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. Materials and Methods: Periodontitis was initiated in 18 New Zealand White rabbits using ligatures around mandibular second premolars, followed by topical P. gingivalis application (10^9 CFU). Following 6 weeks of disease induction (Phase 1), 3 animals were sacrificed to assess the established periodontitis level. P. gingivalis application was discontinued and the remaining 15 animals continued with topical treatment with either 1-TDC (100 mg/ml; N = 5) or placebo (N = 5) or without 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-eosin (HE)-stained sections were used to study cellular inflammatory infiltrate and quantitative histomorphometry. Tartrate-resistant acid phosphatase (TRAP) and osteocalcin was used to identify osteoclast and osteoblast 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 significant reduction in the macroscopic periodontal inflammation, attachment and bone loss (10.1% ± 1.8) while periodontal disease progressed in the untreated and placebo groups (P <0.05). Histological assessment and histomorphometric measurements demonstrated that 1-TDC inhibited inflammatory cell infiltration and osteoclast activity (P <0.05). Conclusions: 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.

Key Words Periodontal inflammation, alveolar bone loss, fatty acids, 1-tetradecanol complex, treatment, animal studies.

Host-mediated immune responses to microorganisms lead to the destruction of periodontal tissues.1-3 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.4-6 High concentrations of the arachidonic acid-derived products leukotriene B4 (LTB4) and prostaglandin E2 (PGE2) and inflammatory cytokines TNF-α, IL-1β, and IL-6 are particularly destructive and are implicated in periodontal diseases as well as other chronic inflammatory diseases such as rheumatoid arthritis and atherosclerosis.7-10 Manipulation of the immune response to suppress unwanted inflammatory reactions has been widely considered as a treatment
for controlling such inflammatory responses in periodontal disease.\textsuperscript{11,12} Several host response-modulating approaches have been described\textsuperscript{6}; however, the control of inflammation with available pharmaceutical agents is still a challenge due to 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\textsubscript{4} from stimulated neutrophils and of interleukin-1 from monocytes.\textsuperscript{13-15} Topical application of omega 3 polyunsaturated fatty acid (ω-3 PUFA) has been shown to be successful in the treatment of inflammatory diseases such as psoriasis as well as experimental periodontitis in animal models decreasing leukocyte chemotaxis, adhesion molecule expression and inflammatory cytokine production.\textsuperscript{16-18} Offenbacher et al. showed that eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) can inhibit the production of PGE\textsubscript{2} to an extent similar to ibuprofen when added to human periodontal homogenates.\textsuperscript{19} Pilot clinical and animal studies with ω-3 PUFA and ω-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 side effects often reported with long-term use of NSAIDs and other anti-inflammatory agents.\textsuperscript{20-24} However, the clinical studies with dietary supplements did not show significant influences on gingival and periodontal inflammation, most probably due to the lack of sufficient concentration of ω-3 PUFA locally when used in reasonable doses.\textsuperscript{22,24} Conversely, it was reported that due to high epithelial penetration of fatty acids,\textsuperscript{25} topical application may be favorable for the treatment of local oral inflammatory diseases including periodontitis.\textsuperscript{26,27}

Parallel to the findings with PUFAs, there is evidence suggesting that the substitution of monounsaturated fatty acids (MUFAs), instead of saturated fatty acids (SFA) may favorably affect cardiovascular risk.\textsuperscript{28-30} Epidemiological studies have demonstrated the protective effects of MUFAs against coronary heart disease (CHD) and evidence from controlled clinical studies has shown that MUFAs favorably affect a number of risk factors for CHD, including plasma lipids and lipoproteins, factors related to thrombogenesis, in vitro LDL oxidative susceptibility, and insulin sensitivity.\textsuperscript{30,31} Experimental evidence further suggests that MUFA-rich diets favorably influence blood pressure, coagulation, endothelial activation, inflammation, and thermogenic capacity.\textsuperscript{32} MUFAs are also beneficial for the prevention of obesity and other metabolic diseases and for immune function.\textsuperscript{33} Recently, we have shown that topical application of 1-tetradecanol complex (1-TDC), a novel monounsaturated fatty acid mixture, which contains a blend of esterified MUFAs, is capable of protecting from inflammatory changes in experimental periodontitis induced by Porphyromonas gingivalis in rabbits.\textsuperscript{34} 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.\textsuperscript{34} In this study, we test 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 Institutional Animal Care and Use Committee (BUMC IACUC) prior to study initiation. In addition, BUMC Institutional Biohazard
Committee (IBC) approved the use of *P. gingivalis* in this animal model to induce periodontal disease.

