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Research Report

# Antioxidant effect of minocycline in gingival epithelium induced by *Actinobacillus actinomycetemcomitans* serotype B toxin

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***ABSTRACT***

***Background****: Actinobacillus actinomycetemcomitans (Aa) serotype B has been associated with aggressive periodontitis. Gingival epithelial cell is exquisitely sensitive to the toxin and may lead to the epithel protective barrier disruption. Experimental models show that minocycline is not related to it's antimicrobial effect and protection against neuron cell apoptosis of a number experimental models of brain injury and Parkinson's disease.* ***Purpose****: This study, examined antioxidant effect of minocycline to inhibit apoptosis of gingival epithelium induced crude toxin bacteria Aa serotype B in mice.* ***Methods****: Thirty adult mice strain Swiss Webster (balb C) were divided randomly into three groups: control group (group A), toxin group (group B) and toxin and minocycline group (group C). The mice were taken at 24 hours after application, and then the tissue sections of gingival epithelium were stained with tunnel assay and immunohistochemistry.* ***Result****: Treatment with these toxin induced apoptosis of gingival epithelium and was associated with DNA fragmentation and reduced gluthatione (GSH). Minocycline 100 nM significantly increased GSH and reduced apoptosis ($p < 0.05$). Minocycline provides antioxidant effect against citotoxicity of bacteria Aa serotipe B.* ***Conclusion****: Nanomolar concentration of minocycline potential as new therapeutic agent to prevent progressivity of aggressiveness of periodontitis.*

***Key words:*** *minocycline, GSH, apoptosis, Aa serotype B crude toxin*

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## INTRODUCTION

Aggressive periodontitis is a disease in dental supporting tissues which is characterized by rapid degeneration of periodontal ligament and alveolar bone in young patient. Periodontitis leads to loss of periodontal attachment to the root surface and adjacent alveolar bone which ultimately results in tooth loss. Recent paradigm shows the importance of balance between oxidant and antioxidant inside the cell.[1] The imbalance between oxidant and antioxidant inside the cell will trigger the reaction of transcription factor which are NFkB, AP-1 and PARP-1, which will cause apoptosis and inflammation reaction. Toxin from Actinobacillus actinomycetemcomitans (Aa) serotype b bacteria will increase the apoptosis and reaction of PARP-1 in gingival epithelium.[2] Aa serotype b crude toxin have cytotoxic effects that will trigger the increase in the amount of apoptosis in gingival epithelial cells, monocytes, lymphocytes, and macrophages.[3,4] Gingival epithelial cells is ten times more sensitive towards apoptosis than macrophage cells.[5] The increase in the amount of apoptosis in host cell triggered by certain pathogenic bacteria is a new phenomenon in the pathogenesis of periodontal disease.[6,7] Minocycline is an antibiotic of choice for periodontitis treatment and often used topically.[8] Minocycline have different activities depending on the concentration. At the concentration above 10 $\mu$M minocycline is toxic towards epithelial cell, while above 100 $\mu$M it became toxic towards fibroblast cell. Minocycline has an effect as antibacterial to Aa bacteria at 4 $\mu$M concentration.[9] Minocycline on nanomolar concentration has bioactivity which is not connected to antibacterial, that is, as strong cytoprotection that protects the neuron cells from hypoxial trauma. Therefore, it is often used in therapies for ischemic stroke, Huntington's disease, multiple sclerosis and Parkinson's disease.[10–13] Nevertheless, so far, the mechanism how minocyclin on

nanomolar concentration functions as antioxidant for gingival epithelial cell is still unclear. An antioxidant that is present in gingival sulcular fluid is reduced gluthatione (GSH) which has quite a high concentration. The existence of periodontopathogen bacteria can decrease in the concentration of GSH.[1] One of the strategies for periodontal disease therapy is to increase the concentration of antioxidant in teeth support tissues cells. The objective of this research is to reveal the mechanism of minocycline as antioxidant caused by Aa serotype b crude toxin in gingival epithelium through the apoptosis and GSH expression.

