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Omega-3 fatty acid regulates inflammatory cytokine/mediator messenger RNA expression in *Porphyromonas gingivalis*-induced experimental periodontal disease

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Introduction: *Porphyromonas gingivalis* is strongly implicated in the etiology of adult periodontitis by inducing inflammatory cytokines, resulting in gingival and periodontal tissue inflammation and alveolar bone resorption. This study tested the hypothesis that supplementing the diet with omega-3 fatty acid (ω-3 FA; i.e. fish oil) would exert anti-inflammatory effects in the gingival tissues of *P. gingivalis*-infected rats.
Methods: Rats were fed either fish oil or corn oil diets *ad libitum* for 22 weeks and infected with *P. gingivalis* strain 381 or strain A7A1-28. After sacrifice, rat gingival tissues were excised and the RNA was isolated and analyzed for proinflammatory mediators [interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), IL-6], T helper type 1 and type 2 cytokines [interferon-γ (IFN-γ), IL-4, IL-10), antioxidant enzymes [catalase (CAT), superoxide dismutase (SOD)], and genes critical for eicosanoid mediator production [cyclo-oxygenase-2 (COX-2), 5-lipoxygenase (5-LO)] by reverse transcription-polymerase chain reaction using rat-specific primers.
Results: Rats on the ω-3 FA diet exhibited decreased proinflammatory cytokine gene expression (IL-1β, TNF-α) and enhanced IFN-γ, CAT and SOD messenger RNA expression compared to rats fed a corn oil diet, supporting a diet-induced modulation of host inflammatory reactions. Analyses of alveolar bone resorption in the rats related to gene expression profiles demonstrated significant positive correlations with IL-1β, IL-6 and COX-2 and negative correlations with CAT and SOD.
Conclusion: These findings suggest that diets enriched for ω-3 FA modulate the local gingival inflammatory milieu of the host following oral *P. gingivalis* infection, which impacts on alveolar bone resorption in rats.

Key words: gene expression; inflammatory cytokine; omega-3 fatty acid; periodontal disease; *Porphyromonas gingivalis*; rat model; reverse transcription-polymerase chain reaction

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Periodontitis is a chronic immunoinflammatory disease with progressive loss of attachment of gingival tissues, reflecting destruction of the periodontal ligament and adjacent supporting alveolar bone. The chronic inflammation is initiated by complex pathogenic subgingival biofilms containing several likely periodontal pathogens, including *Porphyromonas*

gingivalis. A gram-negative anaerobic bacterium that is a commensal opportunistic pathogen of the oral cavity, *P. gingivalis* expresses numerous potential virulence determinants (23).

Interleukin-1β (IL-1β), IL-6 and tumor necrosis factor-α (TNF-α) levels have consistently been reported to be elevated in gingival crevicular fluid and tissues in periodontitis patients (5, 15, 19) and the levels were frequently reduced following periodontal therapy (12). Interferon-γ (IFN-γ), a T helper type 1 cytokine, has routinely been documented as elevated in gingival tissues (21), while IL-4, a T helper type 2 cytokine, was generally low in diseased periodontal tissue (18). IL-10, also a T helper type 2 cytokine, has been evaluated as an anti-inflammatory cytokine that may minimize alveolar bone resorption (44), although IL-10 messenger RNA (mRNA) expression has been found to be increased in the gingival tissues of adult periodontitis patients (50). Cyclo-oxygenase-2 (COX-2) is the rate-limiting enzyme that regulates the synthesis of prostaglandins, thromboxanes and prostacyclins from arachidonic acid and is elevated in inflamed gingiva from patients with chronic periodontitis (36, 51) and other chronic inflammatory diseases. The overexpression of COX-2 and selected matrix metalloproteinases in gingival tissues suggested a possible role in experimental periodontitis in rats (24). The 5-lipoxygenase (5-LO) enzyme is crucial for the biosynthesis of inflammatory leukotrienes. Gingival crevicular fluid samples from patients with localized aggressive periodontitis contained 5-LO-derived products, leukotriene B_4, and the biosynthesis interaction product, lipoxin LXA_4 (28).

Reactive oxygen species are produced under physiological and pathological conditions and their over-production occurs at sites of chronic inflammation, including periodontitis. However, the endogenous enzymatic antioxidant defense mechanisms involving catalase (CAT) and superoxide dismutase (SOD) remove reactive oxygen species products, inhibiting their deleterious effects. Total antioxidant capacity was significantly lower in saliva, gingival crevicular fluid and plasma in patients with periodontitis (6, 11); although both CAT and SOD levels were significantly higher in the gingival tissues of periodontitis patients (37) and scaling and root planing with subgingival application of liposome-encapsulated SOD suppressed periodontal inflammation (38).

