A Randomized Placebo-Controlled Intervention with β-Glucan in the Treatment of Localized Aggressive Periodontitis

Hala H. Hazzaa[1,2], Mai S. Attia[1], Marwa A. El Shiekh[3], Marwa M. Tawfik[4] and Phoebe M. Abd El Massieh[5]

[1]Department of Oral Medicine, Periodontology, Diagnosis and Radiology, Faculty of Oral Medicine, Al-Azhar University (Girls Branch), Cairo; [2]Department of Oral Medicine, Periodontology, Diagnosis and Radiology, Faculty of Dentistry, Nahda University, Beni-Suef; [3]Department of Oral Biology. Faculty of Oral Medicine, Al-Azhar University (Girls Branch), Cairo; [4]Department of Oral Medicine, Periodontology and Diagnosis, Faculty of Dentistry, Beni-Suef University, Beni-Suef; [5]Department of Oro-Dental Genetics, Human Genetics and Genome Research Division, National Research Center, Cairo, Egypt

Abstract

Objective: To investigate the feasibility of β-glucan as an adjunct to non-surgical therapy in patients with localized aggressive periodontitis (LAP).

Materials and methods: Thirty patients were randomly and equally distributed to undergo scaling and root planing together with either placebo pills (Group I) or β-glucan (100 mg/once a day; Group II) for 40 days. Pocket depth (PD), clinical attachment level (CAL), and gingival index (GI) were monitored on days 0 and 91. Histological and immunohistochemical examination were performed using anti-matrix metalloproteinase (MMP)-9 antibodies for gingival samples at the same time points.

Results: A significant clinical improvement was noticed at the end of the study for both treatments ($p < 0.05$). Group II showed a significantly greater reduction in PD (1.57 ± 0.62, $p = 0.005$), an increase in CAL (1.56 ± 0.35, $p = 0.0001$), and a decrease in GI (1.39 ± 0.08, $p = 0.0001$) compared to Group I (0.85 ± 0.66, 0.65 ± 0.25 and 0.82 ± 0.10, respectively). Group II also showed a significantly lower level of MMP-9 expression ($p = 0.0001$), compared to the control treatment.

Conclusions: The results of the non-surgical therapy on patients with LAP were remarkably improved clinically by β-glucan administration, accompanied by a trend for modulation of the MMP-9 profile in gingival tissue samples.

Key words: β-glucan, host modulation therapy, matrix metalloproteinases, randomized double-blind clinical trials

Introduction

There are two principal forms of periodontitis: chronic and aggressive. Aggressive periodontitis (AP) is traditionally known as a periodontal disease that rapidly progresses in healthy patients who show familial aggregation of AP. Aggressive periodontitis can manifest in either a localized or generalized manner. Patients with AP usually express limited microbial plaque deposits that are inconsistent with the intensity of periodontal tissue damage (Armitage, 1999). Patients suffering from AP show rapid loss of clinical attachment and bone destruction that starts earlier in life compared with chronic periodontitis (CP) patients (Albandar, 2014). In AP,

Correspondence to: Hala Helmi Ahmad Hazzaa, 2 Farid Nada St., Benha, Qalubia, Egypt. Mobile number: +02 01014127276; Telephone/fax number: +02 0133249414. E-mail: hala.hazzaa@yahoo.com

patients usually exhibit a hyper-responsive monocyte/macrophage phenotype (Lang *et al.*, 1999) and neutrophil abnormalities (Ryder, 2010).

Activation of natural killer (NK) cells was correlated with excessive alveolar bone resorption (Chaushu *et al.*, 2012). Recently, in AP lesions, a pronounced induction of NK cell-mediated cytotoxicity was reported (Kebschull *et al.*, 2013).

Currently, the management of patients with AP remains a real challenge because of a lack of standardized protocols for effective disease control. These patients often require adjunctive local and systemic chemotherapeutic therapy. The best results were obtained with a systemic route of administration in conjunction with or immediately after conventional therapy (Kaner *et al.,* 2007). Typical systemic antibiotic regimens have included amoxicillin and metronidazole. One group of researchers additionally evaluated the efficacy of adjunctive moxifloxacin in the treatment of AP with significantly greater improvement in bleeding on probing, pocket depth and clinical attachment gain (Ardila *et al.,* 2015).

However, the use of antibiotics usually presents some adverse effects such as gastrointestinal tract problems, renal and hepatic impairment, resistance and allergic reactions (Moreira *et al.,* 2015) that might preclude their use. A potentially powerful and 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 (Cho *et al.,* 2010). β-glucans belong to a group of physiologically active compounds having the ability to stimulate the immune system (De Repentigny *et al.,* 2009). β-glucans are constitutional components of the cell walls of many bacteria, yeast, algae, and cereal grains (Jacob and Pescatore, 2014). Treatment with β-glucans usually results in increased resistance to microbial infections (Yun *et al.*, 2003) and enhanced wound healing (Petravic-Tominac *et al*, 2010). β-glucans are ligands of diverse immune receptors, including dectin-1, complement receptor and toll-like receptor (TLR)-2/6. Additionally, β-glucans can stimulate several types of immune cells, including macrophages, neutrophils, monocytes, NK cells, and dendritic cells. Consequently, β-glucans can modulate both innate and acquired responses (Chan *et al.*, 2009).

The failure to impose a suitable immune response against the microbial plaque deposits has been reported to play a major role in the pathogenesis of periodontal disease (Breivik *et al.*, 1996; Breivik and Thrane, 2001). In this context, Breivik *et al.* (2005) concluded that β-glucan enhances periodontal disease resistance by acting on the immune system and the hypothalamic–pituitary–adrenal (HPA) axis.

Page and Baab (1985) reported that AP is a Th2-mediated lesion. Various researchers have stated that the role of immunity can be promoted by establishing Th1 dominance and by activating macrophages (Inoue *et al.*, 2002; Lee *et al.*, 2002). In this context, it was found that β-glucan can activate macrophages through mitogen-activated protein kinase and the nuclear factor kappa-B signaling pathway (Chang *et al.*, 2010). β-glucan has also a beneficial effect in restoring Th1 function (Suzuki *et al.*, 2001). Thus, β-glucan was hypothesized as an adjunct to scaling and root planing (SRP) for enhancing immune function and restoring the protective Th1 response in patients with AP.

Presently, it is thought that neutrophils are primed to attain a heightened state of readiness due to the continual existence of severe periodontal infections in AP. These primed neutrophils secrete a range of lytic enzymes that can accelerate the native tissue damage (Kantarci and van Dyke, 2005; Ryder, 2010). Matrix metalloproteinase (MMP)-9, a member of the gelatinase family of MMPs, is secreted by neutrophils and is mainly involved in the degradation of gingival collagen type IV (Ingman *et al.*, 1994). Higher crevicular levels of MMP-9 were observed at baseline in diseased sites compared to healthy sites in LAP. A significant reduction in MMP-9 levels was detected following SRP and systemic treatment with antibiotics (Gonçalves *et al.*, 2013). Given that β-glucan can increase the phagocytic activity of leukocytes without inducing the expression of damaging inflammatory cytokines (Stashenko *et al.*, 1995), MMP-9 expression could be regulated using β-glucan in patients with LAP.

Nevertheless, the effective dose of β-glucan remains an important point to be addressed. A dose-response trend in infection incidence among patients who received a *Saccharomyces cerevisiae* yeast strain of β-glucan at doses of 0.1 - 2.0 mg/kg body weight was observed. The authors reported that β-glucan is effective at a dose of 0.5 mg/kg or greater, but that the use of too small a dose (0.1 mg/kg) produces little or no immunomodulatory effect that is similar to a placebo effect (Babineau *et al.*, 1994). Based on that evidence, for the first time, this project was intended to evaluate the daily use of 100 mg β-glucan as an adjunctive treatment to SRP therapy in treating patients with LAP.

