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HISTOPATHOLOGICAL EVALUATION OF RATS INDUCED WITH PERIODONTITIS USING *ENTEROCOCCUS FAECALIS* AND WIRE LIGATION OF THE TEETH

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Abstract: Gingivitis and periodontitis are periodontal diseases that present as an inflammatory periodontium. *Enterococcus faecalis* is a gram-positive, facultative microorganism that can increase periodontal destruction. High levels of medically important pathogens in the periodontitis associated microbiota may pose a risk to the development of infections at distant body sites. This study aims to investigate histological changes within the periodontium, heart and kidney following concurrent infection with ligature wire and *E. faecalis* in rats. Rats were divided into three groups, 0-day (control), 7-day (experimental) and 14-day (experimental), containing four rats each. Sterile 0.2 mm wires were inserted into the inter dental space of the maxillary right first and second molar. A total of 0.5 microliter of *E. faecalis* suspension (1.5 x 10^8 CFU/ml) was injected into the gingival sulcus of the same site once a week for two weeks. Following euthanasia, maxillae, heart and kidney tissue samples were collected, processed for hematoxylin and eosin staining accordingly. The results showed hyperplastic gingival epithelium, attachment loss and inflammatory cell infiltration within the connective tissue after 7-day. However, inflammatory cell infiltration reduced after 14-day. Inflammatory cell infiltration within the myocardium of the heart tissue and congested blood vessels within the glomerulus and inflammatory cell infiltration into the interstitial space were observed in the kidney sample. The findings suggest acute inflammation within the periodontal tissue and developing infection at distant body sites.

Keywords: *E. faecalis*, ligature wire, periodontal disease, periodontitis.

Introduction

Periodontal disease is an inflammatory condition that affects the gingiva, periodontal ligament, radicular cementum and alveolar bone surrounding the teeth (Beck *et al.,* 2020). The disease is initiated by bacterial-host interactions at the biofilm-periodontium interface and is associated with chronic inflammation (Franco *et al.,* 2017). Gingivitis is the first stage of periodontal disease, which can develop to periodontitis if left untreated. It is an inflammation of the gingiva, which develops when the connective tissue is infiltrated by inflammatory cells and is characterised by red and swollen gingiva with spontaneous bleeding (Khuda *et al.,* 2021). As the disease progresses, there is an immune-mediated loss of periodontal tissue, involving the destruction of the periodontal ligament and alveolar bone, which is known as periodontitis (Franco *et al.,* 2017). Subgingival microbiota, specifically periodontopathogenic bacteria and host immuneinflammatory responses are accountable towards the progression of the disease (Preethanath *et al.,* 2020). Oral biofilm or dental plaque is an accumulation of bacteria that form on the tooth surface. The anatomical proximity of this biofilm to the bloodstream may enable systemic spread of the bacteria and their by-products (Souto *et al.,* 2006).

Among the periodontopathogenic bacteria, *Porphyromonas gingivalis, Tanerella forsythia, Treponema denticola* and *Aggregatibacter actinomycetemcomitans* are considered to be the most important pathogen contributing to periodontal disease progression (Yakob *et al.,* 2013). Other than periodontopathogenic bacteria, more than 700 species of oral microorganism are equally responsible for the progression of the disease (de Molon e*t al.,* 2016).

Enterococcus faecalis (*E. faecalis*) is a gram-positive, facultative microorganism and has been associated with periodontal disease, as well as endodontic lesion as demonstrated in several studies (Alghamdi & Shakir 2020; Souto *et al.,* 2006). The virulence factor of *E. faecalis*, such as aggregation substance, surface adhesion, lipoteichoic acid, extracellular superoxide production, lytic enzymes, cytolysins and hemolysins, can be one of the triggering factors for both inflammatory conditions (Kayaoglu & Ørstavik, 2004; Lang *et al.,* 2009).