Targeted modulation of the cytokine network in gingival tissues would be predicted to provide a therapeutic potential for inflammatory periodontal disease. There are three types of fatty acids: saturated, monosaturated, and polyunsaturated. Omega-3 fatty acids fall into the category of polyunsaturated, they include α-linolenic acid, eicosapentaenoic acid and docosahexaenoic acid. Fish oils are rich dietary sources of the omega-3 polyunsaturated fatty acids (ω-3 PUFA) eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3). Fish oil has effects on diverse physiological processes via its distribution to virtually every cell in the body, thus impacting cell functions, cell communication, production of various biomolecules and antioxidant activities (1, 10, 17). Recent studies have examined ω-3 FA effects on the chronic inflammatory diseases such as cardiovascular disease, rheumatoid arthritis, asthma, Alzheimer's disease and Crohn's disease, including periodontal disease; treatment with various host inflammatory modulators has shown some positive effect on the disease process (4, 10, 17). Treatment with fish oil significantly reduced osteoclast levels and osteoclastic activity linked to bone resorption in rats (25, 26). Campan et al. (8, 9) reported a study of human experimental gingivitis treated with n-3 PUFA (i.e. ω-3 FA). The topical application of n-3 or n-6 fatty acids failed to inhibit the development of experimental gingivitis (14) and n-6 PUFA levels in the serum were higher in periodontitis patients, suggesting that an imbalance between n-6 and n-3 PUFA may increase susceptibility to alveolar bone resorption (42). Another study reported that borage oil supplementation, as a source of n-6 PUFA, had beneficial effects on periodontal inflammation (probing depth) compared with either fish oil as a source of n-3 PUFA or the combination of lower doses of both borage and fish oil supplementation in patients with periodontitis (43). A more recent study observed that topical application of resolvin, RvE1, a new family of bioactive products of ω-3 FA, conferred protection against local inflammation and osteoclast-mediated bone destruction in rabbit periodontitis (22). However, the molecular mechanism(s) supporting an anti-inflammatory role for ω-3 PUFA in experimental periodontal disease have not been documented.

The hypothesis of this study was that dietary supplementation with fish oil would alter the gingival tissue cytokine gene expression profiles of inflammatory mediators consistent with decreased alveolar bone resorption.

Materials and methods

Bacterial strains

P. gingivalis 381 and A7A1-28 (ATCC 53977) strains were used in this study (30). Previous studies have clearly documented the differences in virulence of *P. gingivalis* strains, evaluated by soft tissue abscesses and periodontal bone loss in mice and rats (29). *P. gingivalis* A7A1-28 was associated with more bone loss than strain 381 when comparing horizontal bone loss in the maxilla, while strain 381 was associated with more bone loss than strain A7A1-28 when assessing vertical intrabony defects in the mandible (16). *P. gingivalis* strains were grown anaerobically (85% N_2, 10% H_2 and 5% CO_2) on blood agar plates (Remel, Lenexa, KS) for 3 days at 37°C. The bacteria were scraped from the agar surface using sterile cotton swabs soaked in reduced transport fluid, suspended in the reduced transport fluid, and enumerated with a Petroff–Hausser bacterial counting chamber as described previously (30). All bacterial manipulations were carried out under anaerobic conditions to ensure maximum cell viability. *P. gingivalis*, at 2×10^{10} cells/ml, was mixed with equal amounts of sterile 2% carboxymethylcellulose (Sigma Chemical Co., St Louis, MO) and used to infect rats by oral gavage (1×10^{10} cells/rat) within 15 min of removal from the anaerobic chamber.

Animals and diets

Female Sprague-Dawley rats, 8–9 weeks old (weighing 175–200 g; Harlan, Indianapolis, IN) were housed under a 12 h : 12-h light : dark cycle. Rats were maintained in groups under microisolator conditions and provided with normal pellet chow (Harlan Tecklad) as well as water *ad libitum* during 1 week of acclimation. All procedures involving rats were performed in accordance with the approved guidelines set forth by the Institutional Animal Care and Use Committee at the University of Kentucky. The fish oil and corn oil diets were prepared according to the recommendation of the American Institute of Nutrition diet AIN-76A (40) (Dyets Inc., Bethlehem, PA), with the addition of 17% menhaden (fish) oil + 3% corn oil (the fish oil diet) or 5% corn oil only (the corn oil diet). Aliquots of the fish oil and corn oil diets were analyzed for their fatty acid composition by Dyets Inc. (Table 1).

Both diets were received in large quantity, repacked into airtight packets (each packet for 1 day/group) and stored at −20°C. Both diets were supplemented with equal amounts of vitamin E to minimize peroxidative damage during storage. Following acclimation, rats were randomized into fish oil and corn oil diet groups and were provided with these diets for the duration of the experiment (22 weeks). The study groups were: *P. gingivalis* 381 fish oil diet ($n = 21$); *P. gingivalis* 381 corn oil diet ($n = 20$); *P. gingivalis* A7A1-28 fish oil diet ($n = 21$); and *P. gingivalis* A7A1-28 corn oil diet ($n = 20$). Diets remaining from the previous day were discarded. Food consumption was monitored and all rats were weighed weekly until termination of the protocol.

