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ORIGINAL ARTICLE

Immune modulatory and antioxidant effects of locally administrated vitamin C in experimental periodontitis in rats

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ABSTRACT

Background: Vitamin C is an important water-soluble vitamin with antioxidant and immune-modulatory actions. The aim of this study was to investigate the effects of locally applied vitamin C on alveolar bone resorption in rats with experimental periodontitis.

Methods: Twenty-one male Sprague-Dawley rats divided into three groups with seven animals in each group: (1) control, (2) experimental periodontitis and 3) experimental periodontitis-local vitamin C treatment group. After ligature was removed, 50 μL vitamin C was locally administered into the subperiosteum of the buccal gingiva of periodontitis vitamin C (PvitC) group rats for three times in intervals of 2 days. At the end of the study, the animals were scarified, and serum and gingival samples were collected for analysis of serum IL-1β, oxidative stress index (OSI), CTX and malondialdehyde (MDA) levels and gingival MMP-8 immunostaining. Alveolar bone loss and attachment loss were determined based on measurements on histological sections obtained from rat mandibles.

Results: Serum MDA and OSI levels which are related to the oxidative stress were significantly lower in the PvitC group as compared with those in the P group ($p < .05$). Serum CTX levels which are related to the bone resorption were significantly lower in the PvitC group as compared with those in the P group ($p < .05$). The numeric density of MMP-8-positive cells was significantly lower in the PvitC group compared to P group ($p < .05$). Alveolar bone loss and attachment loss were significantly lower in the PvitC group compared to P group ($p < .05$)

Conclusions: The local vitamin C administration provided protection against inflammation-induced alveolar bone resorption by decreasing oxidative stress and inflammation-induced tissue breakdown vitamin C may be a therapeutic agent that can be used in periodontitis treatment.

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Experimental periodontitis; oxidative stress; antioxidant; vitamin C

Introduction

Periodontitis is a chronic inflammatory disease affecting tooth supporting tissues including gingiva, periodontal ligament and alveolar bone [1] and is also the primary cause of tooth loss in humans [2]. Although the cause of periodontitis is subgingival bacterial plaque on the tooth surface, the main cause for the progression of the disease is the abnormal host response against these bacteria and their products [3].

Bacteria and their products affect immune cells in connective tissue and cause osteoclasts to pro-inflammatory cytokines such as interleukin-1 (IL-1)α and IL-1β and tumour necrosis factor α (TNF-α) [4]. Bacterial virulence factors and cytokines in periodontal disease affect the expression and activity of metalloproteinases (MMPs), which give rise to connective tissue and bone destruction [5]. In addition, these cytokines lead to increase in the numbers and activity of polymorphonuclear leukocytes (PMNLs) in the inflamed area. PMNLs release reactive oxygen species (ROS) and proteinases in response to periodontal pathogens [6].

Oxidative stress refers to the disturbance of the oxidant–antioxidant balance which leads to an excessive rise in ROS. An increase in ROS can cause cellular DNA damage, lipid peroxidation of lipid membranes and protein degradation [7]. As ROS can induce periodontal tissue degradation and give rise to osteoclastic bone destruction, detoxification of ROS is extremely important for the preservation of homeostasis in normal tissue and organ systems. In recent decades, interest in the relationship between ROS, antioxidant support and the periodontitis treatment has increased [8].

Vitamin C has antioxidant and immune-modulatory effects in living organisms, especially at the intracellular status [9–11]. Vitamin C is part of the body's antioxidant network and, acts as a scavenger of ROS and reactive nitrogen species [12]. Vitamin C aids the bactericidal activities of PMNLs and macrophages and increases the synthesis of nitric oxide [13]. Vitamin C supplementation can relieve gingival oxidative stress by mitigating the production of pro-inflammatory

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cytokines in infected periodontal tissue [8]. Vitamin C also plays an important role in collagen biosynthesis of connective tissue, which is a primary component of the periodontal ligament, gingiva, periodontal ligament, cement and alveolar bone [14]. Previous studies demonstrated a negative correlation between plasma vitamin C levels and attachment loss [15,16] and the prevalence [10] of periodontitis. Research also reported that vitamin C exhibited immune-modulatory and anti-inflammatory properties due to its ability to modulate nuclear factor kappa B (NF-κB)-DNA binding activity by inhibiting TNF-dependent NF-κB activation [17].

