

# 1-Tetradecanol Complex: Therapeutic Actions in Experimental Periodontitis

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**Background:** The present study was planned to investigate the therapeutic actions of 1-tetradecanol complex (1-TDC), a novel monounsaturated fatty acid mixture, in established periodontitis in rabbits.

**Methods:** Periodontitis was initiated in 18 New Zealand White rabbits using ligatures around mandibular second premolars, followed by topical *Porphyromonas gingivalis* application ( $10^9$  colony forming units). After 6 weeks of disease induction (phase 1), three animals were sacrificed to assess the established periodontitis level. *P. gingivalis* application was discontinued, and the remaining 15 animals continued with topical treatment of 1-TDC (100 mg/ml;  $n=5$ ) or placebo ( $n=5$ ) or no treatment ( $n=5$ ) for an additional 6 weeks (phase 2). Mandibular block sections obtained after euthanasia were decalcified and embedded in paraffin. In addition to the macroscopic analyses, hematoxylin and eosin-stained sections were used to study cellular inflammatory infiltrate and quantitative histomorphometry. Tartrate-resistant acid phosphatase and osteocalcin were used to identify osteoclastic and osteoblastic activity, respectively.

**Results:** *P. gingivalis* application resulted in periodontal disease with gingival inflammation and bone loss (30% compared to baseline) at 6 weeks. Treatment with 1-TDC stopped the progression of the disease and resulted in a significant reduction in the macroscopic periodontal inflammation, attachment, and bone loss ( $10.1\% \pm 1.8\%$ ), whereas periodontal disease progressed in the untreated and placebo groups ( $P < 0.05$ ). Histologic assessment and histomorphometric measurements demonstrated that 1-TDC inhibited inflammatory cell infiltration and osteoclastic activity ( $P < 0.05$ ).

**Conclusion:** The findings suggest that topical application of cetylated monounsaturated fatty acid complex (1-TDC) is a potential therapeutic approach in controlling the progression of chronic periodontal disease. *J Periodontol* 2009;80:1103-1113.

## KEY WORDS


Animal model; alveolar bone loss; fatty acids; periodontal disease; treatment.

Host-mediated immune responses to microorganisms lead to the destruction of periodontal tissues.<sup>1-3</sup> There is substantial evidence that the products of arachidonic acid (AA) metabolism may be pivotal in triggering and perpetuating the inflammatory changes seen in periodontitis.<sup>4-6</sup> High concentrations of the AA-derived products leukotriene B<sub>4</sub> (LTB<sub>4</sub>) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and inflammatory cytokines tumor necrosis factor-alpha and interleukin-1 $\beta$  and -6 are particularly destructive and implicated in periodontal diseases as well as other chronic inflammatory diseases, such as rheumatoid arthritis and atherosclerosis.<sup>7-10</sup> Manipulation of the immune response to suppress unwanted inflammatory reactions has been widely studied as a treatment for controlling such inflammatory responses in periodontal disease.<sup>11,12</sup> Several host response-modulating approaches have been described;<sup>6</sup> however, the control of inflammation with available pharmaceutical agents is still a challenge because of the side effects associated with their chronic use.

Fatty acids have been proposed to reduce chronic inflammation in individuals with arthritis by reducing the release of LTB<sub>4</sub> from stimulated neutrophils and interleukin-1 from monocytes.<sup>13-15</sup> The topical application of omega-3 ( $\omega$ -3) polyunsaturated fatty acid (PUFA) was successful in the treatment of inflammatory diseases, such as psoriasis, as well as experimental periodontitis in animal

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 indicates supplementary slide presentation (with audio) in the online *Journal of Periodontology*.

models by decreasing leukocyte chemotaxis, adhesion molecule expression, and inflammatory cytokine production.<sup>16-18</sup> Offenbacher et al.<sup>19</sup> showed that eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) can inhibit the production of PGE<sub>2</sub> to an extent similar to ibuprofen when added to human periodontal homogenates. Pilot clinical and animal studies<sup>20-24</sup> with  $\omega$ -3 and  $\omega$ -6 PUFA supplementation also showed beneficial results on periodontal inflammation and bone loss, indicating an anti-inflammatory role for these fatty acids without any evidence of the side effects often reported with the long-term use of non-steroidal anti-inflammatory drugs and other anti-inflammatory agents. However, the clinical studies<sup>22,24</sup> with dietary supplements did not show significant influences on gingival and periodontal inflammation, most probably because of the lack of sufficient concentration of  $\omega$ -3 PUFA locally when used in reasonable doses. Conversely, because of the high epithelial penetration of fatty acids,<sup>25</sup> topical application may be favorable for the treatment of local oral inflammatory diseases, including periodontitis.<sup>26,27</sup>

Parallel to the findings with PUFAs, there is evidence suggesting that the substitution of monounsaturated fatty acids (MUFAs) for saturated fatty acids may favorably affect cardiovascular risk.<sup>28-30</sup> Epidemiologic studies demonstrated the protective effects of MUFAs against coronary heart disease (CHD), and evidence from controlled clinical studies showed that MUFAs favorably affect a number of risk factors for CHD, including plasma lipids and lipoproteins, factors related to thrombogenesis, in vitro low-density lipoprotein oxidative susceptibility, and insulin sensitivity.<sup>30,31</sup> Experimental evidence further suggests that MUFA-rich diets favorably influence blood pressure, coagulation, endothelial activation, inflammation, and thermogenic capacity.<sup>32</sup> MUFAs are also beneficial for the prevention of obesity and other metabolic diseases and for immune function.<sup>33</sup> Recently, we showed that the topical application of 1-tetradecanol complex (1-TDC), a novel MUFA that contains a blend of esterified MUFAs, is capable of protecting from inflammatory changes in experimental periodontitis induced by *Porphyromonas gingivalis* in rabbits.<sup>34</sup> 1-TDC was effective in preventing gingival inflammation by controlling the inflammatory cascade initiated by the periodontal pathogen *P. gingivalis* and further protected from more destructive forms of periodontal inflammation.<sup>34</sup> In this study, we tested the hypothesis that MUFA-induced control of inflammation halts the destruction of periodontal tissues after active disease is established.

## MATERIALS AND METHODS

### Animal Model and Experimental Design

The study was approved by Boston University Medical Center (BUMC) Institutional Animal Care and Use

Committee prior to study initiation. In addition, BUMC Institutional Biohazard Committee approved the use of *P. gingivalis* in this animal model to induce periodontal disease.

