

# Inhibition of matrix metalloproteinase activity by a transmucosal patch containing botanical compounds

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## Abstract

**Objectives:** The goal of the study was to evaluate a transmucosal gingival patch (PerioPatch®) containing a combination of botanical extracts. The PerioPatch® has demonstrated a clinical reduction of inflammation and the promotion of healing by reducing inflammatory exudates. The PerioPatch® has also been found to reduce both cellular matrix metalloproteinase (MMP) production and overall MMP activity. A proof-of-concept clinical study was conducted to determine the effect of PerioPatch® on tissue MMP-9 levels and gingival inflammation.

**Methods:** Dry hydrogel patches (PerioPatch®) containing a proprietary botanical extract blend were applied to inflamed gingival tissue. Twenty patients scheduled to undergo periodontal surgery were randomized for treatment with active or placebo patches 24 hours prior to surgery. Gingival inflammation was measured at baseline and 24 hours later, immediately prior to surgery. Tissue levels of MMP-9 were measured in biopsy samples taken during surgery. In-vitro assays were also performed to determine the effect of PerioPatch® on the activity of purified MMP-2 and MMP-9. Fibroblast production of tissue inhibitor of metalloproteinase 3 (TIMP3) activity was also measured by using reverse zymography, as was absorptive capacity of PerioPatch®.

**Results:** There was a significant decrease in tissue levels of MMP-9 within 24 hours after topical administration of the test treatment, as compared to placebo. In a separate study, PerioPatch® was shown to have increased absorptive capacity when compared to placebo patches. In the cell-free assay, the enzymatic activity of MMP-2 and of MMP-9 was directly reduced by the PerioPatch® botanical blend. In addition, fibroblasts exposed to the PerioPatch® were induced to produce TIMP3.

**Conclusions:** This pilot clinical study, which was supported by in-vitro assay results, demonstrates that PerioPatch® can reduce inflamed gingiva and enhance tissue healing by absorbing inflammatory exudates, resulting in a reduction of MMP activity and an induction of TIMP3 activity.

## Introduction

More than 80% of people over the age of 65 years living in the United States suffer from periodontitis [1]. Periodontal diseases are bacterially induced, chronic inflammatory conditions that lead to destruction of soft and hard tissue supporting the teeth. Current understanding of the pathogenesis of periodontitis has shifted away from viewing the bacterial plaque as the primary factor driving disease, to a host-response centered view. The complex immuno-inflammatory response to bacterial plaque, together with genetic and environmental factors that modulate response, is ultimately responsible for disease outcome [2].

## Issues with current therapeutic options for periodontitis

Despite this paradigm shift, currently available therapeutic options for managing the host immuno-inflammatory response in the oral cavity are limited. Low-dose doxycycline requires systemic administration and long-term therapy (>3 months) to reduce matrix metalloproteinase (MMP) levels and to effect changes in the periodontal tissue [3]. Topical anti-inflammatory agents, such as COX-inhibitors, have been shown to irritate mucosal tissue [4], and repeated application of topical steroids raises the risk of infection [5] and reduces the capacity for normal tissue repair. The absence of safe topical agents to control oral mucosal inflammation stands in stark

contrast to the modalities available to manage inflammation when present in other topically accessible sites such as the skin, eyes, or rectum.

The tissue destruction that results from gingival inflammation is induced by host enzymes that damage and degrade the extracellular matrix (ECM). Chief among these enzymes are the MMPs, which are zinc-dependent endopeptidases capable of degrading the collagen and elastin of the ECM. MMP expression is activated by zinc-dependent pro-inflammatory cytokines, such as IL-1 $\beta$ , all of which are involved in the progression of periodontitis [6,7]. The proteolytic activity of MMPs is regulated by tissue inhibitors of metalloproteinases (TIMPs) [8,9] so that remodeling and repair can occur [10]. In pathological states of periodontal inflammation, many studies have shown that the balance between MMPs and TIMPs is disrupted, resulting in increased MMP activity [6,11-13]. MMP-1, -2, -3, -4, -7, -8, -9, and -13

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have been detected in the gingival tissue or crevicular fluid of patients suffering from periodontal disease [14]. Significantly, the tissue or crevicular levels of MMPs increase in experimental gingivitis [15] and decrease in response to periodontal therapy or treatment with low-dose doxycycline [6,16].

