The Impact of Hyperbaric Oxygen Therapy on Serum C-Reactive Protein Levels, Osteoprotegerin Expression, and Osteoclast Numbers in Induced-Periodontitis Diabetic Rats

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Abstract

Objectives This study aimed to examine the impact of hyperbaric oxygen therapy (HBOT) on serum C-reactive protein (CRP) levels, osteoclast numbers, and osteoprotegerin (OPG) expression in periodontitis-induced diabetic rats

Materials and Methods This study constituted an *in vivo* laboratory-based experiment incorporating a posttest only control group design. Thirty male Wistar rats were divided into three groups of research subjects: a healthy group (K0), periodontitis-induced diabetic group (K1), and periodontitis-induced diabetic group treated with HBOT for 7 days (K2). After treatment, the subjects were sacrificed to determine the level of serum CRP by the ELISA method. Immunohistochemical analysis was conducted to check the level of OPG expression, while a histological analysis was undertaken to quantify the number of osteoclasts.

Statistical Analysis The data was analyzed using a one-way ANOVA and Least Significant Difference (LSD) test on which a result of $p < 0.05$ was considered statistically significant.

Results HBOT appreciably decreased serum CRP levels, significantly enhancing OPG expression in periodontitis-induced diabetic ($p < 0.05$) and decreasing the number of osteoclasts in -periodontitis-induced diabetic ($p > 0.05$).

Conclusion HBOT reduced the serum CRP level, increased OPG expression, and decreased osteoclast numbers in periodontitis-induced diabetic rats.

Keywords
- C-reactive protein
- osteoprotegerin
- osteoclast
- diabetes mellitus
- periodontitis

Introduction

Periodontitis is a chronic inflammatory disease which damages tooth-supporting structures, including periodontal ligament and alveolar bone.[1,2] Diabetes mellitus (DM) plays a crucial role as the main cause of periodontitis.[3-5] Several studies have demonstrated that DM can increase the progression and severity of the condition.[6-8] The risk of periodontitis is approximately three times as great in DM compared with that in healthy individuals.[9]

A recent study contended that severe periodontitis increases serum C-reactive protein (CRP) levels.[10] CRP is a pentameric plasma protein produced by the liver in response to various inflammatory stimuli which are detectable in the serum of patients with inflammatory oral disease, for example, in those afflicted with acute alveolar abscesses.[2]

DM induces upregulation of osteoclastogenic factor which stimulates osteoclast activation and differentiation. Diabetic periodontitis increases 2- to 4-fold the number of osteoclasts compared to that in nondiabetic periodontitis.[11] Osteoprotegerin (OPG), a glycoprotein, represents a critical factor in regulating the differentiation and maturation of osteoclasts.[12] OPG levels have been found to be low in cases of periodontitis which increases the receptor

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activation of nuclear factor kappaB ligand (RANKL)/OPG ratio.[13] Furthermore, previous studies have explained that uncontrolled type 2 diabetes in patients with chronic periodontitis presented a higher OPG to RANKL ratio than in healthy individuals.[14,15]

Treatment of diabetic periodontitis necessitates various approaches. One form of therapy that has recently increased in popularity is that of hyperbaric oxygen therapy (HBOT). HBOT constitutes the therapeutic administration of 100% oxygen at an environmental pressure of more than one atmosphere absolute (1 ATA).[16] In 2003, Chen et al reported that a combination of HBOT, scaling and root planning was the most effective treatment of periodontitis.[17] Oxygen at 2.0 ATA can inhibit the growth of the pathogenic bacteria that cause periodontitis. HBOT has been known to have a bactericidal/bacteriostatic effect on actinomyces, bacteroides, and streptococcus.[18]

To the best of the authors' knowledge, no previous study analyzing the effect of HBOT on diabetic periodontitis exists. Therefore, the aim of this study was to examine the impact of HBOT on serum CRP levels, osteoclast numbers and OPG expression in periodontitis-induced diabetic rats

Materials and Methods

Ethical Approval

This study was laboratory-based experimental research with *posttest only control group design* which received approval from the Ethical Clearance for Health Research Committee, Faculty of Dentistry, Universitas Hang Tuah No. EC/018/KEPK-FKGUHT/IV/2019

Animal Model Preparation

Thirty healthy male Wistar rats, aged 8 to 10 weeks and body weight (BW) 180 to 220 g, were acclimated for 7 days. Thereafter, they were divided randomly into three groups, each consisting of 10 rats. The rats were caged separately in groups, and given same standard food and water ad libitum before, during and after treatment. Group 1 (K0) were healthy rats, Group 2 (K1) was periodontitis-induced diabetic rats. Both K0 and K1 constituted as control groups. Group 3 (K2) was periodontitis-induced diabetic rats treated with HBOT.

