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Original Article

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The influence of glycyrrhetinic acid (enoxolone) toothpaste on periodontal treatment outcomes and salivary levels of IL-8, TNF-α, IL-17, MCP-1 and VEGF in patients with chronic periodontitis

Wpływ pasty do zębów zawierającej kwas glicyretynowy (enoksolon) na efekty leczenia przyzębia oraz poziom IL-8, TNF-α, IL-17, MCP-1 i VEGF w ślinie u pacjentów z przewlekłym zapaleniem przyzębia

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Aim: This study evaluates the influence of glycyrrhetinic acid (enoxolone) toothpaste on the results of scaling and root planing as well as salivary levels of IL-8, TNF-α, IL-17, MCP-1 and VEGF in patients with chronic periodontitis. Clinical parameters and biomarkers of periodontitis were assessed longitudinally to determine response to the therapy.

Material/Methods: A 3-month case-controlled study of adults with chronic periodontitis was performed, with 18 patients receiving scaling and root planing and enoxlone toothpaste (group A) and 18 with scaling and root planing with regular toothpaste (group B). Clinical measurements of periodontal disease were recorded and saliva samples were collected at week 0 and 12. Samples were analyzed for immune markers: Interleukin-8 (IL-8), Tumor Necrosis Factor-α (TNF-α), Interleukin-17 (IL-17), Monocyte Chemoattractant Protein -1 (MCP-1) and Vascular Endothelial Growth Factor (VEGF).

Results: All parameters of periodontal health improved significantly in both groups by week 12 ($p<0.01$) with no significant differences between groups. However, improvements in group A were greater than in group B. IL-8, TNF-α, IL-17, MCP-1 and VEGF levels decreased significantly from baseline ($p<0.01$) in group A only.

Conclusions: Salivary levels of IL-8, TNF-α, IL-17, MCP-1 and VEGF seem to reflect disease severity and response to therapy, suggesting their potential utility for monitoring periodontal disease status. Greater improvements of periodontal parameters and significant reduction of salivary biomarkers' levels suggest potential benefits of glycyrrhetinic acid toothpaste in periodontal therapy.

Keywords: glycyrrhetinic acid, chronic periodontitis, IL-8, TNF-α, IL-17, MCP-1, VEGF

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INTRODUCTION

Periodontitis is a chronic complex inflammatory process of periodontal tissues, characterized by the presence of pathogenic bacteria in periodontal tissues, the occurrence of disorders of the immune response and by progressive destruction of clinical attachment. In affected tissues, proinflammatory cytokines are released, which initiate and regulate the occurrence of the inflammatory process. Earlier studies indicated that patients with chronic periodontitis in gingival crevicular fluid and saliva demonstrated increased levels of certain mediators of inflammatory response. These mediators can serve as potential biomarkers that could be used to detect and monitor the disease process [23, 32, 36]. An indisputable advantage of using saliva as a potential biomarker of periodontitis is its non-invasiveness and ease of acquisition. [20, 25, 30].

Interleukin 8 (IL-8) is a chemokine that is a strong chemoattractant and regulator of neutrophil function. Neutrophil activity plays an important role in the process of inflammatory destruction of periodontal tissues, which is largely regulated by IL-8 [11]. IL-8 level seems to correlate with the state of periodontium and react to periodontal treatment [14, 22, 26].

Tumor Necrosis Factor α (TNF-α) is a cytokine released by macrophages, which is considered a key factor in the process of bone resorption associated with periodontitis [27]. TNF-α is also an important biomarker of periodontal disease, and its level correlates with the state of periodontium [1, 8, 38, 42].

Monocyte Chemoattractant Protein 1 (MCP-1) stimulates chemotaxis and expression of other cytokines in monocytes and may cause an oxygen explosive reaction as well as release of reactive forms of oxygen [12, 45]. Studies indicate an association between MCP-1 level in gingival crevicular fluid and the presence and progression of periodontitis [6, 7, 16].

Interleukin 17 (IL-17) is a cytokine secreted by a subpopulation of T helper lymphocytes - Th17 [39]. IL-17 affects secretion of many other proinflammatory cytokines such as interleukin 6 and 8, granulocyte-colony stimulating factor (G-CSF), TNF-α and matrix metalloproteinase [43]. Individuals with periodontitis in deep periodontal pockets demonstrate an increased number of Th17 cells and a higher level of IL-17 in gingival crevicular fluid [9, 10, 34].

