RESEARCH ARTICLE

The Effect Of Sea Cucumber Powder And Hyperbaric Oxygen Therapy On The Expression Of *Tumor Necrosis Factor Alfa* In Rats With Diabetes Mellitus Induced By *Porphyromonas gingivalis*

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ABSTRACT

Background: *Periodontitis have a bidirectional relationship with diabetes mellitus, both can increase oxidative stress that trigger an increase of pro-inflammatory cytokines, such as TNF-α. Sea cucumbers have anti-inflammatory component that act to inhibit the release of inflammatory mediators. Hyperbaric oxygen therapy reduce oxidative stress.* ***Purpose:*** *The aim of this study is to investigate the effect of the sea cucumber powder and hyperbaric oxygen therapy on the expression of TNF-α in rats with diabetes mellitus induced by P.gingivalis bacteria.* ***Materials and Methods:*** *The study was an experimental laboratories research with factorial design. Twenty wistar rats were divided into 5 groups. K0 negative control, K1 positive control. K1-K4 groups were induced with streptozotocin for diabetes condition and P.gingivalis bacteria for periodontitis condition. K2 group was treated with sea cucumber 3% for 7 days, K3 with OHB 2.4 ATA 3 x 30' interval 5' for 7 days, and K4 group was treated with combination of sea cucumber and OHB. At the 51st day all rats were sacrified, then the expression of TNF-α on periodontal macrophages were examined by immunohistochemistry stain. Data were analized by Kruskal Wallis followed by Mann-whitney.* ***Result:*** *Expression of TNF-α were increased in K1 (11.50±1.291) compare K0 ($p<0.05$). Sea cucumber treatment, OHB, and combination treatment decreased expression of TNF-α significantly in amount of 2.50±0.577 (K2), 8.25±2.217 (K3), 3.00±0.816 (K4).* ***Conclusion:*** *Sea cucumber powder and OHB therapy affected the expression of TNF-α in rats with diabetes mellitus induced by P. gingivalis bacteria.*

Keyword: *Sea cucumber powder, TNF-α, periodontitis, diabetes mellitus, OHB therapy*

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BACKGROUND

The relationship between periodontitis and diabetes mellitus has long been discussed, both diseases have a relatively high incidence. Diabetes is a risk factor which increasing the severity of periodontitis. Instead, periodontitis may be a risk factor for glycemic control in people with diabetes.[1,2] Approximately 177 million people worldwide have diabetes mellitus and diabetes has been confirmed as a major risk factor for periodontitis. The risk of periodontitis increased approximately three-fold in individuals with diabetes compared with non-diabetic individuals.[3,4]

Individuals with diabetes have high glucose levels in the gingival fluid and blood more than individuals without diabetes. Increased glucose levels in the gingival fluid and blood in individuals with diabetes can change microflora environment, induce qualitative changes of bacteria that contribute to the severity, periodontal disease in patients with uncontrolled diabetes. Poor glycemic control led to increasing of oxidative stress, advanced glycation end products (AGEs), hyperresponsive innate immune system that contribute to damage factor diabetic periodontitis. In addition, the function of the normal immune cells, such as chemotaxis, cell adherence, phagocytosis, cytokine production and secretion, are all affected by hyperglycemia.[5,6]

Diabetics have more severe periodontal disease because of the presence of glucose which induce accumulation of advanced glycation end products (AGEs) and affect the activity of migration and phagocytic activity of mononuclear and polymorphonuclear, so the formation of a more pathogenic subgingival flora.[7] Many bacteria associated with periodontal disease including Porphyromonas gingivalis one of the most important periodontal pathogens. At the time of periodontal infection, the balance of bacterial flora in the oral cavity become changed, so the species of pathogenic bacteria is grow and survive, even when confronted with the immune response.[6] Lipopolysaccharide (LPS) is one of most important microbial virulence factors in generating a host-mediated periodontal tissue destruction than that, lipopolysaccharide is a potent stimulator of various inflammatory mediators, including IL-1β and TNF-α.[8]

Tumor necrosis factor (TNF α) is reported have a role as a key to the pathogenesis of diabetes mellitus type 2. Research in animals and in vitro human, TNF-α inhibits the action of insulin. In animal and human cell models in vitro, TNF-α inhibits insulin. In one study, an increase in circulating levels of TNF-α is associated with insulin resistance and type 2 diabetes. Individuals with periodontitis disease have high TNF-α response, monocytes from individuals with diabetes and periodontitis release massive amounts of TNF-α.[9,8]