Eighteen male New-Zealand White rabbits were purchased from Pine Acres Research Farm,* equilibrated and housed at the Boston University Medical Center Laboratory Animal Science Center (BUMC LASC) for at least 7 days prior to any experimental procedure. The experiment included two phases and the study design is shown in Fig. 1. Phase 1 (Periodontitis stage) involved the initiation and establishment of periodontal disease over a 6-week period based on our previously established model.26,35 Briefly, rabbits were anesthetized using a combination of xylazine† (5 mg/kg, IM) and ketamine‡ (40 mg/kg, IM) and ligatures (3-0 braided silk suture)§ were placed around the 2nd premolars on both sides of the mandible. Multiple applications (every other day for a period of six weeks) of carboxymethylcellulose gel containing *P. gingivalis* were carried out under inhalation anesthesia** (4% induction and then 2% maintenance) directly to the ligatures. At each application, the sutures were checked, and lost or loose sutures were replaced (Fig. 1). *P. gingivalis* (strain A7436) was grown using standard procedures26 and 10⁹ CFU were mixed with carboxymethylcellulose†† to form a thick slurry on the same day of the experiment and applied topically to the ligated teeth.26,35

Following the periodontitis phase, the animals were randomly grouped for the next phase (Phase 2; treatment stage) 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 3 treatment groups. The test group (N = 5) received 1-TDC at 100mg/ml concentration§§ per tooth delivered with irrigation syringes, while the positive control group (N = 5) received mineral oil 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. The experimental product (1-TDC) used in this study was obtained from Imagenetix, Inc.‡‡ 1-TDC comprises an esterified monounsaturated fatty acid mixture of cetyl myristoleate, cetyl myristate, cetyl palmitoleate, cetyl laurate, cetyl palmitate and cetyl oleate. Mineral oil§§§ was purchased from Sigma Chemicals and served as placebo control. 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, urination, weight gain or loss, and general behavior. At the end of the study, animals were euthanized using an overdose (120 mg/kg, IV) of pentobarbital.*** 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 2 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 histological evaluation.

**Morphometric Analysis**

The left side of the mandible was defleshed by immersion in 10% hydrogen peroxide (3-4 days, 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 buccal, lingual, and mean crestal bone level was calculated. Similarly, for the proximal bone level, measurements were made at mesial and distal aspects of the tooth. The measurements were taken from both the buccal and lingual side on both
proximal aspects of the second premolar and the mean proximal bone level was calculated. The mandibles were then photographed using an inverted microscope at 10 × . The captured image was also analyzed as above and the mean crestal bone level around the tooth was calculated in millimeters using Image Analysis.††† In addition, the soft tissue (pocket) 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. Pocket depth measurements were performed on freshly harvested mandibles prior to any further 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. The radiographs were taken with a digital X-ray. To quantify bone loss, the length of the tooth from the cusp tip to the apex of the root was measured, as was the length of the tooth structure outside the bone, 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 × 100 / total length of tooth). Previous data has established that approximately 90.2 ± 0.3% of a healthy rabbit tooth is within the bone radiographically. The percent bone loss was calculated for each animal and the mean bone loss (mean ± SD) was used for comparisons.

**Histological Analysis**

For histological analysis, the other half of the mandible was immersed in a volume of decalcification solution equal to at least 10 times the size of the section; the solution was replaced every 24 hours for two 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 at least 24 hours before embedding in paraffin. Thin sections (5 µm) were cut and sections were either conventionally stained with Hematoxylin-Eosin (H&E) 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 with minor modification and assessed the density of the inflammatory cells on a grade of 0 to 4 by a blinded pathologist (C.A). Briefly, the grading was as follows: 0: No sign of inflammation and cellular infiltrate; 1: Less than 25% of the studied area showed cellular infiltration; 2: Up to 50% of the studied area showed cellular infiltration; 3: Up to 75% of the studied area showed infiltration; 4: More than 75% of the studied area showed infiltration by inflammatory cells. This measurement was performed using a standard area under the microscope (0.09 mm²); repeated for 3 different slides for each of the 3 levels analyzed, and expressed as the average of 3 slides at the same level to assess coronal, middle, and apical extent of inflammatory infiltrate.

