https://doi.org/10.14368/jdras.2021.37.1.31
ISSN 2384-4353 eISSN 2384-4272

Original Article

Heme effects of hemin on growth of peridontopathogens

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Purpose: The purpose of this study was to investigate effect of heme on periodontopathogens. **Materials and Methods:** The experiment was performed using 7 types of anaerobic bacteria present in the periodontal pocket. The bacteria were cultured using suitable medium in an anaerobic condition with or without hemin, and the growth of the bacteria was measured every 6 hours by a spectrophotometer. **Results:** the growth of *Porphyromonas gingivalis* was different only by the presence or absence of hemin. The growth of other periodontopathogens except *Treponema denticola* was different in a hemin concentration-dependent manner. The growth of *T. denticola* was interfered by hemin. **Conclusion:** Heme may be a factor that leads dysbiosis in the microbial ecosystem of the subgingival plaque and thereby promote a periodontitis-causing environment. **(J Dent Rehabil Appl Sci 2021;37(1):31-8)**

Key words: hemin; periodontopathogen; dysbiosis; growth

Introduction

Periodontal disease is an inflammatory disease in oral cavity and related with gram-negative anaerobic bacteria.[1] In the 19th century, Socranskii group reported that *Porphyromonas gingivalis*, *Tannerella forsythia* (formerly *Bacteroides forsythus*), and *Treponema denticola* are closely related with periodontitis by epidemiological study.[2] After these results, the researchers studied the virulence factors associated with periodontitis in each bacterium.[3] Recently, ecological plaque hypothesis was raised, by which the studies on periodontitis-related bacteria have also changed a lot.[4] According to this hypothesis, the induction of periodontitis is related with dysbiosis of periodontal pocket. In healthy people, microbial ecosystem is balanced by Gram-positive facultative anaerobes like normal flora. However, the microbial ecosystem is broken by changes in external or oral environment, and because of this, the number of Gram-negative obligated anaerobes is increased. Eventually, multi-species pathogens may induce periodontitis. Therefore, the studies on effects of complex bacteria rather than single-species bacteria in periodontitis are being investigated.[5] Regarding changes in the oral environment, A study was recently reported that gingipain of *P. gingivalis* induces initial inflammation and increases the influx of gingival crevicular fluid into gingival pocket.[6] These symptoms can be expected to increase the proportion of Gram-negative obligated anaerobes.

Heme consists of a tetrapyrrole ring with an iron atom in center. Also, heme is linked specific protein such as hemoglobin, myoglobin, hemopexin, albumin and cytochromes[7,8] and reversible binds oxygen and transport electrons in human body. Further-

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Received: March 3, 2021/Last Revision: March 4, 2021/Accepted: March 4, 2021

more, heme is a damage-associated molecular pattern (DAMP) and triggers inflammatory responses of immune cells.[9] Iron is found in the form of heme[10] and a source for bacterial growth.[11] Periodontitis-related bacteria are also used iron for metabolism, toxicity, and pathogenesis.[12-15] In a clinical study, when hemin concentration between healthy and periodontal disease site was compared, the heme in healthy site and the disease site showed range of 0 - 251 nM and 0 - 7723.5 nM, respectively.[16] This data makes that it possible to investigate the effects of hemin on relation with periodontitis. However, the effect of hemin on bacterial growth in periodontal pocket has not been reported.

In this study, the bacteria related with periodontitis was investigated growth in various concentration of hemin.

