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Effect of dietary omega-3 polyunsaturated fatty acids on experimental periodontitis in the mouse

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Background and Objective: Periodontitis is an infective disease caused predominantly by gram-negative anerobes. The host inflammatory response to these bacteria causes alveolar bone loss, which characterizes periodontitis. Omega-3 polyunsaturated fatty acids have recognized anti-inflammatory effects; their oxygenated derivatives are key mediators in reducing inflammation. In this study we tested the hypothesis that dietary supplementation with tuna fish oil rich in the n-3 polyunsaturated fatty acid, docosahexaenoic acid, would reduce alveolar bone loss in mice inoculated with periodontopathic bacteria.

Material and Methods: Adult mice were fed experimental diets containing either 10% tuna oil or Sunola oil for 57 d. After 14 d, 35 mice on each diet were inoculated orally with *Porphyromonas gingivalis*, with a mixture of *P. gingivalis* and *Fusobacterium nucleatum*, with carboxymethylcellulose or remained untreated. The mice were killed, and soft tissue biopsies from the oral cavity of treated mice were used to determine the polyunsaturated fatty acid concentrations. The maxilla was removed, stained and digitally imaged to assess bone loss around the upper molars.

Results: n-3 polyunsaturated fatty acid levels were significantly higher in oral soft tissues of mice fed tuna oil compared with the control group. Mice fed tuna oil and inoculated with *P. gingivalis* or with the combination of *F. nucleatum* and *P. gingivalis* exhibited 72% and 54% less alveolar bone loss respectively, compared with the treatment control group.

Conclusion: Alveolar bone loss was inversely related to n-3 polyunsaturated fatty acid tissue levels. In conclusion, fish oil dietary supplementation may have potential benefits as a host modulatory agent in the prevention and/or adjunctive management of periodontitis.

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Periodontitis is an oral disease that is thought to result from a host immuno-inflammatory response to the proliferation of periodontopathic bacteria in the gingival sulcus. Localized synthesis of prostaglandins and leukotrienes produced from the metabolism of arachidonic acid are important mediators in periodontal tissue destruction (1). In addition to stimulating the production of a range of pro-inflammatory cytokines, prostaglandin E_2 is thought to play key role in the destruction of periodontal connective tissue and alveolar bone by activating osteoclasts and increasing the synthesis of matrix metalloproteinase-1 (2). Leukotriene B_4 has also been implicated in

periodontitis and is believed to contribute to periodontal destruction by causing cellular degranulation and superoxide generation (1). Several other arachidonic acid metabolites, such as thromboxane A_2, have also been shown to have marked pro-inflammatory and pro-thrombotic effects (3).

Eicosapentaenoic acid and docosahexaenoic acid, the major n-3 polyunsaturated fatty acids in fish oil, competitively inhibit the production of arachidonic acid metabolites via the cyclo-oxygenase and lipoxygenase pathways, thus reducing the synthesis of pro-inflammatory arachidonic acid-derived prostaglandins and leukotrienes (4). In addition, metabolism of n-3 polyunsaturated fatty acid results in the production of other anti-inflammatory mediators such as prostaglandin I_3, resolvins and protectins (5).

Various medications that can inhibit these pathways, such as the nonsteroidal anti-inflammatory drugs, have proven beneficial in the treatment of both experimental gingivitis and periodontitis (6,7). However, long-term nonsteroidal anti-inflammatory drug administration is not recommended as it is associated with side effects, particularly in the gastrointestinal tract (8). More recently, cyclo-oxygenase-2-selective nonsteroidal anti-inflammatory drugs have been demonstrated to produce fewer gastric adverse drug reactions, but concerns about potential cardiac side effects have reduced the scope of their use (9). By contrast, the side effects of n-3 polyunsaturated fatty acids are minimal.

As a result of these anti-inflammatory actions and a positive safety profile, n-3 polyunsaturated fatty acid dietary supplementation has become an accepted adjunct in the management of chronic inflammatory diseases such as rheumatoid arthritis and cardiovascular disease. Recently, there has been growing interest in the potential preventive or therapeutic effects of n-3 polyunsaturated fatty acid in the management of periodontal diseases.