The process of periodontal disease is initiated when bacteria colonise the gingival crevice and form a sub-gingival plaque. The bacteria begin to multiply, causing the plaque to mature, resulting in a high percentage of gram-negative bacteria. They can produce specific compounds that can damage the periodontium. Microorganisms, both commensal and pathogenic can enter host tissues and the bloodstream via an entry portal. Once the bacteria have gained access to the sites, their by-products, like lipopolysaccharide, lipoteichoic acid, extracellular vesicles; enzymes such as hyaluronidase, proteinase and collagenase; toxins such as leukotoxins and metabolites such as hydrogen sulphide can trigger periods of aggravation and remissions of inflammation (Ramadan *et al.,* 2020). Periodontal disease is associated with several systemic diseases such as atherosclerotic cardiovascular diseases, diabetes, respiratory diseases, rheumatoid arthritis and kidney diseases (Hajishengallis & Chavakis, 2021). Direct invasion of periodontal bacteria from the periodontal site to systemic circulation through ulcerative epithelium and inflammatory response to the periodontal bacteria or their by-products are the possible pathogenic mechanism describing periodontitis's association with systemic disease (Winning & Linden, 2015).

Animal models are very essential in the establishment of the scientific basis for understanding the pathological processes in periodontal diseases, as well as its association with other diseases. Rats are one of the most widely used species in the laboratory because they can reproduce *in vivo* cellular characteristics and reactions that occur in human (Ionel *et al.,* 2015). Several experimental periodontal disease induction techniques such as ligature suture or wire and oral bacterial inoculation have been widely used to study different hypotheses in periodontal diseases.

E. faecalis is not a normal commensal within the oral cavity, but it can enter through food contaminants or nosocomial infections and is associated with oral microbiota. The presence of high levels of this medically important pathogen within the periodontitis-associated microbiota may pose risk for systemic dissemination and development of infections at distant body sites. The aim of this study is to evaluate the histopathological changes in rat molar periodontium tissue as well as heart and kidney tissue samples following concurrent infection with *E. faecalis* inoculation and 0.2 mm ligature wire.

Materials and Methods

Animal Preparation

A total of 12 Sprague Dawley (SD) rats aged (6 weeks old) weighing about 180 g to 200 g were divided into three groups: 0-day (control), 7-days (experimental) and 14-days (experimental). The rats were kept in ventilated cages under a 12-hour dark/light cycle and at controlled room temperature of ±25°C at the Research Animal Experimental Laboratory, Faculty of Health Science, Universiti Kebangsaan Malaysia, during the experimental period. The rats were given standard rat pellets and drinking water. All

rats were kept for 7-day for acclimatisation prior to the experimental period. The body weight was recorded weekly throughout the study period. All animal study procedure were conducted in accordance with the Animal Ethics Committee of Universiti Kebangsaan Malaysia (UKMAEC) on animal care protocol and approval was obtained before the experimental period (FD/2018/NURRULSHAQINAH/128NOV1961-NOV-2018-JAN 2020).

Experimental Procedure

General anaesthesia was given by intraperitoneal injection with Ketamine 10% 100mg/kg and Xylazine 2% 10mg/kg of body weight (Davis, 2001). A sterile 0.2 mm ligature wire was cut to 5 mm in length. Sterile endodontic file #8, #10 (Dentsply) was bent 90° from the tip and inserted carefully between the proximal space of the upper right maxillary first and second molar tooth area a few times through gentle push and pull movements to create space without damaging the gingival tissue. Then, the 0.2 mm sterile wire was bent and inserted carefully between the space with the help of a needle holder as shown in Figure 1 (a) (Li *et al.,* 2020). The wire was placed in the 7-day and 14-day experimental groups. *E. faecalis* strain was obtain from the American Type Culture Collection (ATCC 29212, USA). The bacteria were grown in a brain heart infusion agar medium in an anaerobic chamber at 37°C for 24 hours. After incubation, the bacterial colonies appeared to be non-transparent, creamy, spherical in shape and normal in size (Atlas of Microbiology, 2015). Then 1.5 x 10^8 CFU/ml of bacteria were standardised using McFarland standards. Following the insertion of a ligature wire, 0.5 µl of the bacterial solution was injected carefully into the gingival sulcus of upper right maxillary first and second molar area using a Hamilton syringe once a week as shown in Figure 1 (b) during the respective induction period.