Vascular Endothelial Growth Factor (VEGF) is a cytokine responsible for the angiogenesis of inflamed tissues and for increasing the permeability of blood vessels [3]. Studies show that induction impact on VEGF production is demonstrated by substances that play a key role in the etiopathogenesis of periodontitis, such as prostaglandin E2, IL-1 and TNF-α [37]. The level of VEGF in gingival crevicular fluid depends on the status of periodontal tissues and is reduced during treatment, which makes it a potential biomarker of periodontal status [2, 35].

An innovative approach in the treatment of periodontitis is a combination of non-surgical treatment with the use of toothpaste containing 1% enoxolone (glycyrrhetinic acid). This natural substance, found in the licorice plant, belongs to the group of nonsteroidal anti-inflammatory drugs and can potentially have a beneficial effect on treatment outcomes by reducing periodontal inflammation and by bacteriostatic action [33, 46].

The aim of the study was to demonstrate the impact of non-surgical treatment of periodontitis and the use of toothpaste containing 1% glycyrrhetinic acid on the status of periodontal tissues and the level of selected cytokines in saliva.

MATERIALS AND METHODS

Qualification of patients

The study included 36 systemically healthy patients diagnosed with advanced chronic periodontitis accor-

ding to the criteria recognized by American Academy of Periodontology (Table 1). The inclusion criteria for in the study were: presence of at least 15 teeth not requiring extraction (excluding third molars) and age from 18 to 75 years. In each quadrant of dentition, at least two teeth with pocket depth (PD) ≥5mm, clinical attachment loss (CAL) ≥3mm and occurrence of bleeding on probing (BOP) were required. The study excluded patients who underwent periodontal treatment during 12 months prior to the study, individuals taking local or systemic antibiotics in the previous 3 months, active smokers or smokers within the previous 5 years, patients diagnosed with systemic diseases that may modulate the course of periodontal disease or affect the systemic inflammatory response (e.g. diabetes) as well as pregnant and lactating women. The study was conducted in the Department of Periodontology and Oral Diseases, Medical University of Warsaw, after approval by the relevant bioethics committee.

Clinical examination and treatment

The clinical examination included measurements at 6 points within each tooth (three lingual/palatal: mesial, central and distal and three buccal: mesial, central and distal) before treatment and 12 weeks after treatment. The clinical examination was performed by one experienced clinician in a double blinded manner and it included an evaluation of bleeding on probing (BOP) according to Ainamo and Bay protocol [21], plaque index (PI) according to O'Leary et al. [19], approximal plaque index (API) according to Lange et al. [18], pocket depth (PD) and clinical attachment level (CAL). Measurements were performed with a manual periodontal probe (PCPUNC 15, Hu-Friedy) and the reference point for evaluation of clinical attachment loss was the cemento-enamel junction (CEJ). If it was not possible to locate the CEJ, the apical margin of the restoration or prosthetic crown was assumed as the reference point.

After qualifying for the study, the hygienisation phase was carried out, including detailed oral hygiene instructions, in order to obtain optimal plaque control (API value ≤25%). By alternating randomization, patients were assigned to two groups (Table 1). Patients in group A (study group) received toothpaste containing 1% enoxolone, whereas patients in group B (control group) received toothpaste without enoxolone. During the study, patients were banned from using additional pharmacological agents that could modulate inflammation and plaque control (e.g. rinses and antiseptic gels). The hygiene regime included mechanical tooth cleaning with the provided manual soft brush using the included toothpaste twice a day for 3 minutes.

The non-surgical treatment of periodontitis included scaling and root planing (SRP) under local anesthesia during two visits with an interval not exceeding 24 hours.

Saliva collection

Five ml of whole unstimulated saliva was collected from all patients at baseline and 12 weeks after the end of treatment. The material was collected in the morning (between 8:00 and 12:00 o'clock), at least two hours after the last meal, according to the technique described by Navazesh [28]. The collected samples were frozen at -80°C, and their analysis was performed within a period not exceeding 6 months from the moment of freezing.

Cytokine analysis

Luminex xMAP technology for multiplexed quantification of cytokines in the saliva samples was used. The multiplexing analysis was performed using the Bio-Plex Luminex™ 200 system (Bio-Rad Laboratories, Hercules, CA, USA). Cytokines were simultaneously measured using a Bio-Plex Pro Human Cytokine Assay (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol.

