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EXPRESSION OF MMP-1 AND MMP-9 IN LOCALIZED AGGRESSIVE PERIODONTITIS PATIENTS BEFORE AND AFTER TREATMENT: A CLINICAL AND IMMUNOHISTOCHEMICAL STUDY

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ABSTRACT

Aim: This study aimed to clinically investigate the efficacy of β-glucan supplementation to non-surgical periodontal therapy in localized aggressive periodontitis (LAP) patients. In parallel, immunohistochemical detection of matrix metalloproteinase-1 (MMP-1) and MMP-9 expression levels has undergone to determine the subcellular changes behind the clinical status and reliability of each antibody in predicting the periodontal condition.

Method: 40 subjects suffering from LAP were randomly and equally assigned to receive scaling and root planning (SRP); either with placebo pills (Group I) or β-glucan (100 mg/ once a day) (Group II), for 40 days. Subjects were clinically monitored on day 0 and day 91. Gingival samples were harvested from hopeless teeth sites to be investigated histologically and immunohistochemically using matrix metalloproteinase (MMP)-1 and 9 antibodies form each participating patient; at the same time intervals.

Results: The experimental intervention showed a greater mean of probing pocket depth reduction (*p=0.1128*), with significant gain in clinical attachment (*p=0.0180*) and reduction of gingival inflammation (*p=0.0207)* compared to the control group at the end of the study. Furthermore, the experimental protocol was able to achieve better modulating effects on the levels of MMP-1 and MMP-9 compared to control therapy, along with enhancing protective healing patterns. MMP-9 was more sensitive indicator of the periodontal condition compared to MMP-1.

Conclusions: The curre nt study demonstrated that the non-surgical treatment of LAP is markedly improved clinically by the adjunctive use of β-glucan, accompanied by a trend for modulation of the cytokine profile in gingival tissue samples with better healing events. The results also suggest that the expression of MMP-9 may be a precise indicator of periodontal disease activity.

KEY WORDS: β-glucan, localized aggressive periodontitis, matrix metalloproteinases, randomized double blind clinical trials.

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INTRODUCTION

Aggressive periodontitis (AgP) causes rapid destruction of the periodontal attachment apparatus and the supporting alveolar bone. It can present in a localized or generalized form. Two common features of both forms are (1) rapid attachment loss and bone destruction in an otherwise clinically healthy patient and (2) a familial aggregation. These patients often present with limited microbial deposits that seem inconsistent with the severity of tissue destruction. However, the deposits that are present often have elevated levels of *Aggregatibacter actinomycetem comitans*, or *Porphyromonas gingivalis*. These patients may also have phagocyte abnormalities and a hyper-responsive monocyte/macrophage phenotype. These clinical, microbiological, and immunologic features would suggest that patients diagnosed with AgP would have poor prognosis.[1]

Over the past years, most of the research was focused on the microbiological aspects of periodontitis. It has been noticed that bacteria alone are not sufficient for the initiation of periodontal disease, although they play an important role in the process. Host response, smoking, stress and other risk factors influence the presence of disease, and the susceptibility to periodontitis is genetically determined.[1, 2] Bacteria play an indirect role in the tissue destruction, through activation of the host response which becomes pathological. [2, 3]

Many host-derived mediators involved in orchestrating the periodontal local immuno-inflammatory processes have been recently monitored by several investigators.[4, 5] One of the main arms known to be involved in periodontal tissue destruction is mediated by the matrix metalloproteinases (MMPs); derived from the host cells during their fight with the periodontopathogens. The MMP family includes 23 human MMPs. Each MMP has distinct and overlapping property, which can cleave all the components of extracellular matrix and basement membrane. Among MMPs, MMP-1 (interstitial collagenase or collagenase-1) is one of the major proteases that can degrade fibrillar bone collagens in concert with MMP-9 (gelatinase).[6]

It is worth noting that fibrillar collagens types I and III, the predominant types of interstitial collagens in periodontium which are resistant to most proteinases, can be degraded by MMP-1. Romanelli et al (1999) [7] determined the neutrophil collagenase activity in GCF of patients with periodontitis and concluded that collagenase activity is positively associated with the severity of periodontal disease.

Regarding MMP-9 belonging to gelatinase family of MMPs; it was suggested to participate in tissue destruction and periodontitis.[4, 8] MMP-9 is mainly secreted by polymorphnuclear leukocytes (PMNs) and they degrade collagen type IV present in gingival tissues.[9] Rai et al (2008) [5] showed that crevicular MMP-9 levels were higher in periodontitis than in healthy controls.

The field of perioceutics was specifically developed to better manage periodontitis. Perioceutics include antimicrobial therapies that can be used to address changes in the microflora, and host modulatory therapy that can be used to address a host response consisting of excessive levels of enzymes, cytokines, and prostanoids and excessive osteoclast function that may be related to certain risk factors.[10] The use of antimicrobials is, however, of concern in relation to accurate diagnosis, appropriate selection, microbial resistance and allergic reactions.[11]

A promising approach in treatment of periodontitis, is the administration of agents that modulate the host response, aiming for achieving clinical benefits out of understanding the host response mechanisms.[12] Current concepts in the treatment make use of natural pathways for inflammation inhibition as well as activation of healing and regeneration. Inhibition or modification of inflammatory/immune response is strived for, using different mechanisms implementing substances like antimicrobial peptides, probiotics, anti-inflammatory lipid mediators and micronutrients.[13]

Beta-glucans (β-glucans), structural components of the cell wall of many bacteria, fungi, algae and yeast as well as cereal grains, that increase the resistance to bacterial infections and cancer cells while stimulating wound healing.[14] In addition, β-glucans were found to have strong immune-modulatory effects in animals and humans,[12, 14] by interacting with innate immune cells such as macrophages, dendritic cells, granulocytes and natural killer cells.[15] These cells are equipped with surface receptors called pattern recognition receptors (PRRs), which discriminate between different ''pathogen-associated molecular patterns'' (PAMPs). The PRRs embrace receptors such as toll-like receptors, the dectin-1 receptor, the mannose receptor, and also the β-glucan binding part of complement receptor 3.[16]

One reason for the augmented colonization of the periodontal pathogens, is believed to be related with a weak specific T helper (Th1)-mediated immunity, [17] and the immune response in patients with periodontal lesions may be inclined towards a strong (Th2)-mediated immunity.[18] Fortunately, it was reported that β-glucan possesses a beneficial action in restoring Th1-function and enhancing the immune functions.[13] Furthermore, numerous studies have shown that β-glucan is a stimulator activating phagocytosis, respiratory burst, and the production of cytokines and chemokines in macrophages.[19] β-glucan has been also suggested to enhance endogenous antibacterial mechanisms in neutrophils and to increase the healing potential of damaged tissues.[20]

A consequential objective for periodontal research would thus be to find a well-tolerated substance that can stimulate protective immune responses, and effectively mount resolution pathways contributing to resolving of the chronic lesion observed in periodontal disease, and hence, improve the healing capacity of the periodontal tissues. In addition, the diagnosis of active phases of periodontal disease and the identification of patients at risk of periodontal disease are still a challenge for periodontal research. Since MMPs may be clearly reflected in periodontal status, [6] the aim of this study was to examine the impact of an adjunctive systemic dose of β-glucan to non-surgical periodontal therapy; on both clinical and patho-physiologic events in localized aggressive periodontitis (LAP)-patients.