Several studies have demonstrated that, during the development of experimental periodontitis lesions in small animals, dietary supplementation with n-3 polyunsaturated fatty acids can suppress gingival levels of arachidonic acid, prostaglandin E_2 and leukotriene C_4 (10,11). Moreover, n-3 polyunsaturated fatty acid dietary supplementation results in a slightly greater reduction in pro-inflammatory mediators compared with the selective cyclo-oxygenase-2 inhibitor Celecoxib (12). This is in agreement with an earlier study which demonstrated that eicosapentaenoic acid or docosahexaenoic acid can inhibit the production of prostaglandin E_2 to an extent comparable to the nonselective cyclo-oxygenase inhibitor ibuprofen (13). In a recent study, animals treated topically with the eicosapentaenoic acid-derived resolvin E1 showed a fourfold decrease in periodontal bone loss compared with control animals (14). As a result of these studies, it has been proposed that a diet rich in n-3 polyunsaturated fatty acids may reduce alveolar bone loss associated with periodontitis. Using an abscess model in rats, Vardar-Sengul *et al*. (15) reported that, although osteocalcin levels were higher in the n-3 polyunsaturated fatty acid groups, no significant difference in alveolar bone loss was seen between control animals and those fed a diet high in fish oil. By contrast, Kesavalu *et al*. (16) measured alveolar bone loss following oral inoculation of rats with *Porphyromonas gingivalis*. They reported that rats fed n-3 polyunsaturated fatty acid had elevated serum concentrations of eicosapentaenoic acid and docosahexaenoic acid and showed significantly less alveolar bone loss compared with corn oil diet controls (16).

Given the above conflicting results, the aim of this study was to investigate further the effect of a high dietary intake of n-3 polyunsaturated fatty acid on alveolar bone loss in a rodent experimental periodontitis model. In contrast with other studies, tuna oil (which is four times richer in docosahexaenoic acid than eicosapentaenoic acid) was used as the n-3 polyunsaturated fatty acid dietary supplement in this study.

Material and methods

This study was approved by the Animal Ethics Committee, Institute of Medical and Veterinary Science, Adelaide, South Australia. All experiments were carried out according to the National Health and Medical Research Council's Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1997).

Experimental diets

Two separate diets were prepared by Specialty Feeds (Glen Forrest, Western, Australia). Both contained standard soft powdered chow, predominantly wheat and barley (full composition available at http://www.specialtyfeeds.com). To the control diet, 10% (w/w) Sunola oil (Goodman Fielder Ltd, Sydney, Australia) was added and evenly distributed. To the experimental diet, 10% (w/w) HiDHA® 25N tuna oil (NuMega Ingredients, Brisbane, Australia) was added and evenly distributed. Analysis of the tuna oil showed that n-3 polyunsaturated fatty acids comprised 35% of total fatty acids, of which 25.5% was docosahexaenoic acid and 6.3% eicosapentaenoic acid. In order to minimize peroxidative damage, fresh dietary mixtures were prepared every 48 h and kept at 4°C before use.

Seventy 6–8-wk-old female BALB/c mice were randomly allocated to either the control or the experimental diet using a table of random numbers. All animals received food and water.

Preparation of inocula

Porphyromonas gingivalis W50 (W83) and *Fusobacterium nucleatum* ATCC 10953 were maintained on anerobic blood agar plates, at 37°C in an atmosphere of $N_2/CO_2/H_2$ (90:5:5, v/v/v). Following anerobic incubation for 72 h on anerobic blood agar (Oxoid, Basinstoke, UK), 1.5 mL of 2% (w/v) prereduced carboxymethylcellulose was placed onto each plate and the cells were harvested directly from the plate using a disposable spreader. Suspended bacteria were then placed in a 1.5 mL microfuge tube. Inoculation of mice occurred within 30 min of cell harvesting. Cell density was estimated to be $> 10^{11}$ organisms/mL (the optical density at 560 nm was > 5.0). The

combined inoculum (*P. gingivalis* and *F. nucleatum*) was prepared by adding equal volumes (0.5 mL) of both preparations followed by gentle mixing.

Induction of experimental periodontitis

Experimental periodontitis was induced in mice using a modified technique described by Baker *et al.* (17). Briefly, after an initial 5 d being fed either the control or experimental diet, 30 animals on each diet received kanamycin (1 mg/mL) *ad libitum* for a 7-d period in their drinking water to suppress the indigenous oral microbiota. Mice were then rested for 3 d before inoculations commenced on day 15.