H&E Staining

At 0-day, 7-day, 14-day post-induction period, the animals were sacrificed using anaesthesia overdose through intraperitoneal injection with a mixture of Ketamine 10% 200mg/kg and Xylazine 2% 20 mg/kg of body weight. Following euthanasia, tissue samples from the maxillae, heart and kidney were collected. The soft tissues for histopathological examination were immediately fixed with 10% neutral buffered formalin for at least 48 hours. The maxillary specimens were decalcified in 10% buffered ethylenediaminetetraacetic acid (EDTA, Sigma) solution for three weeks. After decalcification, all samples were fixed by using an automated tissue processor machine (Leica Model TP1020, USA). Then, samples were embedded with paraffin using the TBS88 Paraffin Embedding System Cool Unit (Medite Model TBS88, Netherlands) and the samples were cut into 5 µm thickness using a rotary microtome (Leica RM2135, Germany). Maxillary specimens were cut coronally perpendicular to the long axis of the alveolar ridge. Each included a complete cross section of the teeth, bone and soft tissue.

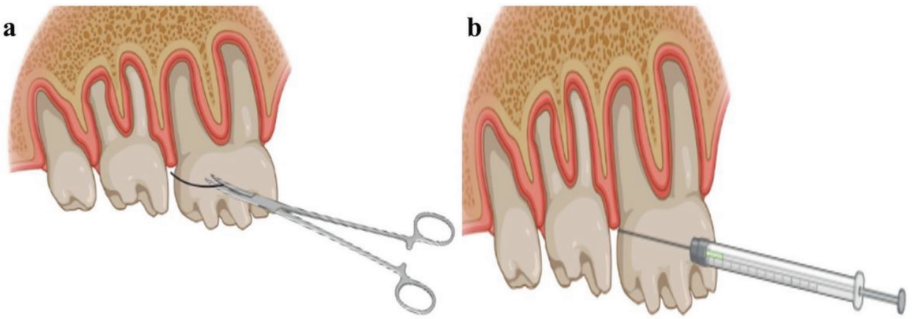

Figure 1: (a) Ligature wire insertion and (b) *E. faecalis* inoculation

Then, the sections were floated in a 60°C water bath (Medite Model TFB 45, Germany) before the good section was fished. The slides were labelled and kept on a hot plate (62°C) (Medite Model TFP 40, Netherlands) for overnight warming. Then the slides were stained with H&E following protocol. After that, slides were mount in DPX with cover slips and viewed under a digital circuit system microscope (Olympus MSAR LED, Japan). The histopathological findings were analysed qualitatively and the descriptions were compared between groups.

Results

Morphology of Periodontium, Heart and Kidney

The periodontium is the specialised tissues that surround and support the teeth to ensure that it remains within the maxillary and mandibular bone. It consists of four principal components, which are the gingiva, periodontal ligament, cementum and alveolar bone as shown in Figure 2 (a, d). The gingival epitheliums have three parts, the oral epithelium, sulcular epithelium and junctional epithelium. The heart wall consists of three-layers, which are the epicardium (the outer part), myocardium (the middle part) and the endocardium (the inner part) as shown in Figure 2 (b). The myocardium is the thickest muscle tissue of the heart as shown in Figure 2 (e). The kidney cross section shows a two-part outer cortex and inner medulla as shown in Figure 2 (c). The two components of the renal parenchyma are the renal corpuscle and loop of Henle. The renal corpuscle has two parts, which are the glomerulus and Bowman's

