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Diabetes & its Complications

The Effect of *Sticopus hermaniii*- Hyperbaric Oxygen Therapy to Osteogenesis of Diabetic Periodontitis

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ABSTRACT

Background: *Diabetes and periodontitis affect a large number of populations worldwide. Osteoprogeterin (OPG) is cytokine regulating osteoclastogenesis, related to osteoclast and osteoblast implicated in various inflammations including periodontitis and diabetes. Hyperbaric oxygen therapy (HBOT) has been used as adjuvant therapy to heal chronic wound problem. Sticopus Hermanii (SH) has been consumed as food rich with content of sulfated glycosaminoglycans (GAGs) which affect wound healing.*

The aim of this study is to examine the effect of HBOT and Sticopus hermanii to the expression of OPG, osteoclast and osteoblast in periodontitis diabetic rats.

Methods and Material: *The research was an experimental laboratory with posttest only control group design. Thirty male Wistar rats aged 8-10 weeks were equally divided in 5 groups of: healthy group (G1), diabetic periodontitis group (G2), diabetic periodontitis group treated SH (G3), diabetic periodontitis group treated with HBOT 2,4 ATA 3x30' interval 5' for 7 days (G4), diabetic periodontitis group treated with combination SH-HBOT (G5). Diabetes was conducted by single dose of streptozotocin 65 mg/kg of BW intraperitoneal (diabetes condition if blood glucose levels >230mg /dL), while periodontitis were induced by Porphyromonas gingivalis ATCC 3327.*

The OPG expressions were examined by immunohistochemistry, while the number of osteoclast and osteoblast by hematoxylin eosin staining.

Results: *It was found that combination SH-HBOT group (G5) in OPG could increase (12.50 ± 2.082) than diabetic-periodontitis group(G2) 2.50 ± 0.577. The combination SH-HBOT group (G5) in osteoblast can increased significantly (28.47 ± 3.20) than diabetic-periodontitis group(G2) 9.22 ± 4.95. Osteoclast was decreased significantly in combination SH-HBOT group (G5) 3.50 ± 0.957 than diabetic-periodontitis group 0.500 ± 816. ($p<0.05$).*

Conclusion: *The combination of SH-HBOT increased OPG expression, osteoblast, and reduced the number of osteoclasts in periodontitis diabetic rats.*

Keywords

OPG, Osteoblast, Osteoclast, Diabetes, Periodontitis.

Introduction

Diabetes and periodontitis have a bidirectional relationship and affected each other. Diabetes has its significant role as a serious threat to global health, affecting 415 millions people worldwide. Diabetes prevalence in Indonesia showed a raising trends, regarding to International Diabetes Federation (IDF) Atlas 2017. Indonesia is the sixth country after China, India, USA, Brazil and Mexico which have 10.3 millions diabetes population of 20-79 years old patients. Based on the data of Indonesian Ministry of Health in 2018, a significant raising trends of diabetes prevalence has been developed from 6.9% in 2013 become 8.5% in 2018.

Periodontitis is a chronic inflammatory disease characterized by the destruction of periodontal ligament and alveolar bone as the supporting structures of the teeth. Data show a strong evidence in favor of considering diabetes as a risk factor for gingivitis and periodontitis [1]. The risk of periodontitis is increased by threefold in patients with diabetes compared to nondiabetics [1,2]. Periodontitis is the sixth major complications of diabetes and the most common complication in the oral cavity with the prevalence of 75%. The level of glycaemic control is the importance key in determining increased risk.The US National Health and Nutrition Examination Survey (NHANES) III result stated that adults with an HbA1c level of >9% had a significantly higher prevalence of severe periodontitis than those without diabetes (OR 2.90; 95% CI 1.40, 6.03) after controlling for age, ethnicity, education, sex and smoking. [3].

Diabetes complication leads to problems of morbidity and mortality in population [1,2]. Diabetes related to infection prone and worse wound healing, is also major risk for severe and aggresive periodontitis resulted in destruction of tooth supporting tissue. Glycemic control plays the important role in periodontitis treatment. It has been stated that in this case, local periodontitis treatment as scaling and root planing could not established the attachment of new connective tissue histologically.

