MMP-7, RANKL mRNA expression in the gingival tissue correlates with periodontitis: An in vivo study

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Introduction
MMP-7 supports the immune response and can have both beneficial and destructive effects. As part of the innate host defense, MMP-7 is connected to the mucosal antimicrobial defense. Even though research on other Matrix metalloproteinase (MMPs) is well-established, understanding MMP-7 expression is required to establish an improved diagnostic strategy. This research investigates the mRNA expression of MMP-7 and RANKL by RT-qPCR assay.
Methods and material
Twelve male Sprague Dawley rats were allocated into three groups, no treatment, experimental (7 and 14 days). Periodontitis is induced by sterile wire insertion (0.2 mm) and *Enterococcus faecalis* inoculation within the gingival sulcus situated between the maxillary right 1st and 2nd molar tooth regions. Following euthanasia, tissue samples from the maxillary gingiva and maxillary jaw were extracted for quantitative real-time PCR assay and histopathological assessment.
Results
Results showed that at 7 days, there was significant upregulation of MMP-7, which was downregulated in 14 days, as well as migration of the junctional epithelium, attachment loss, inflammatory cells, and fibroblasts, as observed by histological analysis.
Conclusions
Thus, the study suggests that MMP-7 is associated with the progression of periodontitis.

Keywords:
Matrix metalloproteinase-7 (MMP-7), MMPs, periodontitis, RANKL

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Key Messages: MMP-7 is expressed in periodontitis.

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Introduction

Matrix metalloproteinase-7 (MMP-7), often referred to as matrilysin-1, is a neutral proteinase that is synthesized by epithelial cells in the sulcular and junctional epithelium of the gingiva [1]. It is the smallest known MMP within the MMP family, and the lack of the hemopexin domain, which is shared by all other MMPs, makes MMP-7 distinct [1,2]. MMP-7 is released by macrophages, endothelial cells, and osteoclasts and is subsequently engaged in inflammatory processes, cell invasion, and angiogenesis [3]. It also induces neutrophil reepithelialization and transepithelial migration, contributing to wound healing and inflammation [4]. The tissue proteins laminin, fibronectin, type IV collagen, gelatin, and elastin can all be broken down by this MMP [4]. MMP-7 is involved in the mucosal antimicrobial defense in addition to its effect on extracellular matrix proteins, which adds to the innate host defense [5]. The receptor activator of nuclear factor Kappa-B ligand (RANKL) and its receptor, RANK, are important molecules that control the growth, recruitment, and function of osteoclasts in periodontitis [6]. It plays a crucial function in periodontal bone resorption and is required for the full development of osteoclast precursor cells. RANKL is present in stromal cells, lymphocytes, and various other cell types in periodontal tissues that play significant direct or indirect regulatory roles [6].

However, several MMPs, such as MMP-2, MMP-8, MMP-9, and MMP-13, have been extensively

investigated to understand their function in the pathogenesis of periodontitis [7]. There is a need to further identify the functions of other Matrix metalloproteinase (MMPs), especially MMP-7. Understanding MMP-7 expression in the etiology of periodontitis is crucial from this perspective as it might help improve the diagnostic process. The current study aims to assess the mRNA expression of MMP-7 at different time points in periodontitis progression.

Materials and methods

Experimental procedure

After obtaining approval from the University Kebangsaan Malaysia Animal Ethics Committee (UKMAEC) (FD/2018/NURRUL SHAQINAH/ 28-NOV./967-NOV.-2018-JAN. –2020), we obtained 12 male Sprague Dawley (SD) rats, which were allocated into three groups: control (0-day), experimental (7- and 14-day) weighing an average of about ~180 g and raised under specific pathogen-free conditions. Studies have been reported in compliance with the ARRIVE 2.0 guidelines [8]. Sample sizes were calculated based on ANOVA calculations using degrees of freedom [9].

