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Expression of Inflammatory Mediators in Periodontitis Over Established Diabetes: an Experimental Study in Rats

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ABSTRACT

Background: Diabetes mellitus is characterized by hyperglycemia ensuing from deficiencies in insulin action, secretion, or both. Hyperglycemia has wide-ranging molecular and cellular effects, leading to oxidative stress, up-regulation of pro-inflammatory responses, and vascular changes. **Objectives:** The aim of this study was to evaluate the expressions of inflammatory markers involved in periodontal destructive process occurring in diabetes, periodontitis (PD), and both coexisting conditions. **Methods:** A rat model was carried out using streptozotocin (STZ) to induce diabetes and Lipopolysaccharides (LPS) with teeth ligature to mimic periodontitis. The animals were distributed randomly into seven groups (n=12) and treated for 10 weeks with alternation between diabetes and PD. The relative quantification analysis of inflammatory markers expression: CRP, MMP-2-14, TIMP-2, IL-4, IFN-γ, was performed at the end of the experiments using western blot after protein isolation from periodontal tissue surrounding the ligation. **Results**: The data showed that CRP, MMP-2, MMP-14, TIMP-2, and IFN-γ are involved in the process of periodontal inflammation associated with diabetes. A significant increase ($p<0.05$) in the expression of inflammatory markers was detected when PD is associated with preexisting diabetes in comparison with diabetes superimposed on preexisting PD. **Conclusion:** This study demonstrated that already established diabetes worsens periodontitis more than diabetes upcoming on existing periodontitis.

Keywords: Diabetes, inflammatory markers, periodontitis, periodontal inflammation, streptozotocin.

1. BACKGROUND

Diabetes mellitus is characterized by hyperglycemia ensuing from deficiencies in insulin action, secretion, or both. Hyperglycemia has wide-ranging molecular and cellular effects, leading to oxidative stress, up-regulation of pro-inflammatory responses, and vascular changes (1). In fact, elevated glucose levels are coupled with diabetic complications throughout the acceleration of advanced glycation end products formation (AGEs) (2). Hyperglycemia is related to multiplied levels of pro-inflammatory mediators like C-Reactive Protein (CRP), tumor necrosis factor alpha (TNF-α), interleukin (IL), interferon-gamma (IFN-γ), and matrix metalloproteinases (MMPs) (3, 4).

Among the pathologies triggered by diabetes, periodontitis is considered as a diabetic complication besides cardiovascular disease, renal disorder, retinopathy, neuropathy, and peripheral vascular diseases (5, 6). Periodontitis is an inflammatory disease of the periodontium, primarily caused by bacterial infection (7). In advanced cases of PD, degradation of the connective tissue and destruction of the bone occur, ultimately leading to tooth loss in adults (8). Some bacterial elements, particularly lipopolysaccharide (LPS), activate macrophages that secrete a good selection and quantity of pro-inflammatory molecules, like IL-1, TNF-α, and prostaglandin E2 (PGE2)(9). In fact, T lymphocytes are activated by microorganism toxins. The cytokines secreted show pro-inflammatory and catabolic activities. They have roles in periodontal tissue destruction that are caused by MMPs (10). The reactive oxygen species can stimulate collagenolytic enzymes and raise interstitial collagenase in inflamed gingival tissue (11). Moreover, circulating CRP level may be a marker of general inflammation and is related to periodontal disease (11-13).

Numerous studies reported that altered immune response, impaired host defense, and increased vulnerability to infection in diabetic patients were the underlying mechanisms for periodontal disease in diabetic patients (14-16). There has been growing evidence of a crucial role of pathogen triggered an inflammatory response from the host in the pathogenesis of diabetes associated to periodontal disease (17, 18). In fact, patients with diabetes and periodontitis have higher production of inflammatory mediators in the gingival tissues compared to non-diabetics (19, 20).

