

EFFECT OF CHITOSAN ON PROTEIN CONTENT IN THE MEDIUM CULTURE OF OSTEOBLASTS EXPOSED TO OXIDATIVE STRESS

(EFEK KITOSAN TERHADAP KANDUNGAN PROTEIN DALAM KULTUR MEDIUM OSTEOBLAS YANG DIPAPAR DENGAN STRES OKSIDATIF)

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Abstract

Chitosan is a derivative of chitin which has potential for use in bone regeneration and has been reported can stimulate bone formation. Oxidative stress as one cause of bone damage, was found increased in osteoporosis, periodontitis and arthritis. One of the species oxygen reactive (ROS), hydrogen peroxide, has been reported can inhibit osteoblast proliferation. This study was aimed to investigate the effect of various chitosan concentrations on protein content in the culture medium of human osteoblast-like cell line, MG 63, which was exposed to hydrogen peroxide. MG 63 cells were exposed to various chitosan concentrations (% w/v) 0.1, 0.2, 0.4, and 1.6%. Culture cells without chitosan were used as a control. Cells were growth with α -MEM medium (37°C, 5% CO₂) until they became confluent, then they were exposed to hydrogen peroxide for 4 hours. The protein content in the culture medium was measured by using Bradford protein assay at 655 nm wavelength. The result showed that hydrogen peroxide decreased protein concentration in the medium culture compared with group without hydrogen peroxide. Treatment group with chitosan concentration 0.4% and 1.6% exhibited a significant increasing of protein concentration in osteoblast culture medium compared with control. In conclusion, in osteoblast culture medium chitosan can inhibit the decreasing of total protein concentration which was caused by oxidative stress.

Key words: osteoblast, hydrogen peroxide, chitosan, protein concentration

INTRODUCTION

Bone resorption is essential for calcium homeostasis, modeling and remodeling of bone.¹ However, excessive resorption is the cause of bone loss observed in common diseases such as postmenopausal osteoporosis, rheumatoid arthritis, and periodontitis. Bone loss occurs because of imbalance between bone resorption and bone formation process. Excessive bone resorption process was caused by the number and activity of bone resorbing cells, osteoclasts, are greater than osteoblasts. bone-forming cells, osteoblasts.^{1,2}

The process of bone resorption is influenced by several factors, one of them is reactive oxygen species (ROS). ROS is metabolites of molecular oxy-

gen (O₂), which can include as radical superoxide (O₂⁻), radical hydroxyl (HO) and hydrogen peroxide (H₂O₂).^{3,4} Small number of ROS is produced by osteoclasts and will help the process of bone remodeling.⁴ However, if the amount of ROS increases due to a variety of exogenous stimuli, such as ultra-violet radiations, toxins, extreme temperatures, pollutants and pathogen infections, it can cause oxidative stress, which can lead into pathologic conditions, such as excessive bone resorption.^{4,5} Marker of ROS was increased in some diseases with manifestation of bone destruction, such as osteoporosis, periodontitis and arthritis.⁶⁻⁸ Moreover, ROS is reported can inhibit osteoblast proliferation and induce apoptosis of osteoblasts, which resulted in disruption of bone formation process.⁹ ROS can also

stimulate osteoclast formation and bone resorption activity, which can enhance bone resorption.^{10,11}

Currently, the number of studies have been performed on biomaterials that can be used for bone regeneration. One of those biomaterials is chitosan, which is a derivative polysaccharide of chitin. Chitosan exhibits excellent biological properties; it is biodegradable, biocompatible, non-toxic and has antimicrobial, antifungal and antioxidant property.^{12,13} In some researches, chitosan is reported can stimulate the formation of osteoblasts and can inhibit bone resorption, make it potential for use in bone regeneration.¹³⁻¹⁶ Although chitosan has been widely investigated and reported to be useful in bone regeneration, but until now it's not been clear about its role in addressing bone loss due to increased ROS.