Materials and Methods

In this study, *Filifactor alocis* ATCC 35896, *Fusobacterium nucleatum* ATCC 25586, *Porphyromonas gingivalis* ATCC 33277, *Prevotella intermedia* ATCC 25611, *Prevotella nigrescens* ATCC 33563, *Tannerella forsythia* ATCC 43037, and *Treponema denticola* ATCC 35405 were used to investigate effect of hemin. For cultivation of periodontitis-related pathogens, *F. alocis* was used columbia broth supplemented with yeast extract, L-cysteine (0.2%), L-arginine (0.4%), hemin (5 μg/ml), and vitamin K (0.2 μg/ml). *T. forsythia* was used modified NOS medium supplemented with N-acetylmuramic acid (0.1 μg/ml), vitamin K (0.2 μg/ml), hemin (5 μg/ml), and fetal bovine serum (4%). *T. denticola* was used GYGVS medium. *F. nucleatum*, *P. gingivalis*, *P. intermedia*, and *P. nigrescens* were used brain heart infusion broth supplemented with hemin (5 μg/ml) and vitamin K (0.2 μg/ml). The bacteria were cultured at 37°C in anaerobic condition (H_2 5%, CO_2 10%, and N_2 85%)

Hemin was purchased from sigmaaldrich and extracted from bovine. Hemin stock solutions were prepared in three concentrations as 0.5, 5, and 50 mg/ml. 5, 50 and 500 mg of hemin were completely dissolved with 0.1, 0.5, and 1 ml of 1 N NaOH in conical tube (SPL LifeSciences, Pocheon, Korea), respectively, and distilled water was then filled onto 10 ml. The prepared solution was filtered with polyvinylidene fluoride (PVDF) filter (0.22 μm of pore size). The stock solution was stored in anaerobic chamber before use.

Each cultured bacterium was harvested by centrifugation at 5,000 × g for 10 min, and fresh medium without hemin was added after removing supernatant. The bacteria were counted with bacterial counting chamber (and adjusted with fresh medium without hemin at 1×10^7 cells/ml. Each medium without hemin (9 ml) was added hemin at final concentration of 0.5, 5, 50 and 500 μg/ml using the stock solution, and then the prepared bacteria were inoculated in the medium. The bacterial growth was measured every 6 hours by optical density using a spectrophotometer at 660 nm of wavelength.

Statistical analysis was performed by IBM SPSS statistics ver. 23 software (IBM, Armonk, USA). The data from the different groups was analyzed by the non-parametric Kruskal-Wallis test. Statistical significance was defined by *P* value of less than 0.05. All data are expressed as the median and interquartile range.

Results

When *P. gingivalis* was cultured BHI broth including vitamin K with various concentration of hemin, the growth of *P. gingivalis* did not showed significant difference in the condition with hemin (Fig. 1), and in hemin free condition, *P. gingivalis* growth was showed slower. The growth of *P. gingivalis* entered the exponential phase after 12 hours, and reached the stationary phase after 36 hours in all concentration of hemin.

P. nigrescens grew the fastest at 50 and 100 μg/ml of hemin condition, and the growth ratio of *P. nigrescens* was changed in a dose-dependent manner of hemin (Fig. 2). Furthermore, the stationary phase of *P. nigrescens* growth showed different concentration by hemin concentration. In case of *P. nigrescens*, the its growth reached the stationary phase after 42 hours, and *P. nigrescens* cultured at 0 and 0.5 μg/ml of hemin did not reach stationary phase.