Materials and methods

Study population

Thirty social class-matched, Egyptian patients with LAP were selected from the outpatient clinic of the Periodontology Department, Faculty of Dentistry, Al-Azhar University. The social class was evaluated using the "Objective socio-economic status evaluation" (Adler *et al.*, 2000). The income extent was evaluated according to the average income of the Egyptian family reported by the Central Agency for Public Mobilization and Statistics, Cairo, Egypt. This study was performed between January 2013 and August 2014.

Patients were diagnosed with LAP according to Armitage (1999). The inclusion criteria included: 1) Patients were healthy as defined by Abramson (1966); 2) Patients had no more than two teeth, other than first molars and incisors, with a probing depth (PD) ≥ 5 mm, a clinical attachment level (CAL) ≥ 5 mm, and bleeding on probing; 3) Patients had a history of a rapid rate of clinical attachment loss; 4) Patients had radiographic evidence of moderate to severe vertical bone loss around the permanent incisors and first molars; 5) Every patient had one tooth indicated for extraction due to periodontal infection; 6) Patients had a familial aggregation of AP.

Our exclusion standards included: patients with previous SRP, allergy to β-glucan, systemic diseases, taking anti-inflammatory medications for a long time, antibiotics in the last 6 months, or allergy to chlorhexidine (CHX). Current or former smokers and pregnant or lactating women were also excluded.

The design of this study (NCT02402296) was accepted by the Ethics Committee of Al-Azhar University. This treatment protocol was applied in accord with the ethical fundamentals described in the Declaration of Helsinki. All patients conferred written informed consent after being informed of the study objectives.

Randomization

In this study, our randomization unit was the patient. Random allocation was performed by an individual not involved in the study by giving every patient a code number. Only one person randomly assigned the patients to the groups using a single allocation ratio (1:1).

Study design

After the last visit of SRP, all the patients were randomly distributed to the following groups (n = 15) in a parallel, double-masked study: Group I (control), in which patients received placebo pills (empty capsules, Faculty of Pharmacy, Al-Azhar University, Girls Branch) for 40 days. Group II (experimental), in which patients received systemic treatment with β-glucan (Imurril capsules, beta-1,3/1,6-D-glucan, manufactured by Sigma Pharmaceutical Industries for Elite Pharma) once a day for 40 days. All the patients used a CHX rinse (Peridex™, chlorhexidine gluconate 0.12% oral rinse, 3M ESPE Dental Products, USA) of 15 ml for 1 min/rinse twice/day for 60 days. Full-mouth SRP was done by a professional periodontist using an ultrasonic scaler (Cavitron® EMZ, Switzerland) and periodontal curettes (Hu-Friedy Instrument Co., USA), in 4 - 6 appointments within 14 days as described.

Supragingival scaling was performed in one visit on day 1. Then, each patient received full-mouth SRP in 2 - 4 appointments on day 7. One or more quadrants were instrumented in each session according to the amount of deposits. Lastly, full-mouth SRP was performed on day 14. This protocol, as well as oral hygiene instructions, was applied to all patients in both groups.

The oral hygiene instructions were as follows: all the patients followed the steps of the Bass method of tooth brushing using soft-bristled toothbrushes (twice/day for 2 min). Patients were asked to use interdental cleaners and the same dentifrice during the period of the study (Parodontax, GlaxoSmithKline plc., United Kingdom).

During the SRP period, all patients took part in a motivation and education program prior to beginning treatment. β-glucan or placebo supplementation and CHX treatment were implemented immediately after the last scaling session.

Method of medication allocation

The placebo and β-glucan supplements were identically packed. Every set of four packs (each pack contained 10 capsules) was labeled with the code number of each subject by the study coordinator according to the treatment assigned. The study members remained blinded to the patient treatment assignment. The randomization code was kept sealed until the end of the study.

Monitoring of compliance

Regular drug uptake and patient compliance were checked every ten days. Each subject returned the old pack, received a new pack, and then completed a questionnaire in which any side effects of the medication or placebo were recorded. At the same visit, all the patients attended an oral hygiene reinforcement session. Oral hygiene was assessed using a graduated probe for evaluating the gingival condition and plaque deposits. The clinical assessment was performed by another trained investigator who remained masked to the treatment administered.

Clinical assessment

The following variables were assessed: 1) gingival index (GI; Löe and Silness, 1963); 2) PD, which was measured from the gingival margin to the bottom of the pocket; and 3) CAL, which was measured from the bottom of the pocket to the cemento-enamel junction using Williams' graduated probe. For each tooth, PD and CAL were measured at three buccal sites (mesio-, mid- and distobuccal), and three lingual sites (disto-, mid- and mesio-lingual). Except for 3rd molars and hopeless teeth, the patients underwent clinical evaluation at baseline (day 0), immediately after the last scaling visit (day 14), and at the end of the study (day 91).

Investigator calibration

The study investigator performed a calibration exercise in non-study periodontitis patients (n = 10) in two separate sessions that were 48 hours apart. The standard error was then calculated, and the intra-examiner variability was 0.147 mm and 0.189 mm for PD and CAL, respectively (kappa value = 0.84).

Light microscopy analysis

Incisional gingival biopsies were collected from the proposed extraction sites under local anesthesia at baseline (day 0), immediately after the last scaling visit (day 14), and at the end of the study (day 91) using a Bard-Parker blade No. 15. Gingival specimens were obtained from two sites along the dentogingival region. Gingival samples were carefully dissected, cut into 1 x 10 mm specimens, and then extraction was done. Fixation was done using a 10% formalin solution. The samples were embedded in paraffin blocks and sections 4 µm-thick were prepared. Serial sections were stained using hematoxylin and eosin (H & E). For each specimen, the total cell count in four separate fields was averaged for each section. The inflammatory reactions were categorized as mentioned by Saghiri *et al.* (2013).

Immunohistochemical analysis

Immunohistochemical staining of the 4-µm-thick sections was done using an anti-MMP-9 antibody (92 kDa collagenase IV Ab-1, clone GE-213; Thermo Scientific, USA) and was performed according to the manufacturers' instructions using MMP-9. Immunoreactivity for MMP-9 was evaluated by measuring the percentage of positively immunostained areas relative to the examined area in each field using a computerized Leica imaging system (Germany). The sites expressing a positive immune reaction with the MMP-9 antibody were identified as brown deposits of the chromogen. Stained regions (nuclear and/or cytoplasmic stain) were regarded as positive regardless of the staining intensity (the stronger the immune reaction, the darker the chromogen intensity).

The imaging system used consisted of a color video camera, a color monitor, and the hard disc of an HP personal computer connected to the microscope, which was controlled by Leica Qwin 500 software. The imaging system was automatically calibrated to convert the measurement units (pixels) produced by the imaging program into micrometers. The percent area of MMP-9 immunoreactivity was assessed with reference to a standard measuring frame 11434.9 μm^2 at 200x magnification. Using the color detector, areas of positive immunostaining were masked with a blue color in a binary manner *(Figure 1)*. Ten fields per section from each patient were serially and successively used for histomorphometric analysis. Mean immunostaining values were then calculated for each specimen.