Experimental Procedures

Diabetic Animal Model Induction

Diabetes condition of the rats in group 2 and group 3 (K1, K2) were induced by a single 65 mg/kg of body weight dose of streptozotocin (STZ) administered intraperitoneally. A diabetic condition was characterized by an increase in blood glucose of 150 to 300 mg/dl or more.[19]

Periodontitis Induction

Periodontitis was induced by oral administration of 10^9 *Porphyromonas gingivalis (P. gingivalis)* ATCC 33277 per subject. The administration of *P. gingivalis* was performed three times in four days. The condition of periodontitis was examined by the clinical manifestation of swelling and redness of the gingiva and confirmed by histopathological examination of periodontal tissue, characterized by damage to periodontal ligaments, gingival sulcus, decreased osteoblast cells and increased osteoclast cells which was performed in preliminary studies.[20]

HBOT Procedure

All animals in group 3 (K2) were put together at a time in a special animal chamber of HBOT with the daily dose of 2.4 ATA, three times, with each period lasting 30 minutes, interspersed by 5-minute intervals to allow the subjects to breathe normal air. Treatments were performed for 7 consecutive days.

Sample Collection

At the end of therapy, all animals were euthanized with 0.2 mL ketamine by the dose of 10 mg/ kg BW. Cardiac punctures were performed to obtain serum sample for CRP level examination by the ELISA method. The expression of OPG and the number of osteoclasts were examined from the mandibular section. Immunohistochemical (IHC) examination was conducted using the monoclonal antibody for OPG (Sigma-Aldrich; St. Louis, MO). Histological examination was subsequently performed to count the number of osteoclasts by means of Meyer's hematoxylin staining (Sigma-Aldrich). Photographs were taken using a phase contrast microscope (CKX41; Olympus, Japan) at 400x magnification.

Statistical Analysis

Data of serum CRP level, OPG expression, and osteoclast number were expressed as mean ± standard deviation (SD), and statistically analyzed with analysis of variance (ANOVA) and LSD test, using SPSS software package version 17.0. Statistical significance was considered when p value < 0.05.

Results

Diabetic Condition Result

Diabetic animal model result was assured as the base condition data of this study. Induction with STZ resulted in confirmed diabetes condition compared with normal rats marked by the increase of blood glucose (BG) level over 150 to 300 mg/dl as the hyperglycemia standard of diabetes condition shown in ►**Table 1**.

Result of paired *t*-test showed no significant difference of BG level in normal group (K0) but there were markedly significant differences in periodontitis-induced groups (K1 and K2). BW data result showed the significant difference in all groups before and after induction (p < 0.05).

Impact of HBOT result on serum CRP levels, OPG expression, and osteoclast numbers result

Treatment of HBOT have been proved to have the impact on serum CRP levels, OPG expression, and osteoclast numbers in periodontitis-induced diabetic rats as shown in ►**Table 2**.

The effect of HBOT on serum CRP levels were examined by ELISA and shown in ►**Fig. 1**. The CRP level were increased on periodontitis-induced diabetic group (K1) compared with normal group (K0). HBOT significantly decreased serum CRP

Table 1 Mean and SD of blood glucose level and body weight before and after STZ induction

Groups	Blood glucose		Body weight	
	Before	After	Before	After
K0	114.81 ± 6.75[a]	119.4 ± 4.55 [a]	197.7± 9.47 [b]	243.1 ± 10.23 [b]
K1	111.8 ± 7.96 [a]	386.5 ± 29.37[b]	196.8 ± 9.42 [b]	171.8 ± 11.05 [b]
K2	118 ± 7.01 [a]	381.5 ± 31.34 [b]	196.7 ± 9.67 [b]	178.3 ± 8.03[b]

[a,b] Difference between the groups with significance level of 5% ($p < 0,05$).
Abbreviations: SD, standard deviation; STZ, streptozotocin.