Hyperbaric oxygen therapy (HBOT) pricipally is a treatment of breathing 100% pure oxygen in a chamber in high pressure condition more than 1 ATA (atmoshere absolute) [4]. Previous study stated that HBOT with the dose of 3x30 minutes for 7 days in diabetes animal model resulted in the decrease of blood sugar level [5]. Wijaya stated that 2.4 ATA HBOT with the dose of 3x30 minutes for 5 days consecutively could raised the number of alveolar osteoblast in diabetic mice and induced by *Porphyromonas gingivalis* [6,7].

Golden sea cucumber (*Stichopus hermanii*) has been known to have medical benefit, it contains organic and inorganic compound with therapeutic properties such as triterpene glycosides, bioactive peptides, fatty acids, amino acids, collagens, gelatins, chondroitin sulfates, vitamins, minerals [8]. The benefit effects of sticopus hermanii have been validated through scientific research and have shown medicinal value as wound healing, neuroprotective, anticoagulant, antimicrobial, antioxidant, antitumor [8], anti inflammation and antibacterial [9]. *Stichopus hermanii* ethanolic extract could inhibit the growth of mixed periodontopathogen bacteria [10].

It also has high antioxidant activity related to its content of Eicosapentaenoic acid (EPA) and Docosehaxaenoic Acid (DHA), indicated by the activity of superoxide dismutase to prevent oxidative stress in hyperglycemic condition, so it could be considered as anti diabetic agent [11]. *Stichopus hermanii* cell growth factor (CGF), one of its component is vascular endothelial growth factor (VEGF) which induced neovascularization, angiogenesis and osteoblas differentiation for bone formation in vivo [9,12].

Osteoprogetegrin (OPG) is a decoy receptor dissolved of RANKL which is produced by osteoblast/stromal `1cells, fibroblasts, lymphocytes, smooth muscles and osteosits [13,14]. Increasing of OPG and decreasing of RANKL can occur due to a decrease cytokine proinflamasi and increased anti-inflammatory mediators (IL-4, IL13 and IFN-γ) [15]. The increasing of OPG is important to inhibit osteoclasts activation so that alveolar bone resorption can be prevented [16].

Osteoblasts are fibroblast which develop differentiations, originated from mesenchymal cells [16]. Osteoblasts are derived from mesenchymal stem cells and are responsible for the deposition and mineralization of bone matrix. Osteoblasts arise from common progenitors which can also form chondrocytes, muscle and adipocytes under the regulation of various hormones and local factors [17]. Osteoblast formation is a consecutive process that involves the development of immature osteoblast before mature cell formation. Osteoblasts can be characterized by several markers with important roles in bone matrix synthesis and mineralization. These include alkaline phosphatase (ALP) and type I collagen. Osteoblasts also secrete further extracellular matrix proteins including osteocalcein (OCN), matrix Gla protein (MGP), bone sialoprotein (BSPs), osteonectin and osteopontin (OPN) [17].

Osteoclasts are derived from myeloid progenitors of haematopoietic cells found within the bone marrow and peripheral blood, it gives rise to both the monocyte-macrophage lineage and the osteoclast lineage [17,18]. During osteoclast differentiation preosteoclasts attach to the bone surface and multinuclear osteoclasts are formed by the fusion of these precursors under the regulation of a complex cell signaling network. Osteoclast formation is depended on many local products which is produced by cytokines and systemic regulators [19].

Materials and Methods

This study is true experimental laboratory research with factorial design. Animal model of periodontitis diabetic were performed on male Wistar rat (*Rattus novergicus Wistar* strain), divided into 5 groups. Control groups were G1 (normal healthy, no treatment) and G2 (diabetes periodontitis, no treatment) while the rest of the groups were diabetes periodontitis groups treated with HBOT (G3), 3% *Sticophus hermanii* gel (G4), and combination of 3% *Sticophus hermanii* gel and HBOT (G5).

Diabetes induction

Diabetes condition were induced by administration of streptozotocin (Sigma-Aldrich) dissolved in citrate buffer pH 4.5 and injected intraperitoneally with single dose of 65mg/kg. Nicotinamide 230 mg/kg were given 15 minutes prior to streptozotocin induction. Overnight after the induction, rats were having 10% dextrose as their drinking water to prevent hypoglycemic, the regular drinking water were served on the next days. The induction resulted in diabetes condition with random blood glucose levels >230mg /dL [19,18].