PATIENTS AND METHODS

In this randomized double blind clinical study, a total of 40 LAP participants were selected from those who referred to the clinics of Department of Periodontology, Dental Medicine Faculty, Al-Azhar University (Girls Branch). Their age ranged from 22-35 years. Medical and dental histories were obtained and intraoral examinations were carried out at pre-screening visit. They were in good general health, had at least 20 teeth excluding third (3rd) molars and teeth indicated for extraction, diagnosed to have LAP based on the clinical and radiographic findings according to World Workshop in Periodontology criteria.[21]

The inclusion criteria were as follows:

- Except for periodontitis, our patients were systemically healthy as evaluated by modified Cornell medical index.[22]
- No more than two teeth other than first molars and incisors, with probing depth (PD) ≥ 5mm, bleeding on probing (BOP), and clinical attachment level (CAL) ≥ 5mm.
- Based on the history; rapid rate of attachment loss and bone destruction.[23]
- A radiographic examination; consisting of anterior and posterior periapical films, revealing an evidence of moderate to severe vertical bone loss around permanent incisors and first molar teeth.
- Every patient should have at least an extraction-indicated tooth for a dento-periodontal affection; that would be excluded from clinical examination.

- Familial aggregation (during the anamneses, the subjects were asked if they had at least one other member of the family presenting or with history of periodontal disease).[23]

Exclusion criteria were as follows:

Previous subgingival scaling and root planning, allergy to β-glucan, smoking, former smoking, pregnancy, systemic diseases that could affect the progression of periodontal disease (e.g. Diabetes mellitus and immunological disorders), long-term administration of anti-inflammatory medication (e.g.; the regular prophylactic use or previous host modulation trials), need of antibiotic coverage for routine dental therapy, antibiotic therapy in the previous 6 months and allergy to chlorhexidine (CHX).

The protocol of the study was approved by the Ethical Committee of Al-Azhar University. Patients who fulfilled the inclusion criteria provided written informed consent and participated in the study, after being informed of the objectives, benefits and risks of the study.

Experimental design, allocation concealment and treatment protocol

This study was a controlled longitudinal intervention attempt that had met the criteria of a randomized clinical trial. All the LAP patients were randomly assigned, using a computer-generated table to one of the following equal treatment groups (20 patients each) in a parallel, double-masked design. **Group (1)** was assigned for patients who had scaling and root planing (SRP) and placebo pills for 40 days (control group). **Group (2)** included those patients who received a systemic β-glucan (100 mg capsule, Imurril, manufactured by Sigma; Pharmaceutical Industries for Elite Pharma) once a day for 40 days (15); after SRP (test group).

Supragingival biofilm control in both groups was achieved by rinsing with 0.12% CHX solution. All subjects were instructed to gargle with 15 ml of CHX twice a day for 60 days for 1 min. in the morning (after breakfast and tooth brushing) and at night (before going to sleep).

Before the study began, all subjects received full-mouth SRP, including the hopeless teeth, with instructions for proper home-care techniques. They were also given the same dentifrice to use during the study period (Paradontax©; Galaxo Smith-Kline, Middlesex, United Kingdom). Full-mouth SRP was performed under local anaesthesia from 4 to 6 appointments. Treatment of the entire oral cavity was completed from 10 to 14 days. SRP was performed by one trained periodontist using ultrasonic scaler (Cavitron® EMZ, Switzerland) and Gracey curettes (Hu-Friedy Ins. Co, USA). The β-glucan/placebo supplementations and the CHX rinses started immediately after the last session of mechanical instrumentation.

The medication allocation method

Both placebo/β-glucan supplements were given for the study coordinator, who marked the code number of each subject on a set of two packs (that looked identical), according to the therapy assigned, and gave them to the examiner. All study personnel, including the examiner, biostatisticians and participants were kept blinded as to patient assignment to treatment. The randomization code was kept sealed until the completion of the study. The treatment allocations were disclosed after the final analysis.

Monitoring of compliance and adverse events

For every patient, 40 capsules were prepared and then put into; 4 identically labeled packs. The packs contained 10 capsules of either placebo or β-glucan, enough for ten days of medication. Each pack was given to the patient who was asked to take 1 capsule/day. The subjects were asked to bring the packs containing the medication once every ten days

when regular uptake and compliance were checked. During these visits, subjects returned the old pack and received a new pack of β-glucan /placebo. They also answered a questionnaire about any self-perceived side-effects of the medication/ placebo. [24] Patients were re-assured to follow the study regimen precisely. Two study assistants conducted this enquiry, and were also responsible for calling the subjects every 2 days to monitor compliance.

In this study, our patients were checked for periodontal maintenance; 2 weeks, 1 month and 2 months post-therapy. This non-standard protocol was followed to reinforce the plaque control measures and maintain a satisfactory oral hygiene. [25]

Reinforcement of oral hygiene during follow-up evaluations was performed by the same periodontist, who remained masked to treatment allocation.

Clinical monitoring

Clinical monitoring was performed by one calibrated examiner and the treatment was carried out by another clinician. Thus, the examiner and the clinician were masked as to the nature of the treatment groups.

In this study, the periodontal clinical measurements included gingival index (GI),[26] in addition to PD measured from gingival margin till depth of the pocket and CAL measured from depth of the pocket till cement-enamel junction. Readings were measured to the nearest mm with a periodontal probe using the Michigan 0 probe with Williams's markings. All parameters were measured at six sites per tooth (mesiobuccal, midbuccal, distobuccal, distolingual, midlingual and mesiolingual) in all the teeth, excluding 3rd molars and those hopeless teeth (indicated for extraction and harvesting of gingival samples). All subjects received clinical monitoring at baseline (day 0), immediately after the last session of SRP, and re-evaluation time (the end of the 3rd month - day 91).[15]

Investigator' calibration

Intra-examiner reproducibility was assessed with a calibration exercise performed on two separate occasions, 48 hours apart. The examiner participated in a calibration exercise that was performed in 10 non-study subjects with periodontitis. The standard error of measurement was calculated and the intra-examiner variability was 0.15 mm for PD and 0.19 mm for CAL.[24] Calibration was accepted if ≥ 90% of the recordings could be reproduced within a difference of 1.0 mm.