Table 2 Analysis of serum CRP levels, OPG expression and osteoclast numbers in periodontitis-induced diabetic rats

Groups	CRP	OPG	Osteoclast
K0	16.76 ± 0.39[a]	6 ± 1.33[a]	1.70 ± 0.95 [a]
K1	99.15±.2.78[b]	2.6 ± 0.52 [b]	6.40 ± 1.65 [b]
K2	16.38 ± 0.72[a]	5.8 ± 0.79 [a]	4.60 ± 1.71 [b]

[a,b] Difference between the groups with significance level of 5% ($p < 0,05$).
Abbreviations: CRP, C-reactive protein; OPG, osteoprotegerin.

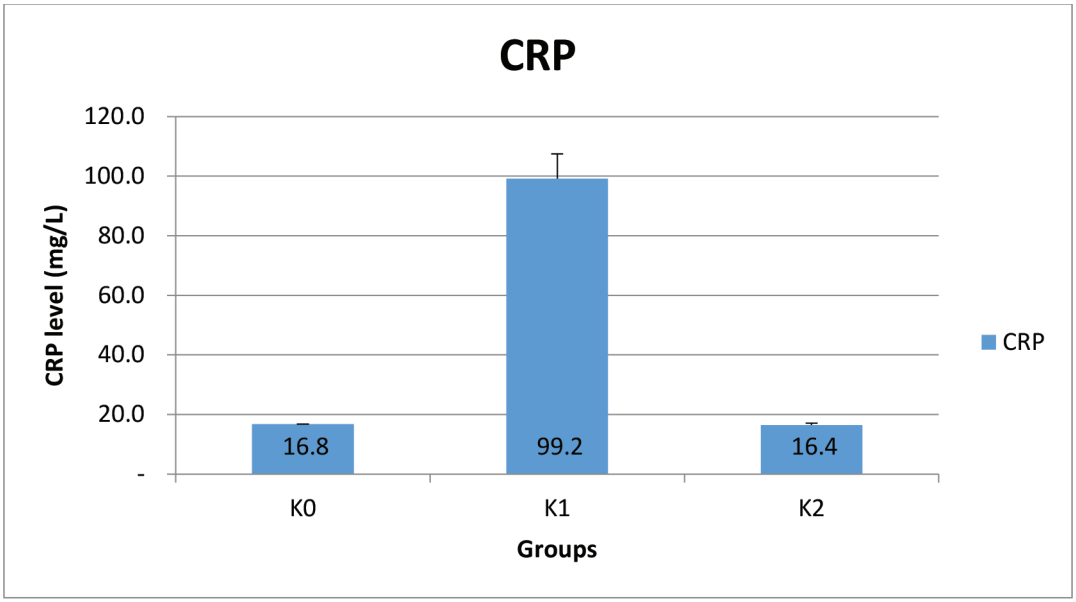

Fig. 1 Average of serum CRP level on normal group (K0) compared with induced-periodontitis diabetic group (K1) and periodontitis-induced diabetic treated with HBOT (K2). CRP, C-reactive protein; HBOT, hyperbaric oxygen therapy.

levels in periodontitis-induced diabetic groupThe post-HBOT level of serum CRP in periodontitis-induced diabetic group was reduced significantly to the same level as in healthy conditions ($p < 0.05$).

The expression of OPG were examined from mandibular section by immunohistochemistry was shown in ►**Fig. 2**.

Results showed that OPG expression in periodontitis-induced diabetic (K1) was lower compared with healthy conditions. Moreover, HBOT was observed in significantly ($p < 0.05$) enhanced OPG expression in cases of periodontitis-induced diabetic (K2) as in ►**Fig. 3**.

The count result of osteoclast number on rat mandibular section in each group is shown in ►**Fig. 4**.