General anesthesia was given through intraperitoneal injection with a mixture of 10% ketamine at 100 mg/kg and 2% xylazine at 10 mg/kg body weight. Experimental periodontitis was induced by sterile orthodontic wire (0.2 mm) insertion, and *Enterococcus faecalis* strain (ATCC 29212, USA) 0.5 µl bacterial infection within the proximal space of the right maxillary 1st and 2nd molar tooth regions [10,11]. At the completion of experiments, all rats were euthanized humanely by anesthesia overdose followed by cervical dislocation.

RT-qPCR assay

Tissue samples of the gingiva around the molar tooth region weighing about 20 mg were obtained for RNA extraction and a quantitative Real-Time PCR (RT-qPCR) assay. RNA was extracted and purified from the gingival tissue using the Innu PREP RNA Mini Kit 2.0 (Analytik Jena, Germany) RNA extraction kit following the manufacturer's instructions. RT-qPCR was carried out using a ready-to-use 2x concentration of ChamQ Universal SYBER qPCR master mix (Vanzyme, China). The 10 µl qPCR reactions had the following components: 5 µl of master mix, 0.5 µl of forward and reverse primers each, 1 µl of DNA template, and 3 µl of RNase-free water. Two-step amplification protocols were performed using a Bio-Rad CFX96 Connect the real-time PCR thermal cycler as follows: pre-denaturation at 95°C for 60 seconds, denaturation at 95°C for 15 s, and annealing at 60°C for 30 seconds. MMP-7 primer was used in the following sequence: forward 5'- CGGA GATGCTCACTTTGACA-3' and reverse 5'-CAT GAGTGGCAACAAACAGG-3', and RANKL forward 5'- CACAGCGCTTCTCAGGAGTT-3' and reverse 5'- GATGGTGAGGTGAGCAAA CG-3' [12,13]. Each reaction was performed three times, and the existence of any contamination was examined using a no-template control, which contains reaction mixtures without template cDNA with every RT-qPCR run. Tissue samples from the inflammation-induced right maxillary tooth were collected, fixed, processed, and stained with hematoxylin and eosin accordingly for histological assessment.

Statistical analysis

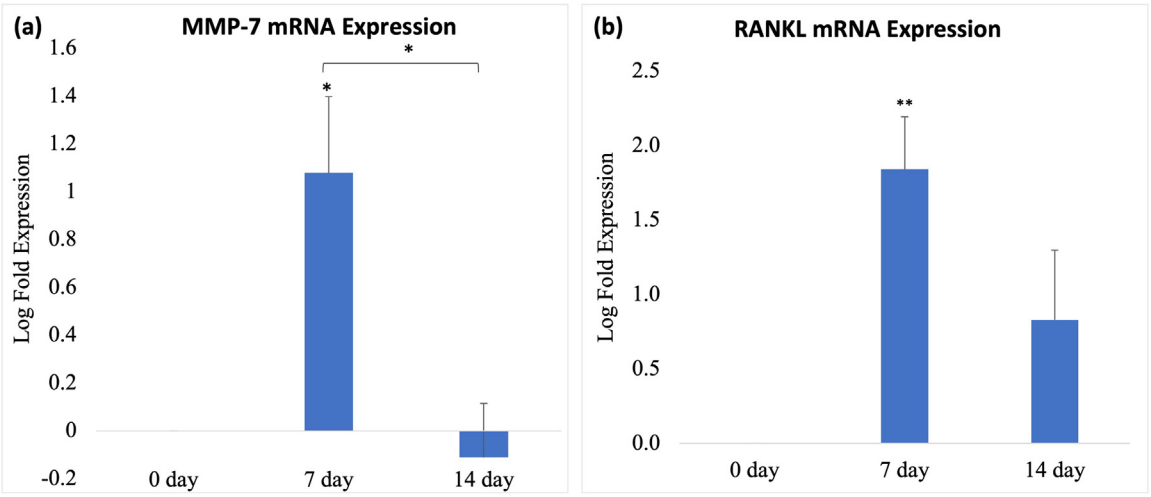
The housekeeping gene (GAPDH) was used to normalize all quantitative RT-qPCR cycle threshold (CT) data, and the fold changes were determined using the $2^{-\Delta\Delta CT}$ method [14]. All data analyses were carried out by IBM SPSS data editor version 23.0 (IBM, USA), and the values were displayed as mean ± standard deviation (SD). ANOVA (One-way analysis of variance) was used statistically to examine the variations in group means, and the Tukey post hoc test was used to identify the means that differed substantially from those of the other groups. The association between the expression of RANKL and MMP-7 was assessed using the Pearson correlation coefficient (r). In this study, statistical significance was defined as P values of <0.05.