Statistical analysis

Categorical variables are presented as a number of observations with percentage and compared with the chi square test or two-tailed Fisher's exact test if any of the expected values in a 2 x 2 contingency table was less than 5. Due to non-normal distribution, continuous variables were presented as a median with quartiles (1st quartile and 3rd quartile, Q1 and Q3) and range without outliers. Comparisons between group A and group B were made using Mann-Whitney U test. For comparisons of repeated measure within each group, a Wilcoxon signed-rank test was employed. Correlations were expressed as Spearman's rho.

Table 1. Patients in the study

Group	Number oj subjects	Mean Age	Number of Females	Number of Males	Number of teeth
Test	16	59.13±10.81	8	8	23.31±3.96
Control	18	59.83±9.31	8	10	20.72±3.99

There were no significant differences between groups

Table 2. Periodontal parameters at baselina and 12 weeks after scaling and root plaining (mean values)

		TEST GROUP (n=16)	CONTROL GROUP (n=18)	p value
PD (mm)	Baseline	4.01±0.98	3.75±0.69	0.054
	12 weeks	3.03±0.91*	3.04±0.6*	0.810
PD>4 (n)	Baseline	49.88±29.19	41.89±36.98	0.115
	12 weeks	20.21±12.9*	17.07±25.41*	0.162
CAL (mm)	Baseline	3.81±1.48	3.57±0.87	0.983
	12 weeks	2.97±1.27*	2.66±0.68*	0.760
BOP (%)	Baseline	60.92±21.17	65.99±19.11	0.913
	12 weeks	29.09±14.46*	37.17±18.63*	0.197
PI (%)	Baseline	44.51±17.42	54.87±22.86	0.559
	12 weeks	22.78±11.30*	26.71±18.07*	0.616
API (%)	Baseline	60.91±26.62	68.32±24.68	0.570
	12 weeks	23.98±18.13*	32±20.89*	0.294

* p<0,01 statistically significant to baseline

A p value <0.05 was considered statistically significant. Calculations were carried out using Dell Statistica 13.1 PL software (Dell Inc. 2016, USA).

RESULTS

The baseline and post-treatment clinical parameters are shown in Table 2. Among the 36 subjects included in the study, only 34 completed the treatment and had a follow-up visit after 12 weeks (16/18, F/M; 59.5±9.89 years; range: 40 to 71 yrs, Fig. 1). The patients were divided into two groups (A/B, 18/16 patients) and there were no significant differences between the groups according to age, gender, number of teeth and clinical parameters (PD, PD> 4, CAL, BOP, PI, API). All clinical parameters in both groups improved after 12 weeks of non-surgical treatment and no adverse effects associated with the applied toothpaste were observed. In group A, the relative improvement in clinical parameters was higher (improvement by 38-66%) than in group B (improvement by 29-65%), but these differences did not reach a statistical significance. During the treatment, postoperative healing proceeded without complications in all patients and no adverse effects associated with the toothpaste were observed.

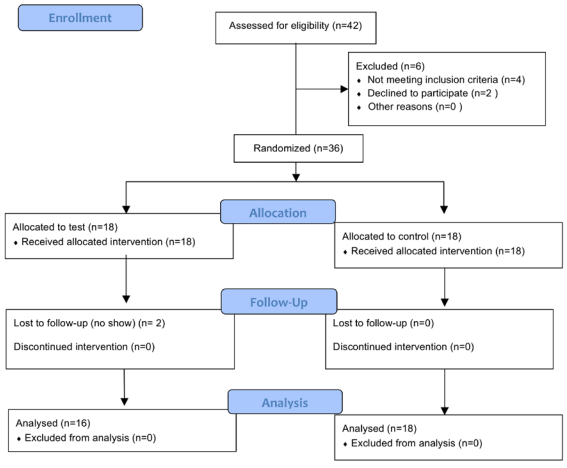

Fig. 1. A CONSORT flow diagram depicting patient recruitment, randomization, patient flow, and follow-up in the study

The levels of studied cytokines in saliva at baseline and after 12 weeks are presented in Table 3 and Figure 2. Baseline cytokine concentrations were similar in both groups. Twelve weeks after treatment, both groups demonstrated a decrease in salivary levels of IL-8, TNF-α, IL-17 and MCP-1. In the case of VEGF, a decrease was observed only in the study group (A). In this group, the levels of all studied cytokines were significantly different from the values measured at baseline (p<0.01). In the case of the control group (B), none of the observed changes was statistically significant. After 12 weeks of treatment, statistically significant differences between the groups in the levels of all studied cytokines were observed.