Both PD and DB share common mechanisms of pathogenesis that are related to altered immune-inflammatory responses at local and/or systemic levels (21). However, despite the remarkable clinical evidence of the negative influence of diabetes on periodontal breakdown, to date there are scarce data concerning the cascade of immune- inflammatory mediators at sites of chronic periodontitis in patients with diabetes (16, 22-27). The two diseases concomitance proposes that there are mutual mechanisms of pathogenesis. It has been suggested that the inflammatory and immune deregulation caused by diabetes could trigger periodontitis and accumulation of dental plaque biofilm (15, 28). However, the exact mechanisms involved in periodontitis after diabetes stimulation is still unclear. The role of IL-4, TFN-γ, and TIMP-2 and their relative contribution to the pathogenesis of periodontitis and alveolar bone resorption in association with diabetes are not entirely established yet (29).

2. OBJECTIVE

The objective of this study was to evaluate the role of periodontitis in the initiation and progression of diabetes and its complications as well as the role of diabetes in the initiation and progression of periodontitis in a rat model using streptozotocin (STZ) to induce diabetes and teeth ligature to mimic periodontitis. The expressions of CRP, MMP-2, MMP-14, TIMP-2, IL-4, TFN-γ was assessed.

3. MATERIAL AND METHODS

Animal's selection

Eighty-Four Male Sprague Dawley rats, weighing 320 ± 20 g were selected. Animals were housed in standard polypropylene cages and maintained under standard conditions: a constant temperature of 22 ± 2°C and humidity (55 ± 5%) with fixed 12:12-h light-dark cycle. They were fed regularly with rat chow and tap water *ad libitum*. Body weight was measured weekly using an electronic balance. The animals used in the present study were maintained in accordance with the principles and guidelines of the Canadian Council on Animal Care as outlined in the Guide for the Care and Use of Laboratory Animals. All the experiments were approved by the Ethics committee of the Lebanese university (CU-MEB/D 126/ 132,018).

Experimental design

The animals were distributed randomly into seven equal groups (n=12) and each group was treated differently over 10 weeks as detailed in Figure 1. Periodontal

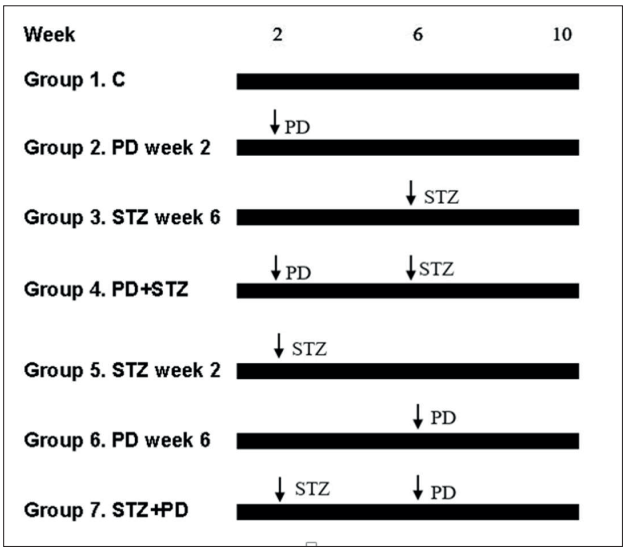

Figure 1. Experimental design (n=12 rats for each group). Periodontal disease (PD). Diabetes induced by injection of streptozotocin (STZ). Group1: Control rats without any intervention; Group 2: PD induction in rats at week 2; Group 3: STZ induction at week 6; Group 4: PD induction at week 2 followed by STZ induction at week 6; Group 5: STZ induction at week 2; Group 6: PD induction at week 6; Group 7: STZ induction at week 2 a followed by PD induction at week 6.

disease (PD) was induced by injection of *A. actinomycetemcomitans* lipopolysaccharide while diabetes was induced by injection of streptozotocin (STZ) as previously described (30).

Animals were grouped as follow:

- Group1: Control rats without any treatment
- Group 2: PD induction in rats at week 2
- Group 3: STZ induction at week 6
- Group 4: PD induction at week 2 followed by STZ induction at week 6
- Group 5: STZ induction at week 2
- Group 6: PD induction at week 6
- Group 7: STZ induction at week 2 followed by PD induction at week 6.

Induction of diabetes

Diabetes was induced by intraperitoneal (i.p.) injection of streptozotocin (STZ) at a dose of 50 mg/kg (Sigma-Aldrich Inc., USA), dissolved in 0.1 M of a citrate buffer (pH 4.5) freshly prepared. Nicotinamide (100 mg/kg) was injected 15 min before STZ. In the control group citrate buffer was injected alone.

