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Decrease of collagen number density in rat facial skin due to *Porphyromonas gingivalis* induction in rat model periodontitis

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Abstract

Background: Periodontitis is a chronic inflammatory disease initiated by bacterial dysbiosis in periodontal tissue. *Porphyromonas gingivalis* (*P. gingivalis*) is the keystone pathogen of periodontitis with the ability to promote systemic inflammation. They can invade other organs outside oral cavity and enter lymphatic and blood vessels. Its virulence factor can affect tissue integrity by disturbing extracellular matrix regulation, resulting in tissue destruction. One of the extracellular matrix components in facial skin is collagen, which is responsible for facial wrinkles.

Objectives: To examine collagen number density in rat facial skin due to *Porphyromonas gingivalis* induction in rat model periodontitis.

Methods: In vivo experimental study conducted with 2 control groups (C1, C2) and 2 treatment groups (T1, T2). The treatment groups received *P. gingivalis* induction for 3 weeks. The treatment group 1 (T1) euthanized 2 weeks after the bacterial induction, along with control group 1 (C1). The treatment group 2 (T2) was euthanized 4 weeks after the bacterial induction, along with control group 2 (C2). The facial skin was taken and prepared for Masson Trichrome staining. Statistical analysis was carried out for the collagen number density.

Results: There was significant difference in collagen number density between treatment groups and control groups ($p < 0.05$). In both treatment groups, the collagen number density is lower than both control groups.

Conclusions: There is a significant difference in collagen number density of rat facial skin between *P. gingivalis* induced and uninduced, and appears lower in rats induced by *P. gingivalis*.

Keywords: Collagen; Facial Skin; Wrinkles; Periodontitis

1. Introduction

Periodontitis is a multifactorial chronic inflammatory disease of tooth supporting tissue and caused by the imbalance between oral microbiota in dental biofilm and host's response. Periodontitis resulting in destruction of connective tissue and alveolar bone, often leads to tooth loss [1,2,3]. *Porphyromonas gingivalis* (*P. gingivalis*), as a key pathogen in periodontitis, is an anaerobic, rod-shaped, gram-negative bacterium that often-colonizes gingival epithelium and has the ability to remodel bacterial compositions in periodontal environments that can lead to dysbiosis [1]. Studies show

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P. gingivalis correlation to development of many systemic diseases and their contribution in the disease severity [1,2,4,5,6].

P. gingivalis can invade other organs by promote persistent local periodontal infection that can heighten the circulating systemic pro-inflammatory cytokines and can leads to low level long term inflammatory state where the circulating proinflammatory molecules increase and affect inflammatory or disease progression in other sites of the body [1,2]. Toxins of *P. gingivalis*, such as LPS; gingipain and OMV, have been found in many organs outside the oral cavity and are suspected to promote local and systemic inflammatory disease progression in many organs. It's been proven that toxins and bacterium materials form *P. gingivalis* has been found in liver, blood-brain barriers, atherosclerosis plaque, and digestive tract. *P. gingivalis* suspected to contribute and promote a number of systemic diseases such as diabetes mellitus, atherosclerosis and other cardiovascular disease, rheumatoid arthritis, Alzheimer disease, non-alcoholic fatty liver disease, respiratory diseases, and cancer [1,7,8,9].

P. gingivalis and its virulence factors appear to affect host cells in so many different ways and help them survive longer. *P. gingivalis* can disrupt epithelium and endothelium junctions and pass through, so that eventually they can enter another site of tissue or circulate in blood. *P. gingivalis* also capable of modifying a cell's function to serve their needs to survive longer in host. They can enter immune cells while inhibiting its function so they can transport inside the cells and remain not affected by its antimicrobial properties [10].

Overexpression of inflammatory mediators can damage collagen and elastin in the skin, causing the skin to lose its elasticity. Depletion of collagen and elastin causes the development of skin wrinkles in the dermis layer. In the skin, aging tissue is contributed by intrinsic and extrinsic aging process. The intrinsic aging process is an unavoidable physiological change that occurs throughout life, meanwhile the extrinsic aging process is escalated by many external factors such as air pollution, sunlight exposure, smoking. Both aging processes happened simultaneously and resulted in skin tissue change like fine lines and wrinkle formation [12,13,14,15].

As the skin tissue aged, fibroblast also aged and went through structural and morphological changes called fibroblast senescence. Change in aged fibroblast will affect the expression of senescence-associated secretory phenotype (SASP) and lead to increased MMP activity and ECM degradation [14,16, 17, 18].