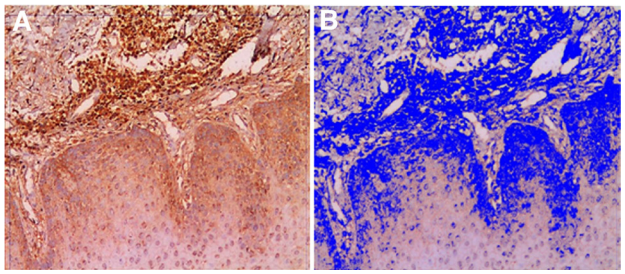

Figure 1. Measurement of immunohistochemical reactivity using a computerized imaging system, where the positively immunostained areas in (A) were masked with a blue color in a binary manner in (B).

Primary outcome variable and sample size calculation

Our research design was based on similar studies (Mestnik *et al.*, 2012); the chosen primary outcome was CAL. The secondary outcomes were PD, GI, and MMP-9. A power analysis was intended to have proper potential to apply a 2-sided statistical test of the research hypothesis (null hypothesis) that there was no difference between the two groups. Using alpha (α) level of 0.05 (5%) and beta (β) level of 0.20 (20%), a post-study power specified that this trial had nearly 80% power and significance level = 5; with the expected minimum sample size of a total of 30 patients (15 patients/group). Analysis was done using IBM™ SPSS™ Sample Power™ Version 3.0.1.

Statistical analysis

All clinical variables were computed per patient and then across the patients in the studied groups. The mean number/percentage of sites with PD ≤ 3 mm, ≥ 4 mm, ≥ 5 mm, ≥ 6 mm, or ≥ 7 mm was also evaluated within the categories of PD per patient and then across the patients in each group. Paired samples *t*-tests were utilized to evaluate the significance of differences in each group over the study course. Comparisons between groups, at each time interval, were done using independent samples *t*-tests. The Chi-square test was applied to compare the distribution of gender between both groups. To explore the outcome variable, a stepwise forward logistic regression model was used: the presence of ≤ 4 sites with PD ≥ 6 mm 3 months after treatment (yes/no). The clinical predictor parameters were age, gender, and β-glucan administration, in addition to the mean PD, CAL, GI, and MMP-9 level at baseline.

The significance level was set at a *p*-value ≤ 0.05. Statistical analysis was done using IBM® SPSS® Statistics version 18 for Windows.

Results

Patient characteristics and adverse effects

No adverse events were reported by any patients. All subjects mentioned that they completed the course of their treatment *(Figure 2)*, as confirmed by pill counting. The groups were statistically comparable in terms of age and gender ($p > 0.05$; *Table 1*).

Study parameters

Table 2 shows the mean data for the clinical and histopathological indicators evaluated at baseline and 3 months after treatment. At baseline, no statistically significant differences were detected in any parameter between groups ($p > 0.05$). However, significant differences were encountered at 3 months between the study groups: specifically, the mean PD, CAL, GI, and MMP-9 level were lower in the test group ($p < 0.05$). Moreover, Group II had statistically significantly fewer sites with PD ≥ 4 mm and ≥ 5 mm ($p < 0.05$). Both groups displayed a remarkable statistically significant improvement in the evaluated clinical variables through the study course ($p < 0.05$).

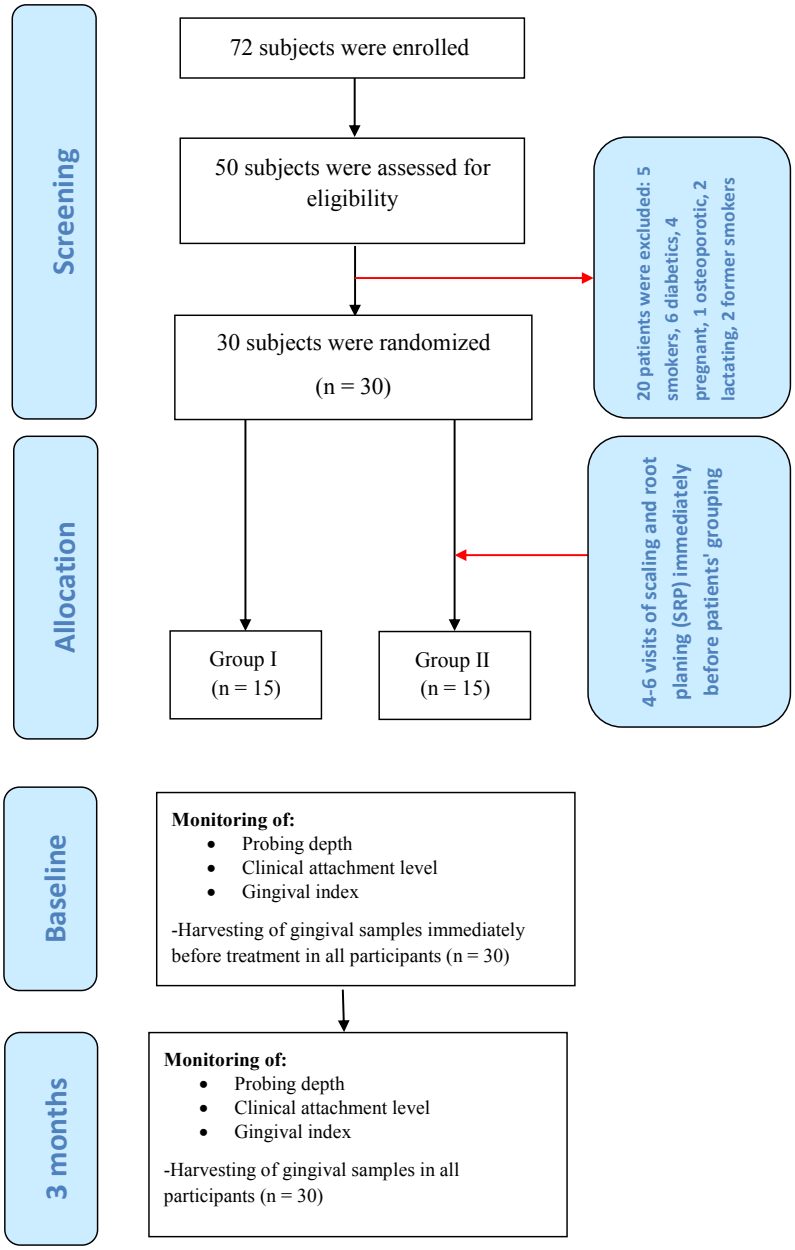

Figure 2. Flowchart showing the study design.

Table 1. Demographic data of the participating patients.

Variable	Group I	Group II	*p* - value
Gender (male/female)	6/9	7/8	0.713
Age (years)	25 ± 1.77	24.4 ± 2.1	0.405

Group I, scaling and root planing alone; Group II, scaling and root planing plus β-glucan

Statistically significant mean differences in the study parameters, including PDs of 4 - 6 mm and ≥ 7 mm and the number of sites with a PD of 4 - 6 mm, were detected between the two time points in Group II ($p < 0.05$; *Table 3*).

Logistic regressions were performed to identify the variables that maximally discriminated between patients prone to develop ≤ 4 sites with PD ≥ 6 mm at 3 months after therapy and those who were not. The presence of ≤ 4 sites with PD ≥ 6 mm at 3 months post-therapy (yes/no) was used as the (binary) dependent variable, and age, gender, β-glucan level, and the following parameters at baseline were entered as independent (predictor) variables: mean PD, CAL, GI, and MMP-9. These variables were entered into the analysis in a forward stepwise fashion. *Table 4* shows that supplementation with β-glucan significantly raised the likelihood of a person having ≤ 4 sites with PD ≥ 6 mm at 3 months after therapy ($p = 0.037$, OR = 17.61). Additionally, the lower the mean CAL at baseline, the greater the possibility of a subject having ≤ 4 sites with PD ≥ 6 mm at 3 months after therapy. Every unit decrease in CAL at baseline was associated with a 58.8 (1/0.017)-unit increase in the likelihood that the subject would have ≤ 4 sites with PD ≥ 6 mm at 3-months after therapy ($p = 0.016$).

