REASEARCH ARTICLE

The Effect of Golden Sea cucumber (*Stichopus hermanii*) and Hyperbaric Oxygen Therapy to The Expression of Osteoprotegerin in Diabetes Mellitus Induce by *Porphyromonas gingivalis* Bacteria

(Pengaruh Bubuk Teripang Emas (Stichopus hermanii) Dan Terapi Oksigen Hiperbarik Terhadap Ekspresi Osteoprotegrin Pada Tikus Diabetes Melitus yang Diinduksi Bakteri Porphyromonas gingivalis)

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ABSTRACT

Background: *Diabetes mellitus (DM) with Periodontitis cause severe alveolar bone resorption. Osteoprotegerin prevent osteoclastogenesis process so that inhibited alveolar bone resorption. Stichopus hermanii powder and hyperbaric oxygen therapy (HBO) have content and good effects in wound healing.* ***Purpose:*** *The aim of this reseach was to analyze the effect of Stichopus hermanii powder and HBO therapy in the increasing of osteoprotegerin expression on DM with periodontitis.* ***Materials and Methods:*** *The research was an experimental laboratories post test only control group design. Twenty Wistar rats were divided into 5 groups. K0 was negative group while K1 was positive group K1-K4 groups were induced with 65mm/kg STZ single dose and 2ml bacterium P. gingivalis., K2 was treated with 3%golden sea cucumber powder in gel form. K3 was treated with OHB therapy 2.4 ATA for 7 days and K4 given a combination of both. Osteoprotegrin expression on osteoblast of alveolar mandible bone were examined by immunohistochemical staining. Data were analyzed by Kruskal-Wallis and Mann-Whitney test.* ***Result:*** *Kruskal-Wallis result showed significant differences from each treatment group ($p < 0.05$). Mann-Whitney test showed the decreasion in the expression of osteoprotegerin between K1(2:50 ± 0.577) compare with K0 ($p<0.05$). Golden sea cucumber powder, HBO therapy and both combination had increased osteoprotegerin expression.significancy in K2(8.25 ± 1.258), K3 (5.75 ± 0.957) and K4(12.50 ± 2.082) ($p<0,05$).* ***Conclusion:*** *Stichopus hermanii powder 3% and 2.4 ATA OHB therapy for 7 days increased the expression osteoprotegrin on DM with periodontitis.*

Keywords: *Diabetes Melitus, Periodontitis, Alveolar Bone Resorption, Osteoprotegerin, Immunohistochemical, Stichopus hermanii, hyperbaric Oxygen .*

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BACKGROUND

People with diabetes mellitus (DM) has the tendency of larger exposed periodontitis than healthy people. Peridontitis is a complication that occurs most often in people with DM and have a high prevalence rate to reach 75%.[1] Survivors periodontitis reached 20% of the world population.[2] Epidemiological Studies concluded that DM increases the risk alveolar bone loss and attachment loss in periodontal tissue three times greater compared with non-diabetic sufferers.[1] Periodontitis with DM who are not cared for will lead to a loosening of the teeth can even be separated from soket.[3]

Diabetes mellitus can be influential on periodontitic therefore effect inflammation.[4] Uncontrolled DM sufferers have levels of Advanced Glycation End Products (AGEs) are high in soft tissues including the periodonsium tissue. The increasing of AGEs, can triggered stress oxidative and increasing the release of product proinflamasi cytokines (IL-1, IL-6 and TNF-α) so can be tissue damage.[5] The damage tissue and bone resorption resulting in the formation of periodontal pocket, provide habitat protection on periodontal microorganisms *Porphyromonas gingivalis*.[2] *Porphyromonas gingivalis* is a black pigmented bacteria anaerobic gram negative, live on subgingival crevice, and has been identified as one of the main periodontal pathogens.

