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ORIGINAL ARTICLE

Oral inoculation of *Enterococcus faecalis*, DNA quantification and histopathological evaluation of gingival, heart and kidney tissue samples in rats

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ABSTRACT

Background and Objectives: *Enterococcus faecalis* is known as common pathogen for endodontic infections and cause secondary and refractory pulp periapical periodontitis. The bacteria can opportunistically colonize periodontal pockets and presents a possibility of infection developing in other organs. This research will investigate the dissemination of *E. faecalis* from the gingival tissue to the heart and kidney.

Materials and Methods: Three groups were formed, consisting of twelve male Sprague Dawley rats: a control group designated as 0-day, and experimental groups labeled as 7-days and 14-days. Periodontitis induced by concurrent infection with sterile wire 0.2 mm insertion and *E. faecalis* inoculation is performed into the gingival sulcus located between the maxillary right 1st and 2nd molar teeth area. After euthanasia, tissue samples around the maxillary gingiva, maxillary jaw samples, kidney and heart tissues were obtained for quantitative Real-Time PCR assay and histopathological analysis.

Results: Results showed at 7-days, there was an upregulation of *E. faecalis* gene expression in the gingiva, heart, and kidney samples as well as infiltration of the inflammatory cells at 7-days post induction, which consequently decreased at 14-days.

Conclusion: Thus, the study suggests dissemination of *E. faecalis* from gingival tissue to the heart, kidney which could be probable link between periodontal disease, heart, and kidney disease.

Keywords: *Enterococcus faecalis*; Gene expression; Heart disease; Kidney disease; Periodontitis

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INTRODUCTION

Enterococcus faecalis is known as common pathogen for endodontic infections can colonize within the periodontal pocket opportunistically, posing a risk of infection spreading to other organs. The oral environment may harbor a variety of ecological factors that are favorable for the development of bacteria that are not typically thought of as members of the normal oral microbiota (1). The intricacy of periodontitis-associated microbiota may favor the colonization of non-oral microorganism (1). Evidence indicates that interactions between oral bacteria and medically relevant pathogens within the periodontal microbiota play a pivotal role in periodontal health and disease (2). Additionally, pockets in the periodontal tissue can serve as reservoirs for microorganisms and their byproducts, which may disseminate to distant organs via the gingival bloodstream due to anatomical proximity (2). Unlike normal commensals found in the oral cavity, *Enterococcus faecalis* is a facultative, Gram-positive pathogen with significant medical implications. They are linked to the oral microbiota and can enter the oral cavity by food contamination or nosocomial infection. Excessive concentrations of medically significant pathogens in the microbiota of the oral cavity may increase the risk of systemic spread and the emergence of infection in other organs (3). This research will investigate the early dissemination of *E. faecalis* from gingival tissue to the heart and kidney that induce inflammatory responses.

MATERIALS AND METHODS

Experimental procedure. After obtaining the ethical approval from UKMAEC (FD/2018/NURRUL SHAQINAH/28-NOV./967-NOV.-2018-JAN. -2020), we acquired twelve male Sprague Dawley (SD) rats, approximately six weeks old, with an average weight of around 180 grams. These rats were bred and maintained under specific pathogen-free conditions in the proper facilities. The rats were categorized into three groups: a control group labeled as 0-day, and experimental groups designated as 7-days and 14-days, respectively. All experiments adhered to the regulations set by the UKMAEC and studies are conducted in accordance with the ARRIVE 2.0 guidelines (4). Rats were housed four per cage under standard conditions, with access to the proper water and laboratory grade food pellets. The animals underwent a seven-day acclimation period. Prior to the start of the experiment and every three days thereafter, the rats' weights were recorded. Sample sizes were determined through ANOVA calculations utilizing degrees of freedom, denoted by the "E" value, which must fall within the range of 10 to 20 (5). We eventually utilized four rats each group; fortunately, none of the rodents were lost. Intraperitoneal injection administered a blend of ketamine (10% at 100 mg/kg) and xylazine (2% at 10 mg/kg) of body weight for general anesthesia. A sterile orthodontic wire, 0.2 mm thick, was gently contoured into a "C" shape and cautiously inserted within the interdental spaces of the upper right maxillary first and second molar areas using a needle holder (6). The *E. faecalis* (ATCC 29212, USA) strain was grown for 24 hours at 37°C in an anaerobic environment using Brain Heart Infusion (BHI) blood agar medium. Then, using the McFarland standard, 1.5×10^8 CFU/ml concentration of bacterial solution was standardized. Following the insertion of the ligature wire, 0.5µl of the bacterial solution was gently injected using a Hamilton microliter syringe into the gingiva sulcus of the upper right first and second maxillary molar tooth regions. All rats were euthanized at the end of the experiment by administering an intraperitoneal overdose of anesthesia (ketamine 10% at 200 mg/kg and xylazine 2% at 20 mg/kg of body weight).