Light microscopic analysis

Gingival tissue samples were taken from extraction candidates' sites at the same time intervals of clinical assessments. Samples were taken out of the interdental area by scalpel, so that the tissue contained the gingival sulcus, junctional epithelium and adjacent connective tissue. Gingival samples were carefully dissected and cut into 1 x 1 mm cubes fixed in 10% neutralized buffered formalin, they were then embedded in paraffin blocks and cut into 4 μm thick sections. Serial sections were stained with hematoxylin and eosin (H&E). Histological analysis with light microscope was performed on these specimens to verify the influence of each treatment on the course of periodontitis, for the qualitative assessment of healing events.

Immunohistochemical procedures

Sections of 4 μm thickness were mounted on electrically positive charged slides and deparaffinized by overnight incubation with xylene then, rehydrated in a gradual descending concentrations of ethanol followed by phosphate-buffered saline (PBS) wash. Blocking the endogenous peroxidase activity was performed by 3% hydrogen peroxide (H_2O_2) for 5 minutes at room temperature. For antigen retrieval, tissue sections were put in glass jars containing 0.01M sodium citrate buffer (pH 6.0) and boiled in a microwave oven twice for 5 minutes each to enhance immunoreactivity (reserve the loss of

antigenicity that occurred with some epitopes of formalin-fixed paraffin-embedded tissues). The slides were allowed to cool and rinsed with PBS, pH 7.2. The immunohistochemical staining for MMP-1 and MMP-9 antibodies was done according to the manufacturer's instructions using a ready MMP-1 (collagenase-1) Ab-1(clone x2A) and MMP-9 (92kDa collagenase IV) Ab-1 (cone GE-213) (Thermo Scientific, USA).

Detection was carried out using the universal kit (DAKO, Denmark) by washing slides in PBS for 5 minutes and incubated with secondary antibody that was biotinylated goat serum conjugated rabbit and mouse sera for 30 minutes. Sections were then washed for 5 minutes in PBS followed by development of antigen–antibody visualization by diaminobenzidine [DAB] in PBS containing 40% H_2O_2. Sections were washed under running tap water for 10 minutes, then counterstained with Mayer's haematoxylin and mounted.

Histomorphometric analysis

Immuno-reactivity, for MMP-1 and MMP-9; was evaluated by estimating the area percentage of positive immunostained cells in relation to the area examined in each field using Leica image analyzer computer system (Germany).The image analyzer consisted of a colored video camera, colored monitor, hard disc of *hp* personal computer connected to the microscope, and controlled by Leica Qwin 500 software. The image analyzer was calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into actual micrometer units. The area and area percentage of MMP-1 and MMP-9 reactive areas were measured with reference to a standard measuring frame of area 11434.9 μm^2 using a magnification (x 200). Using the color detection, reactive areas of positive immunostaining were masked by a blue binary color. Ten fields per each slide section of each patient were successively taken, to be histomorphometrically evaluated. Mean values were then obtained for each specimen.

Our research design was based on similar studies; (25) the chosen primary outcome was CAL. The secondary outcomes were: PD, GI, MMP-1 and MMP-9. A power analysis was designed to have adequate power to apply a 2-sided statistical test of the research hypothesis (Null hypothesis) that there was no difference between the two groups. According to the results of CAL measurements at 3 months follow-up period by Elkhouli (2011), (25) and using alpha (α) level of 0.05 (5%) and Beta (β) level of 0.20 (20%) i.e. power = 80%; the predicted minimum sample size (n) was a total of 40 cases i.e. 20 cases in each group.

Sample size calculation was performed using G*Power Version 3.1.9.2.

Statistical analysis

Analysis of data was performed using SPSS 17 (Statistical Package for Scientific Studies) for Windows. Description of quantitative variables was in the form of mean, standard deviation (SD). Data were explored for normality using Kolmogorov-Smirnov test of normality. The results of Kolmogorov-Smirnov test indicated that most of data were not normally distributed (non-parametric data) so non-parametric tests were used for the comparisons. Significance of the differences over time was evaluated using the Wilcoxon-signed rank test, while significance of the differences between both groups was estimated using the Mann-Whitney U test.

Results were expressed in the form p-values that were differentiated into:

* Non-significant when p-value > 0.05

* Significant when p-value ≤ 0.05

RESULTS

Patient characteristics:

At baseline, 40 patients (28 females and 12 males) participated in this study. They were as

follows: Mean age= 26.4±7.0 years; and acceptable oral hygiene was achieved before the study as seen from the plaque index score (PI), values 0-1 (27) were accepted. Regarding the clinical parameters at baseline; the mean measurements of PD, CAL and GI scores were 6.9mm±1.1, 6.2mm±1.03 and 2.2±0.79 mm, respectively (Table 1). Only two of our participants had two dento-periodontally affected teeth, while the rest of the patients had only one tooth; that were excluded from clinical assessment.

Subject retention, compliance and adverse effects

The study was conducted between, January 2013 till March 2014. On the last day of medication (day 40, following SRP), the subjects were asked to return to the clinic and bring the medication packs, which were checked for any possible remaining pills. About any self-perceived side-effects of β-glucan/ placebo, no adverse events were reported by any of the participants; no gastrointestinal tract (GIT) disturbance (e.g.; nausea, vomiting, diarrhea), and no abnormalities in vital signs (fever, low or high blood pressure) were observed. All subjects reported that they completed the course of the treatment, and this information was confirmed by pill counts. All patients completed treatment and post-treatment phases successfully.

Clinical parameters

In the present study, baseline findings showed no statistically significant difference between the two groups concerning all the clinical parameters *(p>0.05)*. In addition, both treatment modalities achieved a statistically significant reduction in the mean scores of PD, CAL and GI at the treated sites in both groups during follow-up evaluations, as revealed in (Table 1). Regarding the mean PD measurements, the therapeutic intervention group resulted in (2.5mm ±0.64) versus (4.4mm ±0.7) in the control group, with a non-significant difference *(p>0.05)*.

