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Original Article

The effect of adjunctive low-dose doxycycline and licorice therapy on gingival crevicular fluid matrix metalloproteinase-8 levels in chronic periodontitis

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ABSTRACT

Background: This study compared the effect of adjunctive low dose doxycycline and licorice on gingival crevicular fluid (GCF) matrix metalloproteinase-8 (MMP-8) levels in patients with chronic periodontitis.

Materials and Methods: In this *in vivo*, experimental study 39 patients with mild to moderate chronic periodontitis were selected. Samples of GCF were collected from three deepest pockets and MMP-8 concentration was measured. Patients were divided into three groups (n = 13). Groups were treated with doxycycline, licorice and placebo. Sampling and measurement of MMP-8 was repeated after 6 weeks. Data was analyzed by t-paired and ANOVA test. $P < 0.001$ was considered significant.

Results: The decrease in mean of MMP-8 concentration was higher in doxycycline and licorice group in comparison with the placebo group and the difference was statistically significant (P value < 0.001). The decrease in mean of MMP-8 concentration was higher in licorice group than doxycycline group, but the difference was not statistically significant.

Conclusion: The present study showed that licorice extract can prevent the production of MMPs by host cells and can be as useful as antibiotics like doxycycline to cure periodontal and other inflammatory diseases. It must be added that no side-effects were observed in usage of licorice extract.

Key Words: Chronic periodontitis, doxycycline, gingival crevicular fluid, licorice, matrix metalloproteinase enzymes-8

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INTRODUCTION

There are two major etiological factors involved in the pathogenesis of periodontitis. The first is the microbial component[1-3] and the second factor is the host response to periodontopathogens, notably the over production by resident and immune cells of inflammatory mediators and matrix metalloproteinases (MMPs), which can modulate the progression and severity of periodontitis.[1,4] MMP enzymes are a family of neutral proteases that are Zinc/Calcium-dependent with an essential role in the extracellular matrix (ECM) turnover and degradation. MMPs are capable of degrading virtually all components of ECM and increased levels have been associated with periodontal disease in both humans[5-8] and animal models.[9,10] The relevance of MMPs to the pathogenesis of periodontal disease is supported by the decrease in bone loss associated with their nonselective inhibition in animal models of the periodontal disease[11,12] and especially by the improved clinical results observed after periodontal treatment associated with systemic inhibition of MMPs by a sub-antimicrobial dose of doxycycline.[13-16] The concentration of MMPs and their activity is significantly higher in gingival tissue

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and gingival crevicular fluid (GCF) of patients with periodontal diseases compared to ones with healthy periodontium and there is a strong relation between high amounts of MMP-8 concentration in gingival tissues and probing pocket depth, clinical attachment loss and bleeding on probing.[17,18] Longitudinal studies have shown a reduction in amount and activity of MMP-8 in GCF after successful periodontal treatment.[19]

The treatment for periodontitis is aimed at removing dental plaque and calculus and surgical methods.[20,21] The administration of local and systemic antibiotics, anti-inflammatory drugs, or sub-anti-microbial low dose doxycycline has been reported to provide additional benefits.[15,22] Sub-antimicrobial-dose doxycycline is a 20-mg dose of doxycycline (Periostat) that is Food and Drug Administration (FDA) approved and indicated as an adjunct to scaling and root planning (SRP) in the treatment of chronic periodontitis. The 20-mg dose exerts its therapeutic effect by enzyme, cytokine and osteoclast inhibition rather than by any antibiotic effect. Research studies have found no detectable antimicrobial effect on the oral flora or the bacterial flora in other regions of the body and have identified clinical benefit when used as an adjunct to SRP.[15]

Licorice root (radix Glycyrrhizae) is obtained from perennial plant native to Mediterranean countries, central to Southern Russia and certain regions of Asia.[1]

Evidence for a therapeutic application of licorice in oral disease has been reported. Bodet *et al*. showed that human macrophages pretreated with a licorice extract prior to being stimulated with *Aggregatibacter actinomycetemcomitan* or *Porphyromonas gingivalis* lipopolysaccharide (LPS) secrete significantly less pro-inflammatory cytokines, indicating that the extract has an anti-inflammatory property.[1,23]

Since there was no available research, the aim of present study was to compare the therapeutic effect of licorice and doxycycline in periodontitis.