## Use of wound dressings

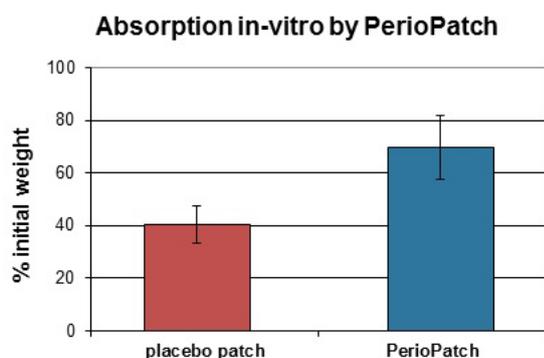
### Mode of action

In general, the activity of wound dressings is based on their physical action, such as protection of inflamed tissue or absorption of fluids from inflammatory sites. This mechanical action can generate physiological changes in the tissue that can be demonstrated clinically and quantified in in-vitro assays.

### PerioPatch®

Among them, PerioPatch® (Izun Pharmaceuticals, <http://izunoralcare.com>) is a hydrogel wound dressing for use in the oral cavity. It is intended for the management of all types of oral wounds, injuries, and ulcerations of the gingiva and oral mucosa. They include stomatitis, minor chafing, and traumatic ulcers, as well as abrasions caused by orthodontic appliances and ill-fitting dentures, and lesions associated with oral surgery. The patch is self-adhesive after contact with the moist tissue of the oral cavity and provides an absorbent and flexible barrier over the affected area. As such, the patch protects inflamed areas and lesions in the mouth from contact with the rest of the oral environment, thereby preventing irritation and painful aggravation of the affected area. The patch absorbs the inflammatory enzymes and allows for natural resolution of the inflammation. This action has been observed clinically, as well as in in-vitro assays not yet published.

The primary effect of PerioPatch® is therefore achieved by physical means, i.e., removal of toxic exudates and protection of healing tissue. There are two aspects of PerioPatch® that influence the absorption of exudates: patch adhesion and the absorptive capacity of the patch. It has also been demonstrated in unpublished studies that the adhesive properties of the basic patch are enhanced by the inclusion of the botanical compounds into the patch. A series of in-vitro experiments were performed, and results indicate that the patch, in the absence of the botanical ingredient, absorbs up to 40% of its initial weight in aqueous fluid; the same patch containing the botanical ingredient absorbs an average 70% of its initial weight in aqueous fluid (Figure 1). This double absorption effect, that of the patch itself augmented by that of the botanical ingredient, is essential for the effective removal



**Figure 1.** In-vitro absorption of fluids by PerioPatch®. Note a close to doubling of absorptive capacity upon inclusion of botanical extracts in PerioPatch® when compared to that of the placebo, an identical patch lacking the botanical ingredient.

of exudates secreted during an inflammatory response of the gingiva.

## PerioPatch® proof-of-concept study

### Background: Botanical extracts and the inflammatory response

Botanical extracts are a known source of bioactive compounds that can inhibit or exacerbate the inflammatory response [17]. They have also been shown to aid in absorption of pro-inflammatory mediators and, as such, support the natural resolution of the inflammation. Since extracts contain multiple chemical compounds, careful evaluation of their effects in preclinical models and in controlled clinical trials is critical. It is believed by researchers involved in the development of PerioPatch® that this treatment could provide a safe and effective means of controlling gingival inflammation topically, providing a needed adjunct for the management of periodontal disease.