Table 2. Mean ± SD of clinical and laboratory measures at both baseline and follow-up settings.

Variable	Time point	Group I	Group II	*p* - value
Mean % (no.) of sites with				
PD ≤ 3 mm	Baseline	38.67 ± 8.78 (52.4 ± 13.32)^{Aa}	36.12 ± 9.54 (53.53 ± 14.67)^{Aa}	0.451
PD ≥ 4 mm	Baseline	61.33 ± 8.78 (82.8 ± 12.3)^{Aa}	63.89 ± 9.54 (94.47 ± 14.02)^{Aa}	0.451
PD ≥ 5 mm	Baseline	40.87 ± 5.22(55.07 ± 6.4)^{Aa}	44.63 ± 5.45 (66 ± 8.19)^{Aa}	0.064
PD ≥ 6 mm	Baseline	22.92 ± 6.1 (30.87 ± 7.84)^{Aa}	23.23 ± 6.2 (34.27 ± 8.86)^{Aa}	0.892
PD ≥ 7 mm	Baseline	9.13 ± 4.01 (12.33 ± 5.46)^{Aa}	9.77 ± 4.67 (14.47 ± 6.91)^{Aa}	0.690
PD ≤ 3 mm	3 mos.	56.87 ± 22.39 (76.8 ± 30.53)^{Ba}	76.70 ± 18.08 (113.8 ± 28.2)^{Bb}	0.013*
PD ≥ 4 mm	3 mos.	43.13 ± 22.39 (58.4 ± 30.79)^{Ba}	23.3 ± 18.08 (34.2 ± 26.13)^{Bb}	0.013*
PD ≥ 5 mm	3 mos.	25.18 ± 17.83 (34.4 ± 24.65)^{Ba}	8.95 ± 12.04 (13.07 ± 17.33)^{Bb}	0.007*
PD ≥ 6 mm	3 mos.	8.71 ± 8.68 (11.93 ± 11.77)^{Ba}	3.08 ± 7.05 (4.47 ± 10.14)^{Ba}	0.062
PD ≥ 7 mm	3 mos.	1.23 ± 1.86 (1.67 ± 2.47)^{Ba}	1.15 ± 2.66 (1.67 ± 3.83)^{Ba}	0.923
Mean measures of clinical and laboratory parameters				
PD (mm)	Baseline	4.18 ± 0.29^{Aa}	4.23 ± 0.44^{Aa}	0.698
	3 mos.	3.33 ± 0.80^{Ba}	2.67 ± 0.67^{Bb}	0.02*
CAL (mm)	Baseline	4.47 ± 0.39^{Aa}	4.33 ± 0.52^{Aa}	0.409
	3 mos.	3.82 ± 0.29^{Ba}	2.77 ± 0.50^{Bb}	0.0001*
GI (mm)	Baseline	2.07 ± 0.04^{Aa}	2.06 ± 0.03^{Aa}	0.846
	3 mos.	1.24 ± 0.09^{Ba}	0.67 ± 0.08^{Bb}	0.0001*
MMP-9	Baseline	29.31 ± 0.81^{Aa}	30.05 ± 1.32^{Aa}	0.072
	3 mos.	22.65 ± 1.45^{Ba}	19.25 ± 1.69^{Bb}	0.0001*

*$p < 0.05$. The assessment of the significance of differences between groups was done at each time point using the independent samples *t*-tests (different small letters indicate significant differences between the two groups, A: stands for the significance at baseline, B: stands for the significance at 3 months). The significance of differences between baseline and the follow-up visit at 3 months was evaluated using paired samples *t*-tests; different capital letters show significant differences at different time points. Group I, scaling and root planing alone; Group II, scaling and root planing plus β-glucan; PD, pocket depth; CAL, clinical attachment level; GI, gingival index; MMP-9, matrix metalloproteinase-9; mos, months.

Table 3. Mean difference in clinical and laboratory variables between the two groups at baseline and 3 months after treatment.

Variable	Time point	Group I	Group II	*p* - value
PD reduction	0 - 3 month	0.85 ± 0.66	1.57 ± 0.62	0.005*
CAL gain	0 - 3 month	0.65 ± 0.25	1.56 ± 0.35	0.0001*
GI reduction	0 - 3 month	0.82 ± 0.10	1.39 ± 0.08	0.0001*
MMP-9 reduction	0 - 3 month	6.67 ± 1.57	10.81 ± 1.78	0.0001*
The mean difference in PD measures between baseline and 3 month				
PD 4 - 6 mm	0 - 3 month	1.02 ± 0.89	1.96 ± 0.71	0.003*
PD ≥ 7 mm	0 - 3 month	2.30 ± 0.94	3.64 ± 1.18	0.002*
The mean difference in number of sites between baseline and 3 month				
PD 4 - 6 mm	0 - 3 month	13.73 ± 28.19	47.47 ± 21.43	0.001*
PD ≥ 7 mm	0 - 3 month	10.67 ± 5.54	12.8 ± 6.27	0.332

*$p < 0.05$; Group I, scaling and root planing alone; Group II, scaling and root planing plus β-glucan; PD, pocket depth; CAL, clinical attachment level; GI, gingival index; MMP-9, matrix metalloproteinase-9.

Table 4. Forward stepwise binary logistic regression model for the presence of ≤ 4 sites with probing depth ≥ 6 mm at 3 months after treatment.

Variables	Coefficient	SE	OR	95% CI	*p*-value
β-glucan	2.87	1.38	17.61	1.19 – 261.1	0.037*
Mean CAL at baseline	-4.099	1.69	0.017	0.001 - 0.459	0.016*

*$p < 0.05$; CAL, clinical attachment level; SE, standard error; OR, odds ratio; CI, confidence interval.

Descriptive histology

At baseline, histopathologic evaluation of the gingival tissue from both groups showed epithelial hyperplasia with elongated rete pegs. The lamina propria, a type of connective tissue, showed increased vascularization together with severe inflammatory cell infiltrates. At 3 months after treatment, Group I (control) revealed epithelial hyperplasia with broad epithelial rete pegs. The connective tissue appeared to have reduced vascularity and moderate inflammation. In Group II, the gingival tissue showed a normal epithelial thickness with elongated, thin rete pegs. The connective tissue appeared to have normal vascularity with inflammation ranging from mild to minimal *(Figure 3)*.

Expression of MMP-9 based on immunohistochemistry

At baseline, MMP-9-immunostained gingival samples in both groups showed cytoplasmic immunostaining all over the epithelial cells, although some cells displayed nuclear staining. The connective tissue showed immunostaining of fibroblasts, collagen fibers, and inflammatory cells. After 3 months, the control group showed cytoplasmic and nuclear immunostaining in the basal and spinous cell layers with clear reduction in the degree of staining in the other cell layers. The connective tissue showed immunostaining of inflammatory cells, fibroblasts, and collagen fibers. Group II showed cytoplasmic and nuclear immunostaining of the basal cell layer, and few spinous cells showed nuclear staining. The remaining cells in these layers lacked MMP-9 staining *(Figure 4)*.