Eighteen male New Zealand White rabbits were purchased,<sup>‡</sup> equilibrated, and housed at the BUMC Laboratory Animal Science Center for  $\geq 7$  days prior to any experimental procedure. The experiment included two phases, and the study design is shown in Figure 1. Phase 1 (periodontitis stage) involved the initiation and establishment of periodontal disease over a 6-week period based on our previously established model.<sup>26,35</sup> Briefly, rabbits were anesthetized using a combination of xylazine<sup>§</sup> (5 mg/kg, intramuscularly [IM]) and ketamine<sup>||</sup> (40 mg/kg, IM), and ligatures (3-0 braided silk suture)<sup>¶</sup> were placed around the second premolars on both sides of the mandible. Multiple applications (every other day for 6 weeks) of carboxymethylcellulose gel containing *P. gingivalis* were applied directly to the ligatures under inhalation anesthesia<sup>#</sup> (4% induction and then 2% maintenance). At each application, the sutures were checked, and lost or loose ones were replaced (Fig. 1). *P. gingivalis* (strain A7436) was grown using standard procedures,<sup>26</sup> and 10<sup>9</sup> colony forming units were mixed with carboxymethylcellulose<sup>\*\*</sup> to form a thick slurry, on the day of the experiment, and it was applied topically to the ligated teeth.<sup>26,35</sup>

After the periodontitis phase, the animals were randomly grouped for the treatment phase (phase 2) of the experiment. At this point, three animals were randomly selected and sacrificed to perform measurements for baseline periodontitis. The remaining 15 animals were assigned to three treatment groups. The test group (n = 5) received 1-TDC,<sup>††</sup> 100 mg/ml<sup>34</sup> per tooth, delivered with irrigation syringes, whereas the positive control group (n = 5) received mineral oil<sup>‡‡</sup> as placebo. The negative control group (n = 5) did not receive any treatment, but the animals in this group received the same dose of anesthesia every other day for standardization purposes. 1-TDC is an esterified MUFA mixture of cetyl myristoleate, cetyl myristate, cetyl palmitoleate, cetyl laureate, cetyl palmitate, and cetyl oleate. The duration of phase 2 was also 6 weeks, and the topical applications were performed every other day without additional *P. gingivalis*. However, the ligatures were maintained throughout the entire experiment and were checked and replaced when necessary. During the study, the animals were monitored daily, including food and fluid intake,

‡ Pine Acres Rabbitry/Farm, Norton, MA.

§ AnaSed, LLOYD Laboratories, Shenandoah, Iowa.

|| Ketaset, Fort Dodge Animal Health, Fort Dodge, Iowa.

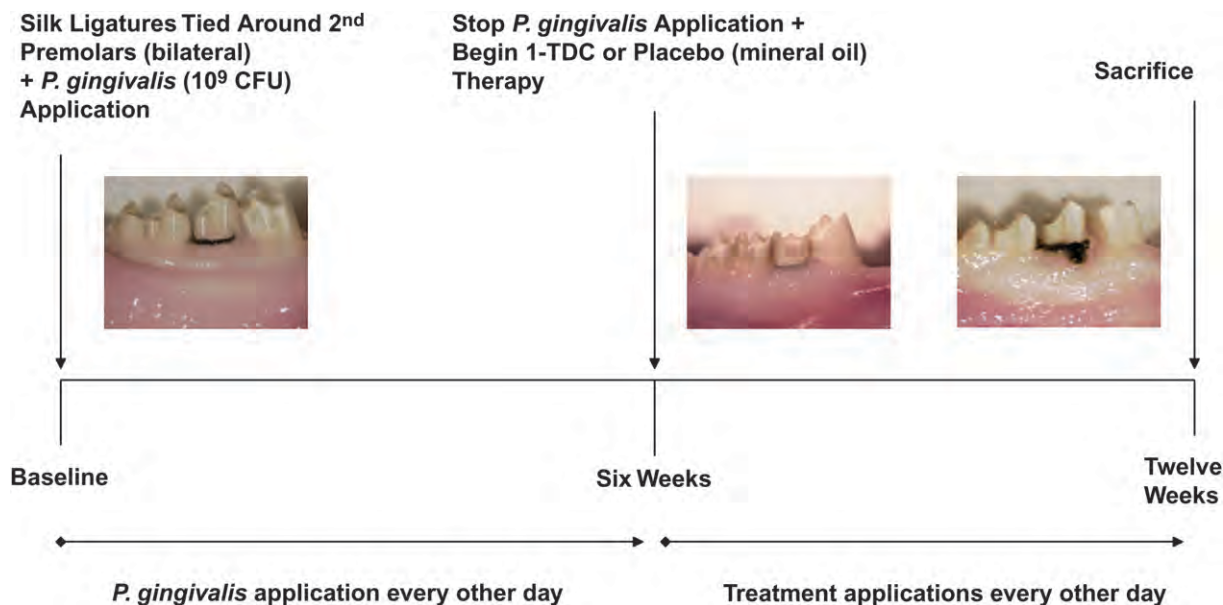
¶ Sharpoint, Surgical Specialties, Reading, PA.

# Isoflurane, Hospira, Lake Forest, IL.

\*\* Sigma-Aldrich, St. Louis, MO.

†† Imagenetix, San Diego, CA.

‡‡ Sigma-Aldrich.

**Figure 1.**

Timeline of experimental design. CFU = colony forming units.

urination, weight gain or loss, and general behavior. At the end of the study, animals were euthanized using an overdose (120 mg/kg, intravenously) of pentobarbital.<sup>§§</sup> The mandible of each rabbit was dissected free of muscle and soft tissue, keeping the attached gingiva–alveolar bone interface intact. The mandibles were split into two halves from the midline between the central incisors. The left half was processed for morphometric analysis of the bone, and the right half was used for histologic evaluation.

#### Morphometric Analysis

The left side of the mandible was defleshed by immersion in 10% hydrogen peroxide (3 to 4 days at room temperature). The soft tissue was carefully removed, and the mandible was stained with methylene blue for good visual distinction between the tooth and bone. The bone level around the second premolar was measured directly with a 0.5-mm calibrated periodontal probe. Measurements were made at three points on both buccal and lingual sides to quantify crestal bone level. A mean crestal bone level around the tooth was calculated. Similarly, for the proximal bone level, measurements were made at mesial and distal aspects of the tooth. The measurements were taken from the buccal and lingual side on both proximal aspects of the second premolar, and the mean proximal bone level was calculated. The mandibles were photographed using an inverted microscope at  $\times 10$ . The captured image was also analyzed as above, and the mean crestal bone level around the tooth was calculated using software.<sup>¶¶¶</sup>

In addition, the soft tissue (probing) depth and bony defect depth were measured in all groups using

a 0.5-mm calibrated periodontal probe. The tip of the tooth at the measured site was used as the reference point for these measurements. Probing depth measurements were performed on freshly harvested mandibles prior to any treatment, including defleshing or fixation. Bleeding on probing evaluations were conducted on the anesthetized animals prior to euthanasia.