### Study objectives

A randomized, placebo-controlled, proof-of-concept clinical trial was conducted to evaluate the capacity of topical treatment with PerioPatch® to reduce tissue MMP levels. This study also investigated whether MMP-2 and MMP-9 activity could be inhibited by a combination of *Echinacea purpurea*, *Centella asiatica*, and *Sambucus nigra*, formulated into an absorptive matrix. In addition, fibroblasts were exposed to PerioPatch® plant extracts to establish whether the production of the MMP inhibitor TIMP3 could be induced.

## Materials and methods

### PerioPatch® matrix

Ethanol/water extracts of the plants *C. asiatica*, *E. purpurea* and *S. nigra* were produced in conformance with European Pharmacopeia standards for analysis and purity, and ultimately incorporated into a dry hydrogel matrix that adheres and conforms to gingival tissue.

### In-vitro assays

**Gelatin zymography:** These experiments were based on gelatin zymography, a cell-free assay whereby 0.06 µg of human MMP-2/9 standards (Chemicon International, [www.chemicon.com](http://www.chemicon.com)) were incubated with increasing concentrations of the extract, ranging from a total of 0.125 mg/ml to a total of 2 mg/ml of extract for 1 hour at 37°C. Samples were mixed with Novex TRIS-Glycine SDS Sample Buffer (2X) (ThermoFisher Scientific, [www.thermofisher.com](http://www.thermofisher.com)) at a dilution factor of 1/100 and loaded onto Novex 10% Zymogram (0.1% gelatin) Gels. Electrophoresis was carried out at a constant current of 40mA and a constant voltage of 125V for 2 hours. Novex TRIS-Glycine SDS Running Buffer (10X) was also used as the electrophoresis buffer. After electrophoresis, the gels were soaked in 2.7% Triton X-100 made up in ddH<sub>2</sub>O for 2 hours and incubated at 37°C overnight in Novex Developing Buffer (10X). The gels were stained for 1 hour in Brilliant Blue R (Sigma-Aldrich, <http://www.sigmaaldrich.com>), and destained for 2 hours in 10% glacial acetic acid prepared in 30% methanol. Enzyme activity was quantified using an F-ChemiBIS 1.6 gel documentation system (DNR Bio-Imaging Systems, <http://dnris.com>). All Novex products mentioned above were obtained from Invitrogen (ThermoFisher Scientific).

**Reverse zymography:** Tissue Inhibitor of Metallo-Proteinase 3 (TIMP3) activity was assayed using reverse zymography with a Reverse Zymography Kit developed by Dylan Edwards, PhD, University of East Anglia, UK. HLF fibroblasts were seeded at a density of  $2.5 \times 10^5$  cells/

well in six-well plates and cultured for 24 hours. Growth medium was replaced with starvation medium (DMEM 4mM glutamine, 1 mM sodium pyruvate, 100 IU/mL penicillin, 0.1 mg/mL streptomycin, and 0.1% BSA) with or without transforming growth factor-beta (TGF- $\beta$ ; R&D Systems, www.rndsystems.com) or test extract for an additional 24 hours. Conditioned medium was removed from each well, vacuum-dried, and re-suspended in 400  $\mu$ l of 0.1% SDS. Samples were electrophoresed on a 12% SDS-PAGE gel that incorporated gelatin and recombinant MMP-2. The gels were then washed in rinse buffer (50 mM Tris, 5 mM CaCl<sub>2</sub> and 25 g Triton-X 100) for 2.5 hours, followed by incubation buffer (rinse buffer without Triton-X 100) at 37°C overnight. The gels were stained in Coomassie Blue (Bio-Rad) for 2.5 hours and destained until a bright blue band was obtained against a clear background. The image was captured with a CCD camera (DNR Bio-Imaging Systems). Densitometry was performed with TotalLab software (Nonlinear Dynamics, <http://www.nonlinear.com>).

**Absorption by PerioPatch®:** PerioPatch® samples were weighed, using an analytical balance. Measured volumes of ¼N saline were applied to the adhesive surface of the patch sample, which was then incubated for 60 minutes at 37°C, 80% relative humidity. After incubation, excess saline was carefully removed using Whatman filter paper (qualitative #1), and the weight of each sample before and after saline absorption was calculated and compared to that of a placebo patch containing no botanicals.