MATERIALS AND METHODS

This experimental study, which is registered in Iranian Registry of Clinical Trials under number IRCT2012121611771N1, was performed on 39 patients with chronic mild to moderate periodontitis. Patients were diagnosed by a periodontist according to clinical examination, radiographic evaluation and their medical and dental history. Patients with any cardiac, renal or infectious systemic diseases did not enter the research. None of the patients either used anti-inflammatory or antibiotic drugs during the last 6 months. None of the patients had undergone scaling and root planning or periodontal surgeries in the last 6 months. Smokers, alcoholics, pregnant, breast feeder patients, patients allergic to antibiotics, patients with poor oral hygiene or patients with poor compliance and patients whom used any herbal drugs with effect on MMP within the last year were excluded from the research.

Patients were asked to wash their mouths. After drying the site and proper isolation, sampling was performed from three deepest pockets (two posterior and one anterior). A #25 paper point was placed in the gingival sulcus of each selected pocket and was removed after 4 min and was reserved in a test tube full of Normal Saline as a medium. Test tubes were kept in an especial box filled with dried ice with ≤20°C temperature and carried to the laboratory. Patients were educated about oral hygiene and scaling and root planning were performed after that. Patients were accidentally divided into three groups (n = 13). Group A were given 20 mg capsules of doxycycline daily, Group B were given 400 mg tablets, containing 380 mg of Licorice and 8-12 mg Glandenic acid daily and group C were served as control and were given placebo tablets. A clinician, completely unaware of group divisions, did the sampling and clinical examination after 6 weeks exactly like the first time. The MMP-8 concentration was measured in the laboratory by means of an especial diagnostic kit (R and D Systems, USA). Samples were coded and entered laboratory process.

All reagents, working standards and samples were prepared as described above due to manufacturer guidelines

Reagent preparation

- Wash buffer: Is consisted of 2 vials of a 25-fold concentrated solution of buffered surfactant with preservatives. To dilute Wash buffer, 20 mL of it was added to deionized distilled water to prepare 500 mL of wash buffer.
- Substrate solution: It is consisted of substrates 1 and 2 available in the MMP8 kit. They were mixed together in equal volumes 2-30 min prior to use and protected from the light by aluminum foil. 50 µL of the resultant mixture was used per well.
- Standard: It is consisted of Standard Cocktail with Calibrator Diluent RD5-37. The standard was allowed to sit for 15 min with gentle agitation prior to making dilutions.

1. 50 µL of assay diluent RD2-1 was added to each well.
2. 50 µL of sample was added per well and the wells were covered securely with a plate sealer and incubated for 3 h at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. A plate layout was provided as a record of standards and samples assayed.
3. Each well was aspirated and washed repeatedly with 400 µL of wash buffer for a total of four washes. At each step the liquid was removed completely for a good performance. After the last wash, any remaining wash buffer was aspirated. The plate was inverted and blotted against clean paper towels.
4. 50 µL of the detection mix was added to all wells and securely covered with a plate sealer and the plate was incubated for 1 h at room temperature on the shaker set at 500 ± 50 rpm.
5. The washing step described in step 3 was repeated.
6. 50 µL of streptavidin-horseradish peroxidase was added to all wells and the plate was securely covered with a plate sealer and incubated for 30 min at room temperature on a shaker set at 500 ± 50 rpm.
7. The washing step described in step 3 was repeated.
8. 50 µL of substrate solution was added to each well.
9. All samples were analyzed with Microplate Reader STAT FAX 2100 (Awareness Technology, Inc. New York, USA) at 540 nm wave length.

To compare the mean of MMP-8 concentration before and after treatment *t*-paired test was used. A one-way ANOVA was used to compare the mean of change of MMP-8 among three groups. $P < 0.001$ was considered as significant.