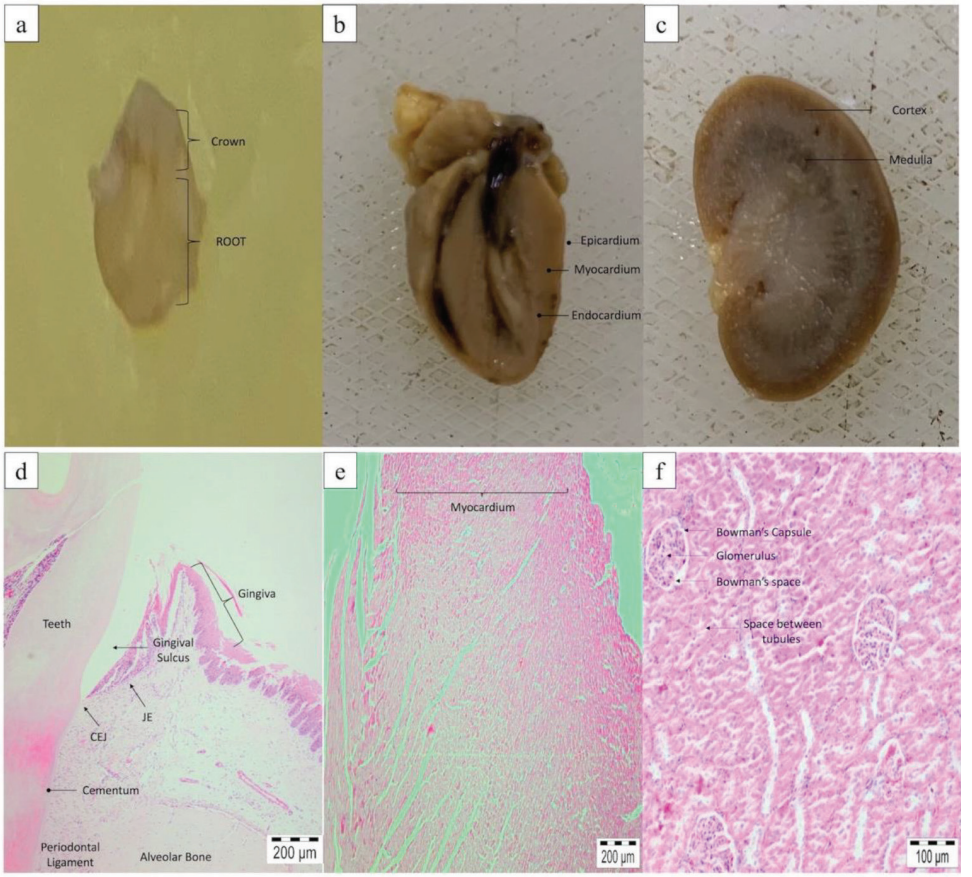

Figure 2: The structures of the periodontium (a, d), heart muscle (b, e) and kidney (c, f)

capsule as shown in Figure 2 (f). There are two types of renal tubules and the space between the tubules is known as the interstitium.

Descriptive Histology of Periodontium

At 0-day (negative control), the histological analysis shows normal gingival epithelium with no epithelial attachment loss. Infiltration of inflammatory cells within the connective tissue, as well as periodontal pocket formation were not observed as shown in Figure 3 (a). At seven day post-induction, hyperplastic gingival epithelium proliferated towards the connective tissue area. Gingival epithelial attachment loss and the formation of a periodontal pocket were observed. More importantly, intense infiltration of inflammatory cells within the connective tissue area, particularly neutrophils, few macrophages, fibroblast and few other cell types, were observed in the 7-day group as shown in Figure 3 (b-d). At 14-day post-induction, the deepening of the periodontal pocket is observed and inflammatory cell infiltration within the connective tissue were less compared with the 7-day group, as shown in Figure 3 (e-f).

Descriptive Histology of Heart Tissue Sample

Histological analysis at 0-day shows no changes within the myocardium as shown in Figure 4 (a-b). Interestingly, we found that following concurrent oral infection infiltration of inflammatory cells of mostly neutrophils, few macrophages invaded the myocardium at 7-day and 14-day as shown in Figure 4 (c-f).