## PerioPatch® clinical trial

### Patient population

Twenty patients between 18 and 75 years of age who were scheduled for periodontal surgery were enrolled in a placebo-controlled, matched-sample study to determine whether topical treatment with a dry hydrogel patch containing a blend of *E. purpurea*, *C. asiatica*, and *S. nigra* extracts would result in a decrease in the tissue levels of relevant MMPs. Trial patients had similar periodontal disease conditions bilaterally and were scheduled to undergo two separately scheduled periodontal surgeries on opposite sides of the mouth within 3 to 4 weeks of each other. Subjects had undergone several courses of routine periodontal treatment consisting of scaling and root planing prior to the decision to proceed surgically. Acceptance criteria included gingival index (GI) scores greater than 1, as assessed by the method of Loe and Sillness [18], and at least two quadrants with three or more periodontal pockets between 5 mm and 12 mm.

### Procedures

Treatment with the experimental patch was compared to treatment with a similar placebo patch that did not contain the botanical extracts. The patch was applied to the buccal aspect of the gingiva three times during the 24 hours preceding the surgical procedure. The first patch was applied by a periodontist, and the following applications were performed by the patient according to instructions from the periodontist.

Patients' GIs were measured before placement of the first patch and 6 to 8 hours after placement of the third patch at the time of the periodontal surgery, at which time tissue biopsies were taken. Both the patient and the individual who performed clinical site assessment and evaluation of tissue samples were blinded as to whether the patch was the test or the placebo patch.

Gingival biopsy specimens were obtained from the buccal side (site of the patch) and the palatal side, with the palatal site used to assess

whether the patch had an impact at tissue sites distant from the patch itself. Excised tissue was frozen and stored until analyzed. After a washout period of at least 3 weeks, the same procedure was repeated on the second side. Although the patch's identity was known to neither the examiner nor the patient, the initial patch for all subjects was placebo, with the second patch for all subjects being the PerioPatch®. The reason for this was to control for the possible therapeutic effect of the PerioPatch® on the contralateral side. Approval to perform the study was obtained from the Institutional Review Board of Shaare Zedek Medical Center (Jerusalem, Israel), and all participating subjects provided informed written consent before the start of the study.

### Determination of MMP-9 levels in biopsy samples

Gingival biopsy specimens were homogenized in PBS, and the concentration of protein per sample was determined by Bradford assay using bovine serum albumin as a standard. Metalloproteinase activity was assayed by gelatin zymography, using 15% gelatin-containing gels as described above. Samples were applied to the gels under non-reducing conditions without heating. Gels were then soaked in 200 ml of 2% Triton X-100 in distilled water on a gyratory shaker for 30 minutes at 20°C. They were then incubated in developing Tris buffer, pH 8.0, for 15 hours at 37°C. Finally, gels were stained and washed according to a protocol described previously by Korostoff *et al.* in 2000 [19]. Gels were photographed and scanned, and activity was measured using NIH Image (<http://rsb.info.nih.gov/nih-image>). For purposes of comparison within the same gel, one sample band was arbitrarily established as having a value of 1 (Group 3), and the band values of the other samples were calculated relative to that sample based on densitometric measurements of the clear band areas.

### Statistical analysis

In terms of clinical evaluation of periodontitis, GI scores were compared before and after treatment with the patch using McNemar's non-parametric test [20] to analyze the nominal data. To confirm the robustness of the analysis, Wilcoxon Signed rank test [21] was applied, as the ordinal scale for GI is limited, and the odds ratio was then calculated. To test for differences in the in-vivo assays for MMP-9, the Fisher Exact test [22] was applied because the sample size was small.

## Results

### Absorption by PerioPatch®

As can be seen in Figure 1, addition of the botanical ingredient to the patch formulation almost doubled the absorptive capacity of the patch.