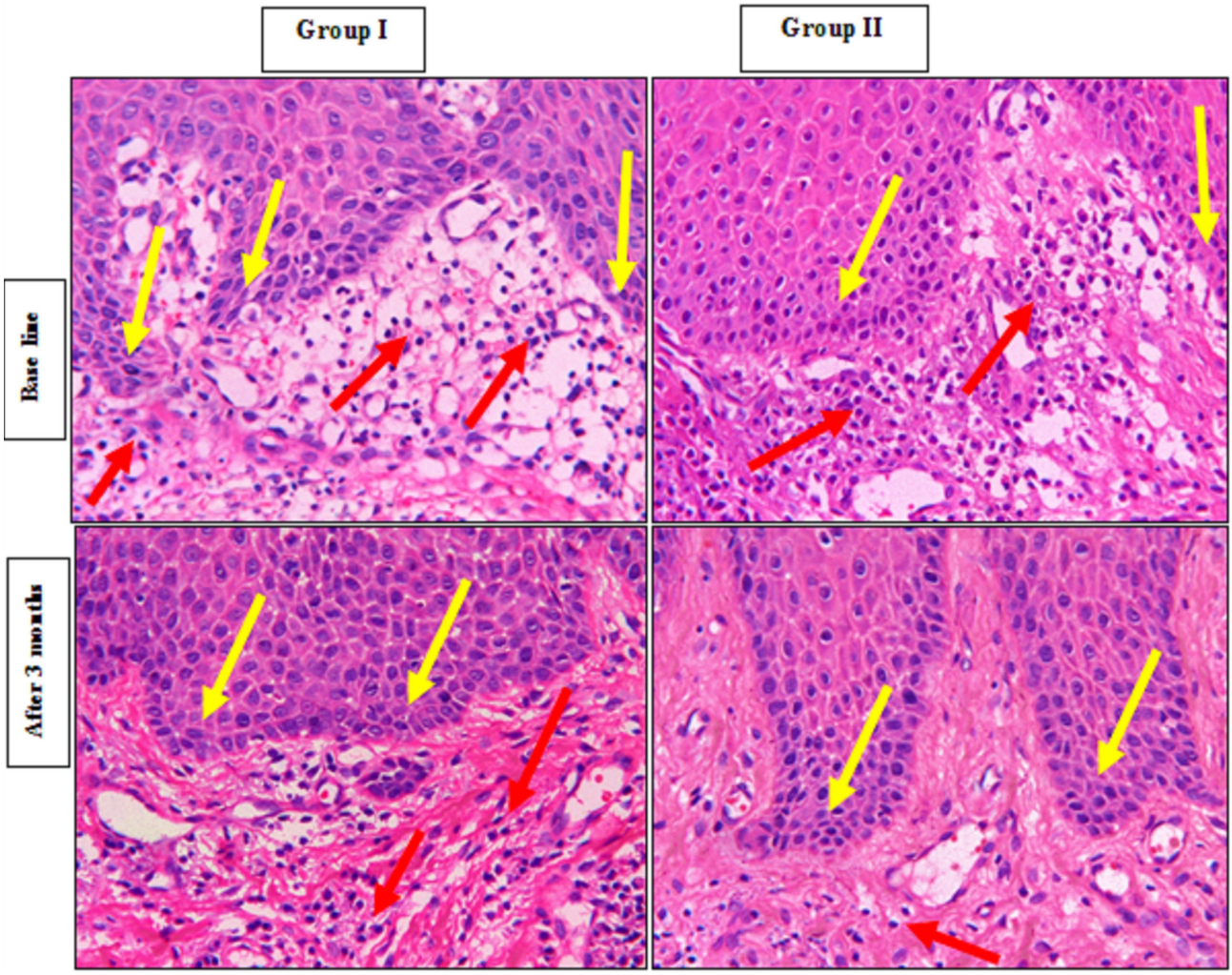

Figure 3. At baseline, the gingival tissue in both groups showed hyperplastic and acanthotic epithelium (A) with elongated rete pegs (yellow arrows). The connective tissue (lamina propria) appeared to have high vascularization and severe inflammatory cell infiltrates (red arrows). After 3 months, gingival epithelium in Group I (control) showed epithelial hyperplasia with broad epithelial rete pegs (yellow arrows). The connective tissue appeared to have apparently decreased vasculature and moderate inflammation. Group II (β-glucan) showed a normal thickness of the epithelium with elongated thin rete pegs (yellow arrows). The connective tissue showed normal vasculature and mild inflammation (red arrow; H & E, magnification 200x).