These bacteria have the virulence called lipopolysaccharide (LPS).[6] LPS make cytokines proinflamasi increases and have affect to the production of Receptor Activator of Nuclear Factor Kappa B Ligand (RANKL) and osteoprotegerin (OPG) on osteoblasts are unbalanced and stimulates osteoclastogenesis.[7]

Treatment of periodontal disease in patients with systemic diseases can potentially improve the general conditions in its entirety, so that the treatment and prevention of periodontitis becomes important.[8] on the treatment of periodontitic accompanied DM remove plaque and calculus with scaling and root-planning (SRP) alone is not enough to eliminate the bacteria as a whole, so there is no effect on the DM controls as well as a decrease in blood glucose levels.[9] Treatment SRP only without adjunvant therapy, give temporary effects on the development of bacteria, thus requiring the giving antibiotics for periodontal status may improve glycemic control significantly.[10,11] Adjuvant therapy on treatment of periodontitic with DM could be utilizing substances contained in the golden sea cucumber (Stichopus hermanii) and the effect of hyperbaric oxygen therapy (OHB).

Hyperbaric therapy is the granting of 100% oxygen at a pressure greater than atmospheric pressure.[16] Oxygen-rich environment is always able to inhibit the growth of anaerobic microorganisms. OHB therapy inhibits the growth of bacteria anaerobe obligate and facultative subgingival anaerobic.[17] OHB Therapy has an effect on RANKL induction decline significantly until decline ratio RANKL/OPG that inhibits bone resorption.[18,19]

Osteoprotegrin is a decoy receptor dissolved of RANKL in a production by Osteoblast/stromal cells, fibroblasts, lymphocytes, smooth muscle and osteosit.[20,21] increasing of Osteoprotegrin and decreasing of RANKL can occur due to a decrease cytokine proinflamasi and increased

anti-inflammatory mediators (IL-4, IL-13 and IFN-γ).[19] OPG's increased is important to inhibits the activation of osteoclasts so that alveolar bone resorption can be prevented.[22]

Based on the above studies, *Stichopus hermanii* has beneficial effects. Therefore, the author would like to do more research to prove the influence of *Stichopus hermanii* powder which will be combined with hyperbaric oxygen therapy as an adjuvant therapy in wistar of diabetes mellitus induced bacterial *Porphyromonas gingivalis* against OPG expression.

MATERIALS AND METHODS

This research is kind of true experimental laboratory research to study design Factorial design. Parameters were seen in this study is the number of OPG expression between treatment groups. Some 20 Wistar (Rattus novergicus Wistar strain) were divided into five groups, where the choosen criterion is the male sex, age 3-4 months with a body weight of 150-200 grams.

The tools used Syringe 5 cc syringe 3 cc syringe insulin 1 cc, blood glucose test strips, gauges blood sugar, cage, container drink rats, scales, mixer, micropipette, micro brush, glass, glass slide, surgical scissors, light microscopy, dry ice, dry freez, blender, container vessel golden sea cucumber, knife, gloves and masks as well as sterile cotton bud, humidified chamber, chamber experimental animals. Materials used Stichopus hermanii, Nicotinamide 230 mg / kg, Streptozotocin 65 mg / kg, 20mg kanamycin, ampicillin 20 mg, klorhesidine gluconate 0.12%, the bacterium Porphyromonas gingivalis ATCC 33 277 2 ml of 1 x 109 cells / ml, phosphate buffered saline (PBS), Dappar citrate, CMC Na 2% distilled water, wistar rats, wistar rat food, beverages wistar rats (ordinary tap water), 100% pure Oxygen 4.13% EDTA, paraffin blocks, mandibular bone, Anti-OPG (N-20) (sc-8468-goat polyclonal igG, Santa Cruz Biotechnology, Inc., Santa Cruz, Ca, USA), Streptavidin-biotin, xylol, alcohols, Proteinase K and 7% low-fat milk, paraffin blocks and biotin – conjugated.