RESULTS

The *t*-paired test showed no statistical significant difference in the mean of MMP-8 in GCF after interference in Placebo group, but a statistical significant decrease was observed in the mean of MMP-8 in both groups, which were given either licorice or doxycycline capsules (*P* value < 0.001) [Figures 1 and 2].

According to Figure 1 and 2 the decrease in the mean of MMP-8 in GCF in licorice group was more than doxycycline group, but this difference was not significant statistically.

The one-way ANOVA test showed no statistically significant difference in the mean of MMP-8 in GCF before interference in the three groups (*P* value = 0.406),

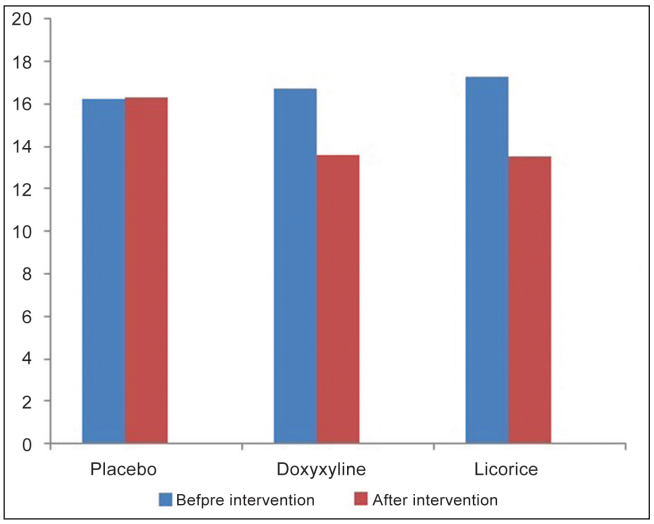

Figure 1: The mean of matrix metalloproteinase-8 in gingival crevicular fluid before and after interference

Drug	Before intervention		After intervention		P-Value
	Mean	SD	Mean	SD	
Licorice	17.31	2.3	13.5	2.8	<0.001
Doxycycline	16.69	1.6	13.6	2.5	<0.001
Placebo	16.22	2.6	16.35	2	0.796
P-Value	0.406		0.002		

Figure 2: The mean of matrix metalloproteinase-8 in gingival crevicular fluid before and after interference

but after interference, the mean of MMP-8 in GCF of licorice group and doxycylcine group was lower than the placebo group and this difference was statistically significant (*P* value = 0.002). It was also shown that the decrease in the mean of MMP-8 in GCF in the groups treated with licorice and doxycyline was more than the placebo group and this difference was statistically significant (*P* value < 0.001). However, there was no statistically significant difference between licorice and doxycycline groups (*P* value = 0.46)

DISCUSSION

Licorice is obtained from the unpeeled, dried roots and stolons of two different plants: *Glycyrrhiza glabra* and *Glycyrrhiza uralensis*. Both plants were well-studied and contain different metabolites. Due to United States FDA (21 Code of Federal Regulation [CFR] 184.1408; 310.528; 310.644; 310.545), licorice and its constituents are recognized as safe materials to be used in foods and as over-the-counter drugs.[24]

Periodontal diseases including periodontitis and gingivitis are chronic infections with two major

etiological factors including by gram-negative anaerobic bacteria and the interaction of these bacteria with host immune system. The most putative pathogens associated by periodontal diseases are *P. gingivalis, Tannerella forsythia* and *Treponema denticola* for chronic forms of periodontitis and *Aggregatibacter actinomycetemcomitans* in aggressive forms.[1]

In vitro studies have shown that licorice and its bioactive ingredients may have potential to be used as phytochemical drugs and can be used as a natural modality to cure periodontal disease.[1] It has been shown that licorice can affect both etiologic factors in periodontal diseases. An *in vitro* study had shown that *G. uralensis* can inhibit the growth and biofilm formation of *P. ginigivalis*.[1] Licorice can also affect the host inflammatory responses. Bodet *et al*.[23] found that pre-treatment of human macrophages with licorice extract before stimulating them with *A. actinomycetomcomitans* or *P. gingivalis* LPS decreases the secretion of pro-inflammatory cytokines (interleukin [IL]-1β, IL-6, IL-8 and tumor necrosis factor-α), which shows the anti-inflammatory effect of licorice on immunologic system.