Protein is a component of cells that plays an important role in cell activities, including growth, division, and differentiation of cells.¹⁷ These activities require proteins derived from proteins contained in the medium culture. In other side, cells also synthesize proteins that can be secreted into the medium culture. Thus, the total protein in the medium culture of cells can be used as an indicator of the response of cells to substances/biomaterials.¹⁷ This study aimed to determine the effect of chitosan on protein content in the medium culture of osteoblast which was exposed to ROS. In this study the conditions of oxidative stress in cell culture is a model of pathological conditions that occur in a number of diseases of bone damage.

MATERIALS AND METHODS

Chitosan used in this study was obtained from BATAN, Indonesia, with specification : degree of deacetylation (72-82); molecular weight 7000-8000 Da; water content less than 10%; and can dissolve in acetic acid. Chitosan was dissolved in acetic acid 0,2% and made into 0.1, 0.2, 0.4 and 1.6% (w/v) chitosan solutions. A total of 200 µl of each chitosan solution was plated on 24 well plates in safety cabinet using sterile techniques. Some wells weren't coated with chitosan and used as control. The coated well plates were allowed to sit in the safety cabinet without lids under UV light for 30 minutes for sterilized purpose.¹⁴

Human osteoblast-like cells, MG 63 cells were obtained from cells stock that stored in cryopreservation on Faculty of Dentistry University of Indonesia Laboratory. Cell was grown in 100 mm petri dish with α -MEM (Gibco) containing 10% FBS (Biowest), antibiotics (100 unit/ml of peni-

cillin G and 100 µg/ml streptomycin) (Biowest), and antifungal (2,5 µg/ml amphotericin B) (Biowest), then it stored in incubator at 37°C and 5% CO₂ conditions. The media was replaced in two-day intervals. After reaching a confluent state, the cells were collected by using cell scraper. 5x10⁵ cells/ml were added on 24 well plates-coated chitosan which has already contained complete medium.

After cells reached a subconfluent state, they were treated by hydrogen peroxide (H₂O₂) 100 µM for 4 hours.¹⁸ Uncoated chitosan-wells were divided into three groups; first group without treatment, second group treated with H₂O₂ 100 µM and third group treated with H₂O₂ 100 µM and 500 U/ml catalase (Sigma) as inhibitor of H₂O₂.

Proliferation of MG 63 cells was measured quantitatively in term of protein content on the medium culture cells. Measurement of total protein concentration in the medium culture osteoblast performed using Bradford protein assay.¹⁹ This method is based on the action changes the color of Coomassie Brilliant Blue G-250 dye (CBBG) when binding to proteins. The value of absorbance of the solution can be measured using a microplate reader in certain wave-length. The value of protein content from the samples can be estimated by using the protein standards as a basis known concentration. A total of 160 µl protein sample was put into 96 well plates. There after, it was added to 40 µl Bradford reagents in each well and left at room temperature for 5 minutes. Absorbance was measured at 655 nm in microplate reader (Biorad) and total protein content was calculated from a standard curve. Data was expressed as µg protein/5x10⁵ cells.

Results were expressed as mean \pm standard deviation (SD). Normal distribution of data was confirmed and the results were checked for homogeneity. Statistical differences between means for different groups were evaluated using one-way ANOVA. Multiple comparisons were performed using the LSD method. Two-tailed *P* value <0.05 was considered as statistical significant.

RESULTS

A significant decreasing in the protein content of medium culture was observed on the cells which exposed to hydrogen peroxide compared to control or group with hydrogen peroxide and its inhibitor (catalase). The difference value between group with hydrogen peroxide and control was significant (*p*<0.05), but the difference value between group with hydrogen peroxide and catalase was not sig-

nificant compared to control ($p>0.05$) (Table 1).

Table 1. The mean value of the total protein concentration of osteoblast medium culture between control and treatment groups exposed to H_2O_2 with/without catalase

Group		N	Means \pm SD ($\mu g/ml$)
Control	Osteoblast	3	33552.28 \pm 8029.52
Treatment I	Osteoblast + H_2O_2	3	13351.57 \pm 2581.92*
Treatment II	Osteoblast + H_2O_2 + catalase	3	24302.73 \pm 1910.55

* significant difference

Chitosan increased the protein content of the medium culture on osteoblast which exposed to hydrogen peroxide. Table 2 showed the total protein concentration of the medium culture in treatment groups were increased in line with increased concentration, but only chitosan 0.4 and 1.6% which had significant difference compared to control ($p<0.05$). Significant difference between groups was seen between chitosan 0.1% with chitosan 0.4 and 1.6% ($p<0.05$).