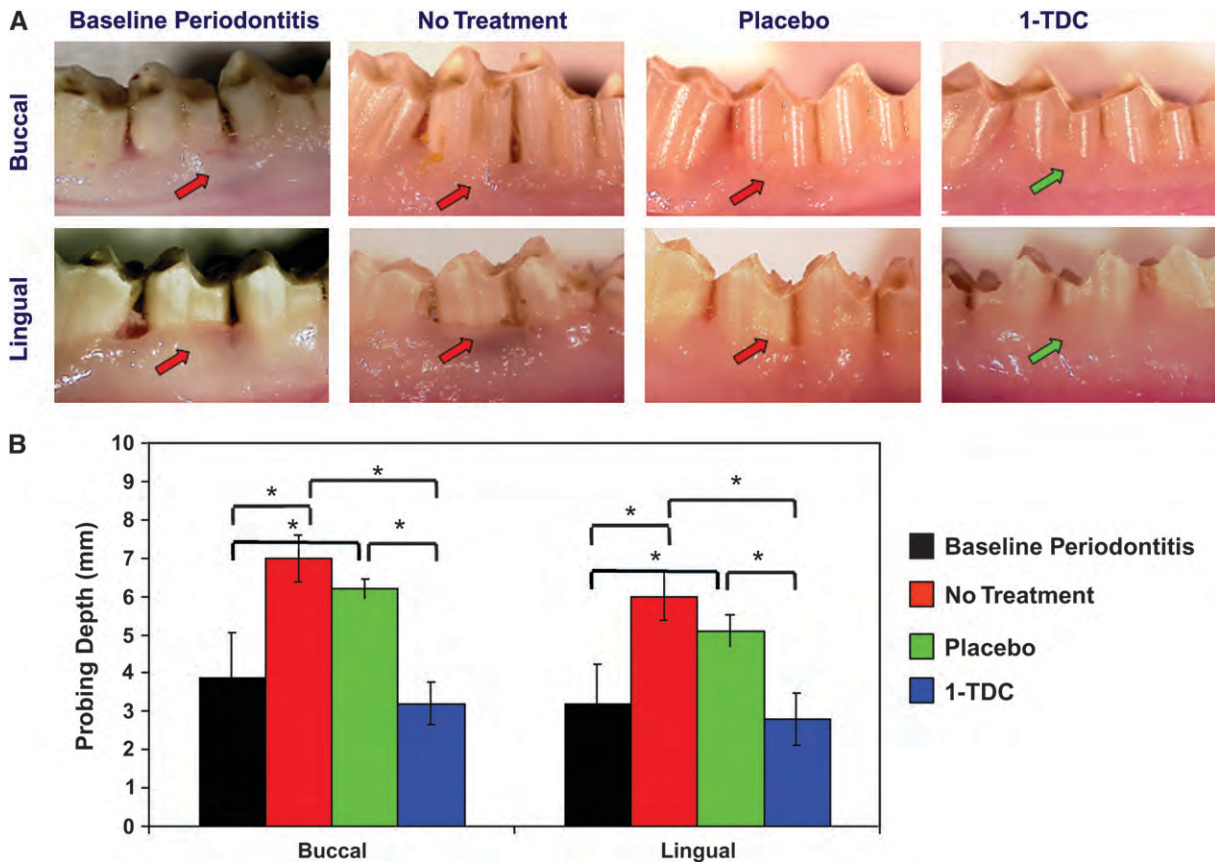
#### Radiographic Analysis

The percentage of the tooth within the bone was calculated radiographically using a modified Bjorn technique.<sup>35</sup> Digital radiographs were taken, and calculations were made using software.<sup>¶¶¶</sup> To quantify bone loss, the length of the tooth from the cusp tip to the apex of the root was measured, and the length of the tooth structure outside the bone was measured from the cusp tip to the coronal extent of the proximal bone. From this, the percentage of the tooth within the bone was calculated. Bone values are expressed as the percentage of the tooth in the bone (length of tooth in bone  $\times$  100/total length of tooth). Previous data established that  $\sim 90.2\% \pm 0.3\%$  of a healthy rabbit tooth is within the bone radiographically.<sup>35</sup> The percentage of bone loss was calculated for each animal,<sup>35</sup> and the mean bone loss (mean  $\pm$  SD) was used for comparisons.

§§ Pentobarbital Euthanasia-5 Solution, Veterinary Laboratories, Lenexa, Kansas.

¶¶ Image-Pro Plus 4.0, Media Cybernetics, Silver Spring, MD; Immagini & Computer, Milan, Italy.

¶¶ Schick Technologies, Long Island City, NY.



**Figure 2.**

Soft tissue changes after treatment with 1-TDC. **A)** After 6 weeks of treatment, the topical application of 1-TDC (100 mg/ml, three times per week) resulted in the restoration of tissues, whereas periodontal disease progressed significantly in the no-treatment and placebo groups (red arrows depict localized bone loss; green arrows depict restored bone). **B)** 1-TDC treatment stopped disease progression and resulted in soft tissue attachment gain, whereas the other treatment groups showed progression that was characterized by increased probing depth (\*P <0.0001).