A correlation analysis showed a statistically significant correlation between IL-8 and BOP baseline levels (r=0.57, p<0.01) for the whole population. There were also significant correlations between TNF-α and PD (r=0.43, p<0.01) as well as between TNF-α and PD> 4 (r=0.53, p<0.01). No statistically significant correlations were observed after completion of the treatment.

DISCUSSION

In clinical practice, outcomes of treatment of periodontitis are assessed only on the basis of clinical parameters such as PD, CAL, BOP, PI, API and radiological examination of the alveolar bone. The applied methodology, despite high effectiveness, is extremely time-consuming, and the results of the tested parameters may

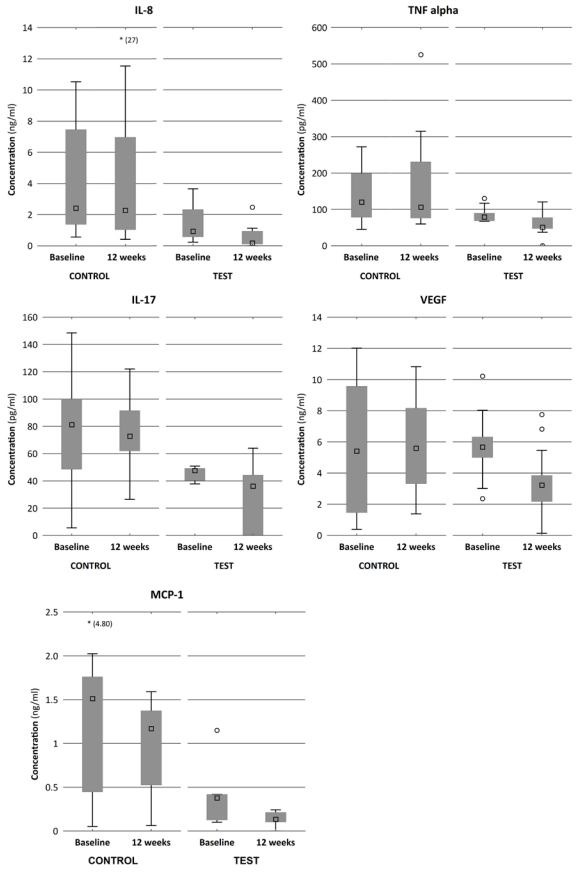

Fig. 2. Boxplots of biomarkers by group and visit. Data are presented as median with interquartile range (box), range excluding outliers (whiskers) and individual outliers with values (*)

be subject to an error related to the lack of inter- and intra-examiner reproducibility [29]. Therefore, alternative methods to identify and monitor the course of the disease are sought for. Studies indicate that saliva can be used in diagnostics of periodontal disease, also as a point-of-care analysis [17, 31, 41].

In the present study we assessed salivary concentrations of IL-8, TNF-α, IL-17, VEGF and MCP-1 before and 12 weeks after the procedure of scaling and root planing in patients with chronic periodontitis. The evaluated cytokines were selected because of their important role in pathogenesis of periodontitis [6, 9, 11, 27, 35]. Among the tested cytokines, previously only IL-8 and TNF-α concentrations in saliva were evaluated. Patients were randomly assigned to two groups comparable in terms of baseline periodontal parameters and cytokine levels. Mean concentrations of IL-8 and TNF-α corresponded to the levels obtained for generalized periodontitis in other studies [15, 24, 40]. Salivary concentrations of IL-17, VEGF and MCP-1 in patients with periodontitis have not been published before. IL-17, VEGF and MCP-1 in patients with periodontitis have not been published before.

The study showed the effectiveness of SRP non-surgical treatment of periodontitis during 12-week observation for a significant improvement in all clinical parameters in both groups. Despite a lack of significant differences between the groups, the improvement of clinical parameters was higher in the case of the study group. Twelve weeks after SRP, IL-8, TNF-α, IL-17, VEGF and MCP-1 levels were decreased in the study group, and IL-8, TNF-α, IL-17 and MCP-1 levels were decreased in the control group. This suggests that the levels of selected cytokines can be used to monitor the response to periodontal treatment. This association can be particularly observed for IL-8, IL-17 TNF-α and MCP-1. The obtained results for IL-8 and TNF-α are consistent with another study evaluating the effect of SRP on the level of these cytokines in saliva [40].