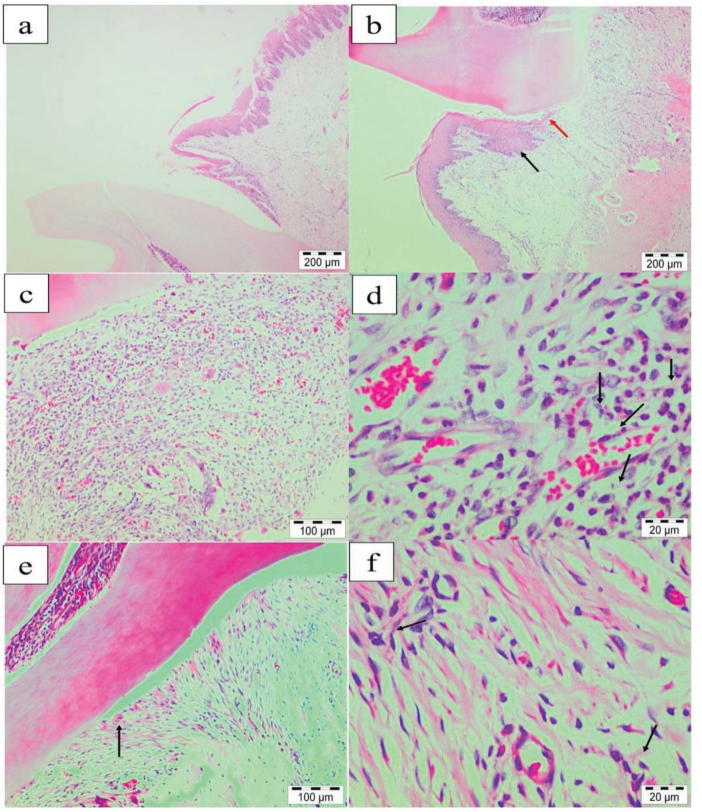

Figure 3: The normal histological structure at (a) 0-day; 7-day, (b, black arrow) hyperplastic gingival epithelium, (b, red arrow) gingival attachment loss, (c-d) inflammatory cells infiltration and at 14-day, the deepening of the periodontal pocket formation (e, black arrow) and inflammatory cell neutrophils (f, black arrow)

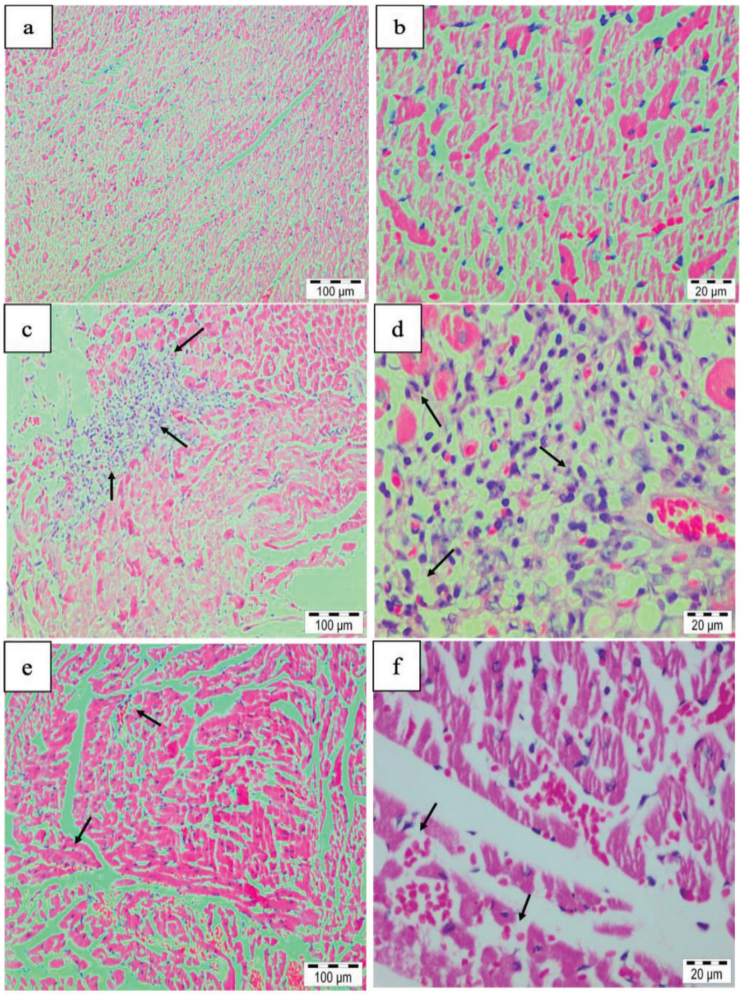

Figure 4: The normal histology within the myocardium at (a-b) 0-day (a-b) and inflammatory cell infiltration of mostly neutrophil (black arrow) within the myocardium at 7 and 14-day (c-f)