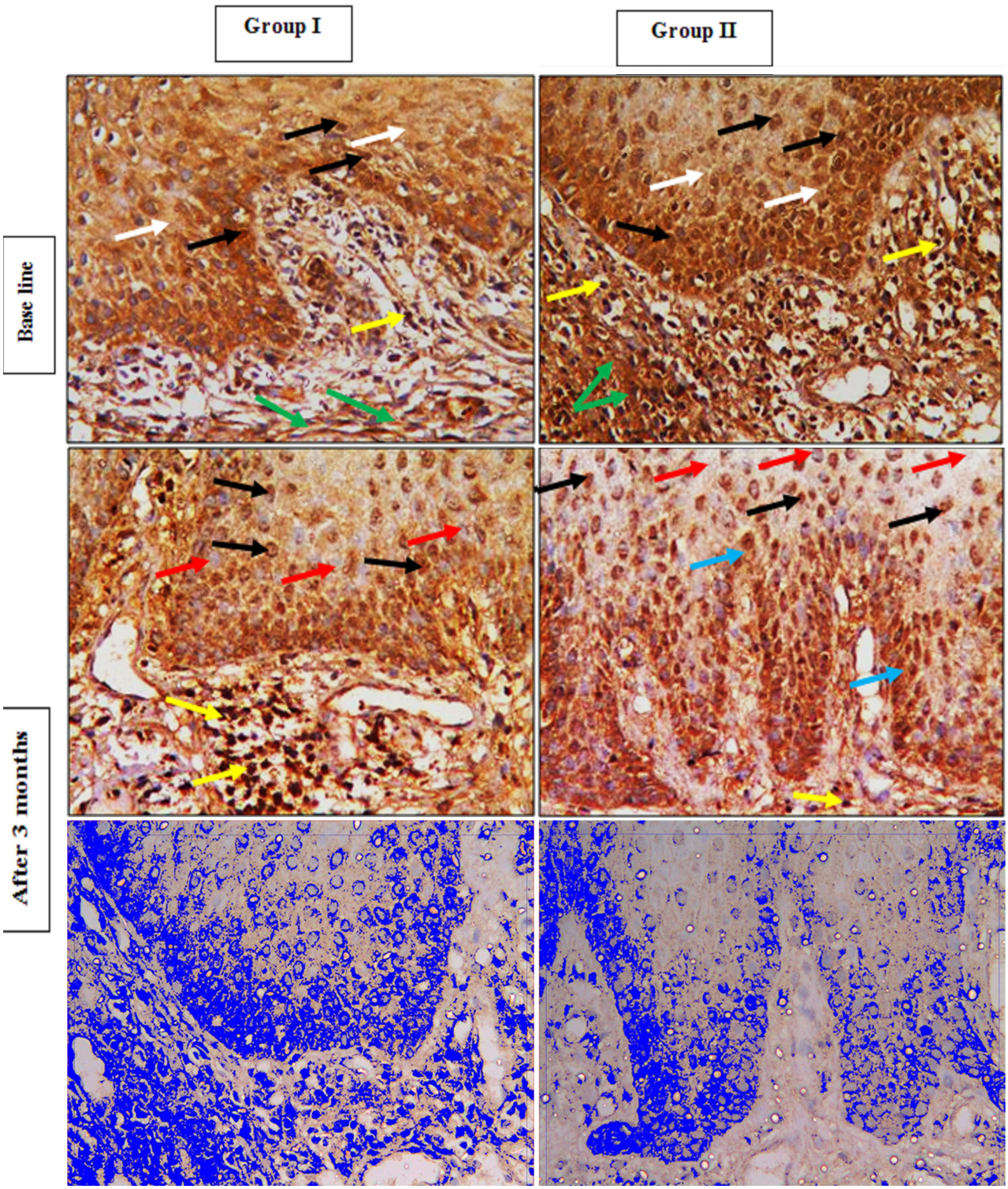

Figure 4. At baseline, the gingival tissue immunostaining with MMP-9 in both groups revealed intense cytoplasmic staining (white arrows), and some epithelial cells showed nuclear immunoexpression (black arrows). The connective tissue showed immunostaining of fibroblasts, collagen fibers (green arrows) and inflammatory cells (yellow arrows). The 3-month results of the placebo group showed cytoplasmic staining and some epithelial cells showed nuclear immunostaining (black arrows). Some areas were negatively stained (red arrows). The connective tissue showed immunostaining of inflammatory cells (yellow arrows). Group II (β-glucan) showed cytoplasmic immunostaining of the basal cell layer (blue arrows) and only a few spinous cells showed nuclear staining (black arrows). The remaining higher cells were negatively stained (red arrows; MMP-9 immunostaining, magnification 200x). An obvious decrease in the staining in Group II compared to Group I and their representation by the masked blue color was also revealed in Figure 1.

Discussion

It is worth noting that this is the first clinical report to provide data about the adjunctive therapy of β-glucan in treating LAP patients. The current findings revealed that the use of this therapeutic protocol offers additional clinical and immunomodulating advantages compared to SRP alone.

The patients in Group II were assigned a β-glucan protocol for 40 days. It has been reported that the β-glucan treatment duration varies from 39 - 90 days in various research models (Kabasakal *et al.*, 2011; Lin *et al.*, 2011; Turunen *et al.*, 2011). In this study, subjects taking β-glucan exhibited significantly greater reduction in PD, gain in CAL and a reduction in gingival inflammation than those receiving SRP alone. Previous studies have demonstrated the efficiency of β-glucan in dampening the microbial-induced gingival inflammation (Rice *et al.*, 2005). Moreover, Breivik *et al.* (2005) stated that β-1,3/1,6 glucan notably decreased periodontal bone loss in a ligature-induced periodontal disease model. Their findings support the conception that β-glucan can reduce periodontal tissue demolition in an *in vivo* model.

Nevertheless, the adjunctive use of β-glucan in combination with SRP therapy showed comparable effectiveness to SRP alone in CP (Acar *et al.*, 2012). This discrepancy could be attributed to the nature of the targeted periodontal disease in each study. It is likely that AP is a Th2-mediated lesion due to its B-cell/plasma cell properties (Page and Baab, 1985). β-glucan is an immunostimulatory agent that enhances host-mediated immune responses and changes the balance of IgG1 antibodies (Th2-dependent antibodies) towards a Th1-dependent IgG2 response (Suzuki *et al.*, 2001). Additionally, β-glucan stimulates the production of the Th1-motivating cytokine interferon-gamma (INF-γ) and suppresses the levels of interleukin-4, which promotes Th2 responses (Inoue *et al.*, 2002). Thus, β-glucan skews the Th1/Th2 balance towards a Th1-predominated response (Suzuki *et al.*, 2001), which is the main requisite for the treatment of AP lesions.

However, the collective evidence from various studies shows a consistency of SRP as a therapeutic line of CP (Cobb, 2002; van der Weijden and Timmerman, 2002), in which the amount of destruction is consistent with the bacterial plaque deposits (Lindhe *et al.*, 1999). The stable lesion in CP is characterized by a Th1 response (Page and Baab, 1985). Thus, SRP therapy alone and coupled with β-glucan resulted in a comparable Th1 response in the treatment of CP.

The possible role of osteoprotegerin, a physiologically important regulator of osteoclast cells (Udagawa *et al.*, 2000), in periodontal diseases warrants discussion. Garlet *et al.*, (2004) reported that patients with AP show a lower level of osteoprotegerin than patients with CP. Silva *et al.* (2015) recently concluded that β-glucan can up-regulate osteoprotegerin expression, with beneficial periodontal effects in diabetic animals. Taken together, the influence of β-glucan may be more pronounced in treatment of patients with AP.

The selected dose of β-glucan is an additional factor that should be considered. Initial *in vitro* studies of yeast-derived β-glucan have demonstrated that it competitively binds to its receptors on human monocytes and neutrophils in a dose-dependent manner (Janusz *et al.*, 1986). In this respect, Dellinger *et al.* (1999) found that systemic administration of placebo, 0.5 mg/kg β-glucan and 1.0 mg/kg β-glucan decreased the frequency of postoperative infection or death in malnourished subjects who had non-colorectal operations (to 44%, 24%, and 17%, respectively).

Recently, a yeast-derived insoluble glucan (100 mg/day for 4 weeks) was efficiently used in the treatment of children with chronic respiratory infections. The authors concluded that systemic β-glucan remarkably increased the salivary levels of C-reactive protein and lysozymes, suggesting that this treatment stimulated mucosal immunity (Vetvicka *et al.*, 2013). Thus, 100 mg/day yeast-derived β-1,3/1,6-glucan appears to be an effective therapeutic dose.

Importantly, the mean reductions in PD and CAL observed in Group I were in congruence with the 3-month outcomes of Chitsazi *et al.* (2014), who reported mean reductions of 0.91 mm and 0.75 mm in PD and CAL, respectively, after SRP. Our results revealed a 29.68% lowering in the number of residual pockets ≥ 4 mm after 3 months in Group I. This result was slightly inferior but comparable to that reported by Guerrero *et al.* (2005). Curiously, a relatively modest improvement in the number of moderate and deep pockets ≥ 5 mm was observed in Group I. This could be explained based on the findings of Abdou and Zaazou (2013), who reported that in Egypt, a revolution with a challenging transition phase characterized by a stressful socioeconomic situation occurred in 2011 which, in turn, can affect the management of periodontitis (Rozlog *et al.*, 1999).

However, the clinical results were significantly improved in Group II. Given that SRP was a basic treatment applied to all participants and that the social class was similar between the two groups, any additional therapeutic benefits in Group II would predominantly be attributable to β-glucan, especially considering the non-significant differences noticed between the study groups at baseline.

Ultimately, in this study, the mean PD reductions reported in deep pockets ≥ 7 mm were 2.3 mm and 3.64 mm in Group I and Group II, respectively. These outcomes were in conformity with those of Feres *et al.* (2012), who reported mean PD decrease of 2.6 mm for the SRP group and of 3.3 mm and 3.7 mm for their two experimental groups after 3 months. Furthermore, the changes induced by our treatments caused most of the deep pockets ≥ 7 mm to diminish below 7 mm. Hence, the remaining mean number of pockets ≥ 7 mm was only 1.67 in both groups, which is too small value for a reliable statistical analysis.

In this study, the light microscopy findings revealed enhanced healing following the experimental protocol compared with the control protocol. These results may be attributed to the immunostimulatory activity of β-glucan through the stimulation of the phagocytic activity of macrophages (Lee *et al*., 2002). In this regard, Chapple *et al.* (1998) noted a lack of macrophage induction and activation in gingival samples taken from intensive periodontal lesions. Hence, the adequacy of β -glucan to activate macrophages is important for the associated enhancement in periodontal healing.