In order to quantify the changes in bone, the mean value (± standard deviation) of the linear distance for bone loss were calculated for each group. Previously developed measurement technique was used to calculate the bone changes at three different sections of the root using the image analysis software. The linear measurements were made at three levels each corresponding to one-third of the root and alveolar bone interface: crestal, mid, and apical.
Linear distance is reported as the distance from the base of the epithelium to the alveolar crest border at the three chosen levels, the 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 3-dimensional understanding of the “change” in the linear measurements at a horizontal distance at 3 different levels (coronal, middle, apical). In other words, the linear distance is used as a quantification of the pocket volume.

Further, osteoblast activity was evaluated by immunohistochemistry in sections stained with osteocalcin as a marker of new bone formation. Briefly, the sections were incubated overnight with primary antibody (Mouse anti-Osteocalcin; 7.5μg/ml) diluted in TBS with 1% BSA at 4°C. The sections were further incubated for 2 hours at room temperature with biotinylated secondary antibody (Goat anti-Mouse) diluted to the appropriate concentration (1/1000) in TBS with 1% BSA. After clearing out the excess buffer solution, the sections were completely covered with Streptavidin-peroxidase and incubated for 30 min. at room temperature and then first stained with TrueBlue peroxidase substrate and finally counterstained with eosin. Osteoblast density was quantified by counting the osteocalcin-stained cells per mm² in each section of the samples, at three levels (coronal, middle, apical) using Image-Pro Plus (version 4.0) software.

**Statistical Analysis**

Mean values for linear measurements were utilized to determine the changes in bone level. Ratio calculations were used and multiple comparisons within groups were made using analysis of variance (ANOVA) with Bonferroni correction. Statistical comparisons were carried out between the two control groups (baseline periodontitis and non-treatment) and treatment groups (1-TDC and placebo) to test the effectiveness of the placebo and the esterified fatty acid mixture.

**RESULTS**

*Macroscopic Analysis*

The gingival tissue and defleshed bone specimens from buccal and lingual aspects of the mandible are shown in Figs. 2 and 3; panel A. The pocket depth was measured as the probing depth from the gingival margin to the base of the sulcus using a UNC periodontal probe at mesial and distal aspects of the teeth treated. At the end of the six weeks, local inflammation and bone loss were clearly observed (Baseline Periodontitis). The probing pocket depth at this time point was measured up to 5 mm (mean pocket depth was 3.9 ± 1.1) 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 was continued as indicated by increased pocket depth up to 7 mm and spontaneous bleeding (No-treatment; Fig. 2, panel B). The placebo-treated group demonstrated similar disease progression to the untreated sites (mean pocket depth 6.2 ± 0.3 mm). Conversely, in the 1-TDC-treated group, probing pocket depth was dramatically decreased and reached to the level below the baseline periodontitis (mean pocket depth was 3.2 ± 0.6 mm) indicating that 1-TDC stopped the disease progression and reduced soft tissue inflammation (1-TDC; Fig. 2, panel B).

In order 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 cusp of the tooth and base of the alveolar bone on defleshed bone specimens (Fig. 3, panel B). Parallel with the soft
tissue pocket depth levels, the bony defect depths indicated the periodontal disease and bone loss observed on the defleshed specimens at the end of the first six weeks (mean bony defect depth 4.2 ± 0.9 mm) (Baseline Periodontitis). Both untreated and placebo-treated sites equally showed increased inflammation and bone loss compared to baseline periodontitis where the bony defect depth reached up to 7 mm (mean bony defect depth was 6.8 ± 0.9 mm and 5.7 ± 0.6 mm, respectively). While the baseline periodontal disease continued to progress in both untreated and placebo-treated groups, 1-TDC showed significant reduction in inflammation and bone loss ($P <0.05$). The areas treated with 1-TDC had significantly less bone loss compared to baseline periodontitis indicating that 1-TDC resulted in bone gain by controlling the inflammation ($P <0.05$) (1-TDC; Fig. 3; panel B).

**Radiographic Analysis**

Fig. 4 illustrates the radiographic images of the animals in all three treatment groups and the baseline periodontitis group (panel a) and the percentage of radiographic bone loss (panel B). Baseline periodontal disease (6 weeks) displayed >30% bone loss (Fig. 4, panel B). Analyses of radiographic images demonstrated that 1-TDC treatment resulted in bone gain, while those treated with placebo and left untreated progressed with >3% and 5%, more bone loss, respectively, compared to baseline periodontitis. No significant difference was found between placebo and untreated groups and the baseline periodontitis group with respect to radiographic bone loss (Fig. 4, panel C; $P >0.05$).