### Direct inhibition of enzyme activity

To measure the inhibitory properties of botanical extracts, different dilutions of extracted PerioPatch® matrix (PPM) were mixed with gelatinase zymography standards of human MMP-2/9 for 1 hour and electrophoresed on gelatin gels. The resulting zymograms showed a concentration-dependent reaction where, at the highest concentration of 2 mg/ml of PPM, the MMP-2 activity was reduced to 28% that of the control, while MMP-9 activity was almost completely inhibited. At this extract concentration, the pro-MMP-9 activity was reduced to 34% of the control (Figure 2).

### Induction of fibroblast TIMP3 production

To confirm induction of TIMP3 by PPM, fibroblasts were incubated with the matrix extract, with or without TGF- $\beta$  added to the growth

medium. The induced protein was visualized using a reverse zymogram technique. The treatment of gingival fibroblasts with PPM induced production of active TIMP3 markedly more than did induction with TGF-β. At a concentration of 1.5 mg/ml, the extract was two- to three-fold more effective as an induction agent than was TGF-β and, at even lower extract concentrations, the generation of TIMP3 exceeded that induced by TGF-β alone (Figure 3).

### Treatment with extract-impregnated patch reduces GI

In terms of safety, there were no serious adverse events reported in this proof-of-concept study, which included 20 patients scheduled for periodontal surgery who were undergoing biopsies. The total of five adverse events reported by four patients were mild in severity. GI scores were obtained in the area where the patch was placed, and an average score was given per visit. A statistical comparison of the GI scores assessed at the area of the placebo and the PerioPatch® were significantly different ( $P = 0.039$ ). The odds ratio was 2.077 (95% confidence interval 1.035 - 4.384), confirming that PerioPatch® had a positive effect in lowering GI scores (Figure 4).

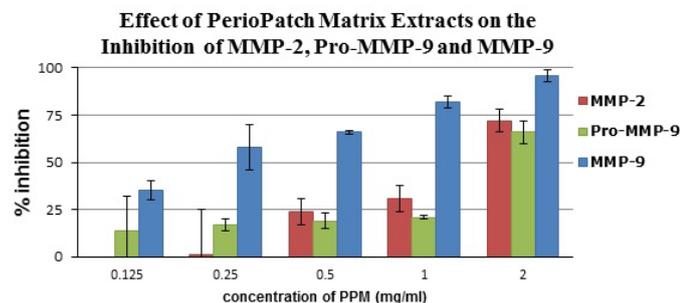
### Application of extract-impregnated patch reduces MMP-9 in vivo

MMP-9 protease activity was measured in biopsy specimens obtained from subjects prior to surgery. Overall, 78.6% biopsy samples in which PerioPatch® was placed had less MMP-9 activity than the areas where the placebo patch was placed. The results of the enzyme

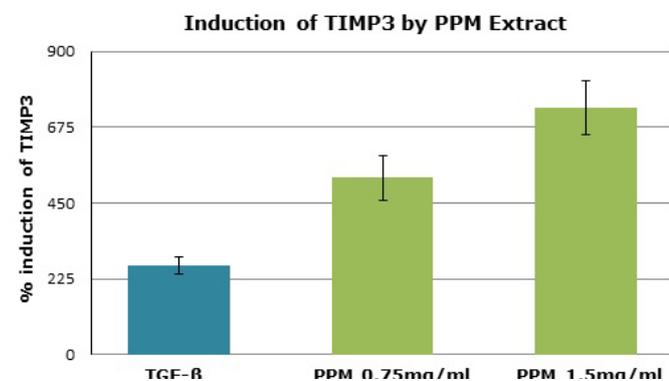
assays were analyzed using two different statistical tests. The Wilcoxon signed rank test [21] was used as a non-parametric test for the case of two related samples or repeated measurements on a single sample. A significant statistical difference was seen in the results of the MMP-9 assay between those samples collected after application of PerioPatch® and the samples obtained after the placebo patches had been on for 24 hours ( $P = 0.017$ ). Similarly, a Fisher Exact test, [22] a contingency table test for small samples, showed that PerioPatch® significantly inhibited the production of MMP-9 ( $P = 0.028$ ) while, for biopsy material taken after the application of the placebo, no inhibition of the enzyme was observed (Figure 5).