Model for experimental periodontitis (PD)

An established rat model of periodontal disease induced by *A. actinomycetem-comitans* lipopolysaccharide (LPS) was followed. Experimental periodontitis was induced under ketamine (Quetamina, VETNIL 10%, Sao Paulo, 70 mg/kg, i.p) and xylazine anesthesia (Calmium 2%, Sao Paulo, 10 mg/Kg, i.p). LPS from *Aggregatibacter actinomycetem comitans* (strain Y4, serotype B) was extracted by the hot phenol-water method as described and diluted in phosphate-buffered saline (PBS) (22, 23). Each rat was injected 3 times with 20 μg LPS through the palatal gingival between the maxillary 1st and 2nd molars. Other groups of rats were injected with PBS. A ligature

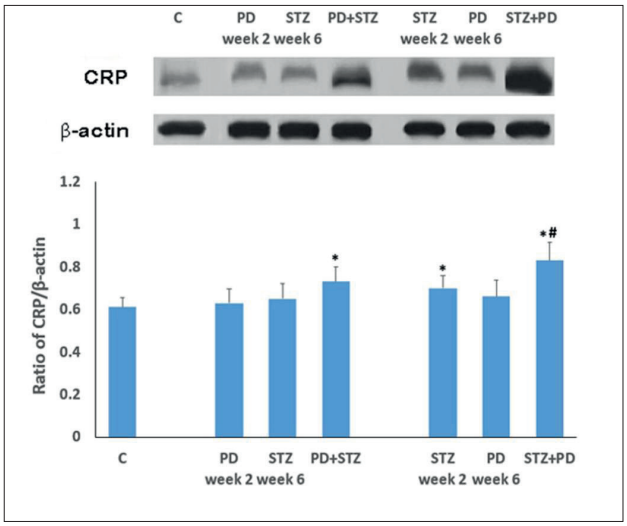

Figure 2. Representative western blot and quantification histogram for CRP expression levels in rats gingiva of 7 groups. The comparative levels of CRP expression were quantified with β-actin normalization. Results are presented as the mean ± standard deviation of pooled data from the 7 groups. * indicates a significant difference (p<0.05) between treatment groups and control. # indicates a significant difference (p<0.05) between (PD+STZ) and (STZ+PD).

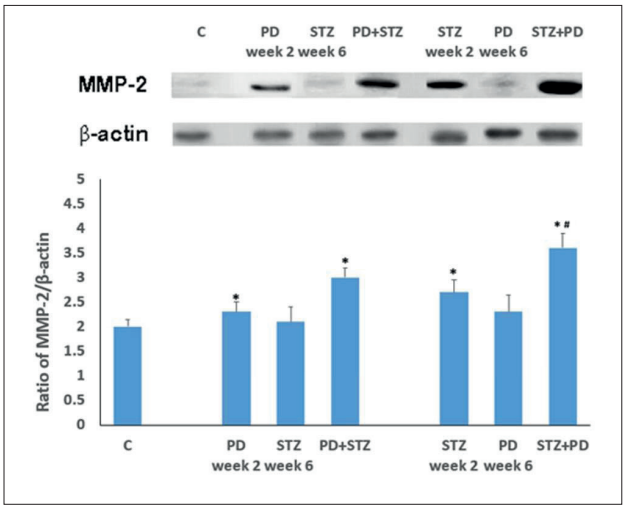

Figure 3. Representative western blot and quantification histogram for MMP-2 expression levels in rats gingiva of 7 groups. The comparative levels of MMP-2 expression were quantified with β-actin normalization. Results are presented as the mean ± standard deviation of pooled data from the 7 groups. * indicates a significant difference (p<0.05) between treatment groups and control. # indicates a significant difference (p<0.05) between (PD+STZ) and (STZ+PD).