Based on the antioxidant, anti-inflammatory and immune-modulatory properties of vitamin C, we hypothesized that vitamin C intake may be beneficial in decreasing periodontal inflammation and destruction in periodontal disease. Therefore, this study was designed to analyse the therapeutic effects of local vitamin C administration on rats in a ligature-induced periodontitis model by biochemical, histological and immunohistochemical assays.

Materials and methods

Animal housing

Twenty-one male Sprague-Dawley albino rats with an initial mean weight of 220–250 g were used in this study. They were housed in an air-conditioned room (23–25 C) with a 12-h light-dark cycle and received human care. The animals were given standard rat chow pellets and tap water ad libitum. The experimental protocol of the study was approved by the Animal Ethics Committee of Atatürk University Animal Experiments Local Ethics Committee (Permit Number: 2017–154).

Randomization and group designation

Twenty-one male Sprague-Dawley rats were randomly divided into three groups of seven animals each: (1) control (C) group: unligated, (2) Periodontitis (P) group: experimental periodontitis (EP) was induced and after the ligatures were removed, physiologic saline was administrated three times in intervals of 2 days locally into the subperiosteum of related teeth, (3) periodontitis vitamin C (PvitC) group: after ligature removal, vitamin C treatment was started, which was administered three times in intervals of 2 days locally into the subperiosteum of related teeth. Schema of experimental design and time course are demonstrated in Figure 1.

Experimental induction of periodontitis

To induce of periodontitis, after being anaesthetized with xylazine hydrochloride (Rompun Bayer, Istanbul, Turkey; 10 mg/kg) and ketamine hydrochloride (40 mg/kg; Ketalar, Pfizer, Istanbul, Turkey), the rats were subjected to EP by tying 3-0 sterile silk ligatures around the right mandibular first molars [18]. In order to experimental periodontitis induction, the ligatures were kept in subgingival position to allow bacterial biofilm accumulation. The ligatures were removed on day 11 when the period of the most severe alveolar bone loss [19]. All EP procedure was made randomly.

Placebo and vitamin C administration

After ligature was removed, 50 μL vitamin C (Redoxon amp 500 mg/5 mL; Bayer Chemical Industry, Istanbul, Turkey) was locally [20,21] administered into the subperiosteum of the buccal gingiva of the right mandibular first molar teeth for three times in intervals of 2 days with insulin needle (0.5 mL, 30 gauge; Becton Dickinson, Franklin Lakes, NJ) in PvitC group [22,23]. The higher doses compared to current dose used in this study have been used several times in the literature without causing any cytotoxicity [24,25]. The same procedure was applied in the periodontitis group by using 50 μL physiologic saline.

Sample collection

At the end of the experimental period, the animals were anaesthetized with xylazine hydrochloride (10 mg/kg) and ketamine hydrochloride (40 mg/kg), cardiac blood samples were collected from the heart for biochemical assays and the rats were then sacrificed, and mandibles and the surrounding tissues were removed for histological and immunohistochemical assays.

Malondialdehyde (MDA) and oxidative stress index (OSI) levels were evaluated in serum samples as oxidative stress markers and CTX was evaluated as a bone resorption marker. The IL-1β level was used as a measurement of infection

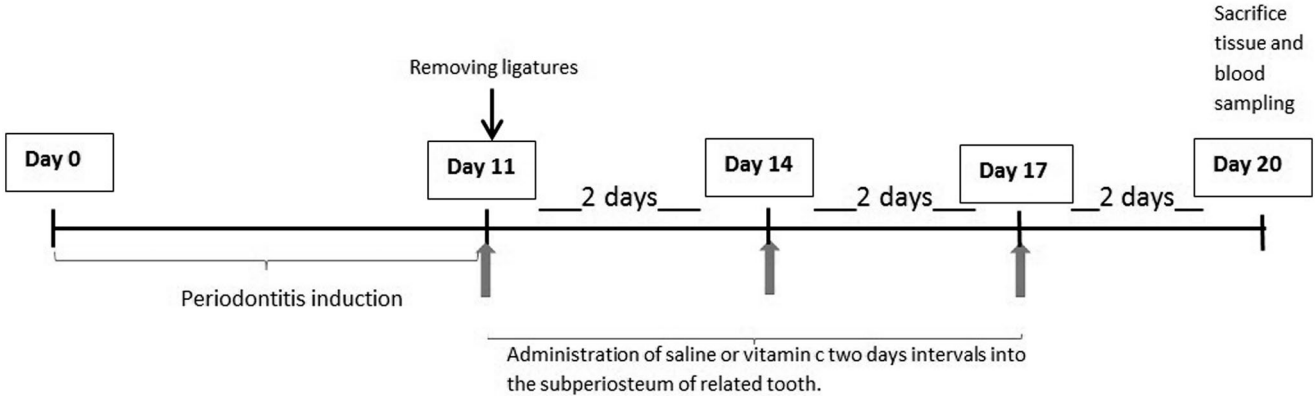

Figure 1. Schema of experimental design and time course.