**Histologic Analysis**

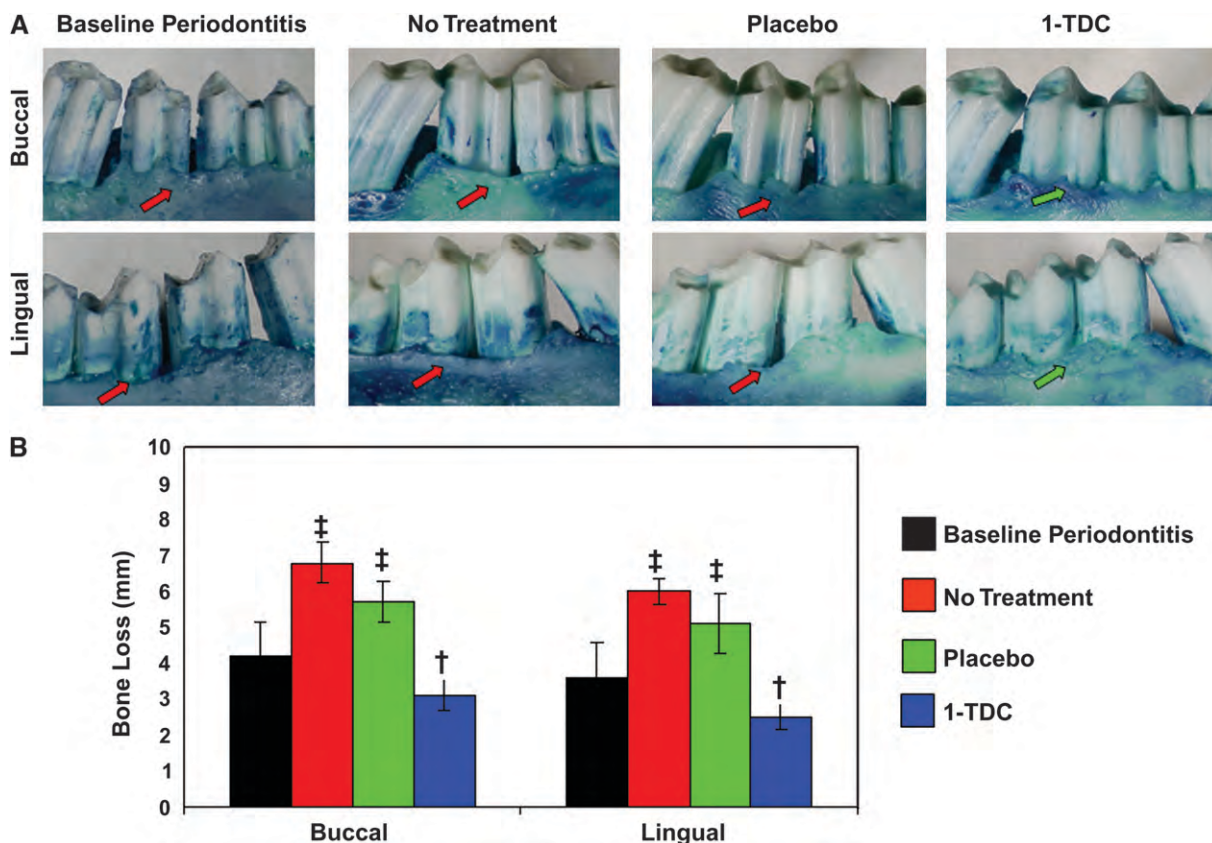
For histologic analysis, the other half of the mandible was immersed in a volume of decalcification solution<sup>##</sup> equal to ≥10 times the size of the section; the solution was replaced every 24 hours for 2 weeks. Decalcification was confirmed by serial radiographs, which were taken every other day. After decalcification, the tissues were rinsed for 3 minutes in flowing deionized water and kept in 10% buffered formaldehyde solution for ≥24 hours before embedding in paraffin. Thin sections (5 μm) were cut, and sections were conventionally stained with hematoxylin and eosin (H&E)<sup>\*\*\*</sup> to identify the cellular composition of the inflammatory infiltrate and for histomorphometric measurements or with tartrate-resistant acid phosphatase (TRAP) to detect osteoclastogenesis. The cellular infiltrate was quantified using a method originally developed in our laboratories<sup>36</sup> with minor modification; the density of the inflammatory cells was assessed by a masked pathologist (CA) on a scale of 0 to 4. Briefly, the grading was as follows: 0 = no sign of inflammation or cellular infiltrate; 1 = <25% of the studied area showed

cellular infiltration; 2 = ≤50% of the studied area showed cellular infiltration; 3 = ≤75% of the studied area showed infiltration; and 4 = >75% of the studied area showed infiltration by inflammatory cells. This measurement was performed using a standard area under the microscope (0.09 mm<sup>2</sup>), was repeated for three slides for each of the three levels analyzed, and was expressed as the average of three slides at the same level to assess the coronal, middle, and apical extent of the inflammatory infiltrate.

To quantify the changes in bone, the mean ± SD of the linear distance for bone loss were calculated for each group. A previously developed measurement technique<sup>37</sup> was used to calculate the bone changes at three different sections of the root using the software. The linear measurements were made at three levels (crestal, mid, and apical), each corresponding to one-third of the root and alveolar bone interface. Linear distance is reported as the distance from the base of the epithelium to the alveolar crest border at the three

<sup>##</sup> Immunocal, Decal, Tallman, NY.

<sup>\*\*\*</sup> Thermo Fisher Scientific, Pittsburgh, PA.



**Figure 3.**

*Bone gain after I-TDC application. A) After 6 weeks of treatment, the topical application of I-TDC (100 mg/ml, three times a week) arrested the bone loss (green arrows), whereas the periodontal disease progressed in the no-treatment and placebo-treated groups, as characterized by increased bone loss (red arrows). Alveolar bone loss for all animals was directly measured on defleshed jaws. (Methylene blue staining.) B) I-TDC treatment stopped the disease progression and resulted in bone gain ( $^{\dagger}P < 0.05$ ), whereas the no-treatment and placebo groups had significant disease progression characterized by more bone destruction compared to baseline periodontitis ( $^{\ddagger}P < 0.05$ ).*

chosen levels (apical, middle, and the coronal third of the root) and is expressed as the difference between treated and untreated sites. This measurement was used to assess the changes in the pocket and to create a three-dimensional representation of the change in the linear measurements at a horizontal distance at three levels (coronal, middle, and apical), i.e., the linear distance is used as a quantification of the pocket volume.

Further, osteoblastic activity was evaluated by immunohistochemistry in sections stained with osteocalcin as a marker of new bone formation.<sup>38,39</sup> Briefly, the sections were incubated overnight with primary antibody (mouse anti-osteocalcin; 7.5  $\mu$ g/ml)<sup>†††</sup> diluted in Tris buffered saline (TBS) with 1% bovine serum albumin (BSA) at 4°C. The sections were incubated for an additional 2 hours at room temperature with biotinylated secondary antibody (goat anti-mouse) diluted to the appropriate concentration (1/1,000) in TBS with 1% BSA. After clearing out the excess buffer solution, the sections were completely covered with streptavidin-peroxidase, incubated for 30 minutes at room temperature, stained with peroxidase substrate,<sup>†††</sup>

and counterstained with eosin. Osteoblast density was quantified by counting the osteocalcin-stained cells per square millimeter in each section of the samples, using the software, at three levels (coronal, middle, and apical).