Periodontitis induction

Periodontitis condition was induced by innoculation of *Porphyromonas gingivalis* ATCC 33277. Premedication performed before induction of *P. gingivalis* bacteria by administering 20 mg ampicillin and 20 mg kanamycin in the animal drinking water along with topical swab of 0.12% chlorhexidine gluconate in the oral cavity, applied for 4 days. Two ml of 10^9 CFU/ml Porphyromonas gingivalis ATCC 33277 were administered by nasogastric tube, swabbed along the gingival sulcus of mandibulary and maxillary molar to molar region, once daily for 3 times in 4 days. Diabetes periodontitis condition were confirmed two weeks after the first day of induction [19].

Treatment Procedure

Sticophus hermanii gel were prepared from the body wall of fresh golden sea cucumber from Raas island coastal, Indonesia. The sea cucumber body were cleaned and cut into small pieces then dried by freeze dry method at temperatures of 2-8°C with the pressure of 5 mTorr, blended into powder and made into gel with 3% sodium carboxymethylcellulose (CMC-Na) [20,21]. Gel were applied in the mandibular gingival sulcus, once daily on groups G3 and G5.

HBOT were taken place in hyperbaric animal chamber on the pressure of 2,4 ATA for 3x30 minutes consecutively, each were paused for 5 minutes interval, once daily for 7 days in group G4 and G5. Alveolar mandible section were performed immunohistochemistry staining using methods steptavidin-biotin-peroxidase labeled streptavidin-biotin (Dako, Carpinteria, USA) for OPG expression (Santa Cruz Biotechnology, Inc., Ca, USA) and hematoxylene eosin staining for the count cell number of osteoblast and osteoclast. Data were analyzed using One-way ANOVA and Pearson's correlation coefficient.

Results

The immunohistochemical expression of osteoprotegrin (OPG) in alveolar mandible section of all groups were shown in Figure 1, while the numbers of osteoblast and osteoclast cells we examined by hematoxylene-eosin staining were shown in Figure 2 and Figure 3.

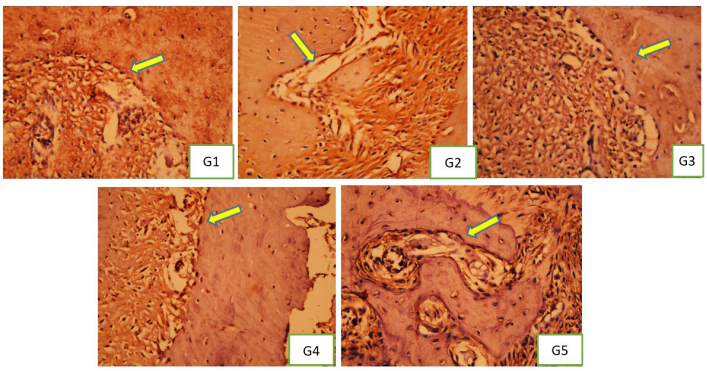

Figure 1: Immunohistochemical expression of OPG (yellow arrow) in osteoblast cells of alveolar mandible in all treatment groups: G1 (normal healthy, no treatment), G2 (diabetes periodontitis, no treatment), G3 (HBOT), G4 (*Sticophus hermanii*), G5 (*HBOT-Sticophus hermanii*).

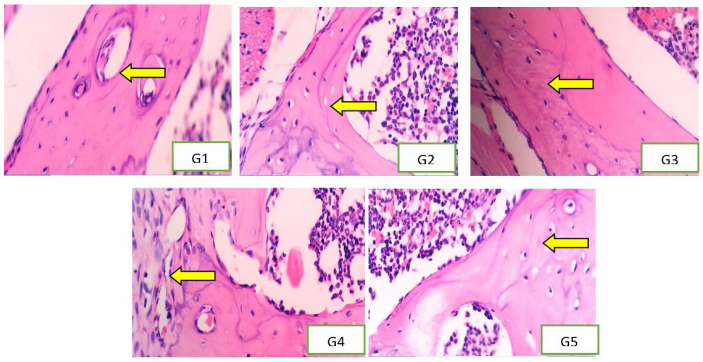

Figure 2: Alveolar mandible osteoblast cells (yellow arrow) stained by hematoxylene eosin of all treatment groups: G1 (normal healthy, no treatment), G2 (diabetes periodontitis, no treatment), G3 (HBOT), G4 (*Sticophus hermanii*), G5 (*HBOT-Sticophus hermanii*).