activity. In gingival samples, MMP-8 immunostaining was evaluated as a periodontal destruction marker.

Biochemical measurement of serum parameters

Measurement of serum total antioxidant status (TAS)-total oxidative stress (TOS) levels and calculation of OSI – TAS and TOS levels were analysed an indicator of the degree of oxidative stress. The percentage ratio of the TOS to the TAS is used to calculate OSI, TAS and TOS levels were measured using ELISA kits (Rel Assay Diagnostics, Gaziantep, Turkey) according to the manufacturer's protocols. The results were expressed as millimolar Trolox equivalent/L (mmol Trolox equivalent/L protein) for total antioxidant capacity and micromolar hydrogen peroxide equivalent/L (μmol H_2O_2 equivalent/L protein) for TOS. The ratio of TOS to total antioxidant capacity was accepted as the OSI and calculated according to the formula previously described by Esen et al. [26] (OSI = [(TOS, μmol/L)/(TAS, μmol Trolox equivalent/L) × 100]).

Malondialdehyde (MDA) assay – Serum MDA levels were measured by spectrophotometric method using a commercial MDA kit (TBARS Assay kit, Item No: 10009055, Cayman Chemical, Ann Arbor, MI). MDA measurement was made according to the manufacturer's recommendations. MDA concentration was expressed as μmol/L.

Serum IL-1β assay – Rat-specific ELISA kits (Invitrogen, Carlsbad, CA) were used to analyse serum IL-1 β concentrations, according to the manufacturer's protocols. The results are expressed as mean SD (pg/mL) of the concentration of each factor in serum.

C-terminal telopeptide of type I collagen (CTX) assay – A rat-specific ELISA CTX Immunoassay Kit (Cusabio Biotechnology, Wuhan, China) was used to determine the levels of serum CTX, a specific resorption marker for degradation of bone type I collagen by osteoclasts, according to the manufacturer's protocols. The results are expressed as mean SD (pg/mL) for all groups.

Histological evaluation

Histological imaging and determination of clinic attachment loss (CAL) and bone support – Mandible sections were prepared at 5 μm thickness, for immunohistochemical and histological procedures, using a microtome (Leica® RM2125RT; Leica Instruments, Nubloch, Germany). Bucco-lingual sections were stained with haematoxylin-eosin. Stained specimens were visualized and examined under a high-power light microscope (Nikon Eclipse i50, Tokyo, Japan). Measurements of the mean distance of the cementoenamel junction–alveolar bone crest (CEJ–BC) and the CEJ–periodontal ligament (CEJ–PL) on the buccal and lingual sides of mandibular first molar teeth were used for determination of bone loss and clinical attachment loss, respectively (Figure 2). All the measurements were performed according to the procedure described by Susin and Rosing [27].

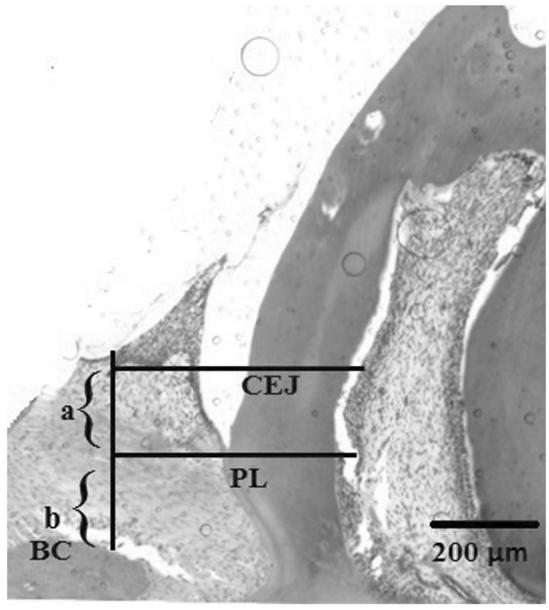

Figure 2. Histologic view of rat periodontal tissue stained with haematoxylin and eosin. Histological measurement of periodontal attachment loss (a: mean distance between the cementoenamel junction and the periodontal ligament) and alveolar bone loss (a + b: mean distance between the cementoenamel junction and alveolar bone crest. BC: alveolar bone crest; PL: periodontal ligament; CEJ: cementoenamel junction.