Osteoclast numbers were elevated in periodontitis-induced diabetic group (K1) compared with healthy conditions (K0) and decreased after the administering of HBOT ($p < 0.05$) as shown in ►**Fig. 5**.

Discussion

This study analyzed the effect of HBOT on serum CRP levels, osteoclast numbers and OPG expression in diabetic rats with periodontitis, and indicated that HBOT has an effect on diabetic periodontitis. HBOT can decrease serum CRP levels and the number of osteoclasts, while increasing OPG expression in cases of diabetic periodontitis. This study indicated that HBOT produces beneficial therapeutic effects in subjects afflicted with diabetic periodontitis.

In this study, the level of serum CRP in cases of diabetic periodontitis was higher compared with that in

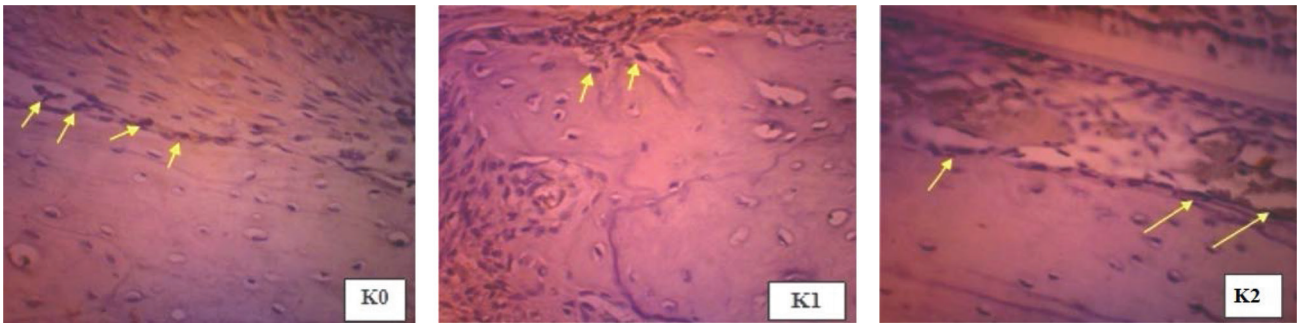

Fig. 2 The expression of OPG in rat mandibular section of normal group (K0), periodontitis-induced diabetic group (K1) and periodontitis-induced diabetic treated with HBOT (K1). Marked arrow showed the OPG expression on rat mandibular section. HBOT, hyperbaric oxygen therapy; OPG, osteoprotegerin.