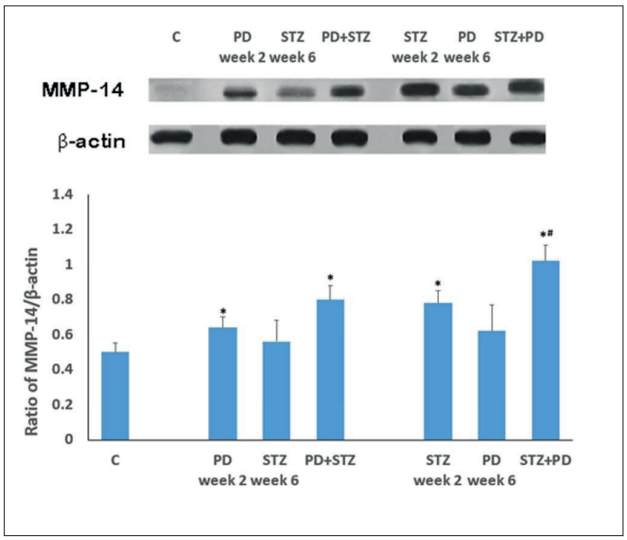

Figure 4. Representative western blot and quantification histogram for MMP-14 expression levels in rats gingiva of 7 groups. The comparative levels of MMP-14 expression were quantified with β-actin normalization. Results are presented as the mean ± standard deviation of pooled data from the 7 groups. * indicates a significant difference (p<0.05) between treatment groups and control. # indicates a significant difference (p<0.05) between (PD+STZ) and (STZ+PD).

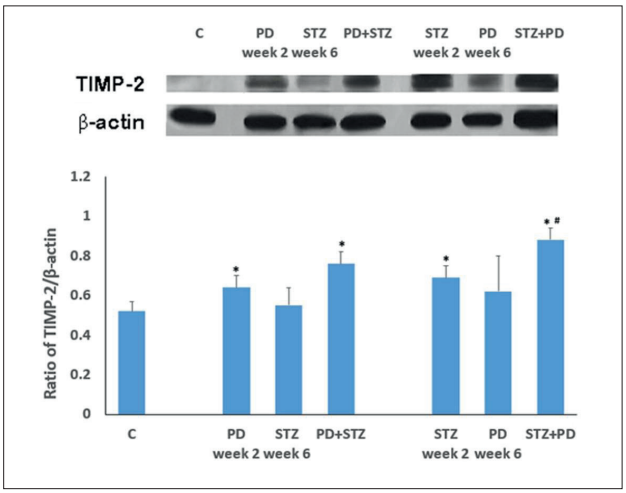

Figure 5. Representative western blot and quantification histogram for TIMP-2 expression levels in rats gingiva of 7 groups. The comparative levels of TIMP-2 expression were quantified with β-actin normalization. Results are presented as the mean ± standard deviation of pooled data from the 7 groups. * indicates a significant difference (p<0.05) between treatment groups and control. # indicates a significant difference (p<0.05) between (PD+STZ) and (STZ+PD).

of 0/0 braided silk was placed around the cervix of the upper right second molar.

At the end of the experimental protocol, and before scarification, animals were anaesthetized and a biopsy from the periodontal tissue surrounding the ligation zone was taken, put in liquid nitrogen and frozen at –80°C until assay. All samples were taken from identical sites in the region surrounding the upper right second molar tooth's cervix.

Protein isolation and western blotting

For western blotting, as previously described by Park and Lee (31), Frozen tissues were homogenized in radio-immunoprecipitation assay lysis buffer (10 mM ethylenediaminetetraacetic acid, 0.15 M NaCl) with 1:30 diluted protease inhibitor cocktail (Roche, Mannheim, Germany). The lysates were sonicated three times for 10 seconds and centrifuged at 12,000 g for 20 minutes. Protein concentrations of the supernatant were routinely determined by a Braford protein assay (Quick Start,