### Discussion

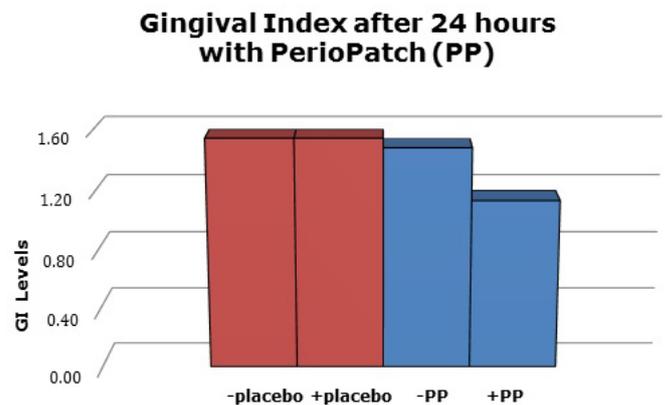
Oral pathogens are capable of triggering tissue destruction by inducing the host cells to increase release of matrix metalloproteinase (MMP), which is one of the mechanisms of indirect tissue destruction and tooth loss. Matrix metalloproteinases are zinc-dependent endopeptidases predominantly derived from polymorphonuclear leucocytes during acute stages of periodontal disease and are the key



**Figure 2.** Inhibition of metalloproteinases MMP-2, MMP-9, and Pro-MMP-9 by PPM extracts, as measured on gelatin zymography gels. Note a concentration-dependent reaction where, at the highest concentration (2 mg/ml of PPM extract), the MMP-2 activity was reduced by 78%, while MMP-9 activity was almost completely inhibited. At this concentration of extract, the pro-MMP-9 activity was reduced by 66%.

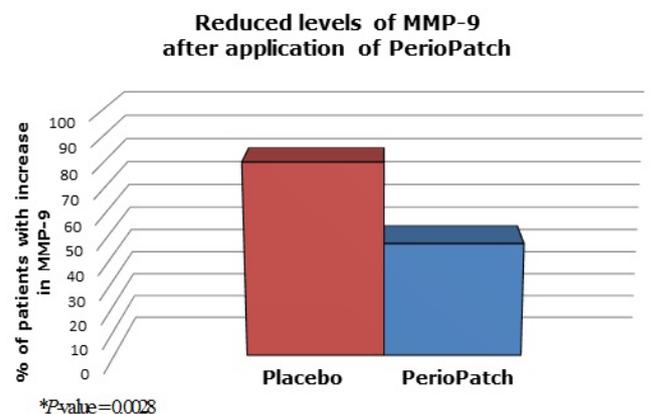


**Figure 3.** TIMP3 induction in HLF fibroblasts incubated with or without PPM extract and measured by reverse zymography. The treatment of gingival fibroblasts with the PPM extracts induced production of active TIMP3 markedly more than did induction with TGF-β. At a concentration of 1.5 mg/ml, the extract was two- to three-fold more effective as an induction agent than was TGF-β and, at even lower concentrations of PPM extract, the generation of TIMP3 exceeded that induced by TGF-β alone.



\* $P$ -value = 0.039

**Figure 4.** Gingival index of 20 patients before and after treatment with a placebo patch or PerioPatch®. A statistical comparison of the GI scores assessed at the area of the placebo and of the PerioPatch® were significantly different ( $P=0.039$ ). The odds ratio was 2.077 (95% confidence interval 1.035-4.384), confirming that PerioPatch® had a positive effect in lowering GI scores.