P. gingivalis oral infection

All rats were given kanamycin (20 mg) and ampicillin (20 mg) daily for 4 days in their drinking water to reduce the oral microflora (31); this was followed by oral gavages with approximately 10^{10} *P. gingivalis* cells for five consecutive days on three alternate weeks during the study period. Oral microbial samples were collected using sterile cotton swabs of all molar and premolar teeth from isofluorane-anesthetized rats. *P. gingivalis* colonization/infection was demonstrated in all rats during the 12-week infection by polymerase chain reaction analysis using *P. gingivalis*-specific primers (31). Blood was collected by retro-orbital access (before infection) and from the heart under anesthesia after 12 weeks of *P. gingivalis* infection (termination of the study period). The sera were used for measurement of fatty acid composition and *P. gingivalis*-specific immunoglobulin G antibody (31). Fatty acid analysis of the serum showed that rats fed diets supplemented with fish oil had significantly elevated eicosapentaenoic acid and docosahexaenoic acid levels, as well as n-6 : n-3 ratios that were significantly decreased and consistent with an elevated peroxidizability index (data not shown) (31). Rats were sacrificed and gingival tissue was excised, frozen in liquid nitrogen and stored at −80°C until RNA isolation. The skulls were removed, autoclaved for 1 h at 121°C and defleshed. Maxillae and mandibles were hemisected and trimmed for the evaluation of periodontal disease by radiographic analysis of alveolar bone resorption (31).

Table 1. Fatty acid composition of the diets

	Diet	
Fatty acid wt%	Corn oil	Fish oil
14:0 (myristic)	TR	9.0
16:0 (palmitic)	10.8	17.1
16:1 (palmitoleic)	TR	12.5
16.2	ND	1.7
16.3	ND	1.7
16.4	ND	1.8
17:0 (margaric)	ND	0.9
18:0 (stearic)	2.1	2.8
18:1 (oleic)	26.5	11.4
18:2n-6 (linoleic)	60.0	1.5
18:3 linolenic	0.6	1.6
18:4	ND	3.5
20:1 (gondoic)	ND	1.6
20:4n-6	ND	2.3
20:5n-3 (eicosapentanoic)	ND	15.5
21:5	ND	0.8
22:1	ND	0.5
22:5n-3 (docosapentanoic)	ND	2.4
22:6n-3 (docosahexanoic)	ND	9.1
Unknown	ND	1.3

Fatty acid analyses were obtained from Dyet's, Inc., Bethlehem, PA. Values are percentages of total fatty acids. Only the most common fatty acids are listed. Minor fatty acids are not listed. TR, trace; ND, not detected.

Radiographic assessment of alveolar bone resorption

The hemisected rat maxillae and mandibles were trimmed and radiographs were taken of each jaw. The crestal alveolar bone resorption for each surface was defined as the distance along the tooth surface from the cemento–enamel junction to the alveolar bone crest. The summation of alveolar bone resorption, also in mm, was tabulated and analyzed for intra- and inter-group comparison (31, 41).

RNA isolation from rat gingival tissue

Total RNA was isolated from the frozen rat gingiva from the fish oil and corn oil diet groups with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols. Quantification of RNA yield was performed by spectrophotometer analysis and the absorbance was evaluated at 260 nm and 280 nm for determination of sample concentration and purity (260 : 280 was always > 1.6). RNA clean-up was carried out with a Qiagen RNeasy mini kit (Qiagen, Valencia, CA). RNA degradation was assessed by visualization of 20 μg total RNA using 1.2% denaturing agarose gels. An ethidium bromide-stained gel was used to verify RNA integrity and loading equivalency. The rat gingival tissue RNA samples were stored at −80°C until gene expression analyses were conducted by reverse transcription-polymerase chain reaction.

Gene expression analyses

Reverse transcription-polymerase chain reaction was performed as previously described (39). Briefly, 1 μg RNA was reverse transcribed into complementary DNA with oligo-dT_{15} and reverse transcriptase (Reverse Transcription System; Promega Corp., Madison, WI). Two microliters complementary DNA were then subjected to enzymatic polymerase chain reaction amplification of selected genes with rat-specific oligonucleotide primers and *Taq*DNA polymerase (Invitrogen Corp., Carlsbad, CA) in a DNA thermal cycler (Fisher Scientific, Pittsburgh, PA). Using the PRIMER EXPRESS Program, rat-specific primers for cytokine/mediators were designed for the reverse transcription-polymerase chain reaction. The rat-specific primers used in cytokine/mediator mRNA expression are shown in Table 2. As an internal control for RNA quantity, the same complementary DNA was amplified using primers specific for rat glyceraldehyde-3-phosphate dehydrogenase mRNA. The amplified polymerase chain reaction products were subjected to 1.5% agarose gel electrophoresis and visualized by ultraviolet fluorescence after staining with ethidium bromide. Densitometry analysis of bands was performed using an ALPHAIMAGER Imaging system (Alpha Innotech Corporation, San Leandro, CA) and normalized with glyceraldehyde-3-phosphate dehydrogenase.