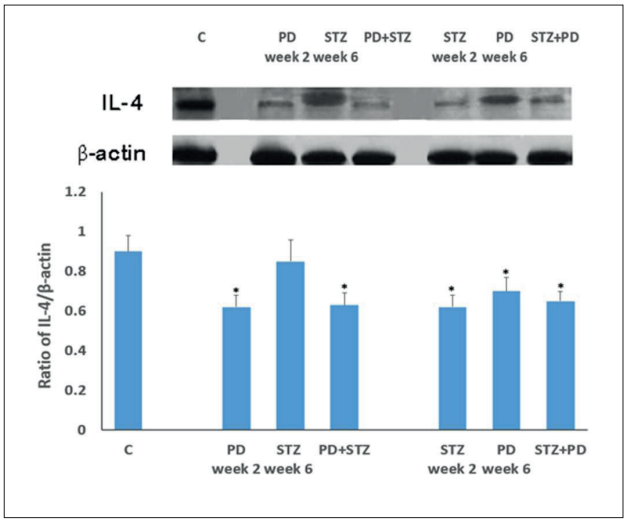

Figure 6. Representative western blot and quantification histogram for IL-4 expression levels in rats gingiva of 7 groups. The comparative levels of IL-4 expression were quantified with β-actin normalization. Results are presented as the mean ± standard deviation of pooled data from the 7 groups. * indicates a significant difference (p<0.05) between treatment groups and control.

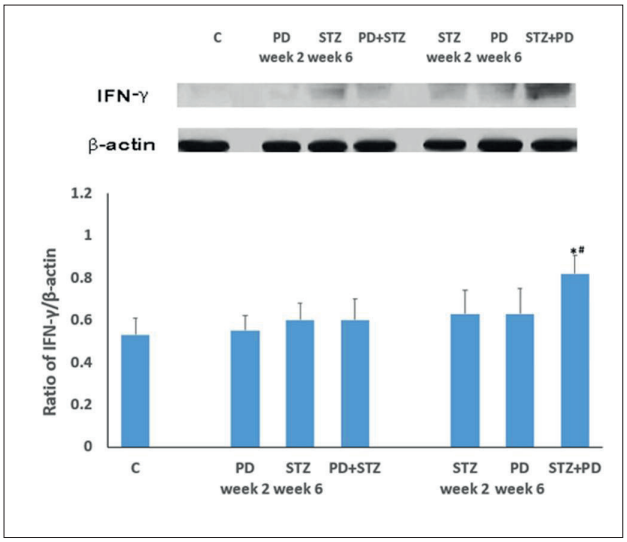

Figure 7. Representative western blot and quantification histogram for IFN-γ expression levels in rats gingiva of 7 groups. The comparative levels of IFN-γ expression were quantified with β-actin normalization. Results are presented as the mean ± standard deviation of pooled data from the 7 groups. * indicates a significant difference (p<0.05) between treatment groups and control. # indicates a significant difference (p<0.05) between (PD+STZ) and (STZ+PD).

BIO-rad Laboratories Inc., Hercules, CA, USA) using bovine serum albumin (BSA) as standard. Lysates were boiled in a sodium dodecyl sulfate (SDS) sample buffer (1 M Tris-HCl [pH 6.8], 40% glycerol, 8% SDS, 2% mercapto-ethanol, 0.002% Bromophenol blue). Prepared samples were separated by 15% SDS-polyacrylamide gels and transferred to a polyvinylidene difluoride membrane. The membranes were subsequently blocked-in tris-buffered saline (TBS) containing 5% powdered milk and 1% BSA for 1 hour, and then incubated with polyclonal anti-CRP antibody, anti-MMP-2 antibody, anti-MMP-14 antibody, anti-TIMP-2 antibody, anti-IL-4 antibody, anti-IFN-γ antibody, (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 1.5 hours at room temperature.

The membranes were washed (five times for 5 minutes with Tween 20) and incubated with a horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. After additional washing (five times for 5 minutes with Tween 20) the western blot procedure was completed with an ECL Plus development kit (Amsterdam, Beckinghamshire, UK).

The relative quantification analysis of CRP, MMP-2, MMP-14, TIMP-2, IL-4, IFN-γ expression was performed using a densitometer (Image Gauge V 3.46, Fuji Photo Film Co., Tokyo, Japan). After normalization to β-actin (Abcam plc, Cambridge, UK) in each sample, levels of CRP, MMP-2, MMP-14, TIMP-2, IL-4, IFN-γ were expressed as a ratio of CRP, MMP-2, MMP-14, TIMP-2, IL-4, IFN-γ to β-actin and the differences in density between groups were determined (31).