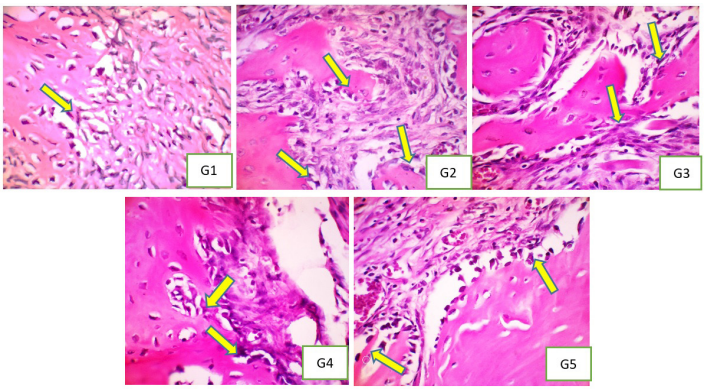

Figure 3: Alveolar mandible osteoclast cells (yellow arrow) stained by hematoxylene eosin of all treatment groups: G1 (normal healthy, no treatment), G2 (diabetes periodontitis, no treatment), G3 (HBOT), G4 (*Sticophus hermanii*), G5 (*HBOT-Sticophus hermanii*).

The average of OPG expression, osteoblast and osteoclast number were shown in Figure 4. Data were analyzed by ANOVA and post hoc LSD with 95% significance level ($p<0,05$).

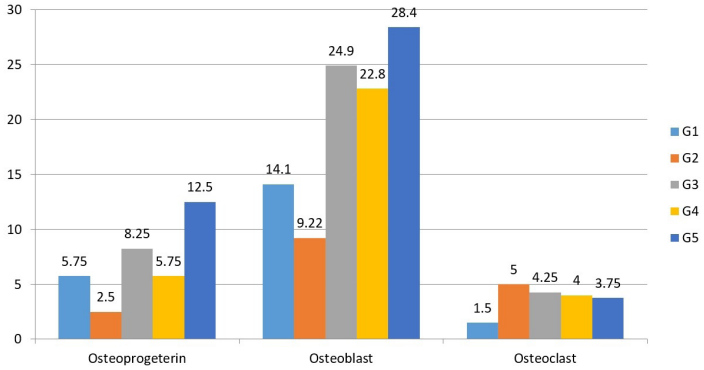

Figure 4: Mean the expression of OPG expression, osteoblast, osteoclast alveolar bone in diabetic-periodontitis rats each treatment group.

OPG expression

The expression of OPG were decreased in diabetes periodontitis condition (G2) compared to normal (G1). Treatment with HBOT (G3) increased the OPG expression, higher than topical treatment

of SH (G4) ($p<0.05$), but the highest result was on the combination of SH and HBOT (G5) ($p<0.05$).

Osteoblast cell number

The number of osteoblast cell were slightly decreased in diabetes periodontitis condition (G2) but not sinificantly compared to normal (G1) ($p>0.05$). Treatment with HBOT (G3) increased the number of osteoblast cell, higher than topical treatment of SH (G4) ($p<0.05$), but the highest result was on the combination of SH and HBOT (G5) ($p<0.05$).

Osteoclast cell number

The number of osteoclast cell were highly increased in diabetes periodontitis condition (G2) compared to normal (G1) ($p>0.05$). Treatment with HBOT (G3) and topical treatment of SH (G4) decreased the number of osteoclast cell ($p<0.05$), but the highest decreasing result was on the combination of SH and HBOT (G5) ($p<0.05$).

The Pearson Corelation Test one way result showed strong correlation between OPG and osteoblast ($R=0.708$), while a negative value between OPG and osteoclast ($R=-0.125$) showed that the greater the OPG expression, the lower osteoclast number. The combination of Sticopus hermanii-Hiperbaric Oxygen Therapy showed the strong correlation between the increasing OPG expression with increasing of osteoblast formation and decreasing the number of osteoclasts in periodontitis-diabetic condition.

Discussion

Streptozotocin induction caused the destruction of pancreatic beta cell and disruption of insulin production, lead to hyperglicaemia. The condition of hyperglycaemia in diabetes mellitus will increase AGEs in the blood and resulted to the increasing production of inflammatory cytokine TNF-α, IL-6 and IL-1. The raising of periodontal bacteria *Porphyromonas gingivalis* endotoxin LPS lead to the increasing of reactive oxygen species and stated the stress oxidative condition. This condition triggered macrophage NF-kB to stimulate inflammatory cytokine to activate TLR2 and TLR 4 as the response to periodontal bacteria. The increasing AGEs release due to hyperglycaemia is followed by the increasing of AGEs receptor (RAGE) also caused oxidative stress. Combination of TLR and RAGE activation stimulate macrophage NF-kB to produce excessive number of proinflammatory cytokines.