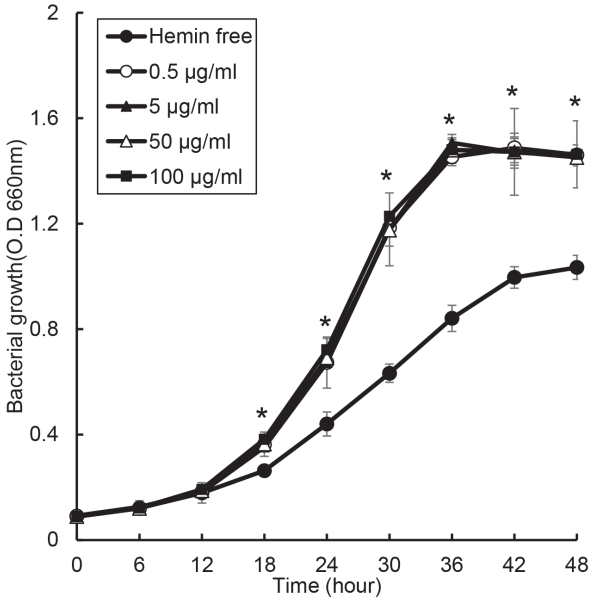

Fig. 1. Effect of hemin concentration on the growth of *P. gingivalis*. *P. gingivalis* was cultured with various concentration of hemin or without hemin. Asterisk (*) indicates statistically significant differences compared with hemin free ($P < 0.05$).

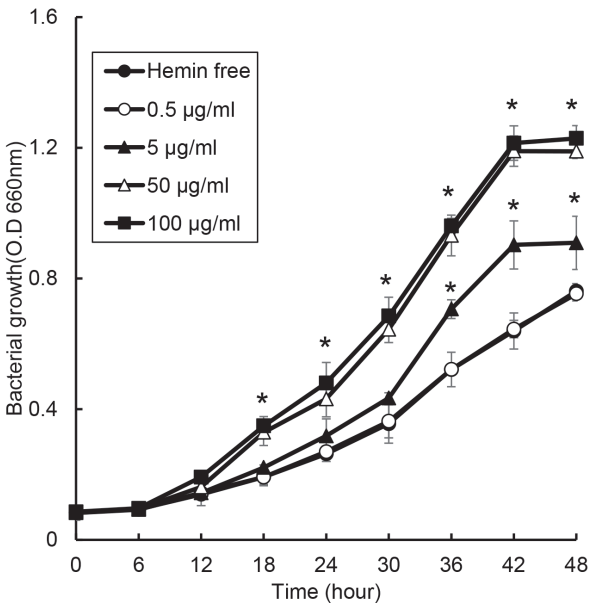

Fig. 2. Effect of hemin concentration on the growth of *P. nigrescens*. *P. nigrescens* was cultured with various concentration of hemin or without hemin. Asterisk (*) indicates statistically significant differences compared with hemin free ($P < 0.05$).

The growth of *P. intermedia* was changed by hemin concentration (Fig. 3). In the condition of 50 and 100 μg/ml of hemin, the growth curve showed typical S shape such as log, exponential, and stationary phase. *P. intermedia* entered stationary phase after 36 hours in 50 and 100 μg/ml of hemin and after 42 hours in 5 μg/ml of hemin.

F. nucleatum slowly grew at 0.5 μg/ml of hemin and hemin free condition. and the growth ratio of *F. nucleatum* was changed in a dose-dependent manner of hemin (Fig. 4). *F. nucleatum* entered stationary phase after 36 hours in 50 and 100 μg/ml of hemin and after 48 hours in 5 μg/ml of hemin.

F. alocis entered stationary phase between 30 and 36 h after inoculation into fresh medium at 50 and 100 μg/ml of hemin (Fig. 5). *F. alocis* slowly grew under 5 μg/ml of hemin and did not showed stationary phase to 48 hours. Up concentration of 50 μg/ml of hemin, the growth of *F. alocis* entered exponential phase after 12 hours and reached stationary phase after 36 hours.