Statistical analyses

Descriptive evaluation of the data is presented as mean ± SD. Statistical analyses of the data were conducted using Student's *t*-test following determination of normal distribution of values. Correlations were determined using the Spearman rank sum analysis with $P < 0.05$ considered statistically significant. All statistical analyses were performed using SIGMASTAT 3.0 software (SYSTAT, Chicago, IL).

Results

General observation

The body weights of the rats fed the fish oil diet were significantly higher ($P < 0.05$) compared to those of the corn oil diet rats (data not shown).

Effects of ω-3 FA on IL-1β, TNF-α and IL-6

The effects of fish oil on proinflammatory cytokine, IL-1β, IL-6 and TNF-α, mRNA expression in the gingival tissues of

Table 2. Rat Oligonucleotide primers used for reverese transcription–polymerase chain reaction

Molecules	Primer sequence	Strand	Size	Annealing temp (°C)
IL-1β	ACA,AGC,AAC,GAC,AAA,ATC,CC	+	417	58
	AGA,CCT,GAC,TTG,GCA,GAG,GA	−		
TNF-α	TGC,CTC,AGC,CTC,TTC,TCA,TT	+	108	60
	CCC,ATT,TGG,GAA,CTT,CTC,CT	−		
IL-6	CCG,GAG,AGG,AGA,CTT,CAC,AG	+	428	60
	GAG,CAT,TGG,AAG,TTG,GGG,TA	−		
IL-10	CCT,GCT,CTT,ACT,GGC,TGG,AG	+	315	58
	TCT,CCC,AGG,GAA,TTC,AAA,TG	−		
IFN-γ	AAA,GAC,AAC,CAG,GCC,ATC,AG	+	212	60
	CTT,TTC,CGC,TTC,CTT,AGG,CT	−		
IL-4	CCA,GGT,CAC,AGA,AAA,AGG,GA	+	269	60
	CAG,TGT,TGT,GAG,CGT,GGA,CT	−		
COX-2	ATC,CTG,AGT,GGG,ATG,ACG,AG	+	381	58
	CTG,CTT,GTA,CAG,CGA,TTG,GA	−		
CAT	GTG,GTT,TTC,ACC,GAC,GAG,AT	+	573	58
	CAA,GTT,TTT,GAT,GCC,CTG,GT	−		
SOD	CCT,AGA,CTG,ACG,CTT,CCC,AG	+	474	58
	GAA,AGA,AAA,CAA,AAG,GGG,GC	−		
GAPDH	GAC,TTT,GCC,TAC,AGC, CTT,GG	+	154	57
	GAT,CGT,GGA,AGG, GCT, AAT,GA	−		

Using the PRIMER EXPRESS Program, rat-specific primers for cytokine/mediators and antioxidant enzymes were designed for the reverse transcription-polymerase chain reaction.

P. gingivalis-infected rats are shown in Fig. 1(A). Infection with both *P. gingivalis* strains 381 and A7A1-28 induced IL-1β mRNA expression in gingival tissues of rats fed the corn oil diet, while the fish oil diet decreased IL-1β gene expression induced by *P. gingivalis* 381 ($P < 0.05$). IL-6 mRNA expression in gingival tissues of rats infected with *P. gingivalis* 381 was significantly greater than levels induced following *P. gingivalis* A7A1-28 infection, although there was no apparent effect of diet (Fig. 1A). Gingival tissues of rats on the corn oil diet and infected with *P. gingivalis* strains 381 or A7A1-28 demonstrated TNF-α mRNA expression that was decreased by fish oil diet supplementation (Fig. 1A).

Effects of ω-3 FA on T helper type 1 and 2 cytokines IFN-γ, IL-10 and IL-4

IFN-γ mRNA expression in gingival tissues has been suggested to be critical for the generation of effective immune responses and for the modulation of inflammatory cytokines (21). Rats fed the fish oil diet demonstrated increased IFN-γ gene expression after infection with *P. gingivalis* 381 compared to the corn oil diet group ($P < 0.05$) (Fig. 1B). Also of interest was the highly significant difference in IFN-γ gene expression, irrespective of diet, when comparing infection with *P. gingivalis* strain 381 to strain A7A1-28 infection ($P < 0.001$). Similar levels of IL-10 mRNA were observed in gingival tissues after infection with both *P. gingivalis* strains, with no diet effect (Fig. 1B). IL-4, a T helper type 2 cytokine, was detected in the gingival tissues of all rats. Following infection, rats fed the fish oil diet exhibited higher levels of mRNA for IL-4 compared to the corn oil diet regardless of which strain was used for the infection. As observed with the IFN-γ, rats infected with *P. gingivalis* 381 demonstrated significantly higher levels of IL-4 mRNA expression ($P < 0.001$) than those infected with *P. gingivalis* A7A1-28 (Fig. 1B).

Effect of ω-3 FA on eicosanoid mediators COX-2 and 5-LO

We evaluated COX-2 and 5-LO enzyme mRNA expression in the gingiva as markers of the modulation of eicosanoid inflammatory pathways following *P. gingivalis* infection. Figure 1(C) shows that COX-2 expression was significantly elevated in gingiva from corn-oil-fed rats, after infection with both strains of *P. gingivalis*. In contrast, 5-LO mRNA expression was increased in the fish-oil-fed rats after both strains of *P. gingivalis* infection. As was observed with IFN-γ and IL-4 cytokine gene expression, *P. gingivalis* 381 elicited significantly greater levels ($P < 0.005$) of mRNA for both COX-2 and 5-LO enzymes when compared to infection with *P. gingivalis* A7A1-28.