The current qualitative analysis of MMP-9 levels in the gingival samples revealed a higher immunoreactivity at untreated sites. Typically, increased expression of MMP-9 has been associated with advanced periodontitis (Smith *et al.,* 2004). Curiously, it was reported that elevated intranuclear MMP-9 activity degrades nuclear DNA repair proteins and enhances the aggregation of oxidative DNA damage in neurons. Furthermore, it was shown that the majority of neuronal and glial cells express MMP-9 in the nucleus and express activated caspase-3 following an ischemic or hemorrhagic incident; these observations indicate a potential connect between nuclear MMP-9 expression and apoptosis (Pirici *et al*., 2011). Prior trials have suggested that apoptosis, a programmed cell death, is involved in the pathogenesis of inflammatory periodontal disease (Zeidán-Chuliá *et al*., 2014). In our model, nuclear staining for MMP-9 was markedly decreased by β-glucan supplementation. Therefore, we speculate that β-glucan supplementation strongly promoted the healing process due to its possible indirect anti-apoptotic activity through the down-regulation of MMP-9.

Ultimately, the logistic regression analysis results reinforced the clinical superiority of β-glucan to placebo. The subjects in Group II were approximately 17.61 times more likely to have ≤ 4 sites with PD ≥ 6 mm at 3 months after therapy than the patients in Group I. Accordingly, use of β-glucan is a significant predictor for a person with LAP to reach a low-risk profile for disease progression (OR = 17.61).

One possible limitation of the present study is the short-term follow-up interval. Indeed, longitudinal trials are required to further grasp the efficiency of the experimental protocol in the therapy of LAP in order to determine whether this protocol can result in valuable changes in the microbial profile and in periodontal clinical indicators over time. In addition, the potential role of several confounders such as smoking and diabetes mellitus should be examined. Furthermore, future prospects for research in this field include studies with larger sample sizes to affirm the current results.

In conclusion, the results of non-surgical treatment of LAP are significantly improved by the adjunctive administration of β-glucan in terms of improved clinical outcomes and down-regulated MMP-9 expression, at least after 90 days follow-up.

References

Abdou DS and Zaazou Z. The Egyptian revolution and post socio-economic impact. *Topics in Middle Eastern and African Economies* 2013; **15**:92-115.

Abramson JH. The Cornell medical index as an epidemiological tool. *American Journal of Public Health Nations Health* 1966; **56**:287-298.

Acar NN, Noyan U, Kuru L, Kadir T and Kuru B. Adjunctive systemic use of beta-glucan in the nonsurgical treatment of chronic periodontitis. In Budeneli N. (Ed): *Pathogenesis and Treatment of Periodontitis*. Shanghai. In Tech China, 2012.

Adler NE, Epel ES, Castellazzo G and Ickovics JR. Relationship of subjective and objective social status with psychological and physiological functioning: Preliminary data in healthy, white women. *Health Psychology* 2000; **19**:586-592.

Albandar JM. Aggressive periodontitis: Case definition and diagnostic criteria. *Periodontology 2000* 2014; **65**:13-26.

Ardila CM, Martelo-Cadavid JF, Boderth-Acosta G, Ariza-Garcés AA and Guzmán IC. Adjunctive moxifloxacin in the treatment of generalized aggressive periodontitis patients: Clinical and microbiological results of a randomized, triple-blind and placebo-controlled clinical trial. *Journal of Clinical Periodontology* 2015; **42**:160-168.

Armitage GC. Development of a classification system for periodontal diseases and conditions. *Annals of Periodontology* 1999; **4**:1-7.

Babineau TJ, Hackford A, Kenler A, *et al*. A phase II multicenter, double-blind, randomized, placebo-controlled study of three dosages of an immunomodulator (PGG-Glucan) in high-risk surgical patients. *Archives of Surgery* 1994; **129**:1204-1210.

Breivik T, Opstad PK, Engstad R, Gundersen G, Gjermo P and Preus H. Soluble beta-1,3/1,6-glucan from yeast inhibits experimental periodontal disease in Wistar rats. *Journal of Periodontol*ogy 2005; **32**:347-352.

Breivik T and Thrane PS. Psychoneuroimmune interactions in periodontal disease. In Ader R, Felten L and Cohen N. (Eds.): *Psychneuroimmunology*, 3rd edition. San Diego. Academic Press, 2001.

Breivik T, Thrane PS, Murison R and Gjermo P. Emotional stress effects on immunity, gingivitis and periodontitis. *European Journal of Oral Science* 1996; **104:**327-334.

Chan G, Chan W and Sze D. The effects of β-glucan on human immune and cancer cells. *Journal of Hematology & Oncology* 2009; **2**:25.

Chang ZQ, Lee JS, Gebru E, *et al*. Mechanism of macrophage activation induced by β-glucan produced from *Paenibacillus polymyxa* JB115. *Biochemical and Biophysical Research Communications* 2010; **391**:1358-1362.

Chapple CC, Srivastava M and Hunter N. Failure of macrophage activation in destructive periodontal disease. *Journal of Pathology* 1998; **186**:281-286.

Chaushu S, Wilensky A, Gur C, *et al.* Direct recognition of *Fusobacterium nucleatum* by the NK cell natural cytotoxicity receptor NKp46 aggravates periodontal disease. *PLoS Pathogens* 2012; **8**:e1002601.

Chitsazi MT, Shirmohammadi A, Pourabbas R, *et al.* Clinical and microbiological effects of photodynamic therapy associated with non-surgical treatment in aggressive periodontitis. *Journal of Dental Research, Dental Clinics, Dental Prospects* 2014; **8**:153-159.

Cho H, Lee KH, Colquhoun AN and Evans SA. Invasive oral aspergillosis in a patient with acute myeloid leukaemia. *Australian Dental Journal* 2010; **55**:214-218.

Cobb CM. Clinical significance of non-surgical periodontal therapy: An evidence-based perspective of scaling and root planing. *Journal of Clinical Periodontology* 2002; **2**:6-16.

Dellinger EP, Babineau TJ, Bleicher P, *et al.* Effect of PGG-glucan on the rate of serious postoperative infection or death observed after high-risk gastrointestinal operations. *Archives of Surgery* 1999; **134**:977-983.

De Repentigny L, Lewandowski D, Aumont F, Hanna Z and Jolicoeur P. Oral mucosal cell response to *Candida albicans* in transgenic mice expressing HIV-1. *Methods in Molecular Biology* 2009; **470**:359-368.

Feres M, Soares GMS, Mendes JAV, *et al.* Metronidazole alone or with amoxicillin as adjuncts to non-surgical treatment of chronic periodontitis: A 1-year double-blinded, placebo-controlled, randomized clinical trial. *Journal of Clinical Periodontology* 2012; **39**:1149-1158.

Garlet GP, Martins W, Fonseca BAL, Ferreira BR and Silva JS. Matrix metalloproteinases, their physiological inhibitors and osteoclast factors are differentially regulated by the cytokine profile in human periodontal disease. *Journal of Clinical Periodontology* 2004; **31**:671-679.

Gonçalves PF, Huang H, McAninley S, *et al.* Periodontal treatment reduces matrix metalloproteinase levels in localized aggressive periodontitis. *Journal of Periodontology* 2013; **84**:1801-1808.