This condition lead to the decreasing ratio of RANKL/OPG in osteoblast where OPG are decreasing. This imbalance ratio serve the opportunity of RANKL to be bound with RANK in the cell surface of osteoclast precusor. The RANK-RANKL activate the process of osteoclastogenesis and lead to alveolar bone resorption. It can be assumed that diabetic periodontitis could reduce the expression of OPG compare to healthy normal control.

Sticopus hermanii contains saponins (triterpene glikosid) which has an antibacterial effect. Saponin also stimulates the activity of macrophages to increase the proliferation of B cells and T cell lymphocytes that are useful to build a defense against bacterial pathogens [22]. Endotoxin is issued by *P. gingivalis* bacteria can be inhibited by flavonoids that activation of NF-kβ not occur and inflammation can be stopped.

Therapy HBO 2.4 ATA 3x30 minutes with intermittent 5 minutes for 7 days in sequence, has been proven in studies carried Prabowo et al. [23] may lower blood sugar levels effectively compared to days 1, 3 and 5. Hyperglycemia occurs due to an increase ROS in mitocondria β cells pancreatic thus formed AGE [24]. During therapy of HBO will be an increase ROS in mitochondria, this will trigger the liver to produce Hsp 70 as a response body to protect cells from damage. This mechanism may improve your insulin receptors are damaged and thus increase GLUT4 translocation of glucose in tissue can reduce blood glucose levels [25]. Therapy HBO induces the formation of antioxidant enzymes such as superoxide dismutase, catalase, glutation, and glutatation reductase [26]. The oxidative stress causes increased activation of NF-kβ. Hyperoxigen can reduce the activation of NF-kβ due to inhibition of oxidative stress resulting in the decreasing production of proinflammatory cytokines.

OPG is one of the key factors that act as a regulator of osteoclastogenesis and bone resorption, which is produced by osteoblasts to inhibit osteoclasts [27]. Osteoblasts regulate osteoclasts through RANKL from the RANK signal pathways. RANKL is expressed in osteoblasts and T cells. The interaction of RANK with RANKL is needed for the formation, differentiation, activation and survival of osteoclasts. Osteoblasts regulate osteoclasts through the RANKL-RANK-OPG signaling pathways, so osteoblasts and osteoclasts are synergistic. The current RANKL and OPG ratio are known as the "modulation of coupled turnover" paradigm. In advanced periodontitis, it shows a high number of RANKL, and a low OPG [28-30].

The osteogenesis process is characterized as creeping substitution, which is a remodeling process that begins with osteoclast resorption and new vascular formation with osteoblasts to produce a new haversian system [31]. During bone remodeling, bone formation is very close and paired with bone resorption, and direct contact between osteoclasts and osteoblasts as maintenance [32].

The results of this study, in the group given combination Sticopus Hermanii gel 3% and HBOT 2,4 ATA 3x30 minute, 5-minute intervals for 7 days showed OPG has a contributes to increase osteoblasts. This showed there was a good cooperation between the contents of *Stichopus hermanii* with a mechanism for the hyperbaric oxygen therapy significantly increased the expression of OPG.

This is because OPG is a receptor protein for RANK and RANKL activity in bone metabolism including osteoblasts. Osteoblasts modulate osteoclast formation and bone resorption by producing OPG and RANKL. OPG is able to bind RANKL, prevent RANKL from binding to RANK, thereby inhibiting osteoclasts maturation and preventing osteoclastogenesis. Cells activity in bone formation, osteoblasts, both OPG and RANKL which have a major role in

maintaining bone balance or dynamics by expressing both proteins in the early stages of healing [33,34].

Conclusion

The effect of combination Sticopus-hermanii gel 3%-Hyperbaric Oxygen Therapy 2,4 ATA 3x30 minute with break interval 5 minute for 7 days administration, there was strong relationship between increased OPG expression with increased osteoblast formation. Whereas increased OPG decreased osteoclast in periodontitis-diabetes rats.

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