**Histological Analysis**

Hematoxylin and eosin (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 (baseline periodontitis, no-treatment and placebo) except the 1-TDC-treated group, where inflammatory changes and bone loss were clearly diminished (Fig. 5, panel A). Inflammatory infiltrate was also quantified at three areas of the root (coronal, middle and apical) using a modified grading system. Increased inflammatory cell infiltrate was obvious in no treatment and placebo groups compared to baseline periodontitis, while 1-TDC treatment significantly reduced the inflammatory cell infiltration compared to periodontitis, no treatment and placebo groups (panel B; $# = P <0.05$).

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

**Osteoclastic Cell Activity**

To evaluate the osteoclastic activity (osteoclastogenesis) during inflammatory changes in the periodontal tissues, osteoclast-like cells were identified using tartrate resistant acid phosphatase (TRAP) staining. Large numbers of TRAP-positive cells were detected in no-treatment and
placebo groups; whereas specimens treated with 1-TDC contained few TRAP positive cells (Fig. 6, panel A). TRAP-positive cells were counted in the coronal 1/3 of the root on each section (cells/mm$^2$) using Image-Pro Plus software program. The results revealed that osteoclastogenesis was increased in untreated and placebo-treated groups parallel to inflammatory changes in the soft tissue ($P <0.05$). The animals treated with 1-TDC showed a statistically significant reduction in osteoclast activity compared to baseline periodontitis, no-treatment and placebo groups ($P <0.05$), indicating that 1-TDC was capable of reversing inflammation in periodontitis (Fig. 6, panel B).

**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. 7, panel A). In the no-treatment group, a few osteocalcin-positive stained cells were detectable on the surface of the alveolar bone; however, alveolar bone resorption and osteoclast activity was clearly dominant as detected in H&E and TRAP stained sections (Fig. 6, panel A). Interestingly, in the 1-TDC treated group, greater density of osteocalcin-positive cells was detected and it was inversely related to the reduced number of TRAP stained cells (Figs. 6 and 7). Osteoblast density was scored at three levels (coronal, middle, apical) using image analysis software program. These results also indicated that 1-TDC resulted in increased osteoblast density at all levels compared to other groups (Fig. 7, panel B). 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 has been shown to be a relevant disease model where the pathological changes in periodontal tissues resemble humans. In this study, we demonstrate that local administration of an esterified monounsaturated fatty acid mixture (1-TDC) halts the progression of *P. gingivalis*-induced periodontal inflammation in rabbits as evidenced by the reduction in inflammatory cell infiltration and bone loss. Both at the macroscopic and histopathological levels, 1-TDC treatment further initiates healing and the reformation of lost periodontal tissue integrity as a result of inflammation.

In a previous study that demonstrated that topical application of 1-TDC provided protection against the periodontitis, we tested three different doses of 1-TDC. Based on those results, the higher dose showed a clear and significant difference in preventing inflammatory cell infiltration and the osteoclast activity. Therefore, in this study, 1-TDC was applied at the 100mg/ml dose. Topical application of 1-TDC arrested the progression of periodontal inflammation and initiated the restoration of the lost periodontium as evidenced by clinical, histopathological and immunohistochemical analysis. However, healing was incomplete and bone and soft tissue levels were not returned to pretreatment levels. More in vitro studies are necessary to elucidate the direct action of 1-TDC on osteoclast 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 experiment and the results obtained with 1-TDC treatment were significantly different compared to the placebo.

Increasing scientific evidence reveals that certain fatty acids have the potential to attenuate inflammation.\textsuperscript{34} Fish oils are the main source of \(\omega-3\) fatty acids where the major polyunsaturated fatty acid (PUFA) components are eicosapentaenoic acid (EPA) and docohexaenoic acid (DHA).\textsuperscript{40} Beneficial effects of PUFAs have been shown in many inflammatory conditions including periodontal disease through regulation of a variety of enzymatic processes. Fatty acids can decrease the amount of arachidonic acid in cell membranes reducing eicosanoid production via cyclooxygenase and lipoxygenase pathways.\textsuperscript{41-43} The integration between arachidonic acid byproducts and their involvement with leukotriene and prostaglandins leads to the control of inflammation.\textsuperscript{5,44,45} Recent studies with dietary \(\omega-3\) fatty acid use in rats showed superior results in reducing the gingival inflammation and bone loss compared to the controls.\textsuperscript{20,21} A limited number of clinical studies with dietary fatty acids also showed improvement in some clinical parameters, especially in gingival index and bleeding index; however, the results were not as profound as those in the animal studies.\textsuperscript{23} A recent pilot study 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 authors suggest that additional studies are necessary to fully assess the potential benefits of dietary fatty acids in periodontal inflammation.\textsuperscript{22}

In this study, we used a topical delivery mode of the monounsaturated fatty acid complex. The results of the study clearly showed that 1-TDC reduces inflammatory changes and initiates 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 actions of 1-TDC are currently being investigated in \textit{in-vitro} experiments in our laboratory.