The division of research subjects. The procedure begins with the division of this study the rats into five groups, namely K0 as a negative control (no treatment), K1 as a positive control (streptozotocin induced and bacteria P. gingivalis), K2 (streptozotocin induced and bacteria P. gingivalis and given a golden sea cucumber powder), K3 (streptozotocin induced and P. gingivalis bacteria and were given hyperbaric oxygen therapy) and K4 (streptozotocin induced and bacteria P. gingivalis and given a golden sea cucumber powder 3% and hyperbaric oxygen therapy).

Making the golden sea cucumber powder. Golden sea cucumber entrails removed and then dried using freeze dry method at temperatures 2-8oC with the pressure of 5 mTorr. The dried sea cucumbers blended into a powder and made into a gel 3% by way of reconstituted with sodium carboxymethylcellulose (CMC-Na) 2% .[23]

The procedure induced diabetes in rats. All groups were weighed, and then fasted for 8-12 hours. Nicotinamide rats given 230 mg/kg 15 minutes before induction of streptozotocin (STZ) dissolved in

citrate buffer pH 4.5 and injected intraperitoneally with single dose of 65mg/kg. Mice drunk dextrose 10% throughout the night after induction. Wistar diabetes showed a random blood glucose levels >230mg /dL.[24]

Induction procedures bacterium P. gingivalis. Premedication performed before induction of *P. gingivalis* bacteria by administering ampicillin 20mg and kanamycin 20mg are mixed in the drinking water of wistar and oral cavity mouth by 0.12% chlorhexidine gluconate is topically administered for 4 hari.[25] Induction of P. gingivalis bacteria carried by imposing 2ml of 1 x 10^9 cells/ml in PBS are live bacteria orally. Bacteria also be smeared along the edge of the gingiva buko-palatal/lingual molar to molar regions above and below, using a cotton bud, and put on the anal area with a syringe colorectal cannula.[26] Giving done 3 times in 4 days and incubated for 3 weeks counted since the first induction bakteri.[23]

Giving *Stichopus hermanii* powder and OHB therapy. Induction of STZ and bacteria is finished, the rats were given a therapy. K2 and K4 group therapy gel *Stichopus hermanii* powder 3% topically on the inflamed gingival sulcus 0.1 ml/day using microbrush for 7 days.[23] Group K3 and K4 receive hyperbaric oxygen therapy which is inserted into the chamber and given 100% pure oxygen is then carried increased pressure within the chamber to 2.4 ATA for 3 x 30 minutes at intervals of 5 minutes inhaling normal air, after which the pressure is stopped and lowered to the original condition (1 ATA).[27] Day 8 after treatment in each treatment group was administered, wistar euthanized and performed neck (cervical) and dislocation to be taken mandible and then buried properly.[28] Mandible taken and do decalcified using 4.13% EDTA solution were replaced every day until soft tissues. Softness tested using needle.[29] Tissue management and by immunohistochemical staining (IHC) was performed using methods steptavidin-biotin-peroxidase labeled streptavidin-biotin (Dako, Carpinteria, USA) and then observed using a microscope and counted OPG expression in osteoblast cells visible brownish in the light microscope with 1000x magnification of 20 roomy pandang.[29,30]

Data were analyzed using parametric statistical tests One-way ANOVA, with a test of Shapiro-Wilk normality test, homogeneity test followed by Levene test statistic. The results of calculations than in each group.

RESULT

Data obtained from the descriptive analyzed, then performed statistical hypothesis testing using the analytic with 95% significance level ($p < 0.05$).

Table 1. Mean and standard deviation of the expression of OPG/visual field in each treatment group

Group	Mean ± Std. Deviation Expression of OPG
K0	5.75 ± 1.708
K1	2.50 ± 0.577
K2	8.25 ± 1.258
K3	5.75 ± 0.957
K4	12.50 ± 2.082