Inoculations consisted of 100 μL of bacteria (either 10^{10} colony-forming units/mL of *P. gingivalis* alone or 5×10^9 colony-forming units/mL of *P. gingivalis* and 5×10^9 colony-forming units/mL of *F. nucleatum*) suspended in 2% carboxymethyl cellulose or, alternatively, carboxymethyl cellulose alone. Each type of inoculation was administered to three groups of 10 mice on each diet by oral gavage. Initially, mice were inoculated every 48 h for 8 d (regime 1), after which inoculations were continued twice weekly for 2 wk before regime 1 was repeated. Mice were then subjected to twice-weekly inoculations until the end of the experimental period (day 57). Following each inoculation, mice were held without food or water for 1 h to minimize elimination of the inoculant from the oral cavity. Five mice on each diet remained untreated throughout.

Recovery of bacteria was conducted at the conclusion of the experiment by plating gingival samples (using sterile paper points) onto anerobic blood agar. Black-pigmented colonies showing cellular morphology consistent with *P. gingivalis* were detected in mice receiving the combined or single culture inoculum (results not shown).

Tissue collection and preparation

Mice were killed after 57 d by CO_2 inhalation and cervical dislocation. Approximately 80 mg of oral cavity soft tissue was collected from each animal by sharp dissection of the palatal gingiva, palatal mucosa, maxillary and mandibular buccal gingiva, and oral mucosa and the tongue. Individual tissue samples were 'snap frozen' in liquid nitrogen and stored at −70°C until tissue assays were performed. The heads were then removed and defleshed by dissection followed by soaking in 1% NaOH. During this stage the mandibular and maxillary portions of the skulls became dissociated. The skulls and detached mandibles were then thoroughly washed in saline and dried at 40°C. Bone loss around the left side and right side upper molars was assessed after staining the maxillary portion of the skull with 1% aqueous methylene blue to identify the cemento–enamel junction and alveolar bone crest of the maxillary molar teeth.

Imaging

For imaging purposes, the skulls were mounted on a rotatable and lockable stage. This allowed all specimens to be loaded and positioned identically so that proper within-group and between-group comparative analysis could be carried out between the specimens. Bone loss analysis was conducted on the buccal surface of all three upper molars on the left side and right side in a method similar to that described by Alayan *et al.* (18). The buccal surfaces of all the upper molar teeth were digitally imaged using a Leica MZ16FA stereo microscope (Leica Microsystems, Wetzlar, Germany) and 32× magnification. The captured images were then further analysed using ImageJ® software (US National Institutes of Health, Bethesda, MD, USA) to calculate the exposed buccal surface area from the cemento–enamel junction to the alveolar bone crest and bound by the mesiobuccal and distobuccal line angles for each of the three upper molars. This area was calculated for the left and right sides and compared between the different experimental groups. It should be noted that this area measurement does not give a strict quantitative assessment of bone loss, as it does not take into account the area occupied by the oral soft tissues i.e. connective tissue and epithelium between the cemento–enamel junction and the alveolar crest (an area commonly referred to as the 'biologic width'). However, increases in the area between the cemento–enamel junction and alveolar crest between groups killed at the same age and time-point were taken to represent loss of crestal alveolar bone. Morphometric analysis of this nature has been shown to be an accurate and effective method of measuring alveolar bone loss in the mouse model (19). In addition to measurements made by an investigator who was not blinded (AB), bone loss calculations were repeated on all specimens from each group by an independent, blinded observer who had no knowledge of the identity of the experimental groups.

Tissue fatty acid analysis

Oral tissue samples were defrosted in 3 mL of chloroform : methanol (2:1, v/v) containing 200 μL of internal standard (C23:0; Sigma Chemicals, St Louis, MO, USA) and butylhydroxytoluene (15 mg/mL), and gently homogenized. A 150 μL aliquot of the homogenate was mixed with 2 mL of methanol toluene (4:1, v/v) in a glass methylation tube, after which 200 μL of acetyl chloride was slowly added with continuous mixing. The tubes were sealed immediately then heated for 1 h at 100°C. After cooling in ice water, 3 mL of K_2CO_3 (10%) was added with vortexing, then the tubes were centrifuged (5 min, 1000 *g*, 4°C) and 300 μL of the upper toluene phase was transferred to a gas chromatograph vial. A 1 μL aliquot was injected onto a Shimadzu Gas Chromatograph (GC) 20A BPX70 capillary column with a split ratio of 5:1 (Shimadzu, Melbourne, Australia). The GC data were then analysed according to the GC software package #211. Data were compared by peak area with the peak area of the internal standard.