Immunohistochemical analysis

For immunohistochemical assay, formalin fixed, paraffin-embedded tissue sections of buccal gingiva surrounding the mandibular right molars removed from rats were stained with anti-MMP-8 (Santa Cruz Biotechnology, Santa Cruz, CA), using the streptavidin–biotin–peroxidase method according to the manufacturer's recommendations. To detect the immunopositive cell intensity of gingival tissue, the high-power light microscope was used. The numerical density values of MMP-8 positive cells in the gingival sections were detected and counted using a stereology workstation, consisting of a modified light microscope (Leica DM4000B; Leica Instruments) and stereology software (Microbrightfield Stereo-Investigator software v. 9.0; Microbrightfield, Williston, VT, USA).

Statistical analyses

For statistical analysis, differences between the groups were tested by ANOVA followed by Duncan's *post-hoc* test using SPSS version 20.0 (IBM, Chicago, IL). All data are expressed as means standard deviation (a value of $p < .05$ was considered significant). For the selection of the statistical analysis technique, Kolmogorov–Smirnov test was used to determine whether the data of each parameter showed normal distribution in all groups. The homogeneity of the data was determined by the Levene's homogeneity test. In immunohistochemical staining, MMP-8 positive stained cell numerical density values were measured with Kruskal–Wallis because of the lack of normal distribution. Serum CTX, IL-1β MDA values, amount of CAL and the rates of alveolar bone support

between the groups were found to be normal distribution the statistical difference was determined by ANOVA analysis and *post-hoc* Tukey test.

Results

Effect of vitamin C on serum biochemical determinations

Serum OSI, MDA, CTX and IL-1β levels were significantly higher in the P group than those in the C group ($p < .05$). In contrast serum MDA, CTX and OSI values were significantly lower in PvitC group compared with those in P group ($p < .05$). Serum IL-1β levels were slightly lower in the PvitC group than those in the P group although this finding was not statistically significant ($p > .05$). The MDA, OSI, CTX and IL-1β levels in the serum samples in the three groups are given in Figure 3.

Effect of vitamin C on MMP-8 immunohistochemistry

As shown in Figure 4 gingival tissues of P group showed strong immunostaining for MMP-8 after the induction of experimental periodontitis as compared with that in the C group ($p < .05$) (Figure 4). Local vitamin C treatment also significantly reduced the numeric density values of anti-MMP-8 positive stained cells comparison with the P group ($p < .05$) (Figure 4).

Effect of vitamin C on histological analysis of rat mandible

Table 1 shows a comparison of the mean values of CAL and PBS in the three groups. Distal CAL and mesial CAL were higher in the P group as compared with these values in the C group ($p < .05$), whereas the distal and mesial CAL values were lower in the PvitC group as compared with those in the P group ($p < 0.05$).In the P group, distal and mesial PBS were lower than the values in the C group ($p < .05$). The mean values for mesial and distal PBS were significantly higher in the PvitC group as compared with the values in the P group ($p < .05$) (Figure 5).

Discussion

In this study, ligature placement induced alveolar bone loss and attachment loss, increased levels of oxidative stress and bone resorption markers in serum and tissue degradation marker levels in gingiva. Our results show that locally applied vitamin C reduces alveolar bone loss and attachment loss, decreases levels of OSI, MDA and CTX in serum and reduces MMP-8 immunostaining in gingival tissue.

