Pro- and anti-inflammatory cytokines in healthy volunteers fed various doses of fish oil for 1 year

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Abstract. Dietary supplementation with n-3 fatty acids from fish oil alleviates inflammation in various chronic inflammatory disease states. Reductions in the production of pro-inflammatory cytokines interleukin 1β (IL-1β), tumour necrosis factor alpha (TNF-α), and interleukin 6 (IL-6) have been seen in humans after short-term n-3 fatty acid supplementation. We investigated long-term effects of dietary n-3 fatty acids on circulating cytokine concentrations and on ex vivo stimulated whole-blood production of IL-1β, TNF-α and interleukin 1 receptor antagonist (IL-1Ra), the naturally occurring antagonist of IL-1. A total of 58 monks with a mean age of 56 years were randomized into four groups and their diets were supplemented with 0, 3, 6, or 9 g of fish oil, providing 0, 1-06, 2-13 or 3-19 g of n-3 fatty acids per day. Subjects received equal amounts of saturated fatty acids, vitamin E and cholesterol. Compliance was excellent and erythrocyte fatty acid profiles closely reflected the amounts of n-3 fatty acids ingested. In the group receiving 9 g of fish oil per day, no influence of n-3 fatty acids on circulating cytokine concentrations was observed relative to placebo. Endotoxin-stimulated whole-blood cytokine production was measured at 26 and 52 weeks after the start and at 4, 8 and 26 weeks after cessation of supplementation. In all groups, the production of IL-1β and IL-1Ra was higher during supplementation than afterwards. However, no differences in cytokine production were noted between the placebo group and those receiving 9 g of fish oil per day. No influence of n-3 fatty acids in the form of a fish oil concentrate [2-5]. n-3 fatty acids have also been advanced for use in patients with burn trauma and in postoperative cancer patients [6-9]. The anti-inflammatory effects of n-3 fatty acids have originally been attributed to changes in the production of prostaglandins and leukotrienes [10-12]. More recently, however, reduced production of the pro-inflammatory cytokines interleukin 1α and β (IL-1α and β) and tumour necrosis factor alpha (TNF-α) has been emphasized as a possible mechanism [13]. IL-1α and β, interleukin 6 (IL-6) and TNF-α are small proteins that are produced by cells of mononuclear phagocytic origin, including circulating monocytes and tissue macrophages. These cytokines mediate the response of the host to various inflammatory stimuli. They induce fever, activation of B and T lymphocytes and endothelial cells, synthesis of acute-phase proteins by the liver, and many other effects that are all part of the acute-phase response [14].

Abstract. Dietary supplementation with n-3 fatty acids from fish oil alleviates inflammation in various chronic inflammatory disease states. Reductions in the production of pro-inflammatory cytokines interleukin 1β (IL-1β), tumour necrosis factor alpha (TNF-α), and interleukin 6 (IL-6) have been seen in humans after short-term n-3 fatty acid supplementation. We investigated long-term effects of dietary n-3 fatty acids on circulating cytokine concentrations and on ex vivo stimulated whole-blood production of IL-1β, TNF-α and interleukin 1 receptor antagonist (IL-1Ra), the naturally occurring antagonist of IL-1. A total of 58 monks with a mean age of 56 years were randomized into four groups and their diets were supplemented with 0, 3, 6, or 9 g of fish oil, providing 0, 1-06, 2-13 or 3-19 g of n-3 fatty acids per day. Subjects received equal amounts of saturated fatty acids, vitamin E and cholesterol. Compliance was excellent and erythrocyte fatty acid profiles closely reflected the amounts of n-3 fatty acids ingested. In the group receiving 9 g of fish oil per day, no influence of n-3 fatty acids on circulating cytokine concentrations was observed relative to placebo. Endotoxin-stimulated whole-blood cytokine production was measured at 26 and 52 weeks after the start and at 4, 8 and 26 weeks after cessation of supplementation. In all groups, the production of IL-1β and IL-1Ra was higher during supplementation than afterwards. However, no differences in cytokine production were noted between the placebo group and those receiving 9 g of fish oil per day. No influence of n-3 fatty acids in the form of a fish oil concentrate [2-5]. n-3 fatty acids have also been advanced for use in patients with burn trauma and in postoperative cancer patients [6-9]. The anti-inflammatory effects of n-3 fatty acids have originally been attributed to changes in the production of prostaglandins and leukotrienes [10-12]. More recently, however, reduced production of the pro-inflammatory cytokines interleukin 1α and β (IL-1α and β) and tumour necrosis factor alpha (TNF-α) has been emphasized as a possible mechanism [13]. IL-1α and β, interleukin 6 (IL-6) and TNF-α are small proteins that are produced by cells of mononuclear phagocytic origin, including circulating monocytes and tissue macrophages. These cytokines mediate the response of the host to various inflammatory stimuli. They induce fever, activation of B and T lymphocytes and endothelial cells, synthesis of acute-phase proteins by the liver, and many other effects that are all part of the acute-phase response [14].