## MATERIALS AND METHODS

This research was categorized as experimental laboratory by using the experimental animal male mice (Mus Muculus) Strain Swiss Webster (Balb C). The design of this research was the completely randomized design. This research was done in the Biomedical laboratory, Faculty of Medicine, Brawijaya University and Pathology Anatomy, Faculty of Medicine, Airlangga University. The unit analysis is the gingival epithelium in buccal anterior of the lower jaw of the Mus Musculus strain Swiss Webster BALB/c male, chosen healthy physically, 2.5 months old with the weight of 25–35 grams obtained from Pusat Veterinaria Farma Surabaya (Pusvetma). The material used is pure minocycline hydrochloride from SIGMA (155718), Actinobacillus actinomycetemcomitans serotype b (ATCC 43718), apoptag detection kit (Chemicon-product S-7101). Monoclonal antibody anti Gluthatione (Stressgen Product, SPA-542), Streptavidine Peroxidase from LabVision Co, TS-060-HR. The tools used are: Incubator, centrifuge with cooler (Eppendorf 5417R), Vortex, Water bath, Micro pipette with sterilized points, Eppendorf tube, Binocular Light Microscope Canon Aplhaphot Y5 connected to a monitor and camera. To ensure that all the procedure done in this research is ethically approved, before it was done, the proposal for this research was given to the Ethical Committee, Faculty of Dentistry, Airlangga University for inspection. The procedure for this research includes the culturing of Aa serotype b bacteria, the creation of crude Aa serotype b toxin, the examination of immunohistochemistry using of assay Tunnel and GSH. Actinobacillus actinomycetemcomitans ATCC 43718 (Y4, serotype b), were cultured in dialysates Todd Hewitt Broth (Difco Laboratories, Detroit, Mich) added with 1% of yeast extract at the temperature of 37° C, 5% $CO_2$ for 4 days.14 Subsequently, the bacteria were centrifuges by 12.000 rpm, and had its supernatant extracted. To separate the non protein from crude toxin, rough toxin that comes from the supernatant was precipitated by adding thick ammonium sulphate 40%. The formed sediment is separated using centrifuges by 3000 rpm for 30 minutes at 4° C. The salt formed from the sediment was then separated by dialysis. The sample was then inserted into a membrane which had its top and bottom secured, after that, the cellophane bag was inserted into a beaker glass which contained buffer Phosphate Buffer Saline (PBS) pH 7.2. The dialysis was stopped when it reached equilibrium.[15]

### Treatment on mice

Group 1: 10 mice is given sterile aquadest topically in the buccal gingival anterior lower jaw every 12 hours using disposable oral sponge swab (Rynel Inc, USA) which is inserted into the sterile aquadest until soaked, and applied by way of two double lateral strokes.[16] After 24 hours, the mice's anterior gingival tissue of lower jaw was extracted as biopsy specimen.[17]

Group 2: 10 mice were induced with bacterial toxin Aa 100 $\mu$g/ml.[14] After 24 hours, the mice's anterior gingival tissue of lower jaw was taken as biopsy specimen.

Group 3: 10 mice were induced with bacterial toxin Aa 100 $\mu$g/ml on the anterior buccal gingiva of lower jaw by using Hamilton Syringe (Reno, Nev)[18] continued with the administering of minociclyne 100 nanomolar topically on anterior buccal gingival of lower jaw every 12 hours using disposable oral sponge swab (Rynel Inc, USA) which are soaked in minocycline, and applied using two double lateral strokes. After 24 ours, the mice's anterior gingival tissue of lower jaw was taken as biopsy specimen.

### Immunohistochemistry examination

The immunohistochemistry examination with the method of Streptavidin –Biotin-Complex and Tunnel Assay were used to get the expression of GSH and apoptosis. The counting of gingival epithelial cells which expressed apoptosis and GSH was done under light microscope with 400 times magnification. Cells which proved to be positive gave brownish color between bluish/greenish epithelial cells. Every reserves were examined at 4 different places clockwise 3, 6, 9, and 12. Each field of vision are examined and counted at two places according to 6 and 12 needle using counting room and counter.[19] The results were then averaged and data were then analized statistically One-Way ANOVA statistic analysis with 95% degree of significance ($p < 0.05$).

## RESULT

To prove the effect of antioxidant minocycline on gingival epithel cell on mice induced with crude bacterial toxin A. actinomycetemcomitans serotype b, the counting of cells which expressed apoptosis and GSH was done. The resulting data were then described and tested within 0.05 degree of significance.