To the best of our knowledge, this is the first study to investigate the effect of locally administrated vitamin C on periodontal breakdown in ligature induced periodontitis, in relation to oxidative stress, inflammatory mediators and cytokines. This study shows that local vitamin C treatment

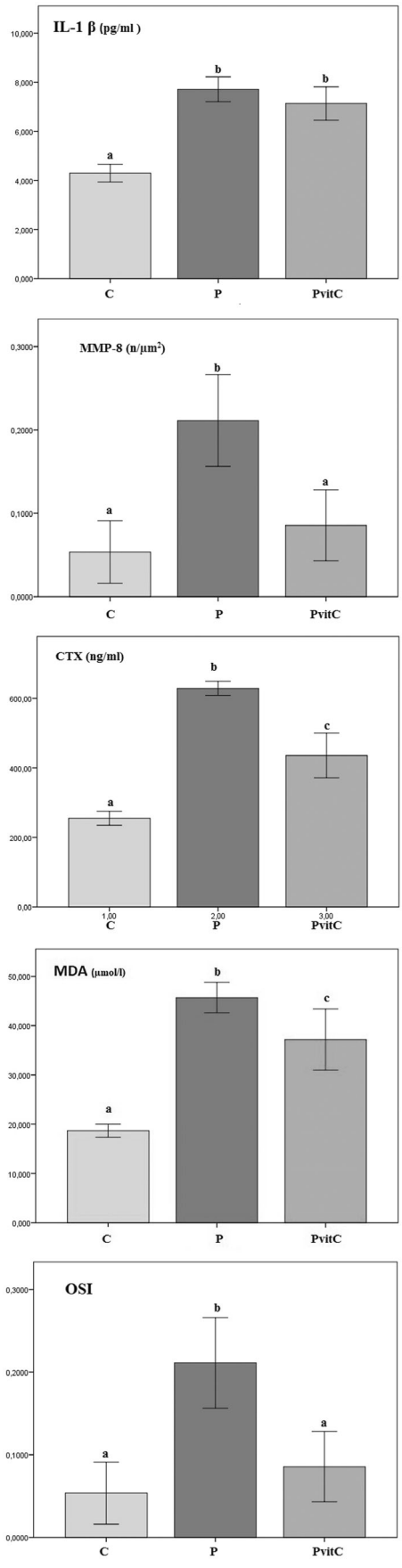

Figure 3. Comparison of biochemical result among groups. Different lowercase letters (^{a,b,c}) show statistically significant differences among groups ($p < .05$).

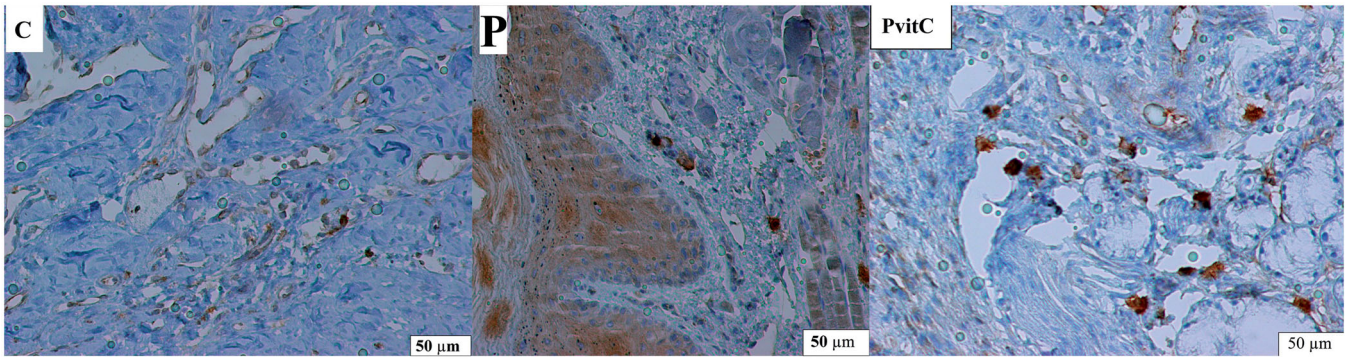

Figure 4. Micrographs of immunostained sections with anti-MMP-8. C: control group; P: periodontitis group; PvitC: periodontitis vitamin C group.

Table 1. Comparison of rates of bone support and amount of clinical attachment loss among groups (mean ± SD).

Group	C	P	PvitC
Bone Support (%)	66 ± 4.47^{a}	50.16 ± 2.71^{b}	59.16 ± 7.65^{a}
Clinical attachment loss	336.33 ± 19.22^{a}	1333 ± 124.98^{b}	961.33 ± 93.92^{c}

Different lower case letters $^{(a,b,c)}$ in the same column indicate statistically significant differences among groups. $p < .05$ by analysis of variance and *post-hoc* Tukey test.