Table 2. The mean value of the total protein concentration of osteoblast medium culture exposed to hydrogen peroxide and chitosan

Group		N	Means \pm SD ($\mu g/ml$)
Control	Osteoblast + H_2O_2	3	13351.57 \pm 2581.92
Treatment I	Osteoblast + chitosan 0,1% + H_2O_2	3	13432.42 \pm 1766.24
Treatment II	Osteoblast + chitosan 0,2% + H_2O_2	3	17649.56 \pm 2421.41
Treatment III	Osteoblast + chitosan 0,4% + H_2O_2	3	18900.18 \pm 3053.12*
Treatment IV	Osteoblast + chitosan 1,6% + H_2O_2	3	19361.31 \pm 4471.25*

* significant difference

DISCUSSION

The increasing of total protein in the medium culture indicated that there was an increasing of the number of living cells which it would increase the amount of protein secreted into the medium culture. Increased total protein concentration of the medium culture may be associated with metabolic processes of cells. Living cells will continue to synthesize and secrete several proteins that reflect the activity of these cells. Some proteins synthesized inside cells

were secreted into the medium culture, such as cytokines, enzymes, and hormones.²⁰ Therefore, the total protein in the medium culture can be used as parameter of proteins secreted by living cells, in this case, osteoblast was exposed by chitosan and H_2O_2 .

This study showed that H_2O_2 had capability to inhibit osteoblast formation and reduced activity of osteoblasts. Our data was consistent with previous work, which had reported that H_2O_2 had an effect on osteoblast proliferation and its activity.^{9,11,20} The decreasing of total protein concentration can also be caused by H_2O_2 itself by influencing the expression of proteins synthesized by osteoblasts. H_2O_2 is reported can inhibit expression of alkaline phosphatase and collagen type I from osteoblast.²⁰ In this study, it also showed that catalase inhibited hydrogen peroxide effect on osteoblast, so it can be concluded that the reduction of osteoblast activity may be happened because of H_2O_2 presence. H_2O_2 exposure resulted in the decreasing of cell synthesis activity. However, treatment with chitosan in the presence of H_2O_2 showed that the amount of protein produced by cells was higher than control and such proteins might be related to cell proliferation process. Chitosan could also stimulate osteoblast to synthesize and secrete a number of proteins, therefore the total protein concentration in the medium would increase.^{13,21} Provision of chitosan on osteoblast culture was reported to increase collagen, alkaline phosphatase, and osteocalcin which represents proteins that plays a role in bone remodeling.^{13,15}

Protective effect of chitosan on osteoblast activity under oxidative stress condition was caused by antioxidant effects of chitosan, as reported by Muresan.²² Mechanism of chitosan's action as antioxidant is still unclear, but presumably the amino and hydroxyl group on the chitosan molecules can react with free unstable radicals and form stable macromolecules.²³ Antioxidant activity of chitosan depends on the molecular weight and degree of deacetylation.^{22,24} Li et. al²⁴ reported that the inhibitory effects of chitosan against ROS increased in line with the decreasing of molecular weight and the increasing of degree of deacetylation. Chitosan BATAN used in this study has molecular weight of 7-8 kDa and degree of deacetylation of 72-82, which can be categorized as low molecular weight and average degree of deacetylation chitosan, so it can provide relative good antioxidant effects on osteoblasts under oxidative stress conditions. Besides those two factors, it seems that the concentration of chitosan also affected the ability of antioxidants from chitosan, as shown in this study. Based on these results, it can be concluded that

chitosan can inhibit the decreasing of total protein concentration in osteoblast the medium culture which was caused by hydrogen peroxide exposure.

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