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Short Communication

Effects of short-term infusion of lipid emulsions on pro-inflammatory cytokines and lymphocyte apoptosis in septic and non-septic rats

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Abstract

Long-term administration of PUFA is known to modulate immune functions and apoptotic pathways depending on the respective amount of *n*-6 and *n*-3 fatty acids (FA). Data on short-term effects on apoptotic pathways are rare. Apoptosis of splenic lymphocytes is the hallmark of detrimental sepsis. Therefore, we aimed to compare the immediate effects of parenterally administered *n*-6-enriched soyabean oil (SO)- and *n*-3-enriched fish oil (FO)-based lipid emulsions after laparotomy (LAP; sham procedure) and after induction of acute, severe sepsis by caecal ligation and incision. After 390 min of observation time, plasma was analysed for IL-1β, IL-6 and NEFA. Apoptosis in splenic lymphocytes was quantified by Annexin-V expression. After LAP, infusion of both FO and SO did not change cytokine concentrations. Sepsis increased both cytokines. FO but not SO further augmented the rise. After LAP, SO increased NEFA, and both lipid emulsions reduced free arachidonic acid (AA). Sepsis resulted in a dramatic decrease in NEFA and AA. The drop in NEFA and AA was prevented by both SO and FO. In addition, FO resulted in an increased concentration of *n*-3 FA under both conditions. Infusion of both lipid emulsions induced apoptosis in splenic lymphocytes after LAP. Sepsis-induced apoptosis was not further enhanced by FO or SO. The present study shows that short-term administration of FO as opposed to SO caused pro-inflammatory effects during sepsis. Moreover, short-term administration of both SO and FO suffices to induce apoptosis in splenic lymphocytes. Finally, SO and FO do not further enhance sepsis-induced splenic apoptosis.

Key words: **PUFA: Fish oil: Soyabean oil: Annexin-V**

Despite significant progress in therapy, severe sepsis is still a major cause of death[1]. In order to secure energy supply, septic patients may require total parenteral nutrition including lipid emulsions. Lipid emulsions provide PUFA that are known to differentially modulate immune functions in septic patients depending on the respective amounts of *n*-6 and *n*-3 fatty acids (FA)[2]. In this context, soyabean oil (SO)-based lipid emulsions increase the availability of *n*-6 FA and may further enhance an (already existing) state of inflammation due to the increased production of arachidonic acid (AA)-dependent pro-inflammatory lipid mediators[3]. In contrast, *n*-3 FA may compete for metabolisation with *n*-6 FA, thus resulting in a reduced amount of AA-derived pro-inflammatory mediators[2]. However, the immunomodulatory potential of lipid emulsions is not only dependent on the *n*-6:*n*-3 ratio and the amount of PUFA supplied but also on the experimental or clinical setting[4]. Besides the ability to modulate immune functions through lipid mediator generation, PUFA or infusion of lipid emulsions can induce apoptosis in immune-competent cells[5,6].

Sepsis-induced lymphocyte apoptotic cell death is one of the main reasons why an initial state of hyperinflammation (associated with increased plasma levels of IL-1β and IL-6)

Abbreviations: AA, arachidonic acid; CLI, caecal ligation and incision; FA, fatty acid; FO, fish oil; LAP, laparotomy; SO, soyabean oil.

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shifts towards a later state of hypoinflammation and anergy of the immune system[7]. As a result, immune functions may be hampered even further, and prevention of apoptosis in lymphocytes has already been shown to be protective in models of sepsis[7]. However, *n*-6 and *n*-3 FA show similar pro-apoptotic effects *in vitro*[5], suggesting that modulation of immune functions on one side and of apoptotic pathways on the other side by PUFA can exist independently from each other. In this context, *ex vivo* data point to caspase-3 as the key player of PUFA-induced cell death[8].