Results

Expression and correlation between MMP-7 and RANKL in gingival tissue samples

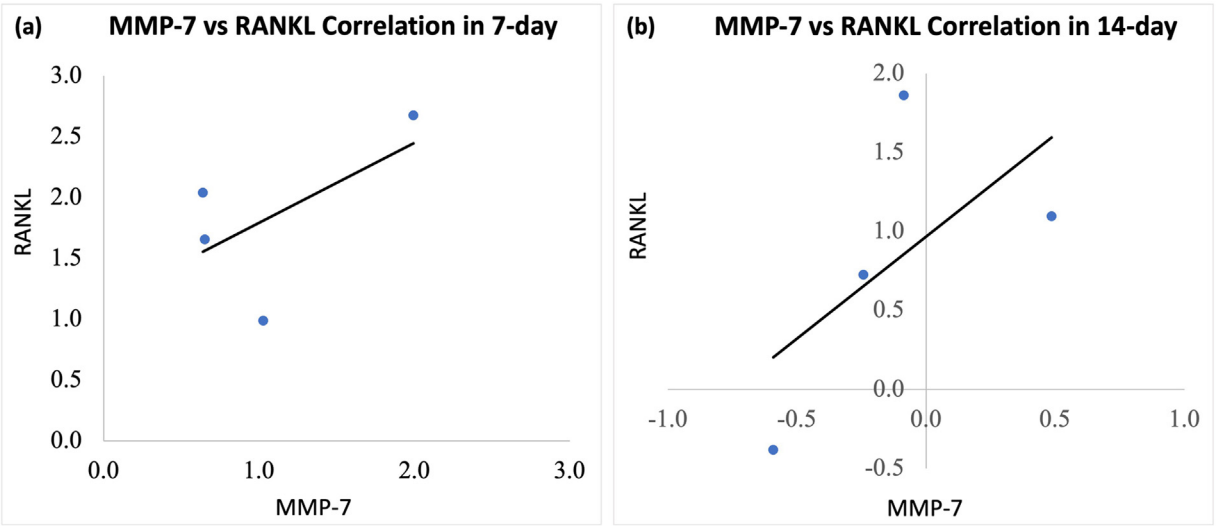
This study evaluated a significant upregulation of MMP-7 ($P<0.05$) and RANKL ($P<0.01$) as shown in Fig. 1a, b in gingival tissue samples at 7 days post-induction. The result showed significantly higher expression of MMP-7 and RANKL at 7 days as compared with the control group. However, at 14 days post-induction, MMP-7 expression was significantly downregulated. Furthermore, RANKL expression was lower as compared with 7-days post-induction, although not statistically significant ($P>0.05$). The Pearson correlation method was applied to reveal a correlation between MMP-7 and RANKL mRNA expression levels at 7 and 14 days. There was a moderate positive correlation between MMP-7 and RANKL at 7 days ($r=0.591$) and 14 days ($r=0.621$) Fig. 2.

Figure 1



Expression of MMP-7 (a) and RANKL (b) in gingival tissue sample. Data are presented as mean±standard deviation, with *P* values denoted by an asterisk: **P*<0.05, ***P*<0.01.

Figure 2



Correlation between MMP-7 and RANKL: (a) moderate positive correlation (r=0.591) at 7 days and (b) moderate positive correlation (r=0.621) at 14 days.