Moreover, group 2 showed (2.8mm ±0.49) gain in the mean measurements of CAL versus (3.6mm ±0.84) for group 1, with a significant gain in favor of the experimental regimen *(p<0.05)*. A significant lower mean value of GI scoring was also recorded in the experimental group (0.6±0.3), compared with that of the control group (1.3±0.82), *(p<0.05)*.

TABLE (1) Mean, standard deviation (SD) of clinical parameters values and area percent of immunoexpression in both groups and significance of the difference using Mann-whitney U test.

		Group 1		Group 2		P value
		Mean	SD	Mean	SD	
(PD)	Baseline	4.9	1.1	4.7	0.74	0.6637
	3 months	4.4	0.7	2.5	0.64	0.1128
(CAL)	Baseline	4.2	1.03	4.6	1.17	0.4658
	3 months	3.6	0.84	2.8	0.49	0.0180*
(GI)	Baseline	2.2	0.79	2.1	0.74	0.7782
	3 months	1.3	0.82	0.6	0.3	0.0207*
MMP1	Baseline	24.289	6.78	23.65	8.08	0.8500
	3 months	18.261	8.861	16.67	7.18	0.6645
MMP9	Baseline	29.115	6.48	30.05	5.27	0.7288
	3 months	22.65	2.797	19.252	2.29	0.0082*

**** Significance is considered at p≤ 0.05***

By the end of this study, the mean percentage (%) of change in clinical parameters with the resultant dampening of inflammation were statistically remarkable and more obvious in the experimental group compared with group 1 (*p<0.05*, Table 2), as following: With respect to PD values, group 2 resulted in a mean % of change (32.8±4.6) versus (21.7±3.9) in the control one, *(p<0.00001)*. Moreover, group 2 showed (27.4± 3.2) as a mean % of change in CAL measurements versus (11.3±1.8) for group 1, *(p<0.00001)*. A significant lower % of GI changes was similarly recorded in the intervention group (71.4±15.5), compared with that of the control group (31.8±8.6), *(p<0.00001)*.

Descriptive histology

At baseline, histopathologic evaluation of the gingival tissue showed epithelial hyperplasia with elongated rete pegs, connective tissue lamina propria showed high vascularization and variable inflammatory cell infiltrates with destruction of collagen fibers in areas associated with more intense inflammation. 3 months results, in the group I (SRP + placebo), gingival epithelium showed epithelial hyperplasia, with broad epithelial rete-pegs, connective tissue appeared with apparently decreased vasculature and moderate inflammation. In group II (SRP + β-glucan), gingival tissue showed normal thickness of epithelium with elongated thin rete peg, connective tissue appeared with well-formed collagen fibers, normal vasculature and inflammation ranged from mild to absent (Figure 1).

Immuno-expression of MM-1, MMP-9

For both groups, the baseline gingival tissue samples immunostained with MMP-1showed cytoplasmic immunoexpression in basal and spinous cell layers while higher prickle cell layers showed membranous immunostaining, connective tissue showed intense staining of both collagen fibers and inflammatory cells. Group I, after 3 months, showing cytoplasmic immunostaining of basal and lower half of spinous cell layers, the higher layers showed faint membranous staining, and connective tissue showed immunostaining of both fibroblast, collagen fibers and inflammatory cells. Group II after 3months, showing cytoplasmic staining of basal and parabasal cell layers the higher layers showed faint membranous staining and the rest of epithelium was negatively stained (Figure 2).

Concerning the immunostained gingival tissue samples with MMP-9, at base line, both groups revealed intense cytoplasmic staining and some epithelial cells showed nuclear immunoexpression and membranous staining of other cells, connective tissue showed immunostaining of fibroblasts, collagen fibers and inflammatory cells. Group I after 3 months showed cytoplasmic and nuclear staining in basal and parabasal cells while the higher cells showed nuclear immunostaining only, connective tissue showed immunoexpression in inflammatory cells. Group II after 3 months showed cytoplasmic and nuclear immunostaining of basal cell layer and few higher layers of spinous cells showed only nuclear staining, while remaining highest cells was negatively stained (Figure 3).