Effect of ω-3 FA on antioxidant enzymes CAT and SOD

Gingival tissue CAT mRNA expression was increased in rats fed the fish oil diet, although the differences were not statistically significant (Fig. 1C). Similarly, expression of SOD mRNA was also increased in rats fed the fish oil diet and infected with both strains of *P. gingivalis*. In contrast to the results observed with the COX-2 and 5-LO responses, *P. gingivalis* strain A7A1-28 infection induced significantly elevated expression of CAT and SOD mRNA compared to *P. gingivalis* 381 ($P < 0.01$) (Fig. 1C).

Inflammatory cytokine/mediator mRNA expression and alveolar bone resorption

Based upon the variations in expression of several cytokine/mediator genes in gingival and periodontal tissues between the infecting strains and the influence of diet, we evaluated the relationship of the expression of inflammatory host responses with alveolar bone resorption in the rats (Fig. 2A–C). *P. gingivalis*-infected rats fed a fish oil diet enriched in ω-3 FA demonstrated significantly ($P < 0.01$ and 0.02) decreased alveolar bone resorption around both the mandibular and the maxillary teeth compared to the rats fed a corn oil diet. Table 3 provides a summary of correlation analyses for the different cytokine/mediator genes, categorized by the infecting *P. gingivalis* strain (381, A7A1-28) and diet (fish oil, corn oil). The results demonstrated positive correlations of IL-1β, TNF-α, IL-6 and COX-2 with the amount of alveolar bone resorption, with IL-1β being consistently significantly correlated under all test parameters. In contrast, generally SOD, CAT, 5-LO and IL-10 mRNA levels were negatively correlated with alveolar bone resorption, with CAT and SOD demonstrating significant relationships, particularly in the rats fed the fish oil diet (Table 3).

Discussion

The anti-inflammatory effects of ω-3 FA have been widely studied in several chronic inflammatory diseases and cancer in both human subjects and laboratory animals. Although there are several reports on the effects of fish oil on inflammatory cytokines and eicosanoid mediators, this is the first study on the periodontium in experimental periodontal disease. The current study was designed to document the characteristics of cytokine/mediator mRNA expression by gingival tissues following *P. gingivalis* oral infection. The results demonstrated for the first time that a fish-oil-enriched diet containing ω-3 FA compared to a corn oil diet significantly