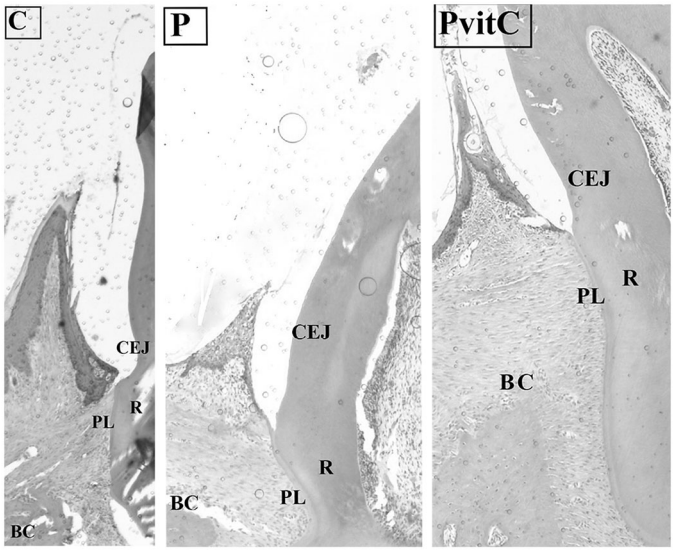

Figure 5. Micrographs of gingival mucosal tissues from all groups in the buccolingual sections of mandibular first molars. Control group section, a normal histologic view (C). Periodontitis group section (P), Periodontitis vitamin C group section (PvitC). CEJ: cementoenamel junction; PL: periodontal ligament; BC: alveolar bone crest; R: root of tooth. Histologic view of rat periodontal tissue stained with haematoxylin and eosin.

reduces alveolar bone loss associated with ligature induced rat periodontitis model.

In normal physiology, there is a dynamic balance between ROS activity and antioxidant defence capacity. In periodontitis, this balance is deteriorated in favour of oxidants and increased oxidative stress causes periodontal tissue destruction. The biochemical results of the current study revealed increased serum MDA levels in the P group, while it was found to be lower in PvitC group compared to the P group. In a previous study, as parallel with our result of OSI levels, it was determined that the levels of plasma reactive oxygen metabolites were decreased when systemic vitamin C support was given to rats with experimental periodontitis [8]. The possible reason for this can be considered as the antioxidant [10] and co-antioxidant [28] effects of vitamin C.

MDA can be used as an indicator of the progression of periodontal destruction caused by ROS [29]. The results of this study indicated increased serum MDA levels in P group, whereas it was found to be lower in PvitC group compared to the P group. Consistent with this finding, a previous study, intra-gastric alpha-lipoic acid and vitamin C administration have been shown to reduce MDA levels in gingival tissues of rats in experimental periodontitis [30]. As a lipid protective antioxidant that inhibits lipid peroxidation [10,31], vitamin C may exert effects on lipid peroxidation in periodontitis by lowering serum MDA levels.

The bone-specific CTX fragment, which is produced during bone destruction and is not present in type 1 collagen in other tissues, is a highly specific and sensitive marker of bone destruction [32]. In this study, serum CTX levels increased in the P group. This finding was consistent with that of previous studies [33,34], which reported that serum CTX levels were related to alveolar bone destruction in periodontitis. However, in contrast to a study by Sanbe et al. [35], CTX levels in the PvitC group were lower than those in the P group. Sanbe et al. [35] suggested that systemic applied vitamin C did not affect serum CTX levels in a high-cholesterol diet-induced periodontitis model. Vitamin C is a cofactor for lysyl and prolyl hydroxylase which is involved in the collagen biosynthesis pathway [36]. In an another study reported that the formation of collagen cross-linkages decreases in vitamin C deficiency and vitamin C may affect serum CTX concentrations [37]. Previous research reported that vitamin C exhibited anti-inflammatory properties by modulating the DNA binding activity of NF-κB [38]. Results of this study may be explained by the role of vitamin C on collagen fiber production and inhibition of NF-κB mediated bone destruction.

Pervious research demonstrated the central role of MMP-8 in the breakdown of the basement membrane, extracellular matrix components of connective tissue and physiological tissue remodelling, especially in the destruction of periodontal tissues [39]. A number of studies showed that MMP-8 was present as high-releasing collagenase in gingival fluid [40] and in inflammatory gingiva [41,42] in periodontitis. In this

study, strong immunoreactivity of MMP-8 was found in P group rats' gingival section whereas weaker immunoreactivity was found in control group, in parallel with the findings of previous reports [41,42]. We also detected weaker immunoreactivity of MMP-8 in the PvitC group comparison with the P group. This finding may be related to the role of vitamin C in the production of collagen fiber.