Descriptive Histology of Kidney Tissue Sample

Histological analysis at 0-day shows a normal glomerulus and no inflammatory cell infiltration within the interstitial tubular space as shown in Figure 5 (a-b). After 7-day, there were losss of architecture of Bowman's capsule, congested blood vessels within the glomerulus and inflammatory cell infiltration, specifically in the neutrophils within the interstitial tubular space as shown in Figure 5 (c-d). However, after 14-day, the severity of the inflammation reduced, although fewer inflammatory cells were observed as shown in Figure 5 (e-f).

Discussion

Experimental periodontal disease induction using ligature suture or wire is commonly used and act as dental plaque accumulation. In this study, 0.2 mm ligature wire is used to induce bacterial plaque accumulation, which facilitates experimentally inoculated *E. faecalis* to invade the periodontal tissue and initiate the inflammatory cascade of events. Gingival hyperplasia, attachment loss, inflammatory cells infiltration and bone resorption are the histological characteristic features of periodontal diseases. Interestingly, in this study it was

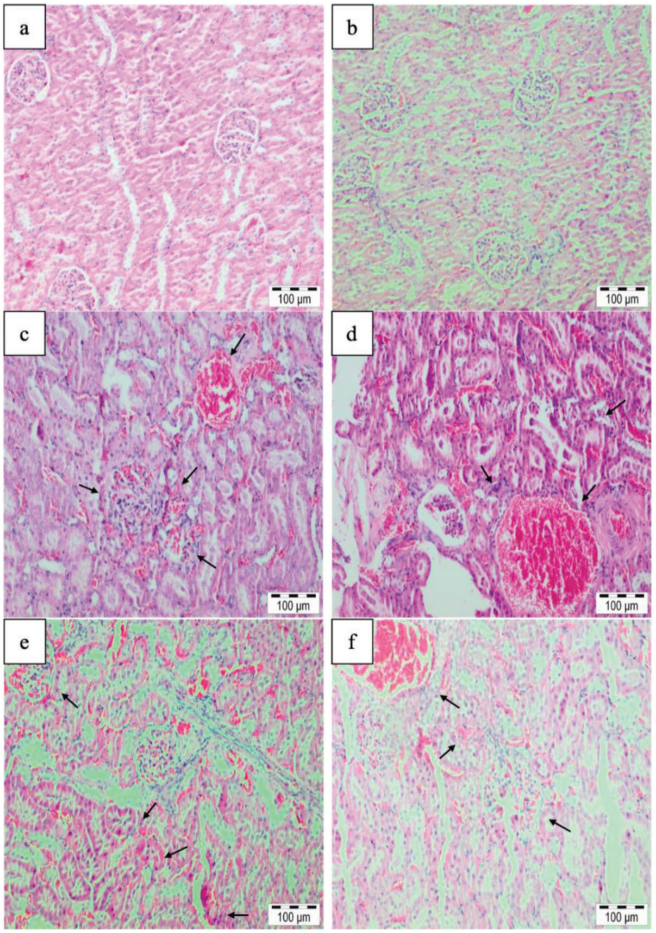

Figure 5: The normal histology within the kidney tissue at 0-day (a-b) and congested blood vessel within the glomerulus, reduced Bowman's capsule space and inflammatory cells infiltration within the interstitial tubular space indicated with black arrow at 7 and 14-day (c-f)

observed that hyperplastic gingival epithelium and gingival attachment loss followed by pocket formation and infiltration of the inflammatory cells of mostly neutrophils, few macrophages follow concurrent oral infection. Liew *et al.* (2020) has reported hyperplastic gingival epithelium by H&E staining following experimental periodontal disease induction by mixed infection with ligature placement and oral bacterial inoculation with a rat model similar to this study. Furthermore, Wu *et al.* (2018) reported infiltration of inflammatory cells within the periodontium by H&E staining following experimental periodontal disease induction using a rat model (Wu *et al.*, 2018). Phagocytic leukocytes such as neutrophils, macrophages, monocytes and dendritic cells are vital defences against invading microorganisms. Neutrophils are found abundantly and have arguably the greatest bactericidal capacity. Following periodontal inflammation, neutrophils are one of the first inflammatory cells to migrate toward the infected site to clear the invading microorganism (Ramadan *et al.,* 2020). The host's defence against bacterial infection depends on pathogen identification and the subsequent recruitment of neutrophils to infection sites. Host cells release a variety of pro-inflammatory cytokines and chemokines through the PAMP/PRR pathway in response to invading