Statistics

Data were assessed for outliers using *Z*-score analysis. There were no significant outliers in the raw data set. The data were also assessed for

normality and were neither significantly skewed or kurtosed. Alveolar bone loss was modelled using a generalized linear mixed model run in SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA) including the fixed effects of diet, inoculant, tooth, side and the interaction of diet by inoculation. Rat identification was fitted as a random effect in the model. *Post hoc* *t*-tests were conducted on all class variables and adjusted for multiple testing. Correlations between tissue fatty acid concentrations and bone loss were determined by linear regression analysis. The level of statistical significance was set at $p < 0.05$.

Results

Alveolar bone loss

Figure 1 shows the mean area per tooth between the cemento–enamel junction and the alveolar crest and bounded by the mesial and distal line angles, in groups receiving different combinations of diet and inoculant. This area represents alveolar bone loss. No significant difference was found between estimates of bone loss obtained by the two independent observers.

Mice infected with either *P. gingivalis* alone or with a combination of *P. gingivalis* and *F. nucleatum* and fed a Sunola oil diet demonstrated a significantly larger area between the cemento–enamel junction and alveolar crest, indicative of greater bone loss in this group ($p < 0.001$), than mice inoculated with carboxymethylcellulose only (sham inoculations) or not inoculated. In both the *P. gingivalis* and combined *P. gingivalis*/*F. nucleatum* inoculation groups, mice fed a diet of tuna oil demonstrated significantly less bone loss (72% and 54% respectively) than animals which received the same inoculation program but were fed the Sunola oil diet. Mice fed the tuna oil diet and then inoculated with *P. gingivalis* showed only a slightly greater area of bone loss than control mice inoculated with carboxymethyl cellulose, with these bone loss differences not reaching statistical significance.

Representative photographs of the defleshed jaws illustrating the effects of treatment are shown in Fig. 2.

The effect of diet on oral soft tissue omega-3 polyunsaturated fatty acid content

Table 1 shows the concentrations of selected polyunsaturated fatty acids in the oral tissues of mice on the control (Sunola) and experimental (tuna oil) diets and which had received either the single or combined inoculations. After 57 d there were marked differences in polyunsaturated fatty acid contents of the intra-oral soft tissues of mice fed tuna oil compared with those fed Sunola oil. The eicosapentaenoic acid levels rose 10-fold and the docosahexaenoic acid levels were doubled, whereas the levels of linoleic acid, an n-6 polyunsaturated fatty acid, were halved. The type of bacterial inoculation received (*P. gingivalis* alone or *P. gingivalis*/*F. nucleatum*) had no significant effect on tissue fatty acid levels.

The extent of bone loss observed with each inoculum was highly correlated inversely with the tissue contents of both eicosapentaenoic acid and docosahexaenoic acid. In mice treated with *P. gingivalis* alone, the correlation coefficients were −0.73 and −0.75 for eicosapentaenoic acid and docosahexaenoic acid respectively. In mice treated with the combination of *P. gingivalis* and *F. nucleatum*, the corresponding values were −0.77 and −0.78.

Discussion

The results indicate that administration of a diet high in tuna oil for 8 wk to mice with experimental periodontitis induced by *P. gingivalis* can reduce alveolar bone loss by up to 72%. Even in mice infected with a combination of *P. gingivalis* and *F. nucleatum*, which caused a greater degree of bone loss, there was still a 54% reduction in bone loss in mice fed the tuna oil diet. In absolute terms, the mean area of bone loss prevented by feeding tuna oil was almost identical in mice receiving the combined inoculation as in those infected with *P. gingivalis* alone. These findings are comparable to those of Kesavalu *et al.* (16), who reported that a fish oil diet inhibited alveolar bone resorption in a similar model of experimental periodontitis in rats.