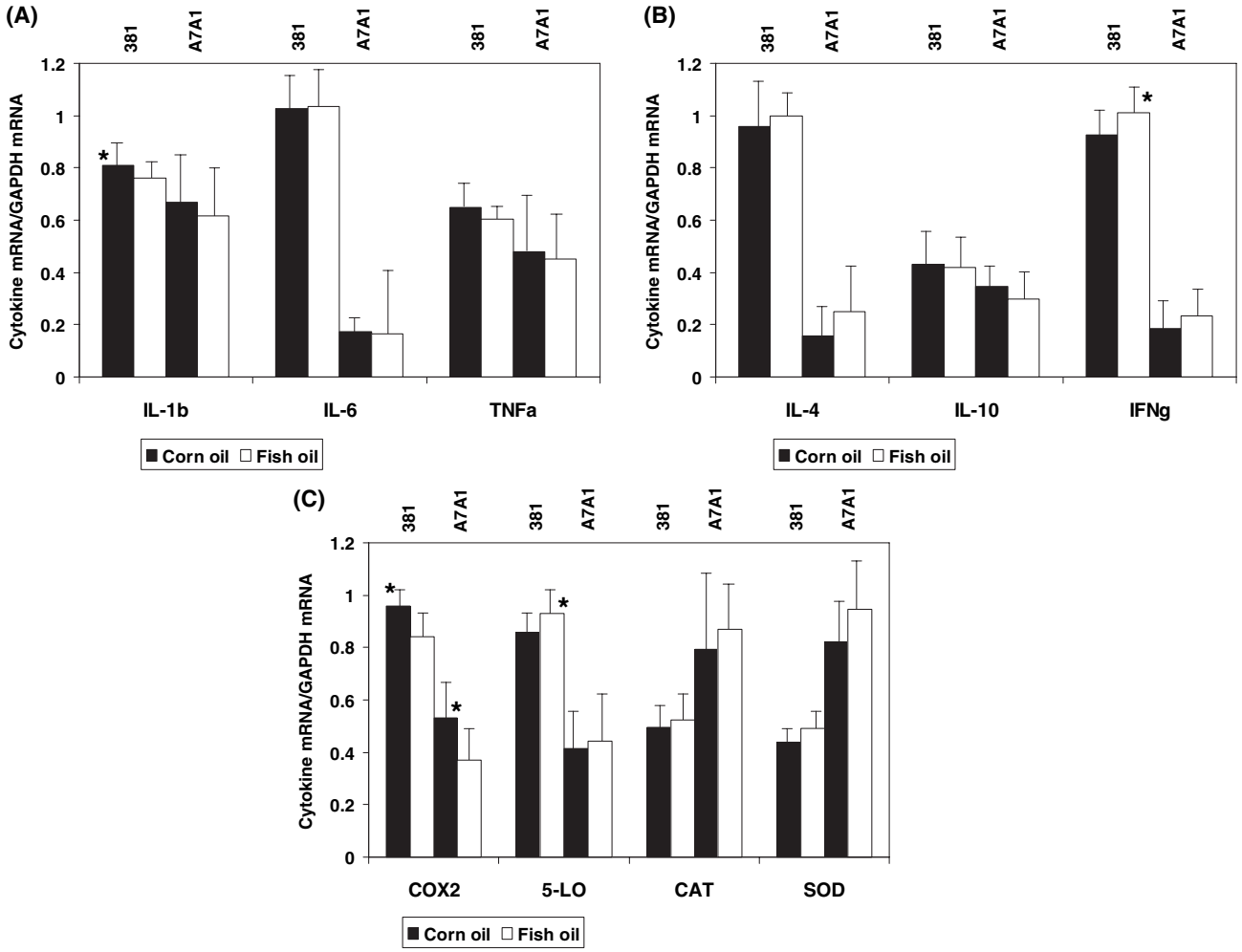

Fig. 1. (A) Fish oil decreases pro-inflammatory cytokines IL-1β and TNF-α gene expression. Expression of gingival tissue mRNA for pro-inflammatory cytokines following oral infection with *P. gingivalis* strains 381 or A7A1-28 (A7A1). Groups of rats were fed a diet supplemented with fish oil or corn oil (CO). The bars denote the mean mRNA expression and the vertical line indicates one SD in 20 rats per group. The asterisk denotes a significant difference ($P < 0.05$) between the corn oil and fish oil diets with each strain of *P. gingivalis*. (B) Fish oil increased IL-4 and IFN-γ gene expression. Expression levels of gingival tissue mRNA for T helper types 1 and 2 cytokines following oral infection with *P. gingivalis* strains 381 or A7A1-28 (A7A1). Groups of rats were fed a diet supplemented with fish oil or corn oil (CO). The bars denote the cytokine mean mRNA expression and vertical lines indicate one SD in 20 rats per group. The asterisks denote significant differences ($P < 0.05$) between corn oil and fish oil diets with each strain of *P. gingivalis*. (C) Fish oil modulation of eicosanoid (COX-2, 5-LO) mediators and antioxidant enzyme (CAT, SOD) mRNA expression. Expression levels of gingival tissue mRNA for eicosanoid mediators (COX-2, 5-LO) and antioxidant enzymes (CAT, SOD) following oral infection with *P. gingivalis* strains 381 or A7A1-28 (A7A1). Groups of rats were fed a diet supplemented with fish oil or corn oil (CO). The bars denote the enzyme COX-2, 5-LO, CAT, and SOD mRNA mean gene expression and vertical lines indicate one SD in 20 rats per group. The asterisks denote significant differences ($P < 0.05$) between corn oil and fish oil diets with each strain of *P. gingivalis*.

modulated the inflammatory cytokine and antioxidant enzyme mRNA expression that accompanied decreased alveolar bone resorption in a rat model of periodontal disease. Kremer (33) observed a reduction of morning stiffness and tender joints in patients receiving dietary fish oil supplementation paralleled by lower serum levels of IL-1β. Similarly, a fish-oil-enriched diet prevented cachectic weight loss and reduced the production of prostaglandin E_2 and proinflammatory cytokines in mice following systemic administration of lipopolysaccharide (32) and eliminated IL-1β, IL-6 and TNF-α mRNA in kidneys from autoimmune lupus-prone NZB/NZW F_1 (B/W) female mice (10). In culture, eicosapentaenoic acid and docosahexaenoic acid inhibited the production of IL-1β, TNF-α and IL-6 (7). Our results with gingival tissues are consistent with the observations from other tissues demonstrating that IL-1β and TNF-α mRNA expression was decreased by feeding rats a diet supplemented with fish oil (10). In the present study, dietary supplementation with ω-3 FA decreased the expression of IL-1β and TNF-α mRNA in gingival tissues, suggesting that decreased proinflammatory cytokines would be reflected in decreased gingival clinical inflammation. Consistent with this potential is the report of Assuma et al. (2), who demonstrated that a combination of IL-1β and TNF-α antagonists blocked experimental alveolar bone resorption in non-human primates.