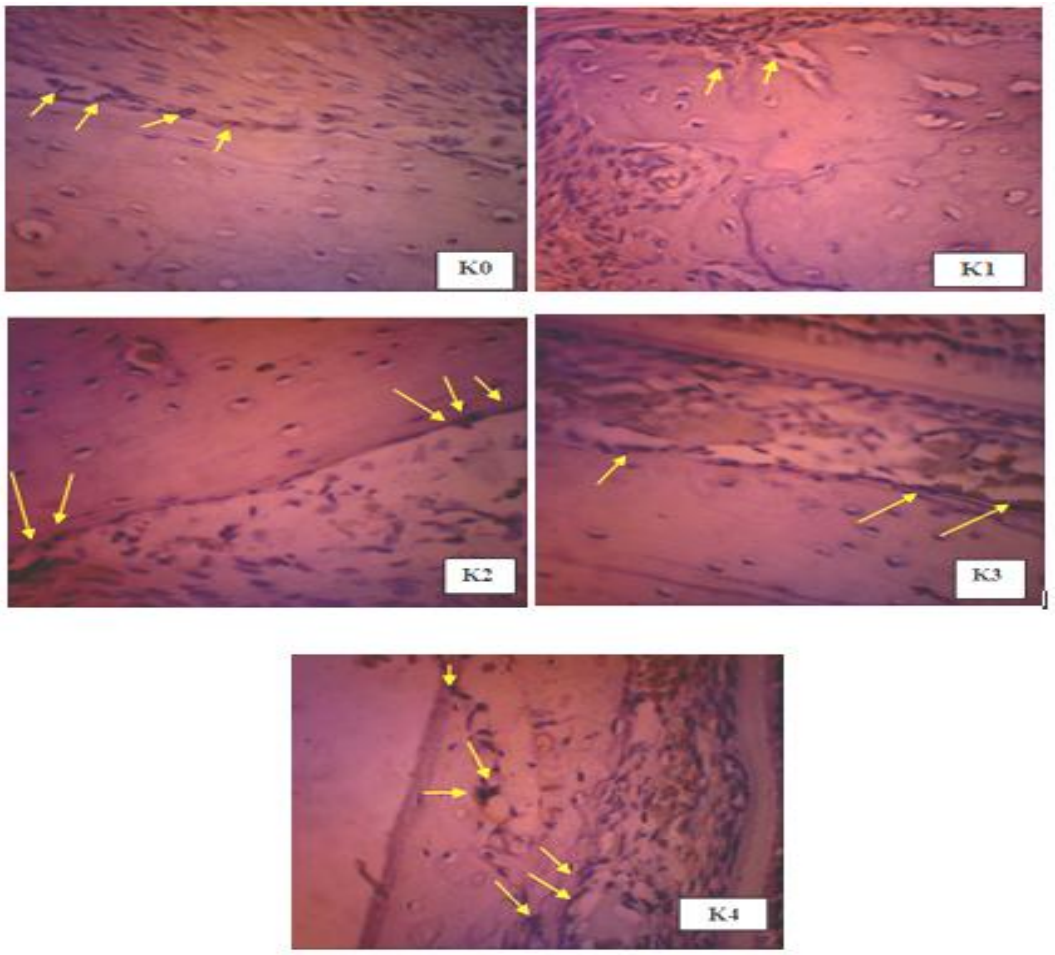

Figure 1. An overview of the immunohistochemical expression of OPG in osteoblast cells (yellow arrow) alveolar mandible with a magnification of 400x. K0 group (negative control), K1 (STZ + PG), K2 (STZ + PG + powder golden sea cucumber), K3 (STZ + PG + Therapy OHB), K4 (STZ + PG + gold + sea cucumber powder OHB therapy).

Significancy homogenity test of variance showed 0.392 ($p > 0.05$) so that we can conclude the whole group has a homogeneous variant data.

The data were not normally distributed can be normalized by transforming data. This study has been carried out the data transformation for the data to be normal. The results obtained in the transformation data is fixed data not normally distributed. Thus, the hypothesis test used in this study is a non-parametric test of Kruskal-Wallis.

Table 2. Result of Kruskall-Wallis Test

Variable	*Sig.*
Osteoklas	0.002

The result of Kruskal-Wallis test obtained significant value 0.002 where the $p<0.05$ so that it can be concluded that there are significant differences in the expression of OPG in five experimental groups.