In conclusion, the results of the present study clearly demonstrate that a monounsaturated fatty acid complex, 1-TDC, stopped the progression of the periodontal inflammation induced by a human periodontopathogen, \textit{P. gingivalis}, in rabbit periodontitis. Furthermore, both the macroscopic and histopathological evaluations indicated that 1-TDC initiated the partial reformation of the soft and bone tissue lost to the periodontal inflammation. Although the data show that 1-TDC has the capacity of inhibiting cytokine release from monocytes in vitro, 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 have significant clinical potential.

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**REFERENCES**


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**Figure 1.** Timeline of experimental design. 3-0 silk ligatures were tied to second premolars in mandibular quadrants at baseline in all groups and *P. gingivalis* was applied in methylcellulose slurry three times per week (M-W-F) for six weeks. At six weeks, 3 animals were sacrificed and the extent of disease determined as baseline periodontitis (represented by black bar in the following figures). *P. gingivalis* application was ceased at this point and the other groups continued for a second six-week with treatment. The treatments were 1-TDC (100mg/ml) and vehicle (mineral oil), which were compared to no treatment. All groups were sacrificed at 12 weeks and the extent of disease determined for comparison to baseline periodontitis and health.

**Figure 2.** Soft tissue changes after treatment with 1-TDC. Periodontitis was induced in New Zealand White Rabbits with 3-0 silk ligature and topical application of the human periodontal pathogen *Porphyromonas gingivalis* for 6 weeks (baseline periodontitis). Periodontal disease was obvious with all characteristics of human periodontitis including soft tissue inflammation (baseline periodontitis) (red arrows depict the localized bone loss). Following 6-weeks of treatment, topical application of 100 mg/ml of 1-TDC three times per week resulted in restoration of tissues, while the periodontal disease significantly progressed in the no treatment and placebo groups (panel A) (green arrows depict the restored bone). Pocket depth was directly measured on harvested jaws (see methods). 1-TDC treatment stopped the disease progression and resulted in soft tissue attachment gain, while other treatment groups showed progression characterized by increased pocket depth (panel B; *=P <0.0001).

**Figure 3.** Bone gain after 1-TDC application. After disease induction over a 6 weeks period (Phase 1) periodontal disease was obvious with all characteristics of human periodontitis including hard tissue destruction (baseline periodontitis). Following 6-weeks of treatment, topical application of 100 mg/ml of 1-TDC three times per week arrested the bone loss (depicted by green arrows), while the periodontal disease progressed in the no-treatment and placebo-treated groups as characterized by increased bone loss (panel A) (depicted by red arrows). Alveolar bone loss for all animals was directly measured on defleshed jaws (see methods). Results indicated that 1-TDC treatment not only stopped the disease progression but also resulted in bone gain (*=P <0.05), while no treatment and placebo groups had significant disease progression characterized by more bone destruction compared to baseline periodontitis (panel B; *= P <0.05).

**Figure 4.** Radiographic analyses of hard tissue components. Radiographic images reveal the bone loss observed in the no treatment and placebo groups (indicated by red arrows) and the bone gain (indicated by green arrow) 1-TDC-treated group compared to baseline periodontitis (panel A). Baseline periodontal disease detected at 6 weeks displayed 30% bone loss (panel B). The ligature alone group in panel B was included from the historical data to indicate the changes in periodontitis groups where *P. gingivalis* used for disease induction. Analyses of radiographic images demonstrated that 1-TDC treatment stopped the progression of bone loss and further resulted in bone gain (*=P <0.05), while those treated with placebo and left untreated progressed with 3% and 5%, more bone loss, respectively, compared to baseline periodontitis (panel C).