In three studies in healthy volunteers, significant reductions in IL-1 and TNF-α production capacity of isolated peripheral blood mononuclear cells were observed after 6 weeks to 3 months of dietary fish oil supplementation [13,15,16]. However, two controlled studies did not find differences in cytokine production capacity after 7 weeks and 4 months of dietary fish oil supplementation [17,18]. Here, we report effects of fish oil on cytokine levels in a 1-year placebo-controlled trial.

Introduction

n-3 (omega-3) polyunsaturated fatty acids have been investigated for use in the treatment of various inflammatory diseases [1]. In patients with rheumatoid arthritis, ulcerative colitis and psoriasis, an alleviation of clinical symptoms has been noted after ingestion of n-3 fatty acids in the form of a fish oil concentrate [2-5]. n-3 fatty acids have also been advanced for use in patients with burn trauma and in postoperative cancer patients [6-9]. The anti-inflammatory effects of n-3 fatty acids have originally been attributed to changes in the production of prostaglandins and leukotrienes [10-12]. More recently, however, reduced production of the pro-inflammatory cytokines interleukin 1α and β (IL-1α and β) and tumour necrosis factor alpha (TNF-α) has been emphasized as a possible mechanism [13]. IL-1α and β, interleukin 6 (IL-6) and TNF-α are small proteins that are produced by cells of mononuclear phagocytic origin, including circulating monocytes and tissue macrophages. These cytokines mediate the response of the host to various inflammatory stimuli. They induce fever, activation of B and T lymphocytes and endothelial cells, synthesis of acute-phase proteins by the liver, and many other effects that are all part of the acute-phase response [14].

In three studies in healthy volunteers, significant reductions in IL-1 and TNF-α production capacity of isolated peripheral blood mononuclear cells were observed after 6 weeks to 3 months of dietary fish oil supplementation [13,15,16]. However, two controlled studies did not find differences in cytokine production capacity after 7 weeks and 4 months of dietary fish oil supplementation [17,18]. Here, we report effects of fish oil on cytokine levels in a 1-year placebo-controlled trial.

Subjects and methods

Subjects

Fifty-eight monks from one Trappist and three Benedictine monasteries in The Netherlands participated in the study. They were all in good health and without known underlying disease such as diabetes, cancer or coronary heart disease. The ages of the subjects ranged from 21 to 87 years with a mean ± SD of 56.2 ± 16.5 years. Body mass indexes were below 30 kg m⁻²; systolic and diastolic
blood pressures were below 160 mmHg and 95 mmHg respectively; concentrations of serum total cholesterol were below 7.0 mmol L\(^{-1}\) and triglycerides below 3.0 mmol L\(^{-1}\).

Ten monks were Trappists and thus vegetarians. During the study period, the subjects did not take medications influencing lipid metabolism or non-steroidal anti-inflammatory drugs. The study was approved by the Ethics Committee of the Department of Human Nutrition, and all subjects gave informed consent.

Experimental design
The study consisted of a 2-week baseline period, followed by a 1-year treatment and a 6-month follow-up period. Within each monastery, the subjects were randomly and blindly divided into four groups, which received zero (n = 14), three (n = 15), six (n = 15) or nine (n = 14) fish oil capsules per day. In each dosage group, the total number of capsules was made up to nine by adding nine, six, three or zero placebo capsules respectively. Periodically, every subject received three bottles with dark-coloured capsules. Depending on the dosage group, none, one, two or all three of the bottles contained fish oil capsules, whereas the remaining bottles contained placebo capsules. At each meal one capsule was taken from every bottle. At regular intervals compliance was established by counting the remaining capsules.