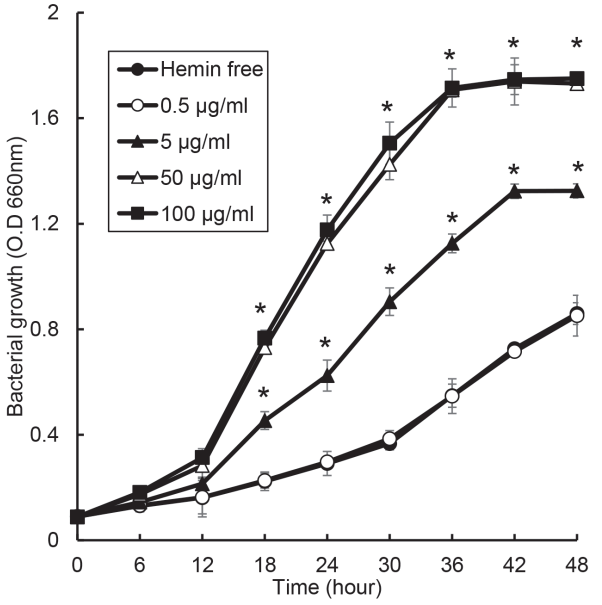

Fig. 3. Effect of hemin concentration on the growth of *P. intermedia*. *P. intermedia* was cultured with various concentration of hemin or without hemin. Asterisk (*) indicates statistically significant differences compared with hemin free ($P < 0.05$).

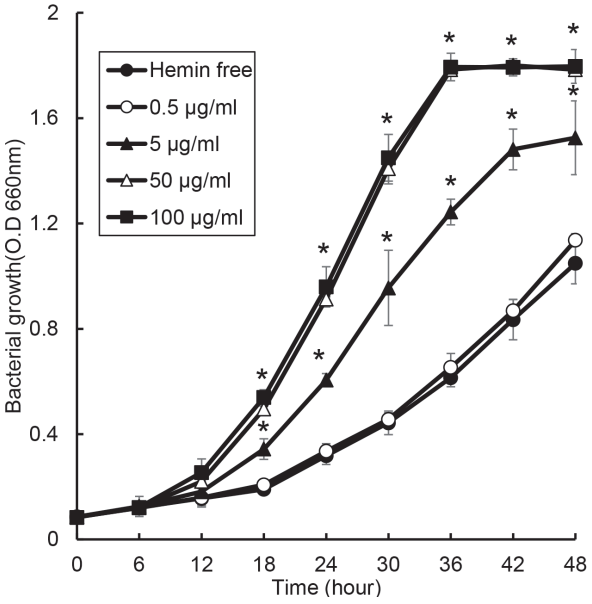

Fig. 4. Effect of hemin concentration on the growth of *F. nucleatum*. *F. nucleatum* was cultured with various concentration of hemin or without hemin. Asterisk (*) indicates statistically significant differences compared with hemin free ($P < 0.05$).

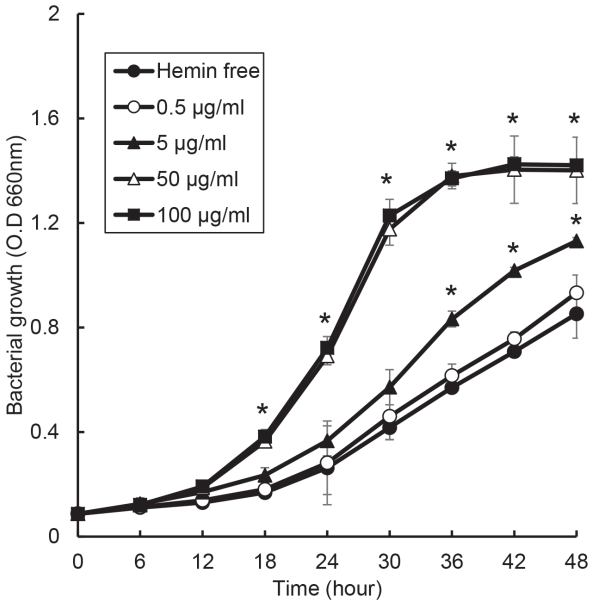

Fig. 5. Effect of hemin concentration on the growth of *F. alocis*. *F. alocis* was cultured with various concentration of hemin or without hemin. Asterisk (*) indicates statistically significant differences compared with hemin free ($P < 0.05$).