Figure 5: Histological analysis and histomorphometric quantification of changes in *P. gingivalis* induced periodontal inflammation and in response to different treatments. Histologic analysis of H&E-stained sections demonstrated clearly diminished inflammation and alveolar bone loss in 1-TDC-treated sections compared to increased inflammatory cell infiltration (depicted by asterisks) in no treatment and placebo groups (panel A). Inflammatory infiltrate was quantified using a modified grading system at three areas of the root (coronal, middle and apical), where 1-TDC has resulted in less inflammatory cell infiltration compared to increased inflammatory cell infiltrate in no treatment and placebo groups (panel B; *=P <0.05). Quantification of histomorphometric changes also revealed that 1-TDC was capable of reducing the bone loss as a result of controlling inflammation compared to baseline periodontitis, no treatment and placebo groups (panel C; *=P <0.05) (magnification at 200×).
Figure 6. Osteoclastic activity by TRAP staining. TRAP-stained sections showed resorbing bone lacunae containing large numbers of osteoclasts in baseline periodontitis, no-treatment, and placebo groups (depicted by arrows) whereas 1-TDC treated specimens contained few or no detectable TRAP positive cells (panel A). TRAP positive cells were counted using Image-Pro Plus software program. 1-TDC significantly reduced osteoclastic activity compared to all other groups (*=P <0.05) where no treatment group showed a dramatically increased osteoclastic activity compared to the baseline periodontitis (panel B; *=P <0.05) (magnification at 200×).

Figure 7: Osteoblastic activity as a marker of bone formation. Osteoblast activity was also evaluated by immunohistochemistry in sections stained with osteocalcin as a marker of new bone formation. Dense osteoblast-like cells (depicted with asterisks) are visible on the surface of the bone in 1-TDC treated areas (panel A). In contrast, resorptive areas with almost no intra- or extracellular osteocalcin activity were detected on bone surfaces in no treatment and placebo groups (depicted by arrow) (magnification at 200×). Osteoblast density (cells per mm²) was also quantified using Image-Pro Plus software program at three levels (coronal, middle, apical) (magnification at 200×). The 1-TDC treated samples showed greater osteoblast density at all levels compared to all other groups (panel B; *=P <0.05).

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† Anased®, LLOYD laboratories, Shenandoah, Iowa.
‡ Ketaset®; Fort Dodge Animal Health, Fort Dodge, Iowa.
§ Sharpoint, Surgical Specialties, Corp., Reading, PA.
**Isoflurane, Hospira, Inc., Lake Forest, IL.
††Sigma-Aldrich Co., St. Louis, MO.
‡‡Imagenetix Inc., San Diego, CA.
§§Sigma-Aldrich Co., St. Louis, MO.
***Pentobarbital Euthanasia-5 Solution; Veterinary laboratories, Inc., Lenexa, Kansas.
†††Image-Pro Plus 4.0, Media Cybernetics, Silver Spring, MD.
‡‡‡Schick Technologies Inc, Long Island City, NY.
§§§Immunocal®; Decal Corporation, Tallman, NY, USA.
††††Abcam Inc. Cambridge, MA.
‡‡‡‡Kirkegaard and Perry Laboratory, Gaithersburg, MD.
Figure 1

Silk ligatures tied around 2nd premolars (bilateral) + P. gingivalis (10^8 CFU) application

Stop P. gingivalis application + Begin 1-TDC or placebo (mineral oil) therapy

Sacrifice

Baseline

Six Weeks

Twelve Weeks

P. gingivalis application every other day

Treatment applications every other day
Figure 2

A

Baseline Periodontitis  No Treatment  Placebo  1-TDC

Buccal

Lingual

B

Pocket depth (mm)

0  1  2  3  4  5  6  7  8  9  10

Baseline Periodontitis  No Treatment  Placebo  1-TDC
Figure 3

A

Buccal

Baseline Periodontitis
No Treatment
Placebo
1-TDC

Lingual

B

Bone Loss (mm)

Baseline Periodontitis
Placebo
No Treatment
1-TDC

* * * # # # #
Figure 4

A. Baseline Periodontitis, No Treatment, Placebo, 1-TDC

B. Bar graph showing bone loss (%)

C. Bar graph showing post-treatment bone gain (%)

Legend:
- * indicates significant difference
- 1-TDC
- Placebo
- No Treatment
Figure 6

A

Periodontitis  No Treatment  Placebo  1-TDC

B

Osteoclast density (cells/mm²)

Periodontitis  No Treatment  Placebo  1-TDC

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Figure 7

A

No Treatment  Placebo  1-TDC

B

Osteoblast Density (cells/mm²)

Periodontitis  No Treatment  Placebo  1-TDC

Coronal  Middle  Apical

*