Guerrero A, Griffiths GS, Nibali L, *et al.* Adjunctive benefits of systemic amoxicillin and metronidazole in non-surgical treatment of generalized aggressive periodontitis: A randomized placebo-controlled clinical trial. *Journal of Clinical Periodontology* 2005**;** **32**:1096-1107.

Ingman T, Sorsa T, Michaelis J and Konttinen YT. Matrix metalloproteinases-1, -3, and -8 in adult periodontitis *in situ*. *Annals of the New York Academy of Sciences* 1994; **732**:459-461.

Inoue A, Kodama N and Nanba H. Effect of maitake (*Grifola frondosa*) D-fraction on the control of the T lymph node Th-1/Th-2 proportion. *Biological and Pharmaceutical Bulletin* 2002; **25**:536-540.

Jacob JP and Pescatore AJ. Barley β-glucan in poultry diets. *Annals of Translational Medicine* 2014; **2**:20-26.

Janusz MJ, Austen KF and Czop JK. Isolation of soluble yeast beta-glucans that inhibit human monocyte phagocytosis mediated by beta-glucan receptors. *Journal of Immunology* 1986; **137**:3270-3276.

Kabasakal L, Şener G, Balkan J, Doğru-Abbasoğlu S, Keyer-Uysal M and Uysal M. Melatonin and β-glucan alone or in combination inhibit the growth of Dunning prostatic adenocarcinoma. *Oncology Research* 2011; **19**:259-263.

Kaner D, Christan C, Dietrich T, Bernimoulin J-P, Kleber B-M and Friedmann A. Timing affects the clinical outcome of adjunctive systemic antibiotic therapy for generalized aggressive periodontitis. *Journal of Periodontology* 2007; **78**:1201-1208.

Kantarci A and Van Dyke TE. Lipoxin signaling in neutrophils and their role in periodontal disease. *Prostaglandins, Leukotrienes and Essential Fatty Acids* 2005; **73**:289-299.

Kebschull M, Guarnieri P, Demmer RT, Boulesteix AL, Pavlidis P and Papapanou PN. Molecular differences between chronic and aggressive periodontitis. *Journal of Dental Research* 2013; **92**:1081-1088.

Lang N, Bartold PM, Cullinan M, *et al.* Consensus report: Aggressive periodontitis. *Annals of Periodontology* 1999; **4**:53.

Lee DY, Ji IH, Chang HI and Kim CW. High-level TNF-α secretion and macrophage activity with soluble β-glucans from *Saccharomyces cerevisiae*. *Biosciences and Biotechnology* 2002; **66**:233-238.

Lin S, Pan Y, Luo L and Luo L. Effects of dietary β-1,3-glucan, chitosan or raffinose on the growth, innate immunity and resistance of koi (*Cyprinus carpio koi*). *Fish and Shellfish Immunology* 2011; **31**:788-794.

Lindhe J, Ranney R, Lamster I, *et al.* Consensus report: Chronic periodontitis. *Annals of Periodontology* 1999; **4**:38.

Löe H and Silness J. Periodontal disease in pregnancy I. Prevalence and severity. *Acta Odontologica Scandinavica* 1963; **21**:533-551.

Mestnik MJ, Feres M, Figueiredo LC, *et al.* The effects of adjunctive metronidazole plus amoxicillin in the treatment of generalized aggressive periodontitis. A 1-year double-blinded, placebo-controlled, randomized clinical trial. *Journal of Clinical Periodontology* 2012; **39**:955-961.

Moreira AL, Novaes AB Jr, Grisi MF, *et al.* Antimicrobial photodynamic therapy as an adjunct to nonsurgical treatment of aggressive periodontitis: A split-mouth randomized controlled trial. *Journal of Periodontology* 2015; **86**: 376-386.

Page RC and Baab D. A new look at the etiology and pathogenesis of early-onset periodontitis: Cementopathia revisited. *Journal of Periodontology* 1985; **56**:748-751.

Petravić-Tominac V, Zechner-Krpan V, Grba S, Srečec S, Panjkota-Krbavčić I and Vidović L. Biological effects of yeast β-glucans. *Agriculturae Conspectus Scientificus (ACS)* 2010; **75**:149-159.

Pirici D, Pirici I, Mogoanta L, *et al.* Matrix metalloproteinase-9 expression in the nuclear compartment of neurons and glial cells in aging and stroke. *Neuropathology* 2011; **5**:492-504.

Rice PJ, Adams EL, Ozment-Skelton T, *et al.* Oral delivery and gastrointestinal absorption of soluble glucans stimulate increased resistance to infectious challenge. *Journal of Pharmacology and Experimental Therapeutics* 2005; **314**:1079-1086.

Rozlog LA, Kiecolt-Glaser JK, Marucha PT, Sheridan JF and Glaser R. Stress and immunity: Implications for viral disease and wound healing. *Journal of Periodontology* 1999; **70**:786-792.

Ryder MI. Comparison of neutrophil functions in aggressive and chronic periodontitis. *Periodontology 2000* 2010; **53**:124-137.

Saghiri MA, Tanideh N, Garcia-Godoy F, Lotfi M, Karamifar K and Amanat D. Subcutaneous connective tissue reactions to various endodontic biomaterials: An animal study. *Journal of Dental Research, Dental Clinics, Dental Prospects* 2013; **7**:15-21.

Silva VDO, Lobato RV, Andrade EF, *et al.* β-Glucans (*Saccharomyces cereviseae*) reduce glucose levels and attenuate alveolar bone loss in diabetic rats with periodontal disease. *PLoS One* 2015; **10**:e0134742.

Smith PC, Munoz VC, Collados L and Oyarzun AD. *In situ* detection of matrix metalloproteinase-9 (MMP-9) in gingival epithelium in human periodontal disease. *Journal of Periodontal Research* 2004; **39**:87-92.

Stashenko P, Wang CY, Riley E, Wu Y, Ostroff G and Niederman R. Reduction of infection-stimulated periapical bone resorption by the biological response modifier PGG glucan. *Journal of Dental Research* 1995; **74**:323-330.

Suzuki Y, Adachi Y, Ohno N and Yadomae T. Th1/Th2-Balancing immunomodulating activity of gel-forming (1→3)-beta-glucans from fungi. *Biological and Pharmaceutical Bulletin* 2001; **24**:811-819.

Turunen K, Tsouvelakidou E, Nomikos T, *et al.* Impact of beta-glucan on the faecal microbiota of polypectomized patients: A pilot study. *Anaerobe* 2011; **17**:403-406.

Udagawa N, Takahashi N, Yasuda H, *et al.* Osteoprotegerin produced by osteoblasts is an important regulator in osteoclast development and function. *Journal of Endocrinology* 2000; **141**:3478-3484.

van der Weijden GA and Timmerman MF. A systematic review on the clinical efficacy of subgingival debridement in the treatment of chronic periodontitis. *Journal of Clinical Periodontology* 2002; **29**:55-71.

Vetvicka V, Richter J, Svozil V, Dobiášová LR and Král V. Placebo-driven clinical trials of yeast-derived β-(1, 3) glucan in children with chronic respiratory problems. *Annals of Translational Medicine* 2013; **1**:26-30.

Yun C-H., Estrada A, Kessel A, Park B-C and Laarveld B. Beta-glucan, extracted from oat, enhances disease resistance against bacterial and parasitic infections. *FEMS Immunology and Medical Microbiology* 2003; **35**:67-75.

Zeidán-Chuliá F, Gursoy M, de Oliveira BHN, *et al.* Focused microarray analysis of apoptosis in periodontitis and its potential pharmacological targeting by carvacrol. *Archives of Oral Biology* 2014; **59**:461-469.