Statistical analysis

All data were presented as means ± SD and results were statistically analyzed using a statistical software (SPSS, V20, USA). The CRP, MMP-2, MMP-14, TIMP-2, IL-4, IFN-γ levels among groups were compared using one-way ANOVA followed by Tukey's test. A *P*-value < 0.05 was considered to be statistically significant.

4. RESULTS

Figures 2 to 7 shows representative western blot and quantification histograms for CRP, MMP-2, MMP-14, TIMP-2, IL-4, IFN-γ expression levels in rat's gingival tissue of 7 groups respectively. The levels of expression were quantified with β-actin normalization. Control rats (C), rats with PD induction at week 2 (PD), rats with STZ induction at week 6 (STZ), rats with PD induction at week 2 in addition to STZ induction at week 6 (PD+STZ), rats with STZ induction at week 2 (STZ), rats with PD induction at week 6 (PD) and rats with STZ induction at week 2 in addition to PD induction at week 6 (STZ+PD).

The comparison of CRP expression levels were studied by detecting a CRP band (molecular weight: 27 kDa) and measuring their density in 7 groups (Figure 2). The mean value of CRP expression (ratio of CRP/β-actin) showed statistically significant increase of CRP expression in groups (PD+STZ), (STZ Week 2) and (STZ+PD) as compared to Control (P<0.05). A significant higher level in (STZ+PD) as compared to (PD+STZ) group (P<0.05) was noted.

The comparison of MMP-2 expression levels were performed by western blot analysis using MMP-2 specific antibody which detected MMP-2 (molecular weight: 72 kDa) in all 7 groups (Figure 3). The mean value of MMP-2 expression (ratio of MMP-2/β-actin) showed statistically significant increase of MMP-2 expression in (PD Week 2), (PD+STZ), (STZ Week 2) and (STZ+PD) groups as compared to Control (P<0.05). We detected also a significant higher level in (STZ+PD) as compared to (PD+STZ) group (P<0.05).

MMP-14 expression levels (molecular weight: 66kDa) were also performed by western blot analysis using MMP-14 specific antibody which detected MMP-14 in all 7 groups (Figure 4). The mean value of MMP-14 expression (ratio of MMP-14/β-actin) showed a statistically significant increase of MMP-14 expression in (PD Week 2), (PD+STZ), (STZ Week 2) and (STZ+PD) groups as compared to control (P<0.05). A significant higher level in (STZ+PD) as compared to (PD+STZ) group (P<0.05) was found as well.

The comparative levels of TIMP-2 expression were similarly quantified in western blot analysis and presented in Figure 5 (molecular weight: 28 kDa). The mean value of TIMP-2 expression (ratio of TIMP-2/β-actin) showed statistically significant increase of TIMP-2 expression in (PD Week 2), (PD+STZ), (STZ Week 2) and (STZ+PD) as compared to Control (P<0.05). In addition, there was a significant higher level in (STZ+PD) as compared to (PD+STZ) group (P<0.05).

Representative western blot detected an 18 kDa molecular weight of IL-4 in all 7 groups (Figure 6). The mean value of IL-4 expression (ratio of IL-4/β-actin) showed statistically significant decrease of IL-4 expression in (PD Week 2), (PD+STZ), (STZ Week 2), (PD WEEK 6) and (STZ+PD) as compared to Control (P<0.05). Nonetheless, there was no significant difference between (PD+STZ) and (STZ+PD) groups.

The comparison of IFN-γ expression levels (molecular weight: 25 kDa) were as well identified by western blot analysis using IFN-γ specific antibody (Figure 7). The mean value of IFN-γ expression (ratio of IFN-γ/β-actin) showed statistically significant increase of IFN-γ expression in (STZ+PD) as compared to Control (P<0.05). Also, a significant higher level in (STZ+PD) as compared to (PD+STZ) group (P<0.05) has been noted.

5. DISCUSSION

The association between diabetes and periodontitis has long been discussed with inconsistent conclusions. Periodontitis is a recognized complication of diabetes (32). On the other hand, the presence of periodontal infection can also affect glycemic control in diabetics.