**Statistical Analysis**

Mean values for linear measurements were used to determine the changes in bone level. Ratio calculations were used, and multiple comparisons within groups were made using analysis of variance with Bonferroni correction. Statistical comparisons were carried out between the two control groups (baseline periodontitis and non-treatment) and the treatment groups (I-TDC and placebo) to test the effectiveness of the placebo and the esterified fatty acid mixture.

<sup>†††</sup> Abcam, Cambridge, MA.

<sup>†††</sup> TrueBlue peroxidase substrate, Kirkegaard and Perry Laboratory, Gaithersburg, MD.

**RESULTS**

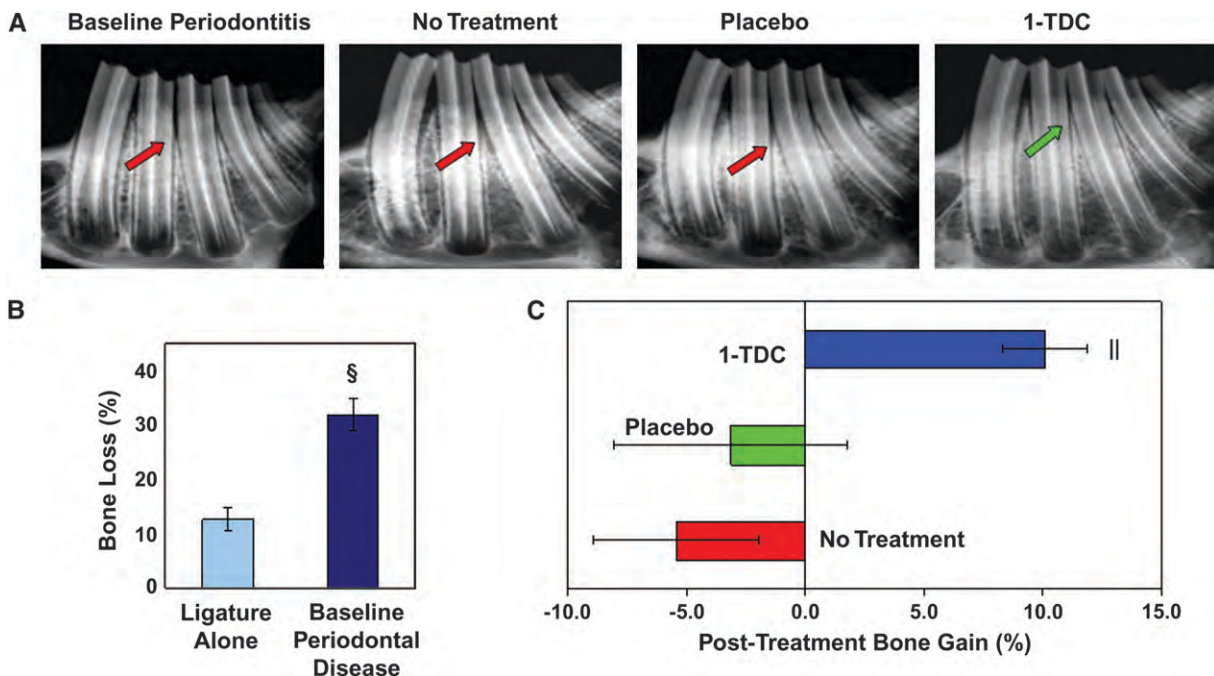
**Macroscopic Analysis**

The gingival tissue and defleshed bone specimens from the buccal and lingual aspects of the mandible are shown in Figures 2A and 3A. The probing depth was measured from the gingival margin to the base of the sulcus using a University of North Carolina periodontal probe at the mesial and distal aspects of the teeth treated. At the end of 6 weeks, local inflammation and bone loss were clearly observed (baseline periodontitis). The probing depth at this time point measured up to 5 mm (mean,  $3.9 \pm 1.1$  mm), and bleeding on probing was present at all sites. When the sites were left untreated over the next 6-week period, the progression of disease continued, as reflected by increased probing depth up to 7 mm and spontaneous bleeding (no treatment; Fig. 2B). The placebo-treated group demonstrated similar disease progression at the untreated sites (mean probing depth,  $6.2 \pm 0.3$  mm). Conversely, in the 1-TDC-treated group, probing depth was dramatically decreased and reached a level below the baseline periodontitis (mean probing depth,  $3.2 \pm 0.6$  mm versus  $3.9 \pm 1.1$  mm), indicating that 1-TDC stopped the progression of disease and reduced soft tissue inflammation (Fig. 2B).

To confirm the action of 1-TDC on periodontal inflammation and bone loss, the depth of the bony defect was calculated by measuring the distance between the cusp of the tooth and the base of the alveolar bone on defleshed bone specimens (Fig. 3B). Parallel with the soft tissue probing depth levels, the bony defect depths reflected the periodontal disease and bone loss observed on the defleshed specimens at the end of the first 6 weeks (mean bony defect depth,  $4.2 \pm 0.9$  mm for baseline periodontitis). Untreated and placebo-treated sites showed equally increased inflammation and bone loss compared to baseline periodontitis, for which the bony defect depth was up to 7 mm (mean bony defect depth,  $6.8 \pm 0.9$  mm and  $5.7 \pm 0.6$  mm, respectively). Although periodontal disease continued to progress in untreated and placebo-treated groups, treatment with 1-TDC resulted in a significant reduction in inflammation and bone loss compared to baseline periodontitis ( $P < 0.05$ ; Fig. 3B).