Treatment of periodontitis with diabetes mellitus did not only include local factors, but also systemic factors such as handling blood sugar.[7] Researchers showed that the local periodontitis treatment such as scaling and root planing histologically not indicate new connective tissue attachment.[5] Periodontitis treatment of diabetes that has been done is the tissue surrounding dental treatments, antibiotics, and blood sugar control. While the primary pathological condition in people with diabetes

mellitus is a disorder of the healing process.[10,11]

Sea cucumbers have long been used as a traditional medicine. One species of sea cucumbers which used as medicine is Stichopus hermanii that contains nutrients in wound healing, as an anticoagulant and antithrombotic, decrease cholesterol level and blood fat, anti-cancer, anti-tumor, immunostimulant, antirheumatic, antimalarials, antiviral, antifungal and antibacterial.[12] The content of omega-3 (EPA and DHA) in Stichopus hermanii can decrease cholesterol levels because infection of mix periodontopatogen bacteria and served to accelerate the healing of wounds and also as an anti-inflammatory that inhibits the production of TNF-α and IL-1β.[13,14] According to research of Rizal (2012)[15], Stichopus hermanii is contained by flavonoids. The content of flavonoids as antioxidants that indirectly supporting anti-inflammatory effects by inhibiting the release of inflammatory mediators.[15,16]

Sea cucumbers are one of the marine life that used in the health field. Sea cucumbers have shown a beneficial effect on periodontal therapy as well as patients with diabetes mellitus. In addition to cucumbers, one therapy that is emerging today is hyperbaric oxygen therapy (OHB).

Hyperbaric Oxygen Therapy (OHB) is a treatment in patients with giving 100% pure oxygen by inhalation method in hyperbaric hyperoxia at the air pressure of more than 1 atmosphere Absolute (ATA) and has been used as adjuvant therapy in various diseases.[10]

Hyperbaric Oxygen Therapy (OHB) shown a beneficial effect on periodontal tissues by increasing oxygen pressure in the pocket. In periodontitis patients with diabetes mellitus who use OHB therapy there is decreased levels of malondialdehyde (MDA), which is one biomarker of oxidative stress.[17,18] In addition, OHB therapeutic effects on monocytes and macrophages are causing disruptions to production of proinflammatory cytokines, resulting in a reduction in pro-inflammatory cytokines in conditions of oxidative stress.[19]

The research results of Fitria et al (2013),[20] cucumbers therapy for 7 days effectively decrease blood sugar levels as well as the golden sea cucumber is used as an antibacterial and antifungal in inhibiting the growth of bacteria. The research results of Prabowo et al (2014),[21] OHB therapy 100% 2.4 ATA 3 x 30 minutes a day with a pause of 5 minutes for 7 days decreased blood sugar levels in diabetes mellitus type 2. It is necessary to investigate the combination of sea cucumber powder gold and OHB therapy against TNF-α expression in diabetes mellitus induced P. Gingivalis bacteria.

MATERIALS AND METHODS

The research is true experimental research with factorial design study. Parameters were examined in this study was the expression of TNF α in diabetes mellitus and periodontitis. The samples were 20 Wistar rats (Rattus novergicus Wistar strain) were divided into five groups, where the selected criteria is the type males, aged 3-4 months with a body weight of 150-200 grams.

The tools used in this research is the syringe 5 cc and syringes 3cc, syringes insulin 1 mL, blood glucose test strips, gauges blood sugar, animal cages, food and drink rat, scales,

measuring cups and a stirrer, test tubes, test tube rack, micropipette, micro brush, glass, glass slide, surgical scissors, microscope, chamber of animals, immunohistochemistry kit.

Materials used nicotinamide 230 mg/kg, 200 mg canamycin, 200 mg ampicillin, 0.6% chlorhexidine gluconate, streptozotocin (to make the rats diabetes mellitus), 10% dextrose, citric acid buffer 0.05M pH 4.5, aluminum foil, bacteria Porphyromonas gingivalis ATCC 33277, powder golden sea cucumber (Stichopus hermanii), phosphate buffered saline (PBS), 4.13% EDTA, xylol, TNF-α antibody (sc-52 746-Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), H2O2, aquadest, Wistar rats, Wistar rats standard food, beverages Wistar rats (ordinary tap water), 100% pure oxygen in a hyperbaric chamber.

The division of research subjects. Those wistar rats were divided into five group: first, negative control group (K0); second, groups of diabetic periodontitis rats (K1); third, group of diabetic periodontitis rats with treatment golden sea cucumber (K2); fourth, groups of diabetic periodontitis rats with treatment of hyperbaric oxygen (K3); and five, groups of diabetic periodontitis rats with treatment combination golden sea cucumber and hyperbaric oxygen (K4).