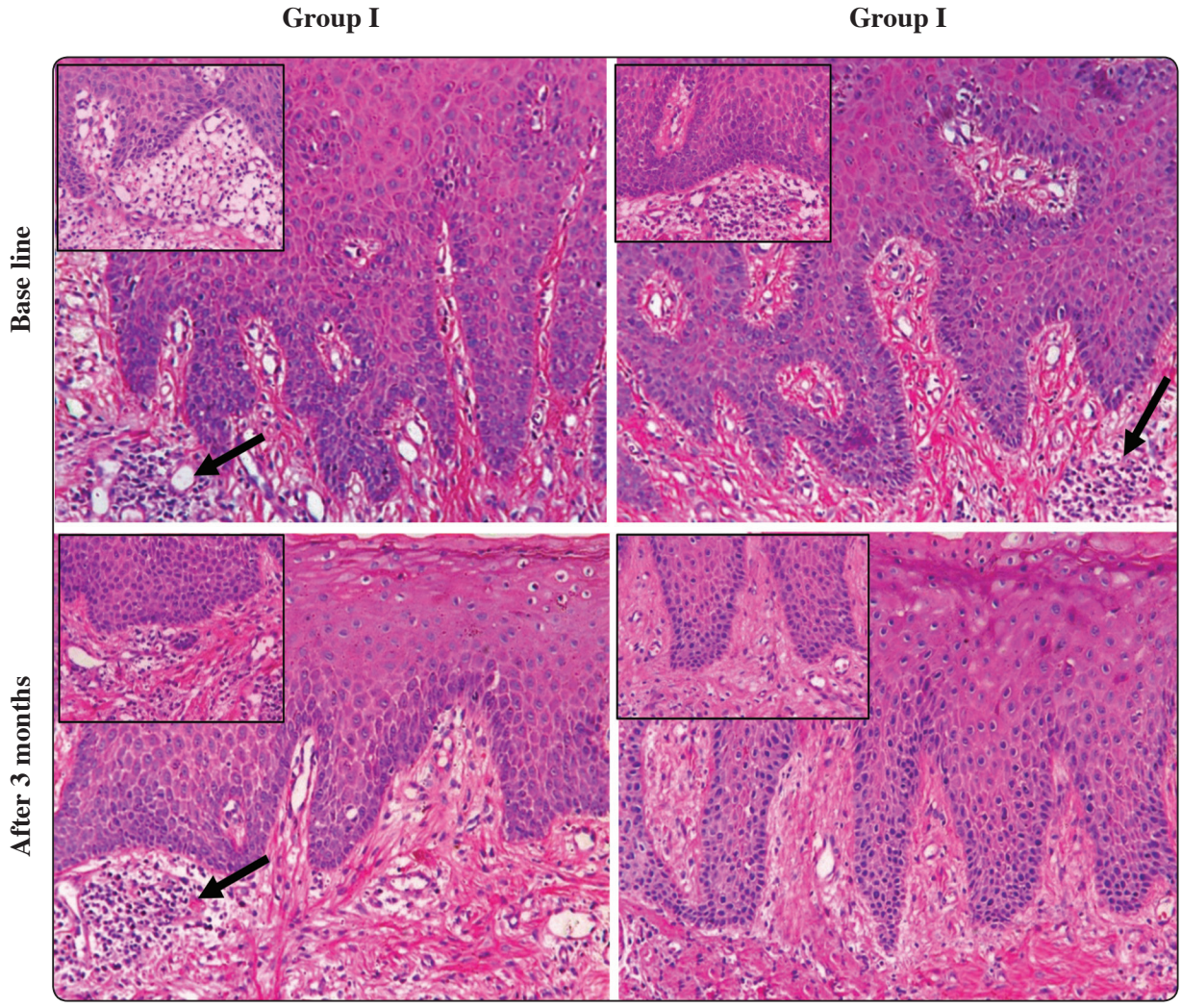

Fig. (1) Gingival tissue of both group I & II at base line, showed hyperplastic epithelium with elongated rete pegs. The connective tissue lamina propria appeared with high vascularization and variable inflammatory cell infiltrates and destruction of collagen fibers in areas associated with intense inflammation (arrow) (inset 200X). Group I after 3 months, gingival epithelium showed epithelial hyperplasia, with broad epithelial rete-pegs, connective tissue appeared with apparently decreased vasculature and moderate inflammation (arrow) (inset 200X). Group II after 3 months, gingival tissue showed normal thickness of epithelium with elongated thin rete peg, connective tissue appeared with well-formed collagen fibers (inset 200X), normal vasculature and mild inflammation (Magnification, 100X).

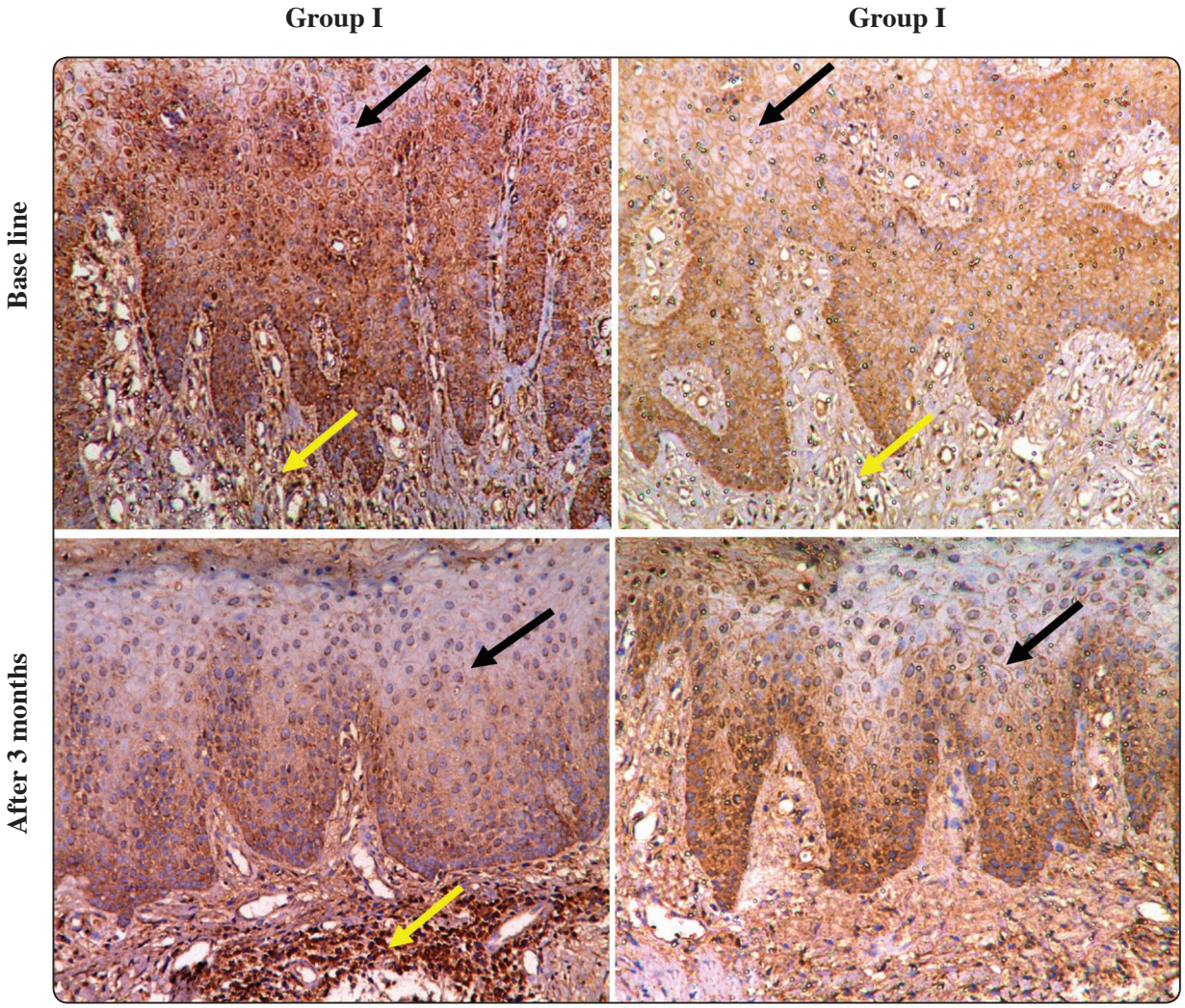

Fig. (2) Gingival tissue immunostaining with MMP1 at base line of both groups showed cytoplasmic immunoexpression in basal and spinous cell layers while higher prickle cell layers showed membranous immunostaining (black arrows), connective tissue showed intense staining of both collagen fibers and inflammatory cells (yellow arrows). Group I, after 3 months, showing cytoplasmic immunostaining of basal and lower half of spinous cell layers, the higher layers showed faint membranous staining (black arrow), and connective tissue showed immunostaining of both fibroblast, collagen fibers and inflammatory cells (yellow arrow). Group II after 3months, showing cytoplasmic staining of basal and parabasal cell layers the higher layers showed faint membranous staining (black arrow) and the rest of epithelium was negatively stained (Magnification, 100x).