**Radiographic Analyses**

Figure 4 shows the radiographs of the animals in the three treatment groups and the baseline periodontitis group and the percentage of radiographic bone loss. Baseline periodontal disease (6 weeks) was equivalent



**Figure 4.**

Radiographic analyses of hard tissue components. **A)** Radiographic images reveal the bone loss observed in the no-treatment and placebo groups (red arrows) and the bone gain (green arrow) in the 1-TDC-treated group compared to baseline periodontitis. **B)** Baseline periodontal disease detected at 6 weeks displayed ~30% bone loss (§ $P < 0.05$ ). The ligature-alone group was included from historical data<sup>26,37</sup> to indicate the changes in periodontitis groups in which *P. gingivalis* was used for disease induction. **C)** Analyses of radiographic images demonstrated that 1-TDC treatment stopped the progression of bone loss and resulted in bone gain ( $P < 0.05$ ), whereas sites treated with placebo and left untreated progressed with ~3% and 5% more bone loss, respectively, compared to baseline periodontitis.

to ~30% bone loss (Fig. 4B). Analyses of radiographs demonstrated that 1-TDC treatment resulted in bone gain, whereas sites treated with placebo or left untreated had ~3% and 5% more bone loss, respectively, compared to baseline periodontitis. No significant difference was found between the placebo and untreated groups and the baseline periodontitis group with respect to radiographic bone loss (Fig. 4C;  $P > 0.05$ ).

**Histologic Analyses**

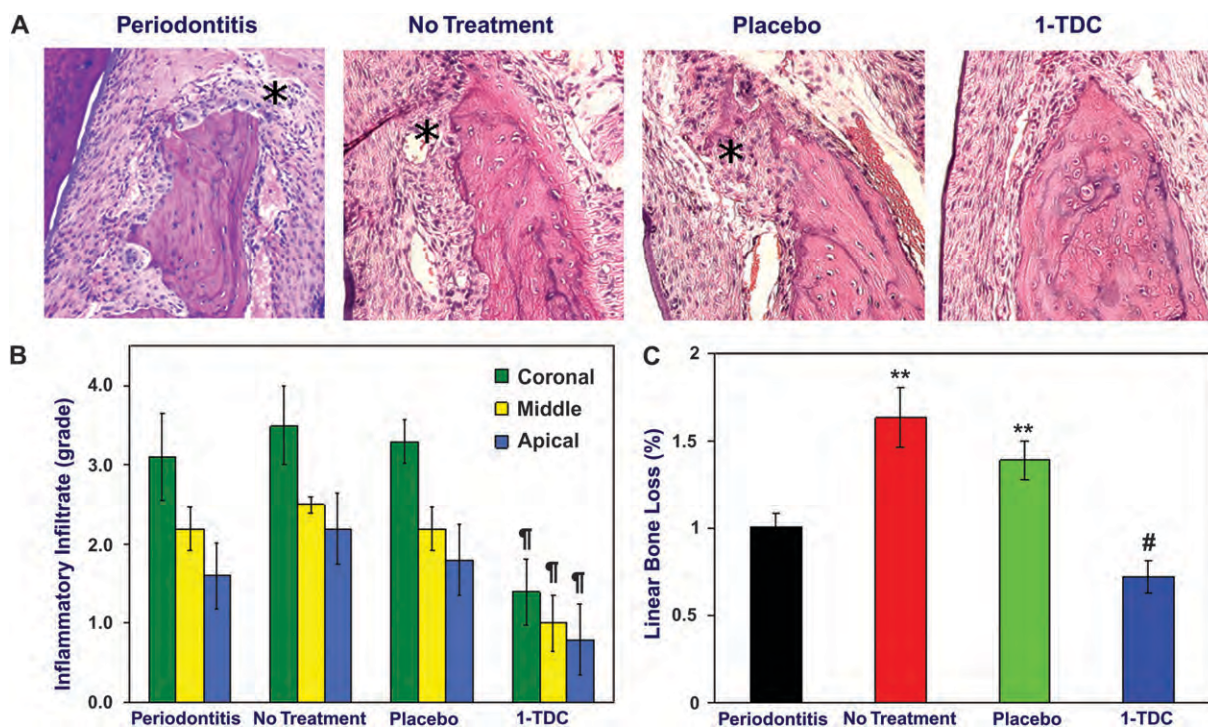
H&E-stained sections were evaluated for changes in soft tissue and alveolar bone (Fig. 5). Three areas were analyzed on each tooth corresponding to the coronal, middle, and apical third of the root. The analyses showed significant inflammatory cell infiltration in connective tissue and bone resorption, with irregular bone surfaces and resorptive lacunae in all groups except the 1-TDC-treated group, where inflammatory changes and bone loss were clearly diminished (Fig. 5A). Inflammatory infiltrate was also quantified at three areas of the root (coronal, middle, and apical) using a modified grading system.<sup>36</sup> In-

creased inflammatory cell infiltrate was obvious in the no-treatment and placebo groups compared to baseline periodontitis, whereas 1-TDC treatment significantly reduced the inflammatory cell infiltration compared to the periodontitis, no treatment, and placebo groups (Fig. 5B).

H&E-stained sections were also evaluated to quantify the alveolar bone changes histologically (Fig. 5C). The linear measurements were made at three levels (crestal, middle, and apical), each corresponding to one-third of the root and alveolar bone interface, and are presented as the percentage of linear bone loss. Histomorphometric analysis of H&E-stained sections complemented clinical assessments and clearly demonstrated that 1-TDC treatment significantly reduced the bone loss compared to the untreated and placebo-treated groups ( $P < 0.05$ ) and resulted in bone gain compared to baseline periodontitis (Fig. 5C).