Although there are several studies dealing with diabetes and periodontitis (6, 15), however, the originality of this work is to show an alternative manner and in the same protocol the worsening effect due to the coexistence of two pathologies. At first glance, it's obvious to suppose that the groups with STZ+PD vs PD+STZ do not follow the same disease path and comparability may have some limitations that were minimized in this study. Thus, days 2 and 6 were chosen to allow the disease to develop and stabilize as described in the literature (30). A period of 4 weeks was respected between all interventions; thus, diabetes was induced at week 2 and PD at week 6 for group 7. Similarly, PD was induced at week 2 and diabetes at week 6 for group 4. Samples were also taken after 4 weeks (at week 10). In addition to group 1 which is the main control, double-checking for the progress of each disease was done also at weak 2 and 6. Thus we used for each case its own control. For example, Groups 2 and 3 are controls for group 4, and groups 5 and 6 are controls for group 7.

The increased destruction of tissue among diabetics may be due to an altered susceptibility to periodontal pathogens induced by the accumulation of glycation end products in the tissues, microvascular changes and impaired lipid metabolism (33, 34). While the postulated mechanism linking the diseases in the other direction is that periodontal bacteria and their products, together with inflammatory cytokines and other mediators produced locally in the inflamed periodontal tissues, enter the circulation and contribute to upregulated systemic inflammation. This may lead to impaired insulin resistance and insulin signaling, thus exacerbating diabetes. Increased HbA1c levels, in turn, contribute to increased risk of diabetes complications (including periodontitis), creating a two-way, bidirectional relationship between the diseases (29, 35).

CRP is produced in response to inflammatory cytokines, IL-6, IL-1, and TNF-α. It is an acute-phase reactant synthesized by the liver. Diabetic patients with periodontal disease have significantly higher levels of IL-1, IL-6, TNFα and CRP as compared to non-diabetic patients. In addition, mononuclear phagocytes isolated from diabetic patients have exaggerated inflammatory responses to LPS (20). This study showed higher levels of CRP when STZ induction was performed at week 2, followed by PD induction at week 6; as compared to Control and PD followed by STZ. Diabetes induction before periodontal treatment would increase inflammation significantly. In addition, CRP levels increased comparably to the severity of periodontal diseases as shown by Shojaee et al.(36).

MMPs belong to the matrixin family, which is able to degrade extracellular matrix proteins. Tissue inhibitor of matrix metalloproteinases (TIMPs) inhibit the proteolytic activity of activated MMPs by forming inhibitory complex with the enzyme (37, 38). The amount of extracellular matrix alteration is controlled by the equilibrium between activated MMPs and TIMPs (39, 40). A disturbance between TIMP and MMP balance can induce pathological progressions like periodontitis and arthritis. Both LPS and pro-inflammatory cytokines are potent stimulators for MMP expression by mononuclear cells (41). Many studies have shown that elevated levels of MMPs, in saliva and mouth rinse samples are associated with the progression of periodontal disease (42). In this study, the analysis of MMP-14 and MMP-2 levels showed that their expression was rather increased in inflamed gingiva, when diabetes induction was performed before periodontitis induction; as compared to Control and PD followed by STZ. Stimulation of gene expression of MMP-2, MMP-9, and MMP-14 was detected after a minor elevation of blood glucose for 6 weeks as reported by Song and Ergul (43). In addition, MMP-2 is shown to be a favorable molecular marker for periodontitis (44).

Cytokines produced by T helper 1 and 2 (Th1, Th2) cells are implicated in the regulation of the immune response against *germs* associated with *periodontal diseases*. The common released products of Th1 cells are

IL-2, IL-12, TNF-β, and IFN-γ; those of Th2 cells are IL-4, IL-5, IL-6, IL-10, and IL-13 (13). Activated T lymphocyte, basophils and mast cells secrete IL-4 which is a glycosylated cytokine. The latter inhibits the secretion of PGE2 and cytokines by macrophage, and bypass inflammation by preventing the synthesis of pro-inflammatory cytokines (45). In human gingival fibroblast isolated from patients with periodontitis, IL-4 might also inhibit the IL-1-induced MMP-3 mRNA and protein expression. This suggested that the absence of IL-4 induces periodontal disease and the presence of IL-4 was implicated in wound healing (38). Our data showed a significant decrease of IL-4 levels in PD+STZ and STZ+PD groups as compared to Control. However, no significant difference in STZ+PD as compared to PD+STZ. This result suggests increased inflammation in groups where diabetes induction was performed before or after periodontitis induction; as compared to healthy gingiva. But, no significant difference for this inflammatory marker between PD+STZ as compared to STZ+PD. Our data are in agreement with Shin et al (44).