Using mice that lacked IFN-γ, Baker et al. (3) demonstrated decreased alveolar bone resorption following oral infection with *P. gingivalis* A7A1-28, suggesting that IFN-γ played a role in periodontal bone resorption. The present study observed an enhanced induction of IFN-γ in gingival tissues of rats fed a ω-3 FA diet

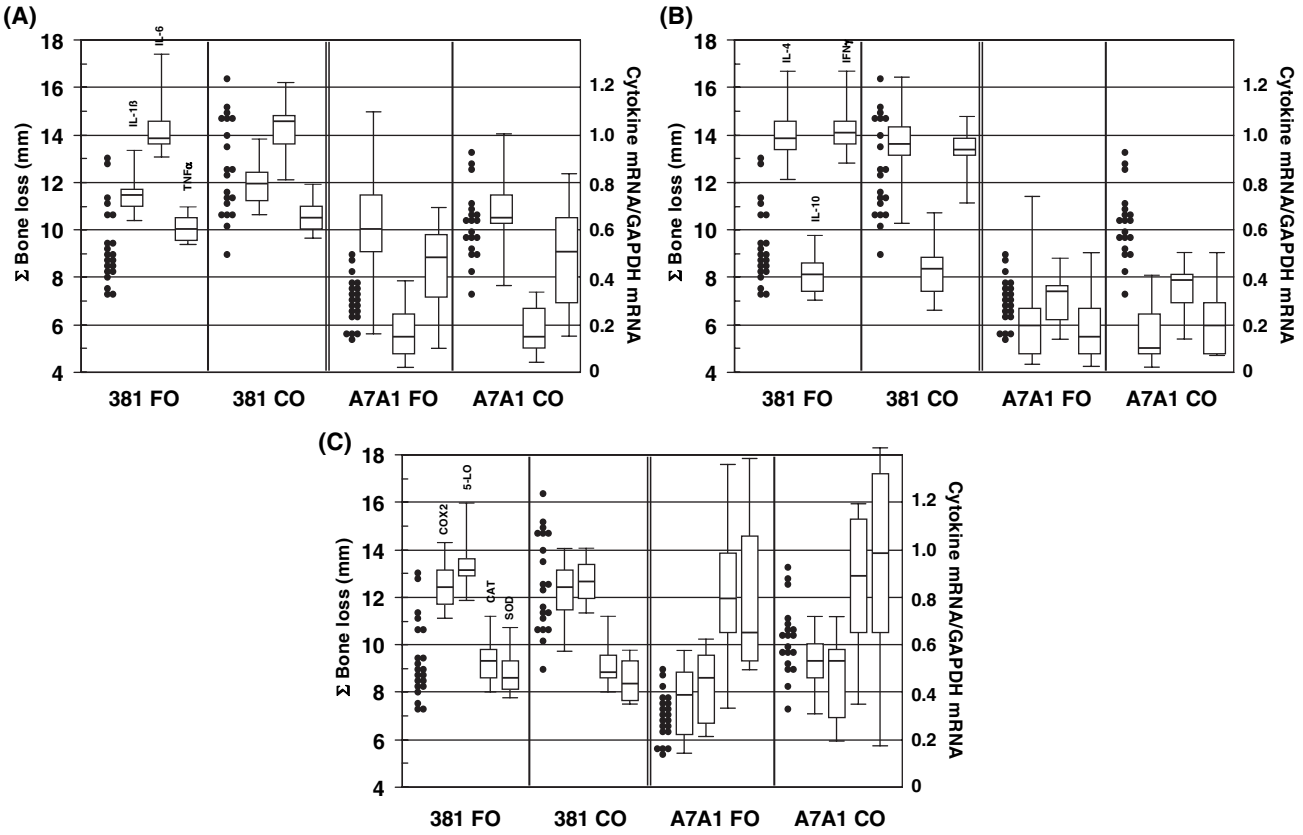

Fig. 2. Total alveolar bone loss levels (each filled circle denotes the mean per rat) related to different diets (FO, fish oil; CO, corn oil) and following infection with *P. gingivalis* strains 381 and A7A1-28. Rats fed a fish oil diet and infected with both *P. gingivalis* strains demonstrated significantly ($P < 0.01$ and $P < 0.02$) decreased alveolar bone resorption compared to the rats fed the corn-oil diet. (A) Relationship to gingival tissue mRNA expression levels of pro-inflammatory mediators (IL-1β, IL-6, TNF-α); (B) relationship to gingival tissue mRNA expression levels of T-cell cytokines (IL-4, IL-10, IFN-γ); and (C) relationship of gingival tissue mRNA expression levels of enzymes controlling lipid mediators (COX-2, 5-LO) and antioxidant genes (CAT, SOD). The boxes denote the limits of the first and third quartiles, the lines denote the group median, and the vertical brackets enclose the minimum and maximum values for each mediator.