Only the study group demonstrated a statistically significant reduction in the level of IL-8, TNF-α, IL-17, VEGF and MCP-1 in saliva. Despite the decrease in IL-8, TNF-α, IL-17 and MCP-1 concentrations in both groups, the levels of all assessed cytokines after treatment were lower in the study group and these values were statistically different as compared to the control group. This suggests an anti-inflammatory effect of enoxolone contained in the toothpaste and a beneficial effect on reducing the level of the evaluated cytokines. There are no other studies assessing the effects of enoxolone on salivary levels of inflammatory cytokines and there is only one study evaluating the effect of enoxolone on IL-8 levels in periodontal tissues. Glycyrrhetinic acid, in vivo and in vitro, inhibits endothelial synthesis of IL-8 stimulated by lipopolysaccharide from *Porphyromonas gingivalis* [13]. These reports seem to be consistent with the results obtained in this study. Glycyrrhetinic acid was shown to inhibit TNF-α synthesis in hepatocytes and associated cellular apoptosis [5]. In addition, studies on neoplastic cells demonstrated enoxolone-induced suppression of VEGF production [44]. These two studies seem to confirm the effect of glycyrrhetinic acid on the reduction of TNF-α and VEGF levels, but the quoted studies do not relate to periodontal tissues.

Glycyrrhetinic acid additionally has antibacterial activity, inhibiting bacterial DNA replication, which reduces the production of enzymes and toxins in the bacterial cell [4]. Although the study did not include microbiological assessment, it should be noted that the decrease in the level of the studied inflammatory cytokines in patients using enoxolone toothpaste may also be associated with its antibacterial activity.

CONCLUSIONS

This is one of the first prospective studies evaluating the effect of periodontal treatment on salivary levels of cytokines, and the first study evaluating levels of IL-17, VEGF and MCP-1. The obtained data, although limited by the size of groups, suggests a relationship between IL-8, TNF-α and MCP-1 and improved clinical status of

Table 3. Salivary cytokines levels in both groups at baseline and 12 weeks after scaling and root plaining

		TEST GROUP A (n=16)			CONTROL GROUP B (n=18)			p value
		Median	Q1	Q3	Median	Q1	Q3	
IL-8 (ng/ml)	Baseline	0.93	0.56	2.32	2.42	1.37	7.46	0.072
	12 weeks	0.18*	0.11	0.94	2.28	1.04	6.98	<0.01
TNF- (pg/ml)	Baseline	78.96	68.16	89.74	119.86	78.02	199.01	0.103
	12 weeks	0.18*	0.11	0.94	2.28	1.04	6.98	<0.01
IL-17 (pg/ml)	Baseline	47.59	40.22	49.22	81.21	48.27	100.05	0.11
	12 weeks	36.13*	0.00	44.31	72.74	61.89	91.60	<0.01
VEGF (ng/ml)	Baseline	5.67	4.99	6.32	5.41	1.46	9.57	0.90
	12 weeks	3.23*	2.17	3.86	5,59	3.31	8.16	<0.01
MCP-1 (ng/ml)	Baseline	0.38*	0.13	0.42	1.51	0.45	1.76	0.10
	12 weeks	0.13*	0.00	0.24	1.17	0.53	1.37	<0.01

* p<0,01 statistically significant to baseline

the periodontium after non-surgical procedures, which may potentially be useful in monitoring response to treatment. Further studies with more numerous groups are necessary to confirm the observed association and to determine possible interfering factors, such as age, gender or race. It is also the first study assessing the effect of glycyrrhetinic acid on salivary levels of cytokines. A greater improvement in clinical parameters, a significant decrease in the level of all evaluated inflammatory cytokines in the study group, may suggest a beneficial effect of glycyrrhetinic acid on 12-week SRP results. Lowering the level of the studied cytokines and possible antimicrobial effects of enoxolone can potentially affect treatment outcomes and the stability of periodontium in the long term. An assessment of possible benefits resulting from the use of toothpaste containing glycyrrhetinic acid requires further research with larger groups and longer observations.

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