ECM components that significantly affect wrinkle formation during aging are collagen. As the body gets older, collagen's production will decrease and their composition will also be altered. Collagen contributes to the skin's strength and integrity and mechanical properties [18,19,20,21].

In this study, we conducted the preliminary research to study the possibility of periodontitis induced by *P. gingivalis* to affect skin condition by inspecting the collagen changes in mice induced periodontitis.

2. Material and Methods

2.1. Animals' preparation

Male Wistar rats aged 4 months were obtained from the Biochemistry Laboratory - Faculty of Medicine - Universitas Airlangga. Rats were caged in a controlled environment with a 12 hours light/dark cycle at 22 ± 0.5°C, 40%–70% relative humidity, with food and water ad libitum. All animals were handled daily for 2 weeks before bacterial induction. During 4 weeks previous to bacterial induction, all rats in each group inhabited the same cage and were fed and hydrated from the same sources. After bacteria induction, they were isolated in separate filter-containing cages [9].

2.2. *Porphyromonas gingivalis* Induction

Wistar rats were divided into four groups (C1, C2, T1, T2), each group consisting of seven rats. Rat model periodontitis in group T1 and T2 received *P. gingivalis* injection procedure every 3 days for 3 weeks (i.e each group received 6 injections in total). Injection procedures were performed under general anesthesia with 2% isoflurane. *P. gingivalis* were injected into the gingival sulcus between the first and second maxillary left molar of the rats as much as 0,1 ml of 1 x 1010 CFU/ml. Group T1 were euthanized 2 weeks after completing *P. gingivalis* injection procedure. Group T2 were euthanized 4 weeks after completing *P. gingivalis* injection procedure.

As controls, group C1 and C2 that consisted of seven rats, each were conditioned for 2 weeks beforehand and did not receive *P. gingivalis* injection. Group C1 euthanized along with group T1 and group C2 along with group T2. After all procedures were completed, tissue sampling from rat facial skin was taken.

2.3. Euthanasia and Tissue Sampling

Group C1 and T1 were euthanized using carbon dioxide asphyxiation at week 2 after completing *P. gingivalis* bacterial injection for group T1. Group C2 and T2 were euthanized at week 4 after completing *P. gingivalis* bacterial injection for group T2. Rat facial skin sampling was taken after euthanization. Rat's hair was shaved with scalpel, and then continued with excision of the rats left facial skin (1 cm x 1 cm dimension) to the subcutaneous depth (Figure 1). Facial skin samples were isolated and placed in 4% paraformaldehyde overnight at 4° C for histological staining [23].

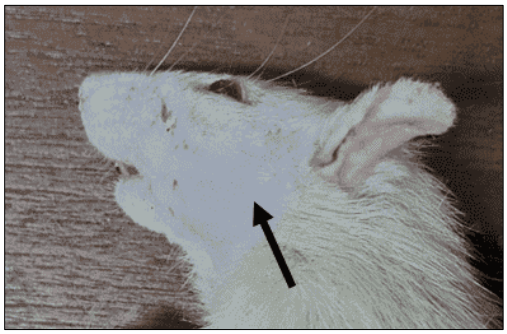

Figure 1 Area of facial skin sampling

2.4. Masson Trichrome (MT) Staining

Skin tissue slides were placed in a staining jar and deparaffinized by submerging them into three series of absolute xylene for 4 minutes each followed by 100%, 95%, 90%, 80% and 70% of ethanol for 4 minutes in each percentage. The slides then were submerged in warmed Bouin's solution at 60°C for 45 minutes. Next, the slides were washed in running tap water until the yellow colour in samples disappeared. To differentiate nuclei, slides are then immersed in modified Weigert's haematoxylin for 8 minutes and washed in running water for 2 minutes. In order to stain cytoplasms and erythrocytes, slides were submerged in anionic dyes, acid fuchsin for 5 minutes and washed with running tap water for 2 minutes. Then, slides were treated with phosphomolybdic acid solution for another 10 minutes as a mordant and immediately submerged into methyl blue solution for 5 minutes in order to stain fibroblast and collagen. After that, slides were washed in running water for 2 minutes and lastly treated with 1% acetic acid solution for 1 minute. Slides then were dehydrated into a series of alcohol of 70%, 80%, 95% and 100% for 1 minute. Before observation, slides were dipped into absolute xylene for 1 minute and finally mounted with cover slip using DPX mounting [24].