Table 3. Correlations between gene expression levels for various cytokines, mediators, and antioxidant molecules and the extent of alveolar bone resorption in rats

	P. gingivalis 381				*P. gingivalis* A7A1-28			
Mediator	Fish oil	*P*	Corn oil	*P*	Fish oil	*P*	Corn oil	*P*
IL-1β	0.553	0.0093	0.727	0.0020	0.690	0.0005	0.532	0.0131
TNF-α	0.213		0.557	0.0087	0.495	0.0225	0.459	0.0363
IL-6	−0.341		0.662	0.0011	0.690	0.0005	0.439	0.0465
IL-10	0.107		−0.475	0.0296	−0.09		−0.297	
IFN-γ	−0.261		0.090		−0.196		0.371	
IL-4	0.149		0.505	0.0195	−0.100		−0.202	
COX-2	−0.331		0.512	0.0177	0.177		0.595	0.0044
5-LO	−0.379		−0.244		−0.287		−0.104	
CAT	−0.550	0.0098	−0.309		−0.628	0.0023	−0.158	
SOD	−0.551	0.0096	−0.083		−0.714	0.0003	−0.258	

and infected with both strains of *P. gingivalis*, suggesting that the fish oil diet up-regulated IFN-γ and decreased alveolar bone resorption (31).

The per cent of IL-10^{+} CD8 cells extracted from adult periodontitis lesions was decreased compared with healthy/gingivitis patients (20). A recent study also demonstrated that *P. gingivalis* infection induced significant periodontal bone resorption in IL-$10^{-/-}$ mice, compared to uninfected IL-$10^{-/-}$ mice or infected wild-type animals, indicating that IL-10 likely contributed to host responses in periodontal disease (44). Dietary fish oil also significantly modulated serum IL-6, IL-10, IL-12, TNF-α, prostaglandin E_2, thromboxane B_2 and leukotriene B_4 levels in autoimmune-prone MRL/lpr mice, a model for rheumatoid arthritis (48). We observed both IL-10 and IL-4 mRNA expression in gingival tissues in the infected rats, although there were no differences in expression between the diets or between the infecting *P. gingivalis* strains.

Arachidonic acid can be metabolized into eicosanoids via lipoxygenases and COX-1 and COX-2 enzymes. Several studies have also shown a relationship between proinflammatory cytokine and COX-2 mRNA and protein expression in inflamed gingiva from periodontitis patients (36, 51). Our observation that a diet enriched with ω-3 lipid-rich menhaden fish oil is capable of enhancing 5-LO mRNA expression and decreasing COX-2 in gingival tissues is consistent with the anti-inflammatory nature of this dietary supplement, and is accompanied by a decrease in IL-1β and TNF-α mRNA expression. Moreover, the decreased gingival inflammation could result from reduced levels of linoleic acid in the fish oil diet and/or the inhibitory effects of eicosapentaenoic acid and docosahexae-

noic acid on metabolic conversion of linoleic acid to arachidonic acid.

Reactive oxygen species are produced at sites of inflammation, primarily related to the normal host protective functions of polymorphonuclear leukocytes (11); however, in chronic inflammation they are deleterious to the wound healing process because of their harmful effects on cells and tissues (35). A study by Petelin et al. (38) evaluated the influence of the reactive oxygen species scavenger enzymes CAT and SOD on periodontal inflammation and demonstrated that scaling and root planing with subgingival application of liposome-encapsulated SOD suppressed gingival inflammation, reduced probing depth, and increased gingival attachment level in experimental periodontitis in beagle dogs. Similarly, a recent study used intraperitoneal injection of rats with a SOD mimetic, M40403, and determined a significant decrease in markers of periodontal inflammation, such as inducible nitric oxide synthase, gingival tissue neutrophil infiltration, lipid peroxidation and extravasations in the tissues, as well as alveolar bone destruction (13). These findings suggest that endogenous antioxidant molecules produced by the genes *cat* and *sod* have a role in reducing, de-activating, and/or removing the reactive oxygen species that affect periodontal tissues. In the present study, we found that the fish oil diet appeared to enhance the CAT and SOD mRNA expression in gingival tissues that accompanied decreased alveolar bone resorption following *P. gingivalis* infection (31). This was similar to findings in kidney tissues of lupus-prone B/W mice, in which a fish oil diet was shown to significantly increase the activities of SOD, CAT and glutathione peroxidase (GSH-Px) compared to corn oil diets (27).

Rats provided with a ω-3 FA diet tended to show increased bone formation rate, bone strength and bone-specific alkaline phosphatase activity, and reduced mineral loss, suggesting a role for these fatty acids in skeletal biology and bone health (49). A recent study showed that 5% dietary fish oil decreased osteoclastogenesis and loss of bone mass in ovariectomized mice (45). Furthermore, eicosapentaenoic acid-deficient animals can develop severe osteoporosis linked to increased renal and arterial calcification related to their role in bone metabolism (34). Our studies are in agreement with these findings and suggested that dietary fish oil appeared to minimize both mandibular and maxillary alveolar bone resorption in *P. gingivalis*-induced experimental periodontal disease in rats (31).

In conclusion, these results indicate that a fish-oil-enriched diet containing ω-3 FA has anti-inflammatory effects in regulating gingival tissue responses to *P. gingivalis* infection. Coupled with reduced gingival tissue levels of lipid-based inflammatory mediators and decreased alveolar bone resorption in rats (31, 46, 47), our results also suggest that a fish oil-enriched diet containing ω-3 FA could be a suitable nutritional prevention and/or intervention strategy in treating the chronic immunoinflammatory lesions of periodontitis.

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Disclosures

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