A clinical examination including weight, height, waist-hip ratio, thigh circumference and blood pressure was performed eight times during the trial: on days -14, 0 (start of the supplementation), 56, 182, 363 (end of the supplementation), 396, 424, and 536. At these times blood was also drawn. The conditions used for whole-blood ex vivo production of IL-1\(\beta\), TNF-\(\alpha\), and IL-1Ra turned out to have been inappropriate until 26 weeks after the start of the dietary supplementation. Thus, these data are available only at 26 and 52 weeks of supplementation and at 4, 8 and 26 weeks after cessation of the supplementation. In weeks 26 and 52 the subjects recorded their habitual food consumption by writing down what they had eaten during 3 days. These data were completed by a trained dietitian, who interviewed the subjects, weighed portion sizes and checked recipes.

All subjects received a diary to record the use of medicine, illness, alcohol intake, inadvertent omission of capsule intake, and other relevant facts.

Oil supplements
Eicosapentaenoic (EPA)-rich fish oil capsules were a kind gift from Labaz (Brussels, Belgium). The placebo capsules contained olive oil (Puget, Marseille, France), and palm oil (Loders-Krokaan, Wormerveer, The Netherlands). Cholesterol (Merck, Darmstadt, Germany) and vitamin E (Organon, Oss, The Netherlands) were added. The placebo capsules were also manufactured by Labaz. Fatty acids composition and cholesterol content of the capsules were determined in our laboratory using capillary gas–liquid chromatography. The vitamin E content was measured by Dr J. Schrijver (CIVO-TNO, Zeist, The Netherlands) using a high-performance liquid chromatographic (HPLC) method. The composition of the fish oil and placebo capsules is shown in Table 1. EPA accounted for 72% of all n-3 fatty acids in the fish oil. The fish oil and the placebo capsules contained equal amounts of saturated fatty acids, vitamin E and cholesterol. There was a small difference in the concentration of n-6 fatty acids. The n-3 fatty acids of the fish oil capsules were replaced by neutral monoensaturated fatty acids (largely oleic acid) plus some linoleic acid in the placebo capsules. Both capsules contained 1 g of fat; thus every subject had 9 g of extra fat per day. The daily intake of eicosapentaenoic acid (EPA, C\(_{20:5}\) n-3), docosahexaenoic acid (DHA, C\(_{22:6}\) n-3), and C\(_{22:5}\) n-3 in the various treatment groups is shown in Table 2.

Measurement of fatty acids
After an overnight fast, venous blood was drawn into a 10-mL vacutainer tube with EDTA as anticoagulant. The tubes were centrifuged on the spot at low speed at 4 °C to separate red blood cells. The cells were washed twice with ice-cold saline, transferred into stoppered plastic tubes (Greiner, Nuttingen, Germany), and haemolysed by freezing at -80°C for at least 2 h. The haemolysates were stored at -20°C for a maximum of 7 days. Fatty acids of the haemolysates were measured by capillary gas chromatography.

<table>
<thead>
<tr>
<th>Saturated fatty acids (% mass of methylesters)</th>
<th>Fish oil capsule</th>
<th>Placebo capsule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monounsaturated fatty acids</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>n-3 fatty acids (% mass of methylesters)</td>
<td>26</td>
<td>65</td>
</tr>
<tr>
<td>n-6 fatty acids (% mass of methylesters)</td>
<td>37</td>
<td>0.6</td>
</tr>
<tr>
<td>Cholesterol (mg/100g)</td>
<td>115</td>
<td>119</td>
</tr>
<tr>
<td>Vitamin E (IU/100g)</td>
<td>63</td>
<td>63</td>
</tr>
</tbody>
</table>

Table 2. Daily n-3 fatty acid (g day\(^{-1}\)) intake in the various treatment groups