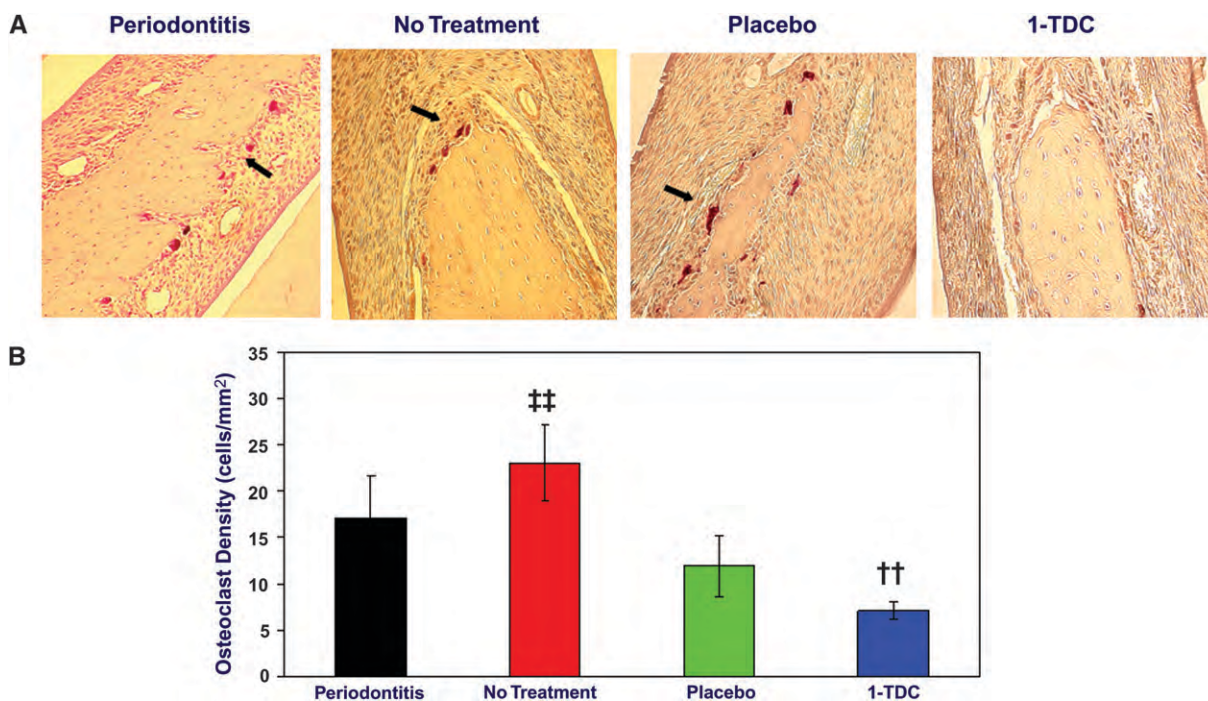
**Osteoclastic Cell Activity**

To evaluate the osteoclastic activity (osteoclastogenesis) during inflammatory changes in the periodontal



**Figure 5.**

Histologic analyses and histomorphometric quantification of changes in *P. gingivalis*-induced periodontal inflammation and in response to different treatments. **A)** Histologic analyses demonstrated clearly diminished inflammation and alveolar bone loss in 1-TDC-treated sections compared to increased inflammatory cell infiltration (asterisks) in the no-treatment and placebo groups (H&E; original magnification  $\times 200$ ). **B)** Inflammatory infiltrate was quantified using a modified grading system at three areas of the root (coronal, middle, and apical). 1-TDC resulted in less inflammatory cell infiltration compared to the no-treatment and placebo groups ( $^{\#}P < 0.05$ ). **C)** Quantification of histomorphometric changes also revealed that 1-TDC was capable of reducing the bone loss as a result of controlling inflammation compared to the baseline periodontitis, no-treatment, and placebo groups ( $^{\#}P < 0.05$ ). The no treatment and placebo groups exhibited significantly increased bone loss compared to the baseline periodontitis group ( $^{**}P < 0.05$ ).



### Figure 6.

Osteoclastic activity. **A)** TRAP-stained sections show resorbing bone lacunae containing large numbers of osteoclasts in the baseline periodontitis, no-treatment, and placebo groups (arrows), whereas 1-TDC-treated specimens contained few or no detectable TRAP-positive cells (original magnification  $\times 200$ ). **B)** 1-TDC significantly reduced osteoclastic activity compared to all other groups ( $^{\dagger\dagger}P < 0.05$ ), and the no-treatment group exhibited a dramatically increased osteoclastic activity compared to the baseline periodontitis group ( $^{\dagger\dagger\dagger}P < 0.05$ ).

tissues, osteoclast-like cells were identified using TRAP staining. Large numbers of TRAP-positive cells were detected in the no-treatment and placebo groups, whereas specimens treated with 1-TDC contained few TRAP-positive cells (Fig. 6A). TRAP-positive cells were counted in the coronal one-third of the root on each section (cells/mm<sup>2</sup>) using the software program. Osteoclastogenesis was increased in the untreated and placebo-treated groups in parallel with inflammatory changes in the soft tissue ( $P < 0.05$ ). The animals treated with 1-TDC showed a statistically significant reduction in osteoclastic activity compared to the baseline periodontitis, no-treatment, and placebo groups ( $P < 0.05$ ), indicating that 1-TDC is capable of reversing inflammation in periodontitis (Fig. 6B).

### Bone Reformation

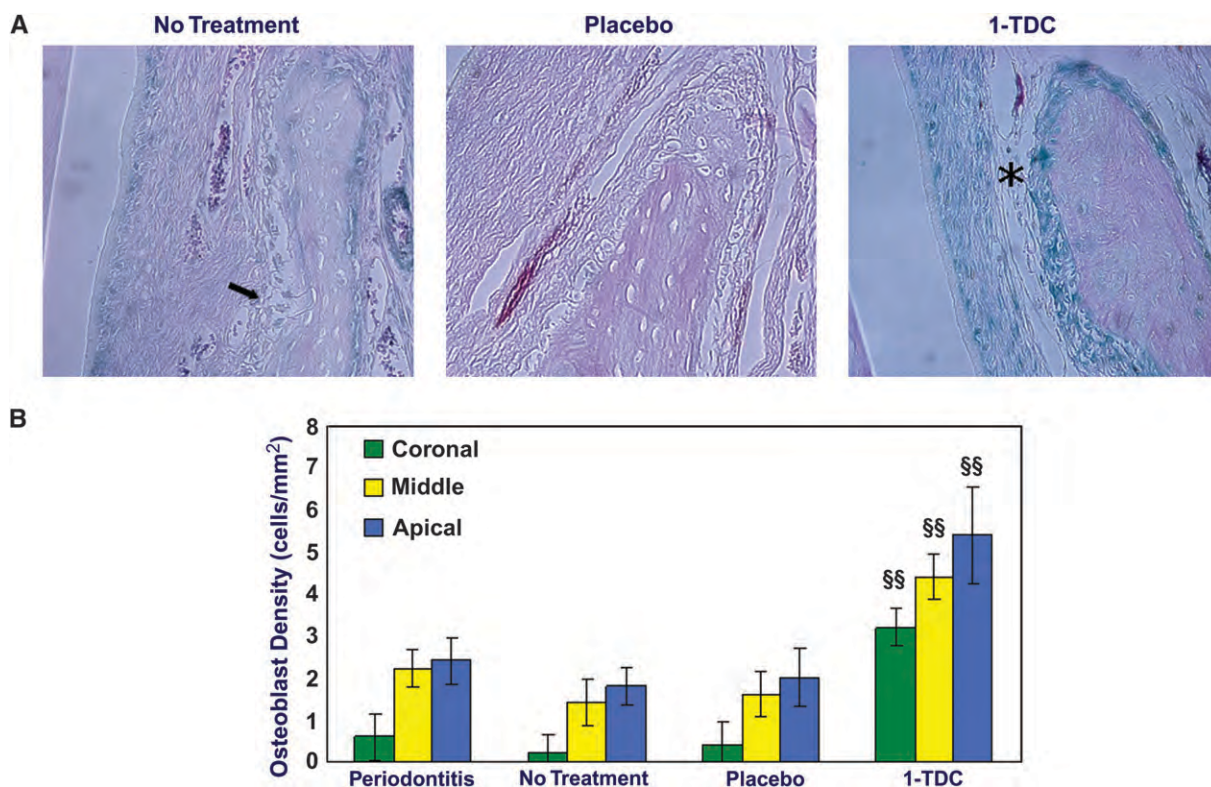
We then examined osteoblastic cell activity to assess healing new bone formation by immunohistochemistry in sections stained with osteocalcin as a marker of new bone formation (Fig. 7A). In the no-treatment group, a few osteocalcin-positive cells were detectable on the surface of the alveolar bone; however, alveolar bone resorption and osteoclastic activity was clearly dominant as detected in H&E- and TRAP-stained sections (Fig. 6A). In the 1-TDC-treated group, a greater density of osteocalcin-positive cells was detected, and