2.5. Collagen Number Density Analysis

After slides were stained using Masson Trichrome (MT), slides were analysed with microscope under 40x magnification. Collagen number density was assessed histopathologically. Scoring system was used for the criteria assessment [25].

3. Results

3.1. Collagen Number Density

In this study, the evaluation of collagen number density was measured by comparing control group C1 with treatment group T1, and control group C2 with treatment group T2. The mean of collagen number density was shown in table 1 and figure 2.

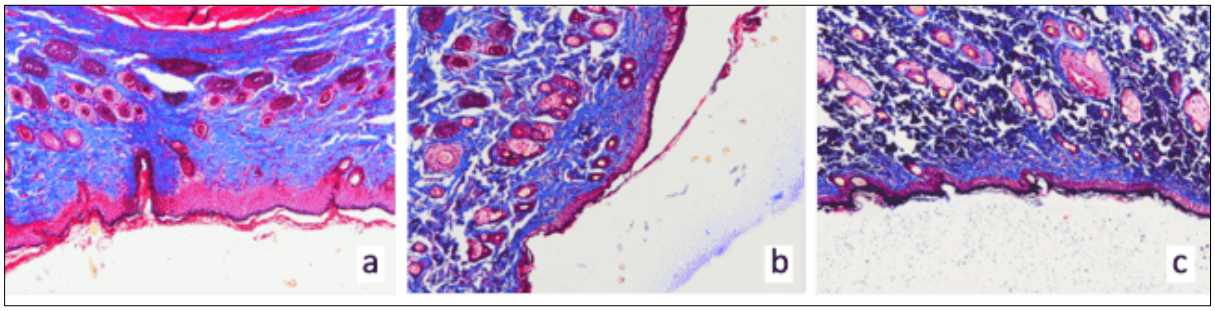

Figure 2 Collagen number density (Masson Trichrome Staining): (a) Control Group, (b) T1 Group; (c) T2 Group

Table 1 Mean of collagen number density in control groups (C1 and C2) and treatment groups (T1 and T2)

Group	N	Collagen number density (Mean ± SD)	Shaphiro-Wilk	ANOVA
C1	7	11.00 ± 2.160	0.949	0.000
C2	7	11.14 ± 1.951	0.549	
T1	7	4.14 ± 1.069	0.294	
T2	7	3.14 ± 1.345	0.873	

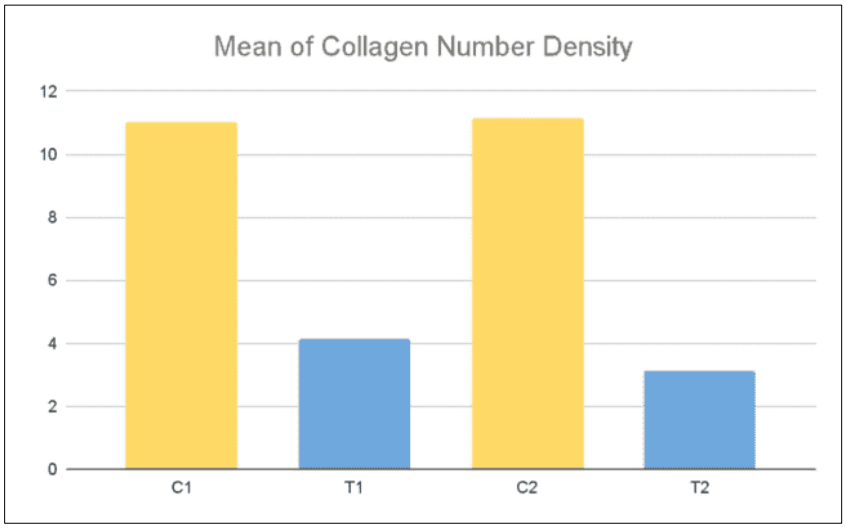

Figure 3 Chart of mean of collagen number density between control groups (C1 and C2) and treatment groups (T1 and T2). It can be seen that collagen density decreased in the treatment group compared to the control group

3.1.1. Note

- C1 : No induction of *P. gingivalis* and euthanized along with group T1
- C2 : No induction of *P. gingivalis* and euthanized along with group T2
- T1 : Induction of *P. gingivalis* for 3 weeks and euthanized 2 weeks later
- T2 : Induction of *P. gingivalis* for 3 weeks and euthanized 4 weeks later