Making the golden sea cucumber powder. Dissected sea cucumber to remove all viscceral organs, then blow-dry sea cucumber using freeze dryer at a temperature of 2-8 ° C with a pressure of 5 mTorr. The dried sea cucumbers are weighed and then blended into a powder. Making the golden sea cucumber powder gel using a mixture of sodium carboxymethylcellulose (CMCNa) 2%.[22]

Diabetes Induction procedures for Wistar rats. Day 7 after adaptation, induction of streptozotocin (STZ). DM induction is done by giving nicotinamide (NAD) of approximately 230 mg / kg, dissolved in the liquid PBS 10 minutes and then given STZ intra-peritoneal for 15 minutes injected a single dose of 65 mg / kg in rat that had fasted overnight between 8-12 hours. Then rats were incubated for 7 days. Otherwise DM rats when blood glucose levels over 220 mg / dl.[23]

Procedure Premedication before induction of bacteria. All the rats were given canamycin (20 mg) and ampicillin (20 mg) daily for 4 days in drinking water, and oral cavity swabbed with 0.12% chlorhexidine gluconate as antiseptic.[24]

Procedure Preparation and Induction Bacteria. Induction of bacteria using ATCC P.gingivalis imposing 33277 with 2 ml of 1 x 109 cells / ml of live bacteria in PBS, and peroral administration. Most bacteria applied using cotton bud along the gingival sulcus and inserted of anal to the colorectal region with syringe canula. Then incubation for 3 weeks since giving bacteria counted first.[25]

After the rats in the induction of STZ and P.gingivalis bacteria, rats were given a therapeutic treatment. For K2 and K4 given group therapy treatment gel golden sea cucumber powder 3% applied topical as much as 0.1 mg / day for 7 days using microbrush on inflamed sulcus gingival. K3 and K4 group treated OHB therapy, administration of 100% oxygen 2.4 ATA 3 x 30 minutes a

day with a pause of 5 minutes for 7 days.[26.21]

On day 8 after therapy gel golden sea cucumber and OHB, all groups were sacrificed then performed euthanasia by the neck (cervical) dislocation, the dosage is taken on the part of the mandible. After that, do the decalcification phase part of the mandible for 4 weeks using 4.13% EDTA. Furthermore, the tissue processing and staining by immunohistochemistry (IHC) was performed using the streptavidin-biotin-peroxidase labeled streptavidin-biotin (Dako, Carpinteria, USA), and then observed using a microscope with 1000x magnification and calculated as much as 20x field view.

Data were analyzed using parametric statistical test one way ANOVA, with a test for normality using the Shapiro-Wilk test, then continued homogeneity statistic test using Levene test. Then the calculation results compared (comparing the expression of TNF-α in each treatment group).

RESULT

Before doing hypothesis test between groups, first of all each group tested the normality by using statistical test of Shapiro-Wilk, because in this study sample size <50 and the results obtained is Shapiro-Wilk test has a significant value of p> 0.05, so that the distribution of data on this research is abnormal.

Table 1. The average and expression deviation standard of TNF-α in each group

Group	Average ± Deviation Std.
K0	5.00 ± 0.816
K1	11.50 ± 1.291
K2	2.50 ± 0.577
K3	8.25 ± 2.217
K4	3.00 ± 0.816