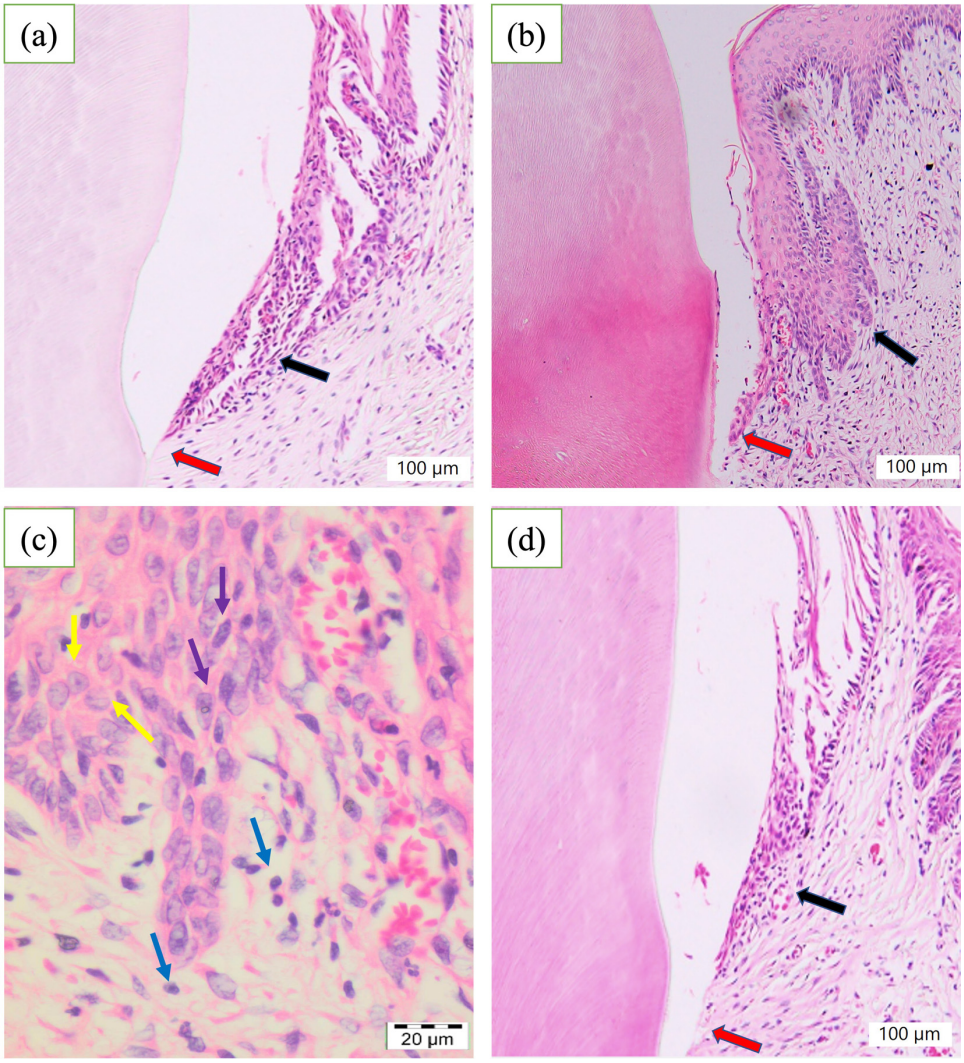
Histological changes in the gingiva

In Fig. 3, in the control group, the junctional epithelium was normal; there was no attachment loss from the CEJ or inflammatory cell infiltration (a). The histopathological analysis of the periodontium of the 7-day post-induction (PI) group showed hyperplastic junctional epithelium, apical migration of the junctional epithelium, and loss of attachment from the cementoenamel junction (CEJ). Furthermore, keratinocytes (yellow arrow), fibroblasts (purple arrow), and eosinophils (blue arrow) were also observed within the gingival tissue in the 7-day PI group (c). In the 14-day PI group, thin ulcerated junctional epithelium and loss of attachment from the CEJ were observed (d).