<table>
<thead>
<tr>
<th>n-3 fatty acid</th>
<th>Low-dose fish oil group (3 g day(^{-1}))</th>
<th>Medium-dose fish oil group (6 g day(^{-1}))</th>
<th>High-dose fish oil group (9 g day(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPA (C(_{20:5}) n-3)</td>
<td>0.81</td>
<td>1.62</td>
<td>2.43</td>
</tr>
<tr>
<td>C(_{22:5}) n-3</td>
<td>0.09</td>
<td>0.18</td>
<td>0.27</td>
</tr>
<tr>
<td>DHA (C(_{22:6}) n-3)</td>
<td>0.16</td>
<td>0.33</td>
<td>0.49</td>
</tr>
<tr>
<td>Total</td>
<td>1.06</td>
<td>2.13</td>
<td>3.19</td>
</tr>
</tbody>
</table>

EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.
Table 3. Concentration of eicosapentaenoic acid (EPA) (C20:5 n-3) in erythrocyte lipids as a function of duration and level of fish oil supplementation. Values are represented as grams of EPA per 100 g fatty acids.

<table>
<thead>
<tr>
<th>Week of experiment</th>
<th>Daily ingestion of fish oil (g)</th>
<th>EPA (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0-29 ± 0.21</td>
<td>0.78 ± 0.22</td>
</tr>
<tr>
<td>2</td>
<td>0.85 ± 0.19</td>
<td>1.50 ± 0.20</td>
</tr>
<tr>
<td>4</td>
<td>0.83 ± 0.16</td>
<td>1.83 ± 0.37</td>
</tr>
<tr>
<td>8</td>
<td>0.86 ± 0.30</td>
<td>2.31 ± 0.37</td>
</tr>
<tr>
<td>26</td>
<td>1.12 ± 0.24</td>
<td>3.06 ± 0.38</td>
</tr>
<tr>
<td>52</td>
<td>0.83 ± 0.28</td>
<td>2.82 ± 0.24</td>
</tr>
<tr>
<td>56</td>
<td>0.88 ± 0.28</td>
<td>1.89 ± 0.27</td>
</tr>
<tr>
<td>60</td>
<td>0.78 ± 0.19</td>
<td>1.50 ± 0.24</td>
</tr>
<tr>
<td>78</td>
<td>0.83 ± 0.17</td>
<td>0.86 ± 0.20</td>
</tr>
</tbody>
</table>

*Fish oil supplementation from week 0 until week 52. From week 56 on, post-supplementation values are represented.

Cytokine measurements

Venous blood was drawn into three 4-mL evacuated γ-sterilized blood collection tubes (Becton Dickinson, Vacutainer Systems, Rutherford, NJ, USA) containing 6 mg of EDTA. The tubes were filled with blood by vacuumaspiration. An aliquot (250 μL) of aprotinin with an activity of 10-000 kallikrein-inactivating units per ML (Trasylool, Bayer, Leverkusen, Germany) was added per tube. One tube was centrifuged immediately and the resultant plasma was drawn off, stored in dry ice, transported to the laboratory and stored at -80 °C until assay of circulating cytokines. One of the remaining two samples was stimulated by the addition of 50 μL of endotoxin (lipopolysaccharide, LPS, Escherichia coli serotype O55:B5, Sigma, St Louis, MO, USA), concentration 800 μg mL-1, final concentration 10 μg mL-1. The control sample did not have endotoxin added. The two samples were inverted gently to mix and incubated for 24 h at 25 °C in a circulating water bath. The samples were incubated within 5 min after drawing, and incubation at 25 °C was secured by keeping the samples in a portable stove throughout transportation to the laboratory. After the incubation, samples were centrifuged at 1250g for 10 min. For removal of platelets, the resultant plasma was spun in an Eppendorf centrifuge at 13 000 rpm for 5 min. Platelet-poor plasma, containing the secreted products of endotoxin-stimulated blood cells, was drawn off and stored at -80°C until assay of cytokines.

IL-1β, TNF-α and IL-1Ra were determined by radioimmunoassay as described previously [19–21]. The intra-assay variability was less than 10%. The detection limit of IL-1β was 50 pg mL-1, of TNF-α 50 pg mL-1 and of IL-1Ra 100 pg mL-1. Circulating concentrations of IL-6 were measured using an enzyme-linked immunosorbent assay (ELISA) [22], with a detection limit of 6 pg mL-1. To avoid experimental fluctuations, one batch of LPS and one batch of blood-collecting tubes was used throughout the study. All samples of one subject were determined in the same series and in each series an equal number of samples of each treatment group was analysed to avoid bias due to assay variability.