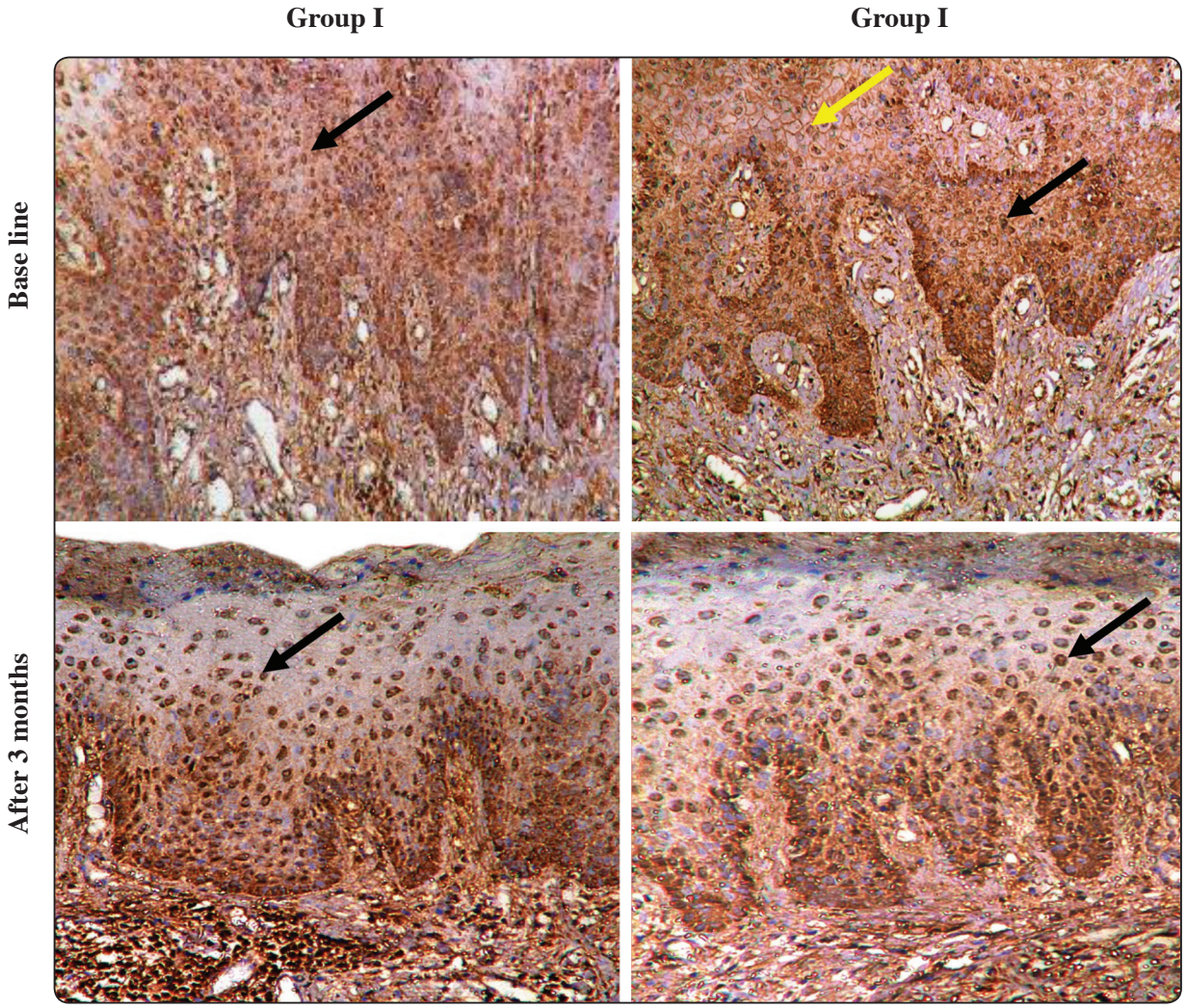

Fig. (3) Gingival tissue immunostaining with MMP9 at base line of both groups revealed intense cytoplasmic staining and some epithelial cells showed nuclear immunoexpression (black arrow) and membranous staining of other cells (yellow arrow), connective tissue showed immunostaining of fibroblasts, collagen fibers and inflammatory cells. Group I after 3 months showed cytoplasmic and nuclear staining in basal and parabasal cells while the higher cells showed nuclear immunostaining only (black arrow), connective tissue showed immunoexpression in inflammatory cells. Group II after 3 months showed cytoplasmic and nuclear immunostaining of basal cell layer and few higher layers of spinous cells showed only nuclear staining (black arrow), while remaining highest cells was negatively stained (Magnification, 100x).

Statistically, the mean value of area percent immunoexpression at baseline for both groups was non-significantly different with regard to both markers. Concerning MMP-1; on 3 months re-evaluation, group 2 recorded a lower mean value, with a non-significant difference as revealed by Mann Whitney U test *(p=0.6645)* (Table 1 and Figure 4). Meanwhile, using the % of change, group 2 revealed a barely meaningful difference *(p=0.0438)*, compared to group 1 (Table 2). Regarding MMP-9; after 3 months, group 2 recorded a lower mean value, with a significant difference *(p=0.0082)*, (Table 1 and Figure 4). Additionally, group 2 revealed a highly significant mean % of change *(p=0.0002)*, compared to group 1 (Table 2).

TABLE (2) Mean± SD in percentage of change from baseline for clinical parameters values and area % of immunoexpression in both groups and significance of the difference using Mann-whitney U test

	Group 1	Group 2	P value
PD	-21.7±3.9	-32.8±4.6	<0.00001*
CAL	-11.3±1.8	-27.4± 3.2	<0.00001*
GI	-31.8±8.6	-71.4±15.5	<0.00001*
MMP1	-24.7±4.2	-29.5±5.6	0.0438*
MMP9	-21.7±3.9	-36.7±9.6	0.0002*

Significant at p≤ 0.05

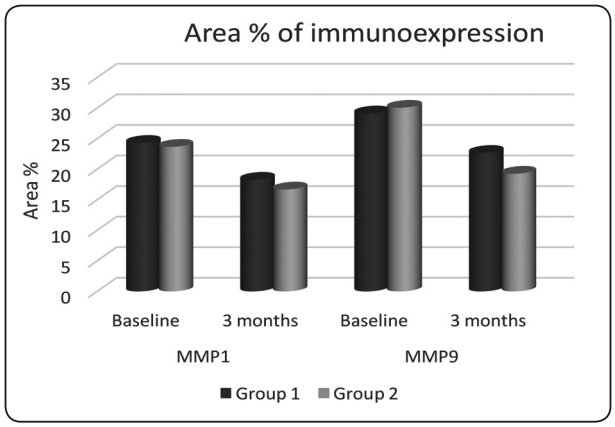

Fig. (4) Column chart showing mean area percent of MMP-1 and MMP-9 expression in both groups.