it was inversely related to the number of TRAP-stained cells (Figs. 6 and 7). Osteoblastic density was scored at three levels (coronal, middle, and apical) using the software program. 1-TDC resulted in increased osteoblast density at all levels compared to the other groups (Fig. 7B). These findings and the normal characteristics of newly formed bone in the 1-TDC sections suggested that 1-TDC was able to stop the progression of the disease and initiate healing, leading to bone reformation.

### DISCUSSION

The rabbit model of periodontitis was shown to be a relevant disease model in which the pathologic changes in periodontal tissues resemble those in humans.<sup>26,35</sup> In this study, we demonstrated that the local administration of an esterified MIFA mixture (1-TDC) halted the progression of *P. gingivalis*-induced periodontal inflammation in rabbits as evidenced by the reduction in inflammatory cell infiltration and bone loss. At the macroscopic and histopathologic levels, 1-TDC treatment further initiates healing and the reformation of periodontal tissue integrity lost as a result of inflammation.

In a previous study that demonstrated that topical application of 1-TDC provided protection against



**Figure 7.**

Osteoblastic activity as a marker of bone formation. **A)** Osteoblastic activity was evaluated by immunohistochemistry in sections stained with osteocalcin as a marker of new bone formation. Dense osteoblast-like cells (asterisk) are visible on the surface of the bone in 1-TDC-treated areas. In contrast, resorptive areas with almost no intra- or extracellular osteocalcin activity were detected on bone surfaces in the no-treatment and placebo groups (arrow). (Original magnification  $\times 200$ ). **B)** Osteoblast density was quantified at three levels (coronal, middle, and apical). The 1-TDC-treated samples showed greater osteoblast density at all levels compared to all other groups ( $^{SS}P < 0.05$ ).

periodontitis, we tested three doses of 1-TDC. Based on those results, the highest dose showed a clear and significant difference in preventing inflammatory cell infiltration and osteoclastic activity. Therefore, in this study, we used the 100-mg/ml dose of 1-TDC. The topical application of 1-TDC arrested the progression of periodontal inflammation and initiated the restoration of the lost periodontium as evidenced by clinical, histopathologic, and immunohistochemical analyses. However, healing was incomplete, and bone and soft tissue levels did not return to pretreatment levels. More *in vitro* studies are necessary to elucidate the direct action of 1-TDC on osteoclasts and osteoblasts to determine whether there is direct action or if the observed healing and bone metabolism changes are the result of limiting the inflammatory cascade.

An important finding in the prior prevention study was that the placebo (olive oil) had the capacity to prevent inflammatory changes. Olive oil contains MUFA. To avoid positive placebo effects and be able to show the true differences between the 1-TDC and placebo treatment, we used mineral oil in the current study. There were no placebo effects in this experi-

ment, and the results obtained with 1-TDC treatment were significantly different compared to placebo.

Increasing scientific evidence reveals that certain fatty acids have the potential to attenuate inflammation.<sup>34</sup> Fish oils are the main source of  $\omega$ -3 fatty acids, and EPA and DHA are the major components of PUFA.<sup>40</sup> The beneficial effects of PUFAs were shown in many inflammatory conditions, including periodontal disease, through the regulation of a variety of enzymatic processes. Fatty acids can decrease the amount of AA in cell membranes, reducing eicosanoid production via cyclooxygenase and lipoxygenase pathways.<sup>41-43</sup> The integration between AA by-products and their involvement with leukotriene and prostaglandins lead to the control of inflammation.<sup>5,44,45</sup> Recent studies<sup>20,21</sup> with dietary  $\omega$ -3 fatty acid use in rats showed superior results in reducing the gingival inflammation and bone loss compared to the controls. A limited number of clinical studies<sup>23,24</sup> with dietary fatty acids also showed improvement in some clinical parameters, especially gingival index and bleeding index; however, the results were not as profound as those in the animal studies. A

recent pilot study<sup>22</sup> with  $\omega$ -6 and  $\omega$ -3 fatty acids in the treatment of periodontal disease showed beneficial effects of dietary  $\omega$ -6 fatty acid (borage oil) on gingival inflammation and probing depth. However, the investigators suggested that additional studies are necessary to fully assess the potential benefits of dietary fatty acids in periodontal inflammation.

In this study, we used a topical delivery mode for the MUFA complex. The study clearly showed that 1-TDC reduced inflammatory changes and initiated the reformation of the periodontal tissues as a result of excessive periodontal inflammation in rabbits, suggesting that the cetylated fatty acids are rapidly and efficiently absorbed. The underlying mechanisms of action of 1-TDC are being investigated in in vitro experiments in our laboratory.

### CONCLUSIONS

The present study clearly demonstrated that a MUFA complex (1-TDC) stopped the progression of periodontal inflammation induced by a human periodontopathogen (*P. gingivalis*) in rabbit periodontitis. Furthermore, the macroscopic and histopathologic evaluations indicated that 1-TDC initiated the partial reformation of the soft and bone tissue lost to periodontal inflammation. Although the mechanisms underlying these actions of 1-TDC remain to be elucidated, it is clear that dietary fatty acids have the potential to alter the level of inflammation, and the therapeutic use of topically applied fatty acid preparations has significant clinical potential.

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