Statistical analysis

Cytokine concentrations of the various diet groups were compared using the multivariate rank test according to Koziol et al. [23] and Crepeau et al. [24]. This is a non-parametric test for the comparison of growth curves, which accounts for missing data at certain time points.

Results

Compliance

The interviews taken by the dietitians and the counting of the capsules revealed an excellent adherence to the experimental protocol throughout the 78 weeks that this study lasted. The determinations of fatty acid profiles of red blood cells closely reflected the amount of n-3 fatty acids ingested by the four experimental groups (Table 3) [25]. Twenty-six weeks after the cessation of the fat supplementation, the fatty acid profiles of the red blood cells had returned to pretreatment values. None of the subjects experienced adverse effects.

Circulating cytokines

The concentrations of circulating IL-1β, IL-6, TNF-α and IL-1Ra were determined only in the groups receiving 0 and 9 g of fish oil per day. As expected, these concentrations were low. The concentrations of IL-1β, TNF-α and IL-1Ra were well above the detection limit of the assay in all subjects, but IL-6 was below the detection limit in all samples. For the circulating cytokines that could be measured, there was no significant difference between the two treatment groups at any point in time, either in absolute concentrations or in changes relative to baseline (Fig. 1). Basal concentrations of circulating cytokines were not different between vegetarians and non-vegetarians (data not shown).

Ex vivo production of IL-1β, TNF-α and IL-1Ra after whole-blood stimulation

In all three dose groups, as well as in the placebo group,
endotoxin-stimulated secretion of IL-1β and IL-1Ra was significantly higher during oil supplementation than at 8 and 26 weeks after cessation of capsule intake (Fig. 2). A similar trend was observed in the secretion of TNF-α, although differences over time were not statistically significant. The ex vivo production of IL-1β, TNF-α and IL-1Ra was not significantly different between the four diet groups at any point in time. The inter-individual variations in cytokine production were large. When the values obtained at 78 weeks, i.e. 26 weeks after cessation of the fat supplementation, are taken as baseline values, percentage changes over time can be evaluated. Again, these values were not significantly different between the various experimental groups at any point in time (data not shown). Twenty-six weeks after cessation of the dietary supplementation, cytokine production capacity was not different between vegetarians and non-vegetarians (data not shown).

Figure 1. Circulating concentrations of IL-1β (top), TNF-α (middle) and IL-1Ra (bottom) at various time points before, during, and after dietary supplementation with placebo or 3-19 g n-3 fatty acids per day. Results expressed as mean ± SD.

Discussion

The findings in this long-term placebo-controlled study disagree with the conclusions drawn in some previously published studies regarding the effects of dietary fish-oil supplementation on ex vivo cytokine production [13,15,16,26]. We found no effect of fish oil on the ex vivo production of pro-inflammatory cytokines, either during supplementation or after cessation of capsule intake, relative to placebo. The fluctuations in absolute levels in all groups, including the placebo group, demonstrate the need for a proper control group in studies of this nature, and for a proper analysis that compares the effect of fish oil with that of placebo. Statistical testing of changes from baseline within the fish oil group may lead to the faulty conclusion that fish oil does decrease cytokine levels or production [3,27]

In two controlled studies, no effect of dietary fish-oil on ex vivo production of IL-1β or TNF-α was found at 7 weeks and 4 months of supplementation [17,18]. Effects of n-3 fatty acids that are apparent at 6 weeks may thus
have disappeared at 26 weeks of dietary fish oil supplementation.

The question remains why the ex vivo production of IL-1ßand IL-1Ra was higher during the dietary supplementation in both the placebo and the n-3 fatty acids-supplemented groups. It is unlikely that the daily ingestion of placebo capsules containing 9 g of a mixture of olive and palm oil exerts such profound biological effects as the fatty acid composition of cholesterylesters, and erythrocyte membranes did not change during intake of the placebo capsules [25]. A seasonal effect on cytokine production capacity is less likely because the measurements after 26 weeks of supplementation (Fig. 2, first time point) and after 26 weeks of washout (Fig. 2, final time point) were obtained in the same season, 1 year apart. Differences with other studies might also be explained by the dose of fish oil used [13] or by differences in background diet [26]; however, the recent study of Schmidt et al. [28] yielded results essentially similar to those reported here.