RT-qPCR assay. Tissue samples around the gingiva of the molar teeth area, kidney and heart tissues were obtained for DNA extraction and quantitative RT-qPCR was done to assess *E. faecalis* gene expression. Extraction and purification of total 25 mg DNA from gingival, heart and kidney tissues were carried out by using Nucleospin DNA extraction kit according to manufacturer protocol. RT-qPCR was carried out using ChamQ Universal SYBR qPCR master mix (Vanzyme, Catalog # Q711). Each 10 µl qPCR reaction comprised 5 µl of master mix, 0.5 µl of reverse and forward primer, 1 µl of DNA template, and 3 µl of RNase-free water. The two-step amplification protocol was executed using the real-time PCR Thermal Cycler (Bio-Rad CFX96 Connect, USA) according to the following steps: 95°C of pre-denaturation at for 60 s, followed by 95°C of denaturation for 15 s, and 60°C annealing for 30 s. *E. faecalis* primer were used following sequence forward 5'-ACACTTGGAAACAGGTGC-3' and reverse 5'-AGTTACTAACGTCCTTGTTC-3' (7). All reactions were performed

in triplicate, and contamination was monitored by including a no-template control in each RT-qPCR run, consisting of a reaction mixture without template DNA. Quantitative RT-qPCR cycle threshold (CT) values were normalized using the housekeeping gene GAPDH, and fold changes were determined using the $2^{-\Delta\Delta CT}$ method (8). The findings of the data analysis were presented as mean ± standard deviation (SD) using SPSS Data Editor version 23.0 (IBM, USA). After evaluating differences in group means using an ANOVA statistical model, Tukey's post-hoc test was applied to determine significantly different group means. The statistical significance was defined in this study as p values less than 0.05.

Samples of tissues taken from the kidney, heart, and maxilla were collected, stored, and stained using hematoxylin and eosin for histological examination. The soft tissues that were intended to be investigated were fixed right away and kept in 10% neutral buffered formalin for a minimum of 2 days. The maxillary jaw samples were decalcified in 10% buffered EDTA solution for a duration of 21 days. Samples were decalcified and processed with a Leica TP1020 tissue processor. Sections of 5 μm thickness were cut using a Leica RM2135 microtome and embedded in paraffin with a Medite TBS88 system. The specimens were cut coronal, perpendicular to the long axis of the maxillary ridge, revealing the teeth, soft tissue, and bone in their entirety.

RESULTS

Inflammatory cells infiltration particularly neutrophils and macrophages in the periodontium observed by histological analysis of 7 and 14 days (Fig. 1b, c). Additionally, the heart tissue sample (Fig. 1e) showed myocarditis along with mild inflammatory cell infiltration (neutrophils and macrophages), and the kidney tissue samples showed congested blood vessels within the glomerulus along with the infiltration of the inflammatory cells, especially neutrophils, observed in 7 -days (Fig. 1h). The severity of lesions in heart and kidney tissues was decreased in 14-days post-infection group. (Fig. 1f, i). No significant histological changes observed in control group (Fig. 1 a, d, g). The RT-qPCR result shows upregulation of the *E. faecalis* gene in the samples from gingival tissue and significant upregulation ($P < 0.01$) in the heart, and kidney at 7-days post-infection (Fig. 2). On the other hand, *E. faecalis* gene expressions were downregulated in the gingival tissue and decreased in the heart, and kidney tissue samples although not statistically significant ($P > 0.05$) (Fig. 2).