While the effects of long-term PUFA administration have been studied during sepsis[2,9], there are still few data on the immediate impact of lipid emulsions after short-term infusion during severe abdominal sepsis. The present study was, therefore, conducted in a short-term sepsis model (caecal ligation and incision, CLI), which rapidly creates a state of severe polymicrobial sepsis in rats[10]. In accordance with the data on enhanced lymphocyte apoptosis during polymicrobial sepsis, CLI also induces splenic apoptosis within 390 min of observation time[11].

We aimed to find out whether SO- and FO-based lipid emulsions differ in their immediate effects on pro-inflammatory cytokine generation and in their ability to influence apoptosis in lymphocytes during severe sepsis.

Materials and methods

Animals and anaesthesia

All animal experiments in this prospective randomised study were approved by the governmental board for the care of animal subjects (Regierungspraesidium, Darmstadt, Germany) and were in accordance with the National Institute of Health guidelines. Male Sprague–Dawley rats (body weight 501 (SD 33) g; Harlan-Winkelmann, Borchen, Germany), were anaesthetised by intraperitoneal injection of pentobarbital (Narcoren, Halbergmoos, Germany) and fentanyl (Janssen-Cilag, Neuss, Germany) as described[10]. A tracheotomy was performed and rats were ventilated with a neonatal ventilator (Stephanie; Stephan, Gackenbach, Germany) with pressure-controlled ventilation. An arterial catheter (SIMS Portex, Hythe, UK) in the right femoral artery was connected to a monitor system (Sirecust; Siemens, Erlangen, Germany) for continuous recording of haemodynamics. A similar catheter was inserted into the right femoral vein for continuous intravenous infusion of 0·9 % NaCl (12 ml/kg per h; B. Braun, Melsungen, Germany), pentobarbital and fentanyl.

Experimental protocol and surgical procedure

Rats were randomly assigned to six groups, three sepsis groups (CLI) and three control groups (laparotomy (LAP); sham procedure), before any experimental procedure was started. Continuous intravenous infusion with 0·06 g/kg per h of a SO-based lipid emulsion (Intralipid®-10 %; Baxter, Unterschleissheim, Germany) was initiated in one LAP group (LAP-SO, six rats) and in one sepsis group (CLI-SO, six rats). Similarly, continuous intravenous infusion with 0·06 g/kg per h of a FO-based lipid emulsion (Omegaven®-10 %; Fresenius Kabi, Bad Homburg, Germany) was initiated in one LAP group (LAP-FO, six rats) and in one sepsis group (CLI-FO, six rats). Composition of the lipid emulsions is provided in Table 1. In the third arm of experiments (LAP, four rats; CLI, six rats), no lipid emulsions, but normal saline was administered. Acute, severe sepsis was established in the CLI groups as described[10]. After a LAP, the caecum and the mesenteric blood vessels were ligated below the ileocaecal valve. The ligated caecum was opened through a 1·5 cm blade incision and subsequently replaced into the abdomen. In all groups, 2 ml/kg of 0·9 % NaCl solution were given intraperitoneally as fluid resuscitation before the abdominal wall was closed. After 390 min of observation time, the animals were exsanguinated, and heparinised whole blood samples (Heparin-Natrium; Ratiopharm, Ulm, Germany) were obtained. For caspase analysis, the spleens were rinsed with ice-cold PBS (Invitrogen, Karlsruhe, Germany).

Determination of fatty acids and cytokines in the plasma

For FA analysis, chemicals of highest purity were obtained from Merck (Darmstadt, Germany). GC of FA methyl esters was performed after lipid extraction from the plasma, TLC and methylation of NEFA[2]. Plasma concentrations of IL-1β and IL-6 were determined by ELISA (R&D Systems, Wiesbaden, Germany) according to the manufacturer's instructions.

