SS; p= 0.03); (*****CG; p= 0.005). The symbols "**#**" and "*****" represent different mean values from 2 hours compared to 12 hours and from 12 hours compared to 24 hours.

Figure 2: Serum ratio of IL-6 to IL-10 concentration in rats treated with different intravenous infusions prior to the induction of acute pancreatitis.



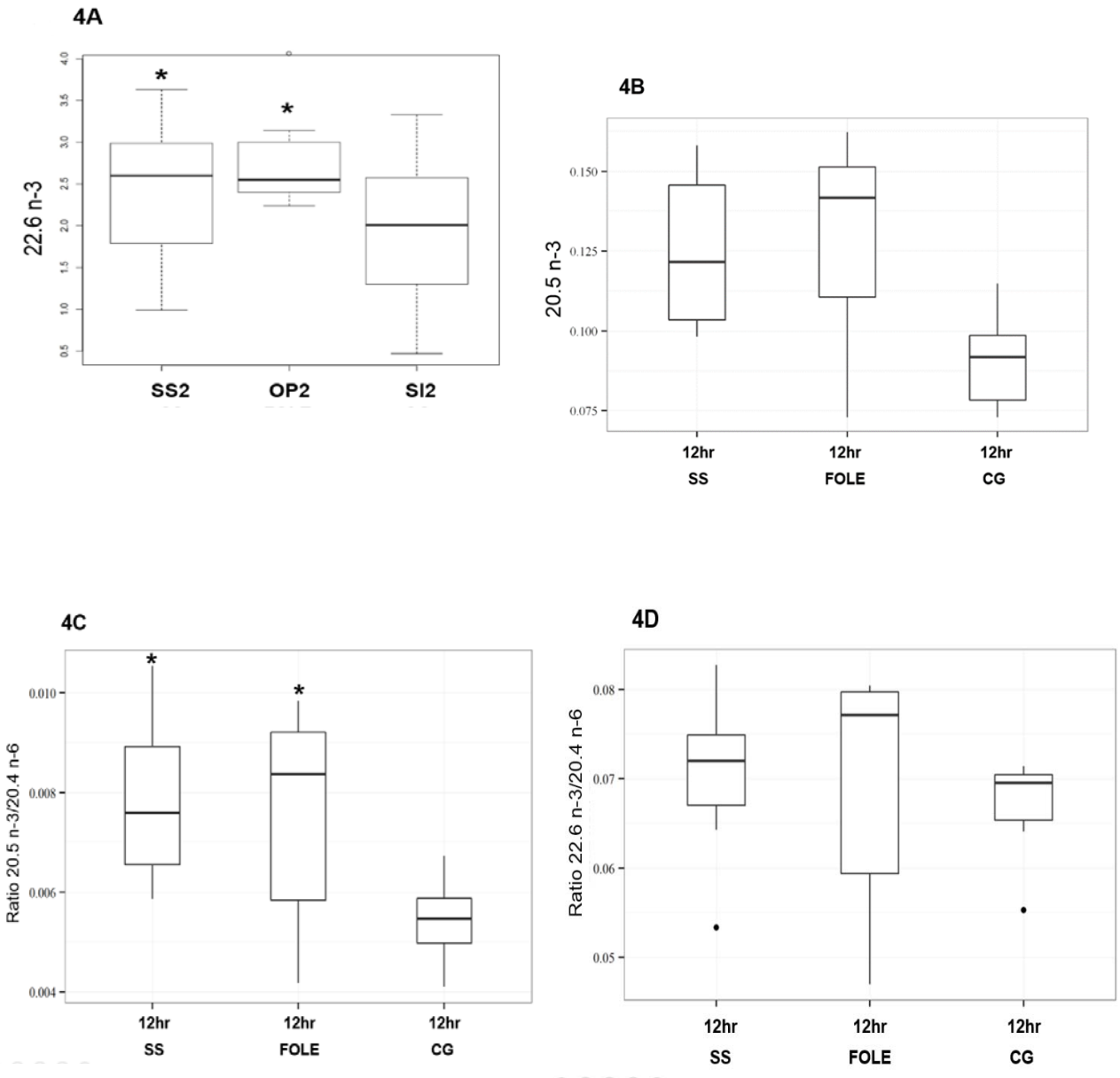
Legend - SS: saline solution; FOLE: fish oil lipid emulsion; CG: control group. 2A: IL-6/IL-10 ratio (*SS; p= 0.04) and (*CG; p= 0.04). The symbol "*" represents different mean values from 2 hours compared to 24 hours.

Figure 3: Lung eicosanoid concentrations (pg/ml) in rats treated with different intravenous infusions prior to the induction of acute pancreatitis.



Legend - SS: saline solution; FOLE: fish oil lipid emulsion; CG: control group. 3A: LTB4 levels (*SS, *FOLE, *CG; p<0.05); 3B: PGE2 levels (*SS, *FOLE, *CG; p<0.05). The symbols "*" represent different mean values from 12 hours compared to 24 hours.

Figure 4: Serum (2 hr) and lung (12 hr) fatty acids (% of total fatty acids) in rats treated with different intravenous infusions prior to the induction of acute pancreatitis.



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586 Legend - SS: saline solution; FOLE: fish oil lipid emulsion; CG: control group.

587 4A: DHA serum (*SS and *FOLE; p<0.05); 4B: EPA lung tissue (*SS; p= 0.001)

588 and (*FOLE; p=0.020); 4C: Ratio EPA/AA (*SS; p=0.001) and (*FOLE; p=0.036);

589 4D: no changes. The symbol "*" represents different or the same mean values

590 between groups.

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