The result shows that there is a difference in the collagen number density between the treatment groups and control groups. In both treatment groups (T1 and T2), the collagen number density is lower than the control groups (CI and C2). Based on statistical analysis, we found that the data is normally distributed and allowed to proceed with the Anova test. Statistical analysis reveals there is a significant difference between control and treatment group with significance value = 0.000 ($p < 0.05$). Post-Hoc test shows significant difference in collagen number density between control groups and treatment groups with significance value = 0.000 ($p < 0.05$), meanwhile there is no significant difference in within-group comparison (C1 vs C2, T1 vs T2).

4. Discussion

This study demonstrates a possible link between periodontitis and systemic disorder, especially facial skin. *P. gingivalis* induction in rat model periodontitis affects the integrity of collagen in rat facial skin. There is a reduction in the number of collagens in rat facial skin induced by periodontitis, as shown in the induced groups the collagen number appeared significantly lower than the control groups. This occurrence may be caused by numerous changes that can happen after induction of *P. gingivalis*, because of its virulence factors, and pro-inflammatory mediator which spreads through the bloodstream.

Porphyromonas gingivalis is a keystone pathogen of periodontitis and greatly affects disease progression. *P. gingivalis* also capable of modifying a cell's function to serve their needs to survive longer in host. They can enter immune cells while inhibiting its function so they can transport inside the cells and remain not affected by its antimicrobial properties [10]. Not only serve for bacterial survival purposes but they can also greatly affect the local environment in other organs that lead to other systemic disease progression. *P. gingivalis* can increase the circulating systemic pro-inflammatory cytokines that lead to the host undergoing a low-level long term inflammatory state [1,2,9].

In tissue cells, *P. gingivalis* can promote the activation of immunological pathways like Toll Like Receptor (TLRs) and NF-ƙB, that leads to heighten pro-inflammatory cytokines and reactive oxidative stress (ROS). Cytokines dysregulation could affect tissue regulation and destruction. Increased pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 will affect MMPs expression and function that lead to tissue degradation and destruction [10,11]. *P. gingivalis* may also participate in disease progression by interfering with mitochondria and increasing the amount of ROS as the byproduct of inflammation process that can promote tissue destruction [12,13].

In the skin aging process, external factors such as sunlight and pollution affect the number of ROS and promote matrix metalloproteinase (MMPs) through increasing MAPK and NFKB pathways activity [16]. Another matter that can increase the level of ROS is inflammation. Extensive research has found that ROS is a key factor in systemic diseases and also serves as the shared pathological link between them and periodontitis. *P. gingivalis* known to have the ability to release ROS in many places they are transported to such as arterial endothelium and brain endothelial cells [13]. The rising ROS level by any means can make the imbalance ratio between matrix metalloproteinase (MMPs) and tissue inhibitors of metalloproteinase (TIMPs) and cause extracellular matrix (ECM) changes by upregulating its degradation process [16].

The imbalance proportion between MMPs and TIMPs will upregulate ECM protein destruction such as collagen and elastic fibers that affect skin morphology and structure. MMPs have significant roles in basement membrane degradation and contribute to epidermis-dermis adhesion. Increased MMPs could greatly affect skin integrity and differentiation, also degrade many components especially basal membrane components including fibronectin, laminin, and elastin [18,19,20,21,26,22].

From this study, we can suspect that there is a possible correlation between local periodontal infection caused by *P. gingivalis* and the amount of collagen in facial skin of rats. It might be caused by *P. gingivalis* or its virulence factors, either they regulate systemically to the skin and enhance the ECM degradation, or the periodontal disease going on cause the low-level long term inflammatory state by increasing the circulating cytokines in the host body that make the ECM regulation disrupted.

5. Conclusion

Based on this study, it can be concluded there is significant difference in collagen number density in rat facial skin between with and without periodontal condition induced by *P. gingivalis*. Decrease of collagen number density occurs in rat facial skin induced by *P. gingivalis* in rat model periodontitis. Collagen is an extracellular matrix responsible for facial wrinkles. Further research is necessary to support better understanding of the relation between periodontal disease and the changes in facial skin, especially wrinkles appearance

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

Statement of ethical approval

The ethical clearance of this study was obtained from Universitas Airlangga Faculty of Dental Medicine Health Research Ethical Clearance Commission.

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