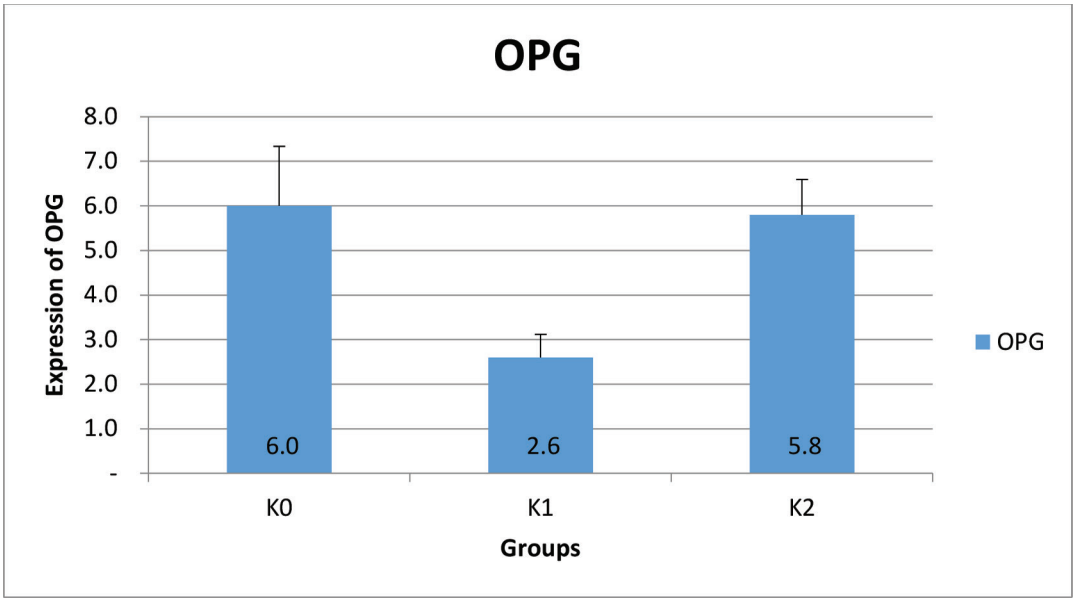

Fig. 3 Average of OPG expression on normal group (K0) compared with periodontitis-induced diabetic group (K1) and periodontitis-induced diabetic treated with HBOT (K2). HBOT, hyperbaric oxygen therapy; OPG, osteoprotegerin.

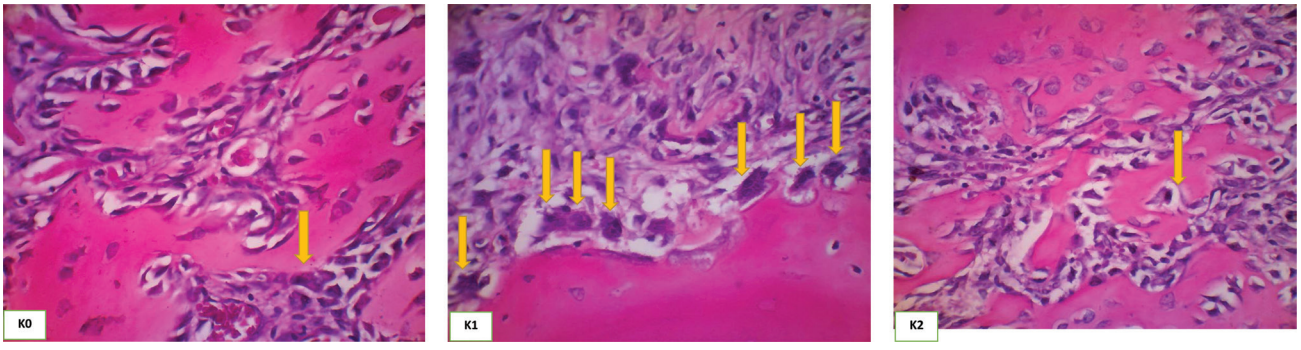

Fig. 4 The number of osteoclasts in rat mandibular section of normal group (K0), periodontitis-induced diabetic group (K1) and periodontitis-induced diabetic treated with HBOT (K1). Marked arrow showed the osteoclast cells. HBOT, hyperbaric oxygen therapy.

healthy individuals due to the resulting inflammation. Hyperglycemia in diabetes produced advanced glycation end products (AGEs) which triggered the secretion of pro inflammatory cytokine IL-1, TNF-α and IL-6.[21] The increase in the number of AGEs resulted in the proliferation of reactive oxygen species (ROS) and led to oxidative stress.[1,22,23] Oxidative stress induced the transcription factor NF-κβ to produce proinflammatory cytokines which, in turn, stimulated hepatocyte cells to secrete CRP in plasma.[23] On the other hand, periodontitis involves chronic inflammatory processes, resulting from the interaction of Gram negative bacteria with the defenses of the host. The inflammatory