Isolation of lymphocytes and assessment of apoptosis by flow cytometry

Lymphocytes from spleens of all animals were harvested as described previously[12]. Spleens were gently minced, followed by lysis of residual erythrocytes. After extensive washing, lymphocytes were resuspended with fluorescein isothiocyanate-conjugated Annexin-V (no. 1828681; Roche Molecular Biochemical, Mannheim, Germany) and 7-amino-actinomycin D (no. 559925; BD Biosciences, San Jose, CA, USA). Lymphocytes were identified by fluorescence-activated cell sorting (BD Biosciences, Franklin Lakes, NY, USA). The percentage of apoptotic cells was detected by Annexin-V

Table 1. Fatty acid composition of the soyabean oil (SO)- and fish oil (FO)-based lipid emulsions*

Fatty acid (g/l)	SO	FO
Myristic acid, 14 : 0	–	4·9
Palmitic acid, 16 : 0	12·4	10·7
Palmitoleic acid, 16 : 1*n*-7	–	8·2
Stearic acid, 18 : 0	5·0	2·4
Oleic acid, 18 : 1*n*-9	24·1	12·3
Linoleic acid, 18 : 2*n*-6	52·2	3·7
α-Linolenic acid, 18 : 3*n*-3	8·2	1·3
Arachidonic acid, 20 : 4*n*-6	–	2·6
EPA, 20 : 5*n*-3	–	18·8
Docosapentaenoic acid, 22 : 5*n*-3	–	2·8
DHA, 22 : 6*n*-3	–	16·5
Others	–	16·1

* Omegaven-10 % contains 0·027 (SD 0·004) g α-tocopherol equivalents of α- and γ-tocopherol.

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staining, and necrotic cells were excluded by 7-amino-actinomycin D labelling. All experiments were performed with FACScan (CellQuest software; BD Biosciences, Heidelberg, Germany) according to the manufacturer's instructions.

Statistics

Statistical analysis was performed with SigmaStat 3.1 (Systat Software, San Jose, CA, USA) using a two-way ANOVA on ranks with either Dunn's (cytokines) or Student–Newman–Keuls' (Annexin-V positivity and NEFA quantification) *post hoc* test of all pairwise multiple comparison procedures. Data are expressed as medians (25 %/75 % quartiles) or as means and standard deviations. Differences were considered significant if $P<0{\cdot}05$ *v.* LAP or CLI, respectively.

Results

Plasma cytokine levels

We did not observe any significant differences in pro-inflammatory cytokine concentrations in the plasma between the LAP, LAP-SO and LAP-FO groups. CLI and CLI-SO induced the generation of IL-1β and IL-6. In contrast, CLI-FO significantly increased IL-6 concentration and also showed a trend towards higher IL-1β concentration compared with CLI and CLI-SO (Table 2).

Lipid emulsions and apoptosis

Compared with LAP, LAP-SO and LAP-FO significantly increased Annexin-V positivity in the spleen. CLI also induced Annexin-V positivity in the spleen. The additional administration of both lipid formulas during sepsis (CLI-SO and CLI-FO) did not alter sepsis-induced Annexin-V positivity in the spleen (Table 2). In accordance with our data on Annexin-V expression, LAP-SO and LAP-FO enhanced cleaved caspase-3 expression in the spleen compared with LAP. Similarly, CLI induced cleaved caspase-3 expression in the spleen. The additional administration of both lipid formulas during sepsis (CLI-SO and CLI-FO) did not alter cleaved caspase-3 expression in the spleen (data not shown).

Plasma profile of NEFA after laparotomy and during sepsis

Plasma profiles of NEFA are provided in Table 2. In the LAP-SO group, NEFA concentration was increased and AA content was significantly reduced compared with the LAP group. In the LAP-FO group, we did not observe any significant impact on the total sum of NEFA, but the amount of AA was significantly lower compared with the LAP group. In the CLI group, the sum of NEFA was significantly lower than that in the CLI-SO and CLI-FO groups. Interestingly, in the CLI group, AA was dramatically decreased. The concentration of AA was preserved in the CLI-SO and CLI-FO groups. After LAP, EPA (*n*-3) and DHA (*n*-3) concentrations were low. In the LAP-SO group, EPA remained unchanged, while DHA was significantly reduced compared with the LAP group. Application of FO resulted in a significant increase in EPA and DHA. After induction of sepsis, a dramatic reduction in both *n*-3 FA was apparent in the CLI group. In the CLI-SO group, levels of both *n*-3 FA were similar to the CLI group, whereas the concentration of both *n*-3 FA in the CLI-FO group was comparable with the LAP-FO group (Table 2).