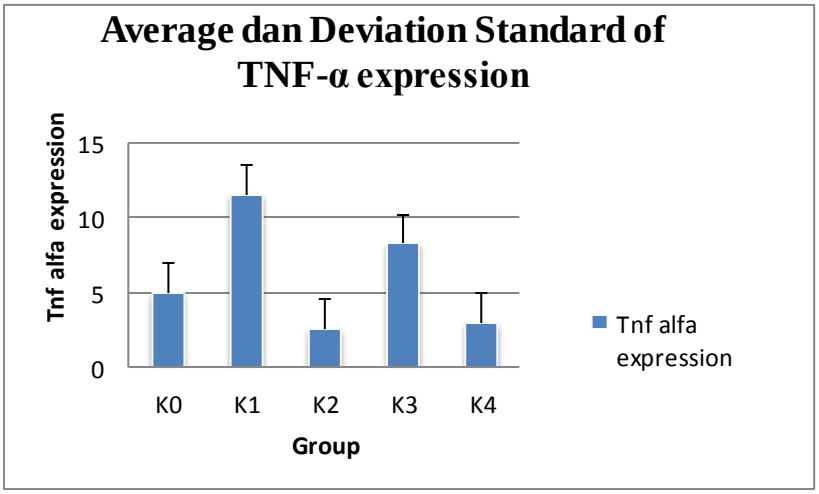

Figure 1. Average and Deviation Standard of TNF-α expression in each group

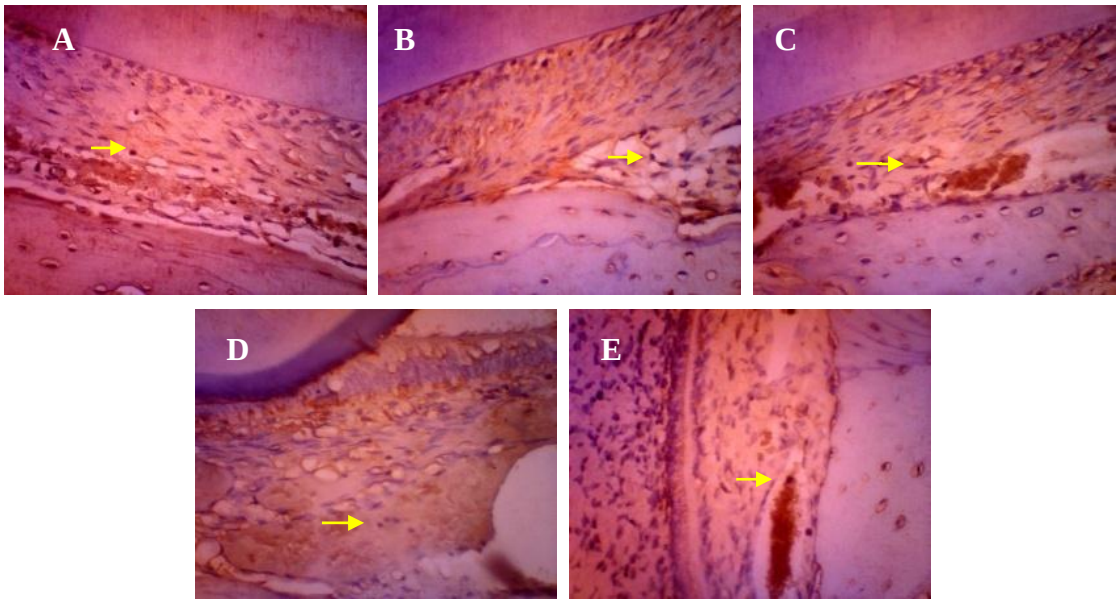

Figure 2. TNF-α expression in macrophages in the periodontal ligament rat (Rattus norvegicus) with 400x magnification. A. K0 (normal); B. K1 (diabetes + bacteria); B. K2 (diabetes + bacteria + sea cucumbers); D. K3 (diabetes + bacteria + OHB); E. K4 (diabetes + bacteria + OHB + sea cucumber). Expression of TNF-α are marked with brown color in macrophages (arrows).

It was found that the expression of TNF-α have variances were not homogenous because p = 0.041 (p<0.05). Therefore, it can be concluded that there is a difference of variance between sets of data are compared.

The research data were not normally distributed, and found that the data are not homogeneous, then doing non parametric Kruskal Wallis hypothesis test to determine the expression of TNF-α.

Table 2. *Kruskal Wallis* test Result

	Sig.
TNF-α expression	0.002

In the table 2. obtained significance value of 0.002 (p <0.05). It shows a significant difference in each group under these conditions, followed by Mann-Whitney test to find out where is the difference in each group with a significance level P <0.05.

Tabel 3. *Mann-Whitney* test Result

	K0	K1	K2	K3	K4
K0		0.020*	0.019*	0.028*	0.027*
K1			0.019*	0.059	0.020*
K2				0.019*	0.343
K3					0.020*

*there is significant difference

DISCUSION

Nicotinamide (NAD) is an antioxidant which exerts protective effect on the cytotoxic action of STZ by scavenging free radicals and causes only minor damage to pancreatic beta cell mass producing type 2 diabetes.[23]

Streptozotocin is a drug that has a specific toxicity in pancreatic β cells. Streptozotcin cause DNA alkylation and β cell death because STZ enter cells through the glucose transporter GLUT2, toxic mechanism is not specific for ß

cells and can cause damage to other tissues including the liver and kidneys.[27,28] Damage to the pancreatic beta cells causes the body can not produce insulin, causing blood glucose levels to rise (a state of hyperglycemia). Conditions of hyperglycemia can result in the formation of reactive oxygen species (ROS) which would then trigger an active nuclear factor kappa β (NFƙβ) that play a role in the increased production of pro-inflammatory cytokines. [23,29] According to research from Swaroop et al (2012) [30] showed that TNF-α can play a potentially important pathophysiological role in the development of insulin resistance. Results of research on groups of bacterial-induced diabetic rats found increased expression of TNF-α compared with normal rats, it was shown that the group of bacterial-induced diabetic rats occurred diabetes.