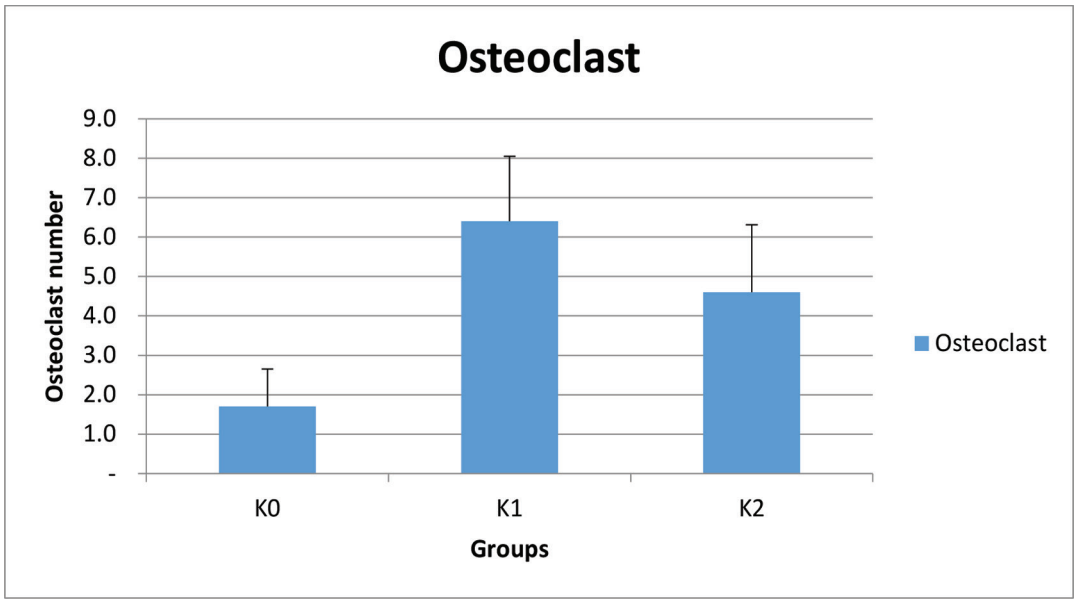

Fig. 5 Average of osteoclast number on normal group (K0) compared with periodontitis-induced diabetic group (K1) and periodontitis-induced diabetic treated with HBOT (K2). HBOT, hyperbaric oxygen therapy.

response increases the levels of cytokines such as IL-1, IL-6, TNF-α which promote activation of the acute phase reactants that subsequently elevate serum CRP levels.[24] Inflammatory mediators such as TNFα and IL-1β increase as a result of diabetes and cause osteoblasts to express RANKL protein and stimulate osteoclast differentiation. This process induces alveolar bone resorption in patients with diabetes.[25,26]

In cases of diabetic periodontitis, HBOT causes levels of CRP to decrease and may significantly reduce the number of bacteria present. This condition induces an improvement in antibody titers and avidity to the specific pathogens with the result that local inflammation is considerably reduced and, ultimately, decreasing the presence of serum CRP.[27] HBOT reduces the amount of bacteria and simultaneously inhibits collagenase secretion.[28] Moreover, HBOT increases local oxygen distribution at the base of the periodontal pocket. This situation inhibits the growth of anaerobic bacteria, while also enabling the ischemic tissues to absorb sufficient oxygen for cell metabolism to occur.[29]

Expression of OPG in the diabetic periodontitis group (G2) decreased significantly compared with that in the healthy subject group (G1), acting as the control. This reduction presumably related to the increasing level of CRP, IL-6, fibrinogen, glycemic status, inflammation, and insulin resistance.[12,30,31]

HBOT increased the OPG expression considerably compared with that present under healthy and diabetic periodontis conditions. OPG is produced by osteoblasts and other cell types, including peripheral blood lymphocytes. OPG, the soluble decoy receptor for RANKL, inhibits RANKL binding to receptor activator of NF-kB (RANK) and prevents osteoclastogenesis and bone resorption.[32-34] RANK-RANKL/OPG ratios and the level of other inflammatory cytokines, such as TNF, constitute critical mediators of osteoclastogenesis in diabetes with periodontal disease.[11,35,36] The RANKL/OPG ratio has been reported to increase in periodontal diseases such as periodontitis.[37] HBOT potentially decreases the RANKL/OPG ratio and inflammatory cytokines that induce a reduction in osteoclast differentiation and formation. Moreover, a decrease in the CRP level in cases of diabetic periodontitis treated with HBOT leads to osteoblast and fibroblast induction and reduces osteoclast formation in periodontal tissue.[2,38] The results of this study showed that the presence of HBOT reduces osteoclast expression in diabetes periodontitis group, while not producing the same result in the healthy control group. This study has a limitation, in that it was only performed on one dose of therapy and was not once measured in a time series examination. Otherwise, this preliminary result was promising and could be explored more for further study.

Conclusion

HBOT 2.4 ATA 3 × 30 minutes at 5-minute intervals for 7 days in periodontitis-induced diabetic rats could decrease serum CRP levels and increase OPG significantly, while osteoclasts have not shown a significant decrease.

Funding

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Conflict of Interest

None declared.

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