T. forsythia slowly grew under concentration of 5 μg/ml of hemin as shown Fig. 6. The growth of *T. forsythia* entered exponential phase between 6 and 12 hours and reached stationary phase after 36 hours in 50 and 100 μg/ml of hemin condition. Especially, comparing 50 and 5 μg/ml of hemin condition, the growth of *T. forsythia* was the same time to reach stationary phase. However, the bacterial concentration was different.

The growth of *T. denticola* was not affect to 50 μg/ml of hemin concentration. Unlike other bacteria, the growth rate of *T. denticola* decreased at a concentration of 100 μg/ml of hemin (Fig. 7). At all concentration of hemin, the growth of *T. denticola* did not reach stationary phase util 48 hours.

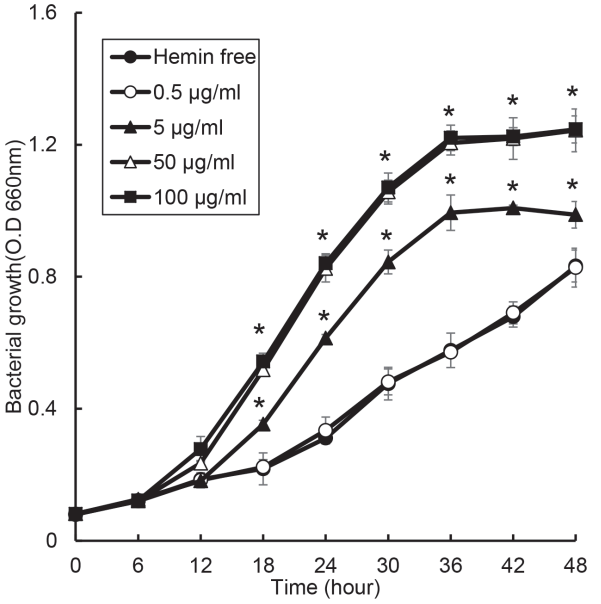

Fig. 6. Effect of hemin concentration on the growth of *T. forsythia*. *T. forsythia* was cultured with various concentration of hemin or without hemin. Asterisk (*) indicates statistically significant differences compared with hemin free ($P < 0.05$).

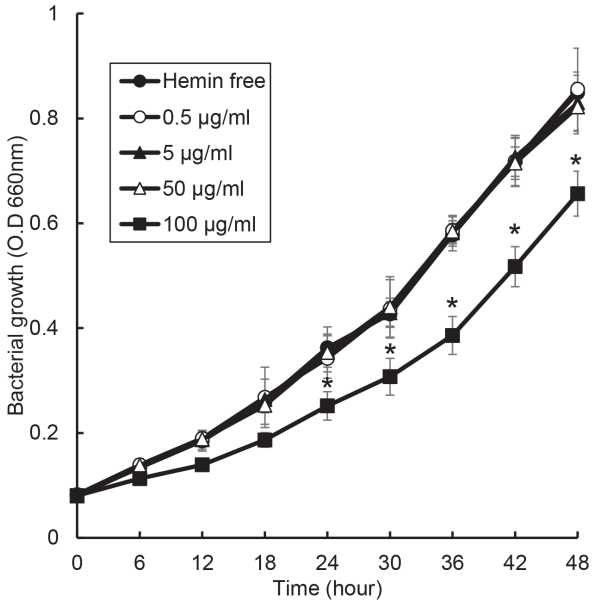

Fig. 7. Effect of hemin concentration on the growth of *T. denticola*. *T. denticola* was cultured with various concentration of hemin or without hemin. Asterisk (*) indicates statistically significant differences compared with hemin free ($P < 0.05$).