Discussion

According to current knowledge of the pathophysiology of periodontal disease, this complex condition is brought on by the activation of the host's inflammatory response to pathogenic microorganisms [15]. In this study, experimental periodontitis was induced by wire insertion, which acts as a plaque retentive factor to colonize the periodontal pathogen, and *E. faecalis* inoculation between the right maxillary

Figure 3



Histological images show no changes within the periodontium at 0-day (a), hyperplastic junctional epithelium, apical migration of the junctional epithelium (black arrow), loss of attachment from the cementoenamel junction (red arrow), keratinocytes (yellow arrow), fibroblasts (purple arrow), and eosinophils (blue arrow) at the 7-day (b) and (c), and at the 14-day, thin ulcerated junctional epithelium (red arrow) and loss of attachment from the CEJ (black arrow) (d) Badiah Baharinb

1st and 2nd molar tooth regions. Periodontitis induction is confirmed by the histological analysis that showed attachment loss from the CEJ and the presence of inflammatory cells, which are the classical features of periodontitis [16]. The current research evaluated significant upregulation of MMP-7 mRNA expression at 7 days, which was downregulated at 14 days using the RT-qPCR essay. Furthermore, histological examination revealed apical migration of the junctional epithelium, the presence of inflammatory cells, fibroblasts, and keratinocytes, as well as attachment loss after 7 days. If the two data were compared, it is hypothesized that all of the inflammatory symptoms were more pronounced in the 7-day period and that the expression of MMP-7 and RANKL was significantly higher. MMP-7 can be linked to the junctional epithelium's antimicrobial defense because it participates in innate host defense by activating defensins in their latent form in the healthy, undamaged epithelium [17]. The current study shows that 7 days after experimental periodontitis induction, MMP-7 mRNA expression is upregulated. Lundmark *et al.* (2015) have also reported upregulation of MMP-7 in gingival tissue biopsies in periodontitis patients as compared with healthy controls [18]. The mucosal immune response involves MMP-7, whose expression rises quickly in the injured epithelium. Periodontitis is caused in the current study by concurrent infection-induced bacterial accumulation at the periodontal site. Yet, after 14 days, the disease's symptoms started to subside. Examining MMP-7's expression pattern reveals that it varies as the disease condition changes. However, on day 14, MMP-7

downregulation, decreased RANKL expression, and a lack of inflammatory cells showed that the inflammation had subsided. The protective function of periodontal tissues and the likely development of the host's immune response as a result of bacterial invasion might be attributed to the decrease in inflammation intensity during the 14-day period. Similar research was done by de Molon *et al.*, (2014) who found that inflammatory symptoms peaked in the first week and then gradually subsided [19]. Moreover, Lundmark *et al.* (2016) have also reported higher MMP-7 levels in patients with periodontitis as compared with a healthy group, which is similar to this study [20]. The development, recruitment, and function of osteoclasts are controlled by the molecules RANKL, RANK, and a decoy receptor called osteoprotegerin (OPG). Periodontitis progression is characterized by bone resorption, and the degradation of bone regulates RANKL expression [21]. Moreover, at the 7-day and 14-day time points, there was a moderately positive correlation between the expression of MMP-7 and RANKL mRNA, suggesting that MMP-7 is influenced by the progression of periodontitis.

However, other MMPs, such as MMP-8, MMP-9, and MMP-13, have established roles as biomarkers for the development and detection of periodontitis [7]. Yet, a group of biomarkers will probably be more accurate than a single one. Moreover, a number of systemic diseases linked to periodontitis may raise the levels of MMPs in saliva or gingival crevicular fluid [22]. Thus, it is crucial to look at several MMP groups to track the development of periodontal disease (gingivitis, periodontitis). Our findings imply that MMP-7 may be crucial for periodontal tissues' early defense. Moreover, this study demonstrates that MMP-7 expression is linked to the establishment of periodontitis. Therefore, future studies should focus on MMP-7 to establish it as a potential biomarker in periodontitis alongside other MMPs. One of the study's limitations is that no other MMP expression that had already been determined by research to be correlated with MMP-7 was investigated.

Conclusion

In conclusion, our study showed that the expression of MMP-7 is associated with the progression of periodontitis, which may help us understand the function of this MMP in periodontitis. This study also suggests MMP-7 as a potential biomarker in the periodontal inflammatory process.

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Conflicts of interest

There are no conflicts of interest.

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