DISCUSSION

A number of host modulatory agents have been investigated in clinical trials for their potential use as adjuncts to mechanical SRP. The most studied drugs are antibiotics used in non-antimicrobial doses (e.g. tetracycline derivatives), anti-inflammatory agents and bone-sparing drugs.[28] However, most of these traditional drugs are associated with significant unwanted side effects, including hemorrhage, GIT problems, renal and hepatic impairment that preclude their use. Another important consideration is the protective role of immune response and the potential hazards of its negative modulation, especially aggravation of infection. [28, 29]

A potentially powerful, and fairly novel, alternative approach to the problem of infection control is the use of biological response modifiers that enhance the host phagocyte response without the expression of damaging inflammatory cytokines.[30] β-glucans are belonging to a group of physiologically active compounds; having the ability to stimulate the immune system,[31] in addition to its good margin of safety.[32] To our knowledge, this was the first double-blinded placebo-controlled randomized clinical trial to provide data about the short-term effects of the adjunctive use of β-glucan in non-surgical treatment of LAP.

Unfortunately, most β-glucans are insoluble resulting in limited applications in humans. Injectable microparticulate preparations cause granuloma formation, inflammation, pain [33] while, the oral administration is more suitable to consumers; with proven efficacy. β-glucan, taken orally, passes through the stomach virtually unchanged, because it is an acid-resistant. Moreover, intestines lack enzymes that break it down to molecules that can be absorbed through the intestinal wall; [34] so that the oral route was selected in our study.

Regarding the duration of β-glucan usage, it has a good safety margin and no data is available so far on its systemic usage in periodontal diseases. Treatment duration with β-glucan varies from 39-90 days in animal and human studies according to the type and severity of the problem.[35-37] Biagini et al., (1988) [38] documented soft tissue healing after SRP and found precisely oriented collagen bundle fibers by 30-60 days. Therefore, our study patients were put on β-glucan medication for 40 days according to the aforementioned data. Furthermore, our participants underwent a tight control schedule to check any self-perceived side-effects of the medication (every 10 days). Notably, no adverse reactions were reported during the regular checking

visits; [24] supporting the safety of our treatment protocol (dose, route and duration).

In the present study, on clinical re-evaluation, the experimental group revealed a significant PD reduction, CAL gain and reduction of gingival inflammation, compared to the control group. A possible explanation for the better clinical outcomes in group II might be related partly to the value of SRP; in resolution of inflammatory response and arresting the disease progression;[39] in addition to the β-glucan adjunct. In accord, previous studies have demonstrated the therapeutic efficacy of β-glucan dietary supplementation in reducing alveolar bone resorption through the reduction of osteoclastic activity and dampening of gingival inflammation through their anti-inflammatory properties,[15] as well as modulating specific cytokines involved in periodontal wound healing. [40]

Similar results have been also reflected by other researchers. Breivik et al. (2005) evaluated the effect of β-1,3/1,6 glucan on the progression of ligature-induced periodontal disease in animals. Their findings showed that orally administered β-glucan significantly reduced periodontal bone loss. [20] Moreover, Stashenko et al. (1995) tested the effect of this biological response modifier on infection-stimulated alveolar bone resorption in an in vivo model. Their findings supported the concept that β-glucan can decrease hard and soft tissue destruction in animals. [41]

On the other hand, the impact of β-glucan adjunctive therapy to periodontal treatment was nothing highlighted as reflected by a recently demonstrated report. The authors reported no additional effect of β-glucan on the clinical parameters, compared to SRP only. This disagreement could be attributed to the difference between their administered dose and ours. They evaluated the effects of incorporating 10 mg dietary supplement given once daily for 40 days as a therapeutic adjunct plus SRP, [15] while we use 100 mg capsule once a day within the same time frame.

Traditional periodontal diagnostic methods including the assessment of clinical parameters are of limited usefulness because they are not sufficiently accurate to discern between previous periodontal disorders and present disease activity. A qualitative approach can be used to assess the accuracy of clinical diagnoses, and may be of use for more diagnostic accuracy.[42]

In this study, the light microscopic findings reflected better regenerative features; in the form of well-organized transverse bundles of collagen fibers, preserved fibroblasts and normal vasculature, with absence of any inflammatory changes; following our experimental protocol, compared to control group. Since β-glucan affects immune function with quickened macrophage activation and establishment of Th-1 dominance, the tissue destruction seen in periodontal disease might be inhibited through its immune-modulating effect, as agreed by *Acar et al. (2012).* [15] Moreover, *Chaple et al.* (1998) had reported that; there was a failure of the recruitment and activation of macrophages in the gingival samples obtained from untreated advanced periodontitis patients. Thus, the ability of β-glucan to stimulate macrophages seems to be very crucial.[43]

Extracellular matrix remodeling is a complex and essential process for the development, homeostasis and repair of connective tissues. Temporal, concerted regulation of genes coding MMPs and their tissue inhibitors (TIMPs) is required for those processes to occur. [44]

Quantitative analysis of MMP-1 and MMP-9 immuno-expression in the gingival tissue samples from our control sites revealed higher staining reaction of these cytokines than at the experimental sites. Increased expression of MMPs in diseased periodontal tissues seems to be the consensus in the literature and is thought to account for the destruction of soft and mineralized tissues that result in some of the clinical symptoms of periodontal disease. [45, 46]

In the current study, MMP-1 immunostaining at base line was more intense and expressed in the cytoplasm and cell membrane of the basal and spinous cell layers but higher layers showed only membranous staining. Immunoreactivity for MMP-1 extended from the epithelial layers to the underlying connective tissues, and was essential in both inflammatory and fibroblast-like cells. These results were in consistence with other studies on aggressive periodontitis; supporting that over-expression of MMP-1 in human diseased gingiva might contribute to periodontal pathological processes. [47] A nuclear staining of MMP-1 was also revealed in our study, it is an unusual finding because MMP-1 is a cytoplasmatic or membrane-bound protein. In accord, an atypical nuclear staining was observed by *Limb et al.,* who found that MMP-1 was strongly expressed in the nuclei of retinal Müller cells, suggesting that MMP-1 may play an important role in the control of Müller cell proliferation and differentiation during retinal proliferative disease.[48]