Table 5. Result of Mann-Whitney Test

Kel.	Rata-rata	Kel.	Rata-rata	*Sig*
K0	5.75	K1	2.50	0.019*
		K2	8.25	0.076
		K3	5.75	0.882
		K4	12.50	0.021*
K1	2.50	K2	8.25	0.019*
		K3	5.75	0.019*
		K4	12.50	0.019*
K2	8.25	K3	5.75	0.027*
		K4	12.50	0.028*

Description: * (significant differences)

Mann-Whitney test showed that K2 (8.25 ± 1.258) and K3 (5.75 ± 0.957) have a difference of OPG expression that does not mean compared to K0 (5.75 ± 1.708) with p> 0.05, while in the other group there were significant differences (p <0.05). Mann-Whitney test was done to conclude that all groups had no significant differences.

DISCUSSION

The results showed that there were significant differences (P <0.05) in the amount of expression of OPG negative control group (5.75 ± 1.708) and STZ-induced group and *P. gingivalis* (2:50 ± 0.577). This is due to the induction of STZ resulting in damage to pancreatic beta cells so that insulin secretion was no resulting blood sugar levels in cells decreases.[31,32] Hyperglycemia in diabetes can increase the AGEs in the blood resulting in increased production of proinflammatory cytokines (TNF-α, IL-6 and IL-1). Bacteria have that LPS endotoxin. Lipopolysakarida *P. gingivalis* bacteria released into the tissues resulting in increased concentrations of superoxide and expression of Reactive Oxygen Species (ROS), causing oxidative stress in the body. Oxidative stress happens trigger NF-κβ to stimulate macrophages to produce cytokines proinflamasi.[33] issued bacterial endotoxin captured by TLR2 and TLR4 on the surface of cells that TLR2 and TLR4 activated.[7]

The condition of hyperglycemia in diabetes mellitus increased AGEs and followed by an increase in AGE receptor (RAGE). When AGEs bind to the RAGE, this can lead to stress oksidatif.[34] Combination activated of TLR and RAGE can stimulate macrophage NF-κβ so that the excess production of proinflammatory cytokines. This condition can lead to a decrease in the ratio of expression of RANKL / OPG in which there is an imbalance between the production of RANKL and OPG in osteoblasts, it showed decreased expression of OPG. The imbalance can be an opportunity for RANKL binding to RANK on the surface of cells that are precursors osteoklas.[10] Bonding that occurs in RANKL-RANK will result in osteoclastogenesis that osteoclasts are formed and cause bone resorption.[22] It can be concluded that the DM and periodontitis may decrease OPG expression in groups of mice induced by STZ and *P. gingivalis* bacteria when compared to the negative control group.

In the wistar group and STZ-induced bacteri *P.gingivalis* with *Stichopus hermanii* powder 3% (8.25 ± 1.258) as well as in groups of wistar induced by STZ and the bacteri *P. gingivalis* with OHB therapy (5.75 ± 0.957) compared with the group of wistar induced by STZ and the bacteri *P. gingivalis* (2.50 ± 0577) obtained results showed an increase in expression of OPG were significant ($p<0.05$). Saponins (triterpene glikosid) which contained the *Stichopus hermanii* has an antibacterial effect. Saponin 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.[35] Endotoxin is issued by *P. gingivalis* bacteria can be inhibited by flavonoids that activation of NF-kβ not occur and inflammation can be stoped.[13,36]

Oxidative stress that occurs due to the formation of ROS induced in the body during bacterial pathogens can be inhibited by flavonoids, this is because their strong antioxidant effect owned flavonoid.[13] the formation of a strong defense against the bacteria causing the decrease inflammation that can increase the expression OPG.[36,37] Glycosaminoglycans contained in *Stichopus hermanii* can increase osteoblast proliferation by releasing the hyaluronic acid. Hyaluronic acid increases TGF-β as a growth factor that affects the differentiation and proliferation of osteoblasts quickly. The increased proliferation of osteoblasts affect the expression OPG.[38,39] Chondroitin sulfate contained in *Stichopus hermanii* acts as an anti-inflammatory because it can suppress the production of inflammatory cytokines (IL-1 and TNF-α).[40] This condition makes chondroitin sulfate can increase the expression of OPG.[14]