During inflammatory conditions, immune system associated cells are activated to release a variety of proinflammatory cytokines such as IL-1β and TNF-α [43]. Many previous studies demonstrated increased levels of IL-1β in gingival fluid [44], serum [45] and saliva [46] in periodontitis. Tomofuji et al. [8] showed that systemic vitamin C administration suppressed IL-1β gene expression. Our results are inconsistent with their findings. In this study, serum levels of IL-1β were slightly lower in the PvitC group as compared with those in the P group, although this finding was not statistically significant. The short duration of this study and type of model of induced periodontitis may explain this finding, with local inflammation not affecting the cytokine status at the systemic level in the short term. This is one of the major limitations of EP studies whether the levels of inflammatory markers are not changed in serum because of ligature induced periodontitis is related only one tooth. In addition, due to the low body weight and blood volume of the rats used in the studies, limited local inflammation around only one tooth may have been insufficient to induce changes in the serum levels of the markers. Also, we think systemic evaluation of IL-1beta may not reflect the local inflammatory status of periodontitis therefore the lack of differences between groups may be because of this.

The histological results of this study revealed elevated alveolar bone resorption and clinical attachment loss in the P group as compared to the other groups. These findings were in line with those of previous studies [18,34]. The histological results also showed that vitamin C therapy inhibited alveolar bone loss and supported the periodontal tissue remodelling. In response to bacterial stimuli in periodontitis, due to PMNL-induced ROS and tissue destructive mediators, alveolar bone destruction occurs. In an *in vitro* study by Xiao et al. [47], vitamin C inhibited the differentiation of receptor activator of NF-κB ligand-induced osteoclast precursor cells to produce mature osteoclasts. In other research, vitamin C promoted osteoblastic differentiation in periodontal ligament cells by reducing oxidative stress and modulating type 1 collagen production [48]. However, Abou Sulaiman et al. [49] reported that systemic use of vitamin C did not have additional benefit in conventional non-surgical treatment in term of attachment level. On the contrary to this study, it was suggested that systemic vitamin C administration may be useful in preventing alveolar bone resorption in a high-cholesterol diet-induced periodontitis model [8,50]. In addition, Yussif et al. [51] applied local vitamin C treatment after phase 1 periodontal therapy. Their clinical and histological findings have shown that vitamin C is an effective adjunctive treatment in reducing various degrees of chronic gingival inflammation. We may think local vitamin C therapy minimized alveolar bone and periodontal ligament destruction by inhibiting oxidative stress and modulating the host to suppress of inflammation.

We preferred the local application path, unlike previous vitamin C studies. Advantages of local applications include the conversion of low doses of the drug into high concentrations at the related region, less administration, less systemic adverse effects and higher patient acceptance than systemic drugs [52].

A potential limitation of this study is the lack of dose-dependent drug groups. Studies evaluating the therapeutic effect of vitamin C on alveolar bone loss in periodontitis are needed to determine the appropriate dose for *in vitro* experiments. Another limitation of our study is that the use of rats having ability to produce their vitamin C may not be the best model to monitor vitamin C as a treatment method. Furthermore, the ligature model is an additional limitation. This model induces acute inflammation, which is not directly equivalent to that observed in chronic periodontitis in humans. Also, considering the fact that this was based on an experimental animal model, the administered dose of vitamin C and findings of this study cannot adapt directly to humans. Furthermore, repeated local injections of vitamin C are not suitable for clinical use in patients. Additional studies are needed to shed light on the effects of local administration of vitamin C in term of different doses, time intervals and local release agents as route of administration.

Conclusion

In this study, it was shown that locally applied vitamin C reduces alveolar bone loss and attachment loss, decreases levels of OSI, MDA and CTX in serum and reduces MMP-8 immunostaining in gingival tissue.

Despite the limitations of this study, the beneficial effects observed herein support the use of locally applied vitamin C as an immune modulatory and antioxidant agent in periodontitis and suggest that it may have a therapeutic benefit in periodontal treatment. In light of this informations, it may be useful to select local release agents as a means of administering vitamin C in future studies.

Disclosure statement

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