Similarly, it was noticeable that nuclear staining of MMP-9 was more expressed at baseline tissue samples. It was previously reported that elevated intra-nuclear MMP-9 activity degrades nuclear DNA repair proteins and promotes accumulation of oxidative DNA damage in neurons. The nuclear localization of gelatinases and their nuclear substrates support a novel role for intra-nuclear gelatinase activity in an intrinsic apoptotic pathway. [49] Furthermore, different active MMPs have been proven to be located in the intra-cytoplasmic or intra-nuclear compartments, with no clear-cut function. It was shown that majority of the neuronal and glial cells following an ischemic or hemorrhagic event expressing MMP-9 in the nuclear compartment also co-expressed activated-caspase 3, indicating a possible link between nuclear MMP-9 localization and apoptosis. [50]

Apoptosis, or programmed cell death is a form of physiological cell death. It is increased or decreased in the presence of infection, inflammation or tissue remodeling. Previous studies suggested that apoptosis is involved in the pathogenesis of inflammatory periodontal disease. [50, 51] In our study model, it was noticed that nuclear staining of MMP-9 decreased markedly after β-glucan treatment. Therefore, we could speculate that β-glucan supplementation to periodontal therapy has a tremendous driving force for the healing process; due to its possible indirect anti-apoptotic ability through its down regulating effect on MMP-9 expression.

Furthermore, neutrophils have evolved to respond rapidly and aggressively to external stimuli and release large quantities of destructive enzymes very rapidly in inflamed periodontal tissues. [52] Aggressive periodontal disease has been consistently associated with a decrease in either the number or function of PMNs.[53] Given that β-glucans are belonging to a group biological response modifiers; having the ability to stimulate the immune system;[31] consequently, the reduction of both MMP-1 and MMP-9 expressions following β-glucan regimen can be explained in light of the findings of *Stashenko et al*., who reported that β-glucan enhances; not only the leucocyte number or phagocytic activity; but also demonstrates its strong immunomodulatory effects that can increase the resistance to a specific periodontal pathogen without the expression of damaging inflammatory cytokines.[41]

Overall, in the current study, recovery of periodontal tissues at the treated sites in both groups was accompanied by a reduction in the levels of MMP-1 and MMP-9; with a remarkable improvement in favor of group 2. This finding showed that augmenting the non-surgical periodontal therapy with the immune regulatory actions of β-glucan, is advisable for better modulation of specific cytokines with known detrimental effects on periodontal healing. [15, 41]

It is worth mentioning that the % of change in MMP-1 immune expression level reflected a barely significant difference after 3 months between group 1 and group 2. This finding might be attributed to the synthesis of MMP-1 by a wide variety of normal cells, such as fibroblasts, macrophages, endothelial and epithelial cells. In addition, MMP-1 is the most abundant component of the periodontal tissue matrix, [54] regulating the degradation of native interstitial collagens.[55] Reviewing the literature, early studies on tetracycline use as a host modulatory therapy; interestingly revealed that MMPs which were produced in excessive quantities in inflamed periodontal tissues were more sensitive to inhibition than those constitutively expressed. [56]

Hence, this conclusion could explain our results; concerning the significant sensitivity of MMP-9 to β-glucan compared to MMP-1. Given that patients with periodontitis may have elevated levels of specific inflammatory markers that can be correlated to the severity of the disease,[57] MMP-9 could be reported as a more sensitive indicator of the periodontal status compared to MMP-1 according to our preliminary results in this regard.

Ultimately, there is a compelling evidence that adjunctive antibiotic treatment frequently results in more favorable clinical response than conventional therapy alone in treatment of LAP; [2] owing to the presence of periodontal pathogens, specifically *Aggregatibacter actinomycetem comitans*, known to remain in the tissues after therapy and re-infect the pocket. [58]As antibiotics have many side effects e.g.; drug resistance, interaction,… etc., many researchers have focused on new therapeutic agents, as alternatives to antibiotics.[15] Recently, *Arweiler et al*., in 2013 assessed the use of photodynamic therapy plus SRP in treating a group of patients with AgP without antibiotic therapy. The resultant 3 months-clinical improvements following their regimen; [59] could encourage the designation of other non-antibiotic-based therapeutic combinations for AgP patients.

Actually, protocols for treating AgP are largely empirical. In our preliminary short-term study, β-glucan was used alone, without antibiotic therapy, as an adjunct to SRP. Fortunately, numerous studies have proved that β-glucans can improve resistance to bacterial infection when used solely. Antimicrobial activity was proven against *Staphylococcus aureus* resistant to antibiotics; in mice.[2] Furthermore, orally-administered β-glucan provided a maximal antrax-protective effect in mice, without using antibiotics;[34] it was also reported that β-glucan therapy reduces postoperative infections by 39%. [60]

Finally, it was reported that β-glucan stimulates the production of precursor cells in bone marrow, resulting in a bloodstream flow of new immunocytes into the various lymphoid organs throughout the body. The increased amount of immunocytes in circulation means increased protection from potential invaders. It is important particularly in case of stressed tissues with weak immune response. Moreover, β-glucan therapy showed reduced mortality, lowered infection, stronger tensile strength of scar tissue; meaning that immunomodulators; which enhance macrophage activity; support the repair of damaged tissues and have positive effects on collagen biosynthesis in the healing of wounds in experiments using animal and human models.[61] Taken together, it is conceivable that our proposed host-oriented regimen could be particularly beneficial for patients who are susceptible to periodontal disease due to a reduced phagocyte function, increased cytokine response and virulent microbial staffs; such as aggressive forms of periodontitis, with an expected clinical efficacy.

CONCLUSION

The current study confirm that β-glucan regimen may provide therapeutic beneficial clinical effects to augment the results following non-surgical periodontal therapy. A combined potential of these agents could be suggested to modulate the

expression of MMP-1 and MMP-9 known to have detrimental effects on periodontal healing. The expression of MMP-9 may be a more precise indicator of periodontal disease activity, compared to MMP-1.

RECOMMENDATION

As β-glucans are inexpensive and have a good margin of safety, their potential therapeutic value deserves further detailed investigations for clarifying the paucity of information in the literature aiming to design a proper strategy for their possible use in clinical periodontal practice. Moreover, further studies are warranted to evaluate the extent of its clinical efficacy, in addition to assessing its antibacterial activity against the periodontal putative pathogens.

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