Therapy OHB 2.4 ATA 3x30 minutes with a pause of 5 minutes inhaling normal air for 7 days in sequence, has been proven in studies carried Prabowo et al (2014) may lower blood sugar levels effectively compared to days 1, 3 and 5. The condition of hyperglycemia occurs due to an increase ROS in mitocondria β cells pancreatic thus formed AGEs, this is known as free radicals, causing stress oxidative.[5] During treatment of OHB will be an increase ROS in mitocondria, this will trigger the liver to produce Hsp 70 as a response body to protect cells from damage. This mechanism may improving your insulin receptors are damaged and thus increase GLUT4 translocation of glucose in tissue can get into that blood sugar decreases.[41]

Administering 100% oxygen in the tissues created conditions hyperoxigen that give the effect bacteriosid to bacterial *P.gingivalis*.[16] Oxidative stress that occurs during the induction of pathogenic bacteria and AGEs could be reversed by the balance between oxidants and antioxidants. OHB therapy stimulates the formation of antioxidant enzymes such as superoxide dismutase, catalase, glutasi, and glutasi reduktase.[42] Occurrence of oxidative stress causes increased activation of NF-kβ. Hyperoxigen that occurred during OHB therapy may decrease the activation of NF-kβ due to inhibition of oxidative stress resulting in the decreasing production of proinflammatory cytokines.[43] Increased oxygen in the body can cause an increase in the proliferation of osteoblasts so as to induce the expression of proinflammatory cytokine production OPG.[42,44] Declining production of

proinflammatory cytokines can lower RANKL, this is according to research conducted Al Hadi et al (2013) in which the mechanism of hyperoxigen at OHB therapy can reduce the production of RANKL. RANKL/OPG ratio decreased, so that expression of OPG can be decrease.[19]

OPG expression group that received *Stichopus hermanii* powder 3% (8.25 ± 1.258) and the group given therapy OHB 2.4 ATA 3x30 5 minute intervals for 7 days (5.75 ± 0.957) showed no significant difference when compared to the negative control group wistar (5.75 ± 1.708) ($p > 0.05$), it is proved that the expression of OPG in the alveolar bone of wistar DM with periodontitis were given *Stichopus hermanii* 3% only and by therapy OHB just undergone a recovery until the situation returns to normal as the expression of OPG the alveolar bone in normal wistar.

In the group given a combination of *Stichopus hermanii* powder 3% and 2.4 ATA OHB therapy 3x30 5 minute intervals for 7 days (12.50 ± 2.082) when compared to all groups experienced a significant difference ($p < 0.05$). This indicates there is a good cooperation between the contents of *Stichopus hermanii* with a mechanism for the hyperbaric oxygen therapy significantly increased the expression of OPG. OPG expression increased significantly inhibit binding of RANKL-RANK causing apoptosis of osteoclasts and inhibit alveolar bone destruction.[19]

Thus, the given *Stichopus hermanii* powder 3% which combinated with OHB therapy 2.4 ATA 100% 3x30 minutes at intervals of 5 minutes of breathing air, which also performed for 7 consecutive days were able to increase the expression of wistar alveolar bone induced DM *P.gingivalis* better when compared to the treatment group who received *Stichopus hermanii* powder 3% and the treatment group were treated OHB 2.4 ATA 3x30 minute interval of 5 minutes for 7 consecutive days.

CONCLUSION

There is the influence of Stichopus hermanii powder 3% and 2.4 ATA OHB therapy with 3x30 minute and 5 minutes breathing air for 7 days against osteoprotegrin expression in wistar alveolar bone induced DM and *P. gingivalis* bacteria.

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