pathogens, which serve as a chemoattractant and enhance neutrophil migration to infected tissues. Neutrophils can phagocytose bacteria and microorganisms through oxygen-dependent and oxygen-independent processes (Kobayashi *et al.,* 2018). The generation of superoxides, which is converted to other reactive oxygen species that are efficient in killing bacteria, is involved in oxygen-dependent bactericidal activity. Interestingly, both cell types were observed, predominantly neutrophils within the periodontium, following the experimental-concurrent oral infection. However, we did not observe severe alveolar bone losses, which is one of the important hallmarks for late-stage periodontitis, possibly due to the short induction duration.

The current study evaluated the histopathological changes within the heart and kidney following experimentally induced concurrent oral infection. The oral cavity is a reservoir for numerous oral and non-oral pathogenic microorganisms. *E. faecalis* is a medically important pathogen that can enter the oral cavity and is associated with oral microbiota. Studies have demonstrated the association of this microorganism with endodontic lesions, chronic periodontitis and infective endocarditis. In this study, we have found that inflammatory cell infiltration of mostly neutrophils within the myocardium of the heart tissue samples, as well as the interstitial tubular space of the kidney tissue samples at 7-day and 14-day post-induction following concurrent oral infection. Furthermore, congested blood vessels within the glomerulus, as well as loss of architecture of Bowman's capsular space were also observed.

All these pathological signs indicate the initiation of acute inflammatory conditions in the heart and kidney, probably in response to bacterial invasion. Kholoud *et al.* (2013) has reported the infiltration of inflammatory cells within the myocardium after 7-day following the experimental inoculation of *E. faecalis* using the rat model (Kholoud *et al.,* 2013). Furthermore, Kau *et al.* (2004) reported pathological changes within the kidney tissue samples following experimental *E. faecalis* inoculation in the mice model (Kau *et al.,* 2005). Inflammation of the periodontal tissue leads to the deepening of the gingival crevice, which results in the formation of a periodontal pocket that can act as a reservoir of a large number of microorganisms.

Periodontal diseases affect the oral tissues and bacteria, which can enter the bloodstream through the affected periodontal site. The circulatory system, which consists of blood, blood vessels and the heart is usually free from microbial organisms. In the current study, it can be assumed that orally inoculated *E. faecalis*, which is probably associated with other periodontal pathogens, had invaded the periodontium. Then, it entered the bloodstream through the ulcerated gingival epithelium and gingival blood vessel following oral infection and probably developed bacteraemia. Bacteria enter the heart and kidney through systemic circulation, which results in the activation of the innate immune system, deploying inflammatory cells, particularly neutrophils, in response to microbial invasion.

Although we have used same induction stimulus for 14-day, the pathological changes in the periodontium, heart and kidney were reduced, probably due to sufficient immunity responses by the host to clear up the infected site or the induction stimulus was not adequate to initiate further inflammatory responses. However, inflammatory changes were acute, probably due to the short induction time. In case of a prolonged induction period, probably severe inflammatory cell infiltration and pathological changes will be observed. De Mololn *et al.* (2014) has reported bone loss and upregulation of pro-inflammatory cytokines on day 7 of periodontitis induction, with a consequent reduction on day 15 using the ligature method with oral bacterial inoculation in the mice model (de Molon *et al.,* 2014). Thus, this study evaluates the dissemination of the microorganism in the early stage of the locally infected periodontal site towards the heart and kidney, which initiate acute inflammation.

Conclusion

This study suggests that *E. faecalis* in the periodontitis, associated with microbiota, can initiate acute inflammation within the periodontal tissue and develop infection at distant body sites. Further research is needed to understand its complete role in periodontal diseases, as well as non-oral diseases.

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