Porphyromonas gingivalis is an anaerobic gram-negative rod bacteria and include black-pigmented bacteria, these bacteria cause inflammation and tissue damage in diseases periodontal.[31,32] P.gingivalis get into crevices using fimbriae and attached gingival epithelial cells digingiva, proteases have the ability to destroy periodontal tissue directly or indirectly, and LPS can cause inflammatory responses and resulting in the production of cytokines in periodontal tissue, cause the activation of genes and cause bone damage alveolar.[31,6] Based on the results of the research showed that there were significant differences (p=0.020) on TNF-α expression between groups of normal rats and a group of bacterial-induced diabetic rats (see Table 5). The test results showed that the expression of TNF-α in the group of bacterial-induced diabetic rats (11:50 ± 1.291) increased compared with normal rats (5.00 ± 0816), this is due to streptozotocin-induced rats to make samples of rats became diabetic and bacteria capable P.gingivalis inducing the sample into periodontitis.

Some research it is known that the golden sea cucumber (Stichopus hermanii) as a potential therapy in the field of dentistry, besides the golden sea cucumber (Stichopus hermanii) itself has long been used as a traditional medicine. Golden sea cucumber powder used on research using the golden sea cucumber (Stichopus hermanii) which are found in waters of Indonesia.[33]

Based on the results of the research showed that there were significant differences on the expression of TNF-α between groups of normal rats, groups of bacterial-induced diabetic rats and rats treated groups of sea cucumbers (see table 5). The test results showed that the expression of TNF-α in groups of rats treated cucumbers decreased compared to the group of normal rats and a group of diabetic rats induced bacteria, because the content of the golden sea cucumber that is therapeutic as it helps wound healing, antibacterial, antifungal, antitumor, antianaphylactic, anti-inflammatory, antinociceptive and antioxidant.[34]

Teripang gold (Stichopus hermanii) examined contains the active ingredient antibacterial, antifungal (antifungal), antitumor and anticoagulant (anti-clotting) and it also contains compounds that can reduce cholesterol and lipid, anticancer and antitumor compounds, as well as antibacterial compounds.[35] Golden sea cucumber has a various kinds of content, one of the active ingredients of the golden sea cucumber is Triterpenoid

strong role as an immunostimulatory. In general Triterpenoid group capable of damaging the cell membrane, deactivate enzymes and proteins denature so that the cell wall is damaged due to a decrease in permeability. Changes permeability of the cytoplasmic membrane allows ions important organic entry into the cell resulting in poor growth and even to kill cells.[36.35] In addition, the gold content of sea cucumbers that PUFAs (DHA and EPA) may block the formation of prostaglandins and proinflammatory cytokines (IL-6, IL-1β and TNF-α).[37,38]

Hyperbaric oxygen increases the formation of oxygen free radicals, then oxidize proteins and lipid membranes of bacteria, destroys DNA and inhibits bacterial metabolic functions. The enzyme superoxide dismutase, catalase, glutathione and glutathione reductase inhibiting free radical formation, until the oxygen concentration exceeded enzyme concentration.[39] Based on the results of the research showed that there were significant differences between groups of normal rats and rats treated group OHB, but there is no significant difference between groups of bacterial-induced diabetic rats and rats treated group OHB. The test results obtained by the expression of TNF-α in groups of rats treated group OHB increased compared to normal rats, but decreased the expression of TNF-α in groups of rats treated OHB compared with bacterial-induced diabetic rats.

Based on the results of the study showed that there were significant differences between groups of bacterial-induced diabetic rats and mice combination treatment group due to the content of one of the golden sea cucumber are flavonoids that act as antioxidants and indirectly support the anti-inflammatory effects by inhibiting the release of mediators inflamasi.16Penurunan expression TNF-α at 2.4 ATA hyperbaric oxygen 100% oxygen 3x30 minutes with 5 minute intervals for 7 days clinically recommended, because it can lower blood sugar levels, mRNA expression and activity of antioxidant enzymes significantly.[21,40,26]

CONCLUSSION

According to the research, it can be concluded that there is the influence of the golden sea cucumber powder 3% and hyperbaric oxygen therapy 3 x 30 minute interval of 5 minutes for 7 days on the expression of TNF-α in rats with diabetes mellitus induced by the bacterium Porphyromonas gingivalis.

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