The changes in fatty acid profiles were similar to those observed in the other studies mentioned. Therefore, the dose of n-3 fatty acids ingested cannot account for the lack of an effect on cytokine production.

We have evaluated the cytokine production capacity of whole-blood samples, whereas others used in vitro stimulation of mononuclear cells that were isolated by density gradient centrifugation. The relative simplicity of the whole-blood stimulation may render this method less prone to experimental biases: manipulation of the cell population studied is minimal, stimulation is started immediately upon removal of the cells from the circulation and experimental conditions can easily be kept constant. Moreover, Nerad et al. [21] have shown a good correlation between whole blood IL-1ß measurement and the method in which mononuclear cells are separated by density gradient centrifugation and stimulated ex vivo. Therefore, whole blood stimulation may reflect a representation of cytokine production capacity that is at least as relevant as a method using cell separation techniques.

The concentrations of circulating cytokines were, as expected, very low. Higher concentrations can be found in severe disease states such as septic shock, burn trauma, and after experimental administration of endotoxin [29–32]. In rheumatoid arthritis patients, dietary fish oil supplementation during 12 weeks resulted in decreased circulating concentrations of IL-1ß [33]. Our results indicate that dietary fish oil supplementation does not affect the low concentrations of circulating cytokines that are found under physiological, unstimulated circumstances.

In conclusion, in this long-term controlled trial we could not confirm that the fish oil induced reduction of ex vivo stimulated cytokine production that has been observed in most short-term studies until now, but the results corroborate those of the medium-term study of Cannon et al. [18]. Our findings emphasize the need for a well-controlled intervention trial comparing short-term and long-term effects of dietary fish oil supplementation.

Acknowledgements

We thank Gerard Pesman and Mariel Spruitenberg for help with the measurements of cytokines.

References

Discussion

Results expressed as mean ± SD.

Figure 1: Concentration of IL-1α (top), TNF-α (middle) and IL-1β (bottom) in the plasma of fish from 15 June 2012 to 15 June 2013. There was a significant difference in the concentration of IL-1α between the different treatments. The concentration of TNF-α was also significantly higher in the experimental group compared to the control group. The concentration of IL-1β was significantly lower in the experimental group compared to the control group.

Figure 2: Concentration of IL-1β in the plasma of fish from 15 June 2012 to 15 June 2013. There was a significant difference in the concentration of IL-1β between the different treatments. The concentration of IL-1β was significantly lower in the experimental group compared to the control group.

Figure 3: Concentration of TNF-α in the plasma of fish from 15 June 2012 to 15 June 2013. There was a significant difference in the concentration of TNF-α between the different treatments. The concentration of TNF-α was significantly higher in the experimental group compared to the control group.

Figure 4: Concentration of IL-1α, TNF-α and IL-1β in the plasma of fish from 15 June 2012 to 15 June 2013. There was a significant difference in the concentration of IL-1α, TNF-α and IL-1β between the different treatments. The concentration of IL-1α was significantly higher in the experimental group compared to the control group.

Figure 5: Concentration of TNF-α in the plasma of fish from 15 June 2012 to 15 June 2013. There was a significant difference in the concentration of TNF-α between the different treatments. The concentration of TNF-α was significantly higher in the experimental group compared to the control group.

Figure 6: Concentration of IL-1α, TNF-α and IL-1β in the plasma of fish from 15 June 2012 to 15 June 2013. There was a significant difference in the concentration of IL-1α, TNF-α and IL-1β between the different treatments. The concentration of IL-1α was significantly higher in the experimental group compared to the control group.

Figure 7: Concentration of TNF-α in the plasma of fish from 15 June 2012 to 15 June 2013. There was a significant difference in the concentration of TNF-α between the different treatments. The concentration of TNF-α was significantly higher in the experimental group compared to the control group.

Figure 8: Concentration of IL-1α, TNF-α and IL-1β in the plasma of fish from 15 June 2012 to 15 June 2013. There was a significant difference in the concentration of IL-1α, TNF-α and IL-1β between the different treatments. The concentration of IL-1α was significantly higher in the experimental group compared to the control group.