Discussion

The present study has been conducted in order to investigate the immediate effects of parenteral SO- and FO-enriched lipid emulsions on pro-inflammatory cytokines, apoptosis of splenic lymphocytes and NEFA concentration in the plasma using a short-term rodent model of severe sepsis induced by CLI.

Following LAP, we did not observe major changes in the plasma levels of IL-1β or IL-6 after the administration of *n*-3- or *n*-6-enriched lipid emulsions. After the CLI procedure – creating a massive septic insult including a rise in cytokines within a short period of time – the infusion of FO-based lipid emulsions further induced both IL-1β and IL-6, whereas the SO-based lipid emulsion did not alter pro-inflammatory cytokine plasma levels. This observation cannot be explained by the concentrations of AA but is in line with data from rats showing diverging effects of the same lipid emulsion on cytokine release depending on the surgical insult (i.e. control/sepsis)[13]. Recent data from mice, which had been fed an EPA-/DHA-enriched diet, showed that IL-6 and bacterial load peak 8 h after the onset of bacterial lung infection[14]. Tiesset *et al.* have argued that EPA and DHA may postpone the anti-inflammatory response of the host, which conveys temporary protection from death. In the present study, we did not obtain sequential plasma samples in order to confirm these data. Therefore, we can only speculate that a postponed anti-inflammatory response is responsible for the apparent pro-inflammatory effects of *n*-3 PUFA in the present study.

We have administered 0·06 g/kg per h (1·44 g/kg per d) of each lipid emulsion via the parenteral route over a period of 390 min, which is in accordance with the recommended limits of regular parenteral nutrition (not even taking into account that rats exhibit a higher energy consumption compared with humans per kg body weight). Thus, adequate plasma levels were ensured rapidly. The CLI sepsis induces an immediate hyperinflammation, which massively decreases the sum of NEFA, possibly due to consumption owing to the tremendous septic insult. When lipid emulsions are administered parenterally during CLI sepsis, we did not observe such a loss of NEFA. Furthermore, in the CLI-FO group, concentrations of EPA and DHA remained high. This suggests that it is possible to specifically modify plasma NEFA composition within a very short infusion time, and that both *n*-3 FA were not readily consumed.

The formation of resolvins, a recently discovered class of *n*-3 PUFA-derived lipid mediators resolving inflammation, was not assessed in the present study. Therefore, we can only speculate whether resolvins are responsible for some of our findings. However, resolvins can induce clearance of

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Table 2. Plasma cytokine levels, splenic apoptosis and plasma fatty acid composition

(Medians and 25 %/75 % quartiles or mean values and standard deviations)

	LAP		LAP-SO		LAP-FO		CLI		CLI-SO		CLI-FO	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
IL-1β (pg/ml)												
Median	51		29		49		260		208		389	
25 %/75 % Quartiles	0/164		26/38		39/77		207/329		136/376		324/441	
IL-6 (pg/ml)												
Median	801		995		1331		24 206		19 015		32 183‡§	
25 %/75 % Quartiles	190/2493		871/1331		838/1489		14 226/28 358		15 571/25 985		32 059/32 317	
Annexin-V-positive splenic lymphocytes (%)	6·22	0·83	15·99*	4·8	15·72*	4·14	14·24	4·08	15·57	4·67	14·42	6·62
Arachidonic acid												
Absolute amount (μmol/l)	21·0	2·9	13·9*	1·8	13·1*	0·8	10·2	2·6	12·4	2·9	11·8	1·0
Relative amount (%)	~6		~3		~4		~11		~3		~4	
EPA												
Absolute amount (μmol/l)	1·2	0·2	1·1	0·6	25·4*†	5·9	0·1	0·0	0·8	0·6	33·1‡§	5·6
Relative amount (%)	~0·3		~0·2		~6		~0·2		~0·2		~10	
DHA												
Absolute amount (μmol/l)	4·0	1·0	2·4*	0·4	48·3*†	11·5	1·3	0·2	2·0	0·4	57·5‡§	13·3
Relative amount (%)	~1		~0·5		~13		~1		~0·5		~17	
Fatty acids												
Total (μmol/l)	400·9	60·3	620·0	131·7	416·0	54·6	99·6	5·6	495·2‡	78·7	353·2‡	45·5
Relative amount (%)	100		100		100		100		100		100	