TIMPs consist of four members: TIMP-1, 2, 3, and 4. They exhibit basic similarities but structural and biochemical differences. TIMP-2 is able to bind non-covalently to MMP-2 away from its active sites. This can prevent its activation and inhibit the enzyme activity [46, 47]. In our study, the quantitative analysis of TIMP-2 levels showed that TIMP-2 expression increased significantly when diabetes induction was performed before periodontitis induction; as compared to Control and PD+STZ. This might suggest that the host with periodontitis and underlying diabetes is producing TIMP-2 as an anti-proteolytic protection to overcome the tissue-destructive effects of MMPs in gingival tissues. In fact, many studies reported that TIMP-2 may be involved in the periodontal inflammation associated with type 2 diabetes [44].

IFN-γ is an antiparasitic and antiviral mediator. CD4+/CD8+ lymphocytes and natural killer cells activated by antigens or mitogens will produce IFN-γ [13]. IFN-γ controls numerous aspects of the immune response; it is a mediator of mononuclear phagocytes [48]. These data show significantly increased level of IFN-γ in STZ+PD as compared to Control and PD+STZ. In fact, Franco et al. [49], reported a significantly elevated levels of IL-4, IL-10, IL-17 and INF-γ in patients with type 2 diabetes and chronic periodontitis.

The results of this study showed that diabetes worsens periodontitis, but in a more severe way when periodontitis occurs after well-established diabetes. It shows that underlying diabetes upregulates the production of inflammatory cytokines and chemokines, CRP, MMP-2, MMP-14, TIMP-2 and IFN-γ; leading to increased inflammation and tissue damage. Tissue destruction and bone resorption is due to inflammatory mediators. In fact, alveolar bone, cementum resorption (discontinuous cementum) and inflammatory cell infiltration is shown in periodontium of rats presenting diabetes after periodontitis. Furthermore, a significant increased inflammation and alveolar bone loss was detected in the periodontium of rat presenting periodontitis after diabetes [30]. Experimental trials for diabetes and/or periodontal diseases are fundamental for understanding their etiology and evolution in humans. Thus, adopting animal models in medical research concerning these diseases is a necessary step before starting clinical trials. In this context, rat is the most extensively-studied rodent for the pathogenesis of periodontal diseases, especially molar teeth. In fact, the organization of the dental gingival tissue in rats is relatively similar to that observed in humans. Results from the current studies support the hypothesis of diabetes and pre-diabetes predisposing to periodontitis; as well as periodontitis being a factor capable of disturbing metabolic control and maybe influencing the development of diabetes-related complications.

Finally, as a perspective and human extrapolation, the inter-relationship between diabetes and periodontitis concerning the biomarkers common to both these diseases may enable earlier detection, targeted preventive measures and therapeutic intervention of these chronic conditions. Data has shown a three-to-four-time amplified risk for progressive periodontal damage in diabetic patients as compared with non-diabetic [4, 50]. Timely detection and diagnosis of disease may significantly affect the clinical management of periodontal patients by offering earlier, less invasive, and more cost-effective treatment therapies.

- **Ethics approval**: The animals used in the present study were maintained in accordance with the principles and guidelines of the Canadian Council on Animal Care as outlined in the Guide for the Care and Use of Laboratory Animals. All the experiments were approved by the Ethics committee of the university (CUMEB/D 126/ 132,018).
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- **Authors' contributions**: Charbel Choubaya: concept and design of the study and managed funds. Nathalie Chahine: design of the study, performed the experiments and analyzed data. Georges Aoun: revised the work critically. Sukumaran Anil: drafted the work and revised it critically. Pierre Zalloua : participated to experiments and wrote the paper. Ziad Salameh : designed the experiments and managed funds. All authors approved the final version to be published.
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