Sasaki *et al*.[25] showed in an *in vitro* study that 18β-glycyrrhetinic acid (a metabolite of licorice) can suppress the LPS and receptor activator of nuclear factor kappa-B ligand (RANKL) induced phosphorylation of Nuclear Factor Kappa B (NF-KB) P105 and showed that licorice can modulate host immune system response.

La *et al*.[26] showed the inhibitory effect of licorice on production of MMP-7, 8, 9 and IL-6, 8 and declared that licorice extract can reduce the concentration of these inflammatory factors due to reduction in activity of NF-KB-P65 and its effect on inhibition of producing cytokines and MMPs. These findings were confirmed by the earlier researches showing that licorice extracts have anti-inflammatory properties by inhibiting NF-KB P65 from phosphorylation.[27,28]

The resorption of alveolar bone is a typical sign of periodontal diseases, which happens by the recruitment, differentiation and activation of osteoclasts. They attach to the bone surface and cause mineral dissolution of bone. Osteoclast-derived MMPs can degrade demineralized organic matrix of bone. A recent study had shown that isoflavonoid glabridin (a metabolite of licorice) can inhibit the differentiation of human osteoclast precursors into mature osteoclasts by inhibiting the RANKL-dependent differentiation pathway. An *in vitro* study also confirmed that glabridin is able to reduce bone degradation induced by mature osteoclasts.[1] Choi *et al*.[29] had earlier reported about the direct stimulatory effect of glabridin on bone formation by enhancement of osteoblasts proliferation.

Emingil *et al*.[30] in 2004 found out usage of low doses of doxycycline can reduce the amount of MMP-8 in GCF of periodontal patients. In another study Sorsa *et al*.[31] declared that therapeutic dosage of doxycycline can reduce the activity and amount of MMP-8 in ginigva, GCF and saliva of periodontal patients. Ashley [32] found out usage of 20 mg of doxycycline once or twice/day after phase one of periodontal treatment can reduce anti-collagenase activity and usage of the drug twice/day can even lead to more reduction in anti-collagenase activity. The same results were found in different researches.[33-36]

Górska and Nedzi-Góra[37] declared usage of 20 mg doxycycline twice/day for 3 months, can result in enhancement of clinical parameters like probing pocket depth, bleeding index in patients with severe periodontitis in comparison with routine periodontal treatment, but has no effect on concentration of MMP-8 in saliva. It must be added that scaling and root planning can reduce the concentration of MMP-8 due to reduction in the amount of bacteria responsible for migration of inflammatory cells and stimulation of MMP-8 secretion, the reduction in the amount of bacteria may reduce the inflammatory response of periodontal tissue after hygiene control, but the host response may not return to the normal status.[37]

Lee *et al*.[38] showed that the inhibitory action of MMP inhibitors can be enhanced by the addition of non-steroidal anti-inflammatory drugs in treatment of periodontal patients. This fact shows that treatment strategies that aim on preventing the periodontal apparatus from breakdown can be successful treatment modalities.[38]

The present study had shown that the decrease in the mean of MMP-8 in GCF in the groups treated with licorice and doxycyline was more than the placebo group and this difference was statistically significant, but there was no statistically significant differences found between licorice and doxycycline groups. This means the licorice extract can be useful in treating periodontal diseases due to its MMP-8 inhibitory property.

Some limitations of this study were the incompliance of patients during the study, since the time of drug

usage was long and some patients said they forgot to take some dosages of their drugs. According to previous *in vitro* and *in vivo* studies on therapeutic effects of licorice and the results of the present study, it seems legitimate to say that licorice extract is a herbal drug which doesn't have the side-effects of a chemical drug, so it seems reasonable to use it as an addition or a replacement to the chemical drugs used to treat periodontal diseases.

CONCLUSION

The present study showed that licorice extract can prevent the production of MMPs by host cells and can be as useful as antibiotics like doxycycline to treat periodontal and other inflammatory diseases and it must be added that no side-effects were observed in usage of licorice extract.

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