In contrast with the results of the current study and those published by Kesavalu *et al.* (20), Vardar-Sengul *et al.* (15), using an *Escherichia coli* endotoxin-induced periodontal abscess model, found that alveolar bone loss was greater in rats fed fish oil compared with control animals. However, the differences were small and not statistically significant. This lack of effect may be partially explained by their n-3 polyunsaturated fatty acid dosage, which was only a small frac-

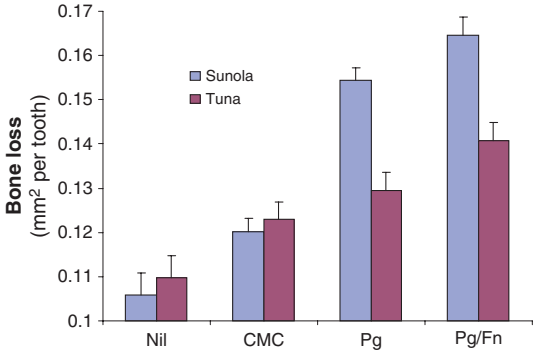

Fig. 1. Alveolar bone loss, represented by mean area per tooth (mm^2) between the alveolar crest, cemento–enamel junction and medial and distal line angles, in mice fed Sunola oil or tuna oil diets. Data are shown as mean ± standard error of the mean; $n = 10$ except for groups receiving no inoculations ($n = 5$). CMC, carboxymethyl cellulose; Nil, no inoculation; Pg, *Porphyromonas gingivalis*; Pg/Fn, *Porphyromonas gingivalis* + *Fusobacterium nucleatum*.

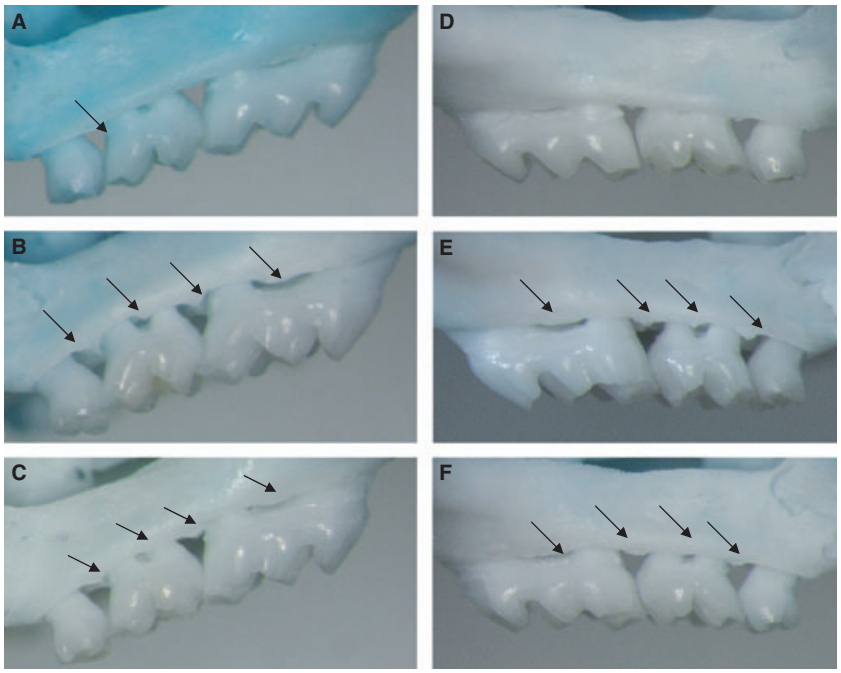

Fig. 2. Representative photographs of the defleshed jaws. (A) Sunola oil diet and carboxymethyl cellulose inoculations; (B) Sunola oil diet and *Porphyromonas gingivalis* inoculations; (C) tuna oil diet and *P. gingivalis* inoculations; (D) Sunola oil diet and carboxymethyl cellulose inoculations; (E) Sunola oil diet and *P. gingivalis* + *Fusobacterium nucleatum* inoculations; and (F) tuna oil diet and *P. gingivalis* + *F. nucleatum* inoculations. Arrows indicates sites of observable bone loss. There is less bone loss evident in (C) than (B) and in (F) than (E).