DISCUSSION

In the current study, 0.2 mm orthodontic ligature wire worked as a dental biofilm retentive factor for the development of periodontal pathogen and facilitated experimentally inoculated *E. faecalis* invading within the periodontium. In gingival tissue, bacterial gene expression was significantly upregulated at 7-days after induction as compared to the control group; however, *E. faecalis* gene expression was downregulated at 14-days post induction. As opposed, the bacterial gene was upregulated in the heart and kidney tissues at both induction periods. The higher expression level of *E. faecalis* in the 7-days post induction suggests the establishment of this organism in the respective organs. It is believed that the *E. faecalis* preferably colonizes in the periodontal pocket since it provides a very rich environment for bacterial viability (9). After colonization has been established, the enterococci produce virulence factors that may be relevant to the inflammation process, which includes surface adhesion, aggregation substance, production of extracellular superoxide, lipoteichoic acid, lytic enzymes, hemolysins, and cytolysins (10). Apart from the virulence factors, the mechanical procedures also may contribute to the damage of oral tissues and lead to the entering of bacteria into the bloodstream through the ulcerated and inflamed crevice, pocket epithelium, as well as gingival micro ulceration to induce bacteremia (11). In this study, evidence of elevated expression levels of the bacterial gene within the heart and kidney tissue samples suggest that bacteria disseminated from the periodontal inoculation site to the heart and kidney through the systemic circulation. The finding was in agreement with a study that demonstrated the presence of *E. faecalis* in the periodontal tissue and heart in a dog study model (12). Moreover, the findings are also similar to a study by Kau et al. (2005), who isolated this microorganism from an inflamed kidney following experimental inoculation in a mice model (13). The development of an immune response in the host following concurrent oral infection is likely to be the cause of the downregulation of *E. faecalis*