\* $P$ -value = 0.0028

**Figure 5.** MMP-9 levels in patients treated with placebo or with PerioPatch®. MMP-9 protease activity was measured in biopsy specimens obtained from subjects prior to surgery. A Fisher Exact test showed that PerioPatch® significantly inhibited the production of MMP-9 ( $P = 0.028$ ) while, for biopsy material taken after the application of the placebo, no inhibition of the enzyme was observed.

enzymes responsible for extracellular collagen matrix degradation. A significant increase in MMP-9 enzyme activity and TIMP induction has been reported in patients suffering from inflamed gingival disease [23].

In this proof-of-concept study, PerioPatch® had a dramatic effect on the gingival index of the individuals in the study group. PerioPatch® was applied three times over a 24-hour period and, on visual inspection of the gingiva, the signs of periodontal disease had abated in many of the patients. A placebo patch that had been applied in a similar manner 3 weeks earlier had no effect on the GI. The etiology of the inflammation had already been previously removed, and the tissues were in a stagnated, chronically inflamed state; yet, with PerioPatch®, there were substantial positive changes. The rapid improvement of the inflammatory lesions, as measured by the reduction in GI within a 24-hour period, was significant.

The gingival biopsies taken from both the buccal and palatal sides were analyzed for MMP-9 activity after application of both the placebo patch and PerioPatch®. In the biopsies obtained after the treatment with PerioPatch®, MMP-9 activity was sharply reduced in a majority of the samples. The major effect of PerioPatch® appears to be its ability to remove pro-inflammatory exudates from the affected area, with the botanical ingredient greatly enhancing this effect.

An additional effect of the botanicals can be observed in the *in vitro* study, wherein direct inhibition of the two gelatinases, MMP-2 and MMP-9, was achieved by incubating the enzymes with PPM. The highest inhibition was observed when 2 mg/ml of PPM was used, resulting in 96% and 72% reduction in MMP-9 and MMP-2 enzyme activity, respectively. PPM inhibited the enzyme activity in a concentration-dependent manner.

TIMPs regulate the activities of MMPs, and an imbalance between MMPs and TIMPs results in the pathological tissue destruction observed in periodontitis. The constitutive expression and evolutionary conservation of TIMP3 indicate its important function. TIMP3 induction by the cytokine TGF- $\beta$  suggests that the role of this factor and TIMP3 in cartilage remodeling has important implications for periodontal disease. Induction of this natural inhibitor to improve the imbalance would aid the healing process. In this study, PPM induced TIMP3 far more efficiently than did TGF- $\beta$ , even at the lower concentration of 0.75mg/ml of test material, demonstrating yet another effect of this patch.

Long-term longitudinal studies of patients with periodontal disease demonstrate that, despite receiving constant maintenance therapy, they suffer from continued periodontal attachment loss in areas of persistent inflammation [24,25]. This clearly indicates the need for additional modalities of therapeutic intervention to control the inflammatory process. PerioPatch® could be used to augment current therapies, as well as to improve treatment outcomes and the success of long-term maintenance therapy. In addition, it has a strong role to play in supportive therapy in any indication where reducing inflammation and localized tissue destruction through the MMP pathway would improve the clinical situation or assist in patient management.

## Conclusion

While inflammation is necessary to initiate repair of injured tissue, when dysregulated, it can damage tissue and prevent normal healing. Thus, the effective management of inflammation such as that demonstrated in chronic inflammatory disease—including gingivitis and periodontitis—is critical to promote healing. This is particularly

the case after surgical procedures such as implant placement, periodontal surgery, ridge augmentation, and extractions.

The available topical approaches to managing inflammation in the oral environment have been associated with adverse events, including irritation of the mucosal tissue, increased risk of infection, and reduction in normal tissue repair. In contrast, the topical herbal patch described (PerioPatch®) demonstrated reduction of inflammatory cell activity in the crevicular fluid and significant reduction of the MMP activity in the gingiva—all without serious adverse events.

This clinical trial suggests that botanical extracts offer a promising approach to topically reducing inflammation and promoting healing after surgical procedures and may have numerous additional applications in oral care as well.

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