Table 1. Polyunsaturated fatty acid concentrations (mg/100 g) in oral soft tissues of mice fed Sunola oil or tuna oil diets and inoculated with *Porphyromonas gingivalis* (*Pg*) or *Fusobacterium nucleatum* + *P. gingivalis* (*Pg*/*Fn*)

Fatty acids	Sunola + *Pg*	Tuna + *Pg*	Sunola + *Pg*/*Fn*	Tuna + *Pg*/*Fn*
LA (18:2 n-6)	310 ± 12	148 ± 12*	216 ± 12	116 ± 12*
EPA (20:5 n-3)	2 ± 1	19 ± 1*	2 ± 1	16 ± 1*
DHA (22:6 n-3)	336 ± 16	621 ± 16*	333 ± 16	536 ± 16*

Data are mean ± standard error of the mean, $n = 10$ in all cases.
*significant differences between the effects of Sunola oil and tuna oil diets on polyunsaturated fatty acid concentrations ($p < 0.001$). There were no significant differences between the effects of the inoculants.
DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid.

tion of the 10% tuna oil added to the rodent feed in the current study and that used by Kesavalu and co-workers (20). In other rodent studies, dietary concentrations in the range of 5–25% (w/w) fish oil are commonly used (21,22). The pathophysiology of bone loss in the endotoxin-induced periodontal abscess model used by Vardar-Sengul *et al.* (15) also differs from that in our experimental periodontitis model in which mice were gavaged with whole live bacteria. In the abscess model, lipopolysaccharide derived from *E. coli*, an organism not considered to be a major periodontal pathogen, was used.

Eicosapentaenoic acid and docosahexaenoic acid can be mobilized within tissue exudates and converted to different classes of bioactive compounds that act as local regulators of inflammation. The E-series resolvins derived from eicosapentaenoic acid were the first to be identified from self-limiting and resolving murine exudates (24). Among them, resolvin E1 has been demonstrated to promote resolution of inflammation by hindering the recruitment and migration of white blood cells. Topical application of resolvin E1 in rabbit periodontitis has been shown to confer dramatic and almost complete protection against bone loss in experimentally induced periodontitis in rabbits (14).

Subsequent to the discovery of E-series resolvins, D-series resolvins and docosatrienes (termed protectins) derived from docosahexaenoic acid were described. D-series resolvins have been shown to block tumor necrosis factor-α-induced interleukin-1β transcripts in microglial cells (24) and to act as regulators, limiting the infiltration of neutrophils into inflamed tissues, including those of experimental periodontitis (25,26). The protectin, PD1, also seems to have an additive effect in concert with resolvin E1 and to reduce the amount of polymorphonuclear infiltrate early after the initiation of inflammation (27,28).

Previous studies investigating the potential periodontal therapeutic effects of n-3 polyunsaturated fatty acids have evaluated fish oils that are high in eicosapentaenoic acid but relatively low in docosahexaenoic acid. Given recent evidence highlighting the anti-inflammatory effects of docosahexaenoic acid, particularly with regard to decreasing prostaglandin E_2 production (23), and the importance of docosahexaenoic acid-derived resolvins and protectins in the resolution of inflammation (5), a tuna oil diet with a high docosahexaenoic acid : eicosapentaenoic acid ratio was used in the present study.

The results show a significant reduction in alveolar bone loss in mice fed a diet high in docosahexaenoic acid. Further investigation into the anti-inflammatory effects of high docosahexaenoic acid : eicosapentaenoic acid ratio fish oil supplementation in the periodontal field is warranted, particularly given the emerging evidence demonstrating the effect of its metabolites in triggering the resolution of inflammation.

The recognition that periodontal tissue breakdown is primarily caused by the host response to certain bacteria has created interest in treatments to modulate this response as an adjunct to conventional periodontal therapy.

Fish oil has many potential advantages for use as an adjunct for host modulation therapy. It is widely available for purchase 'over the counter', has potential collateral health benefits and is relatively inexpensive. In contrast to other agents that have been proposed as possible candidates for systemic use in host modulation of inflammatory conditions, large-scale long-term data suggest that n-3 polyunsaturated fatty acid supplementation is essentially free of side effects.

In conclusion, dietary supplementation with docosahexaenoic acid-rich tuna oil caused a marked elevation of the n-3 polyunsaturated fatty acid levels in oral soft tissues. This diet significantly reduced alveolar bone loss in a murine periodontitis model. Given recent advances in our understanding of the way in which n-3 fatty acids may exert their anti-inflammatory effects, further human research to evaluate the possible benefits of their use in patients with periodontitis is warranted.

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