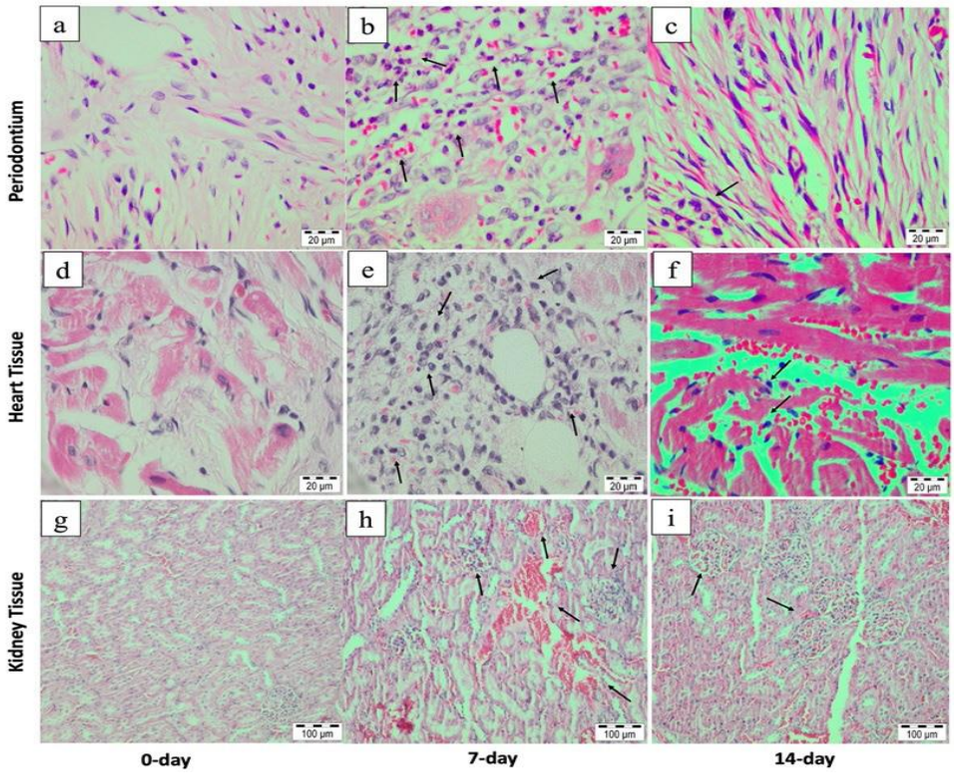

Fig. 1. Histological analysis of Periodontium (a, b, c), Heart (d, e, f), Kidney (g, h, i) with 40× magnifications. Inflammatory cells (neutrophil, macrophages) indicated with black arrow and (a, d, g) are the negative control.

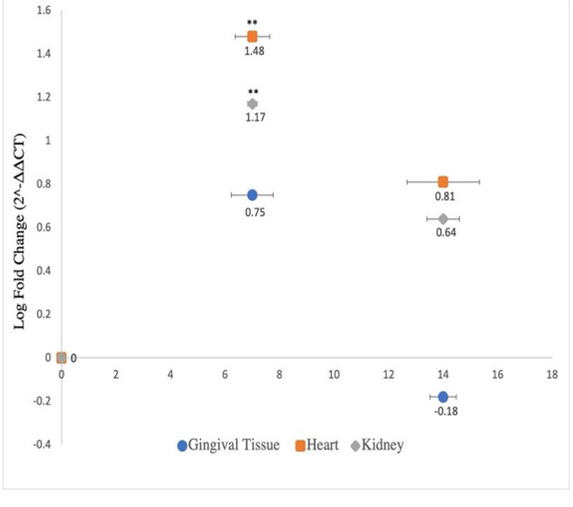

Fig. 2. *E. faecalis* gene expression by RT-qPCR. Data are shown as the mean ± standard deviation and p values are indicated by asterisk, **p < 0.01.

gene expression in the gingival tissue at 14-days post infection. However, it is suggested that the presence of dead bacterial genes detected by the RT-qPCR assay might justify the upregulation of bacterial gene expression in the heart and kidney after 14-days. Meanwhile, there was no bacterial gene expression in the tissue from gingiva, kidney and heart tissue samples in the control group.

The histopathological findings were consistent with the bacterial gene expression results. In the current study shows that inflammatory cells infiltration mostly neutrophils in the gingival tissue, heart and kidney tissue were higher in 7-days as compared to 14-days. The *E. faecalis* gene expression is also elevated in the gingival, heart, and kidney tissues in the 7-days groups, indicating a higher bacterial load in the tissue from gingiva, kidney and heart tissue samples. Inflammatory cells were also higher in the 7-days groups, suggesting the immune response due to invading microorganisms in these three organs. Furthermore, the absence of *E. faecalis* at the periodontal site reduced inflammatory signals in the tissue from gingiva, kidney, and heart after 14-days, as evidenced by the RT-qPCR assay and histological analysis. One of the limitations of this study is that the induction period is short enough to cause severe inflammatory changes in distant organs. Additional strong inflammatory alterations might be observed in the distant organs if the induction duration was

extended, and more stimulation were given.

CONCLUSION

In conclusion, the research showed that *E. faecalis* can migrate from an infected periodontal area to the heart and kidney, causing inflammation. As a result, the study suggests that periodontal disease causes systemic dissemination of microorganisms to distant organs which could be a probable link between periodontitis, heart and kidney disease. Future research is required to corroborate the conclusions gained from this study, since this is an important step toward future work on the bacteria's systemic dissemination.

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