Discussion

In relation of the outbreak of periodontitis, the type of bacteria is previously considered more important, but in recent years, not only the type of bacteria but also the number of total bacteria is considered important. In the 1970s, a specific plaque hypothesis was suggested that specific bacteria were associated with periodontitis,[17] and later, Socranskii group suggested *P. gingivalis*, *T. forsythia*, and *T. denticola* as periodontitis-causing bacteria.[18] Philip D. March proposed ecological plaque hypothesis that combined specific pathogens and bacterial composition.[4] This hypothesis is that oral diseases are the result of an imbalance in the total microflora due to ecological stress, resulting in an enrichment of oral pathogens as periodontitis related bacteria. Also, according to the recent "The Keystone-Pathogen Hypothesis", *P. gingivalis* induces initial inflammation of periodontal tissue using gingipain.[6] The initial inflammation increases the influx of gingival crevicular fluid (GCF) into the periodontal pocket and causes dysbiosis in the microbial ecosystem. Therefore, this study was first examined the effect of hemin as a component of GCF on the growth of periodontitis-related bacteria to investigate the factors related to the dysbiosis.

The growth of *P. gingivalis* was not significantly changed by hemin concentration although the growth was slower in hemin free condition. Except for *T. denticola*, the growth of anaerobic bacteria present in other periodontal pocket was found to be rapid at certain hemin concentration. In most studies, when cultivating periodontitis-related bacteria, the concentration of hemin is 5 μg/ml.[19-21] This concentration is not the best condition for the growth of periodontopathogens. When predicted based on the experimental results, it was found that periodontopathogens grow best at least 50 μg/ml of hemin. In case of *P. gingivalis*, the optimal condition was when the hemin concentration was 5 μg/ml or higher. Unlike other bacteria, *P. gingivalis* grew well in low concentration of hemin. In clinical study about measurement of hemin concentration, the average hemin concentration is 46.6 nM in healthy sites and 1116.6 nM in diseased sites.[16] Based on this clinical study and the results, it is possible to explain "The Keystone pathogen hypothesis" and "Ecological plaque hypothesis". *P. gingivalis* induced initial inflammation, by which GCF is flowed into periodontal pocket and heme concentration may be increased. This elevated heme condition may promote the growth of other periodontopathogens. The heme is a factor that cause dysbiosis in the microbial ecosystem of the periodontal pocket. Eventually, the rapid growth of the bacteria related to periodontitis occupies a large part in the microbial ecosystem and induces periodontitis.

Recently, ecological plaque hypothesis has been recognized as the theory regarding the occurrence of periodontitis. Although it is known that dysbiosis in periodontal pocket occurs due to the influx of GCF, there is little information about which factors cause the dysbiosis.

Conclusion

The present study showed that periodontopathogens rapidly grow above a specific concentration of hemin. Eventually, heme may be a factor that leads dysbiosis in the microbial ecosystem of the subgingival plaque and thereby promote a periodontitis-causing environment. The further study will be necessary to investigate the change in virulence of periodontopathogens caused by changes in hemin concentration.

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치주병원균의 성장에서의 heme의 영향

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목적: 이 연구의 목적은 치주병원균에 대한 헴의 영향을 살펴보기 위함이다.

연구 재료 및 방법: 치주낭에 존재하는 7종의 혐기성세균을 이용하여 실험을 진행하였다. 세균을 혐기환경에서 배지를 이용하여 hemin의 있고 없음으로 하여 배양을 하였다. 세균의 성장은 매 6시간마다 분광광도계를 이용하여 측정하였다.

결과: 헤민의 존재여부에 따른 성장의 차이는 *Porphyromonas gingivalis*에서만 관찰되었다. *Treponema denticola*를 제외한 치주병원균의 성장은 헤민의 농도에 의존적인 것으로 관찰되었다. *T. denticola*의 성장은 헤민에 의해 방해를 받았다.

결론: 헴은 치은연하 치태의 미생물 생태계에서 미생물분포의 불균형을 유도하여 치주염을 유발하는 환경을 조장할 것이다.

(구강회복응용과학지 2021;37(1):31-8)

주요어: 헴; 치주염세균; 성장; 세균불균형

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접수일: 2021년 3월 3일 | 수정일: 2021년 3월 4일 | 채택일: 2021년 3월 4일