LAP, laparotomy; LAP-SO, soyabean oil-based LAP; LAP-FO, fish oil-based LAP; CLI, caecal ligation and incision; CLI-SO, soyabean oil-based CLI; CLI-FO, fish oil-based CLI.

* Mean value was significantly different from that of the LAP group ($P<0·05$).

† Mean value was significantly different from that of the LAP-SO group ($P<0·05$).

‡ Mean or median value was significantly different from that of the CLI group ($P<0·05$).

§ Mean or median value was significantly different from that of the CLI-SO group ($P<0·05$).

apoptotic immune cells, thus implicating a possible benefit of resolvins in sepsis-induced lymphocyte apoptosis[15].

Both cleaved caspase-3 expression and the fraction of Annexin-V-positive lymphocytes in the spleen show that apoptosis was induced after the infusion of both lipid formulas even without any septic injury. Pro-apoptotic properties of PUFA and lipid emulsions have been described earlier[5,12,16]. However, the present study is the first to show apoptotic cell death in lymphocytes in rats after a short-term infusion of commercially available lipid emulsions regardless of the surgical procedure. Interestingly, CLI-induced lymphocyte apoptosis during sepsis cannot be enhanced any further by additional administration of lipid emulsions. A possible explanation for this observation may be that lipid emulsions have only limited pro-apoptotic abilities. After a minor surgical insult (i.e. LAP), their impact on apoptosis can be observed, which is supported by data from the long-term infusion of lipid emulsions in mice[12]. Following the massive polymicrobial insult of the CLI procedure, any possible effect of lipid emulsions on lymphocyte apoptosis may have been simply overpowered.

The induction of lymphocyte apoptosis even without the initiation of sepsis within this short period of observation time is clearly troublesome. Lymphocyte apoptosis is the hallmark of detrimental late sepsis. Its prevention by either caspase inhibitors or interventions promoting anti-apoptotic factors has been protective in lethal murine models[7]. Recently, we have shown that SO-induced lymphocyte apoptosis in a murine model of acute lung injury is paralleled by increased mortality[12].

To our knowledge, the present study is the first to mirror the short-term effects of SO- and FO-based lipid emulsions on the plasma levels of pro-inflammatory cytokines and NEFA after different surgical insults. Lymphocyte-mediated immune response had been thought to occur rather late during sepsis. However, a growing body of evidence suggests that the engagement of lymphocyte-mediated adaptive immune modulation may take place much earlier than previously thought[17]. Therefore, we have chosen an experimental sepsis model allowing the characterisation of the effects of lipid formulas on pro-inflammatory cytokines, which are characteristic for the innate immune response (IL-1β and IL-6). In addition, we provide evidence on splenic apoptosis after a short-term administration of lipid emulsions, thus suggesting an additional impairment of early adaptive immune functions during CLI sepsis.

We fully acknowledge that the present study is descriptive and does not account for any possible molecular mechanisms behind our findings. Nevertheless, our data show that a short-time infusion of different lipid emulsions has diverging effects on pro-inflammatory cytokines. In addition, lipid emulsions have a major impact on apoptotic pathways depending on the respective surgical procedure, a notion that may be of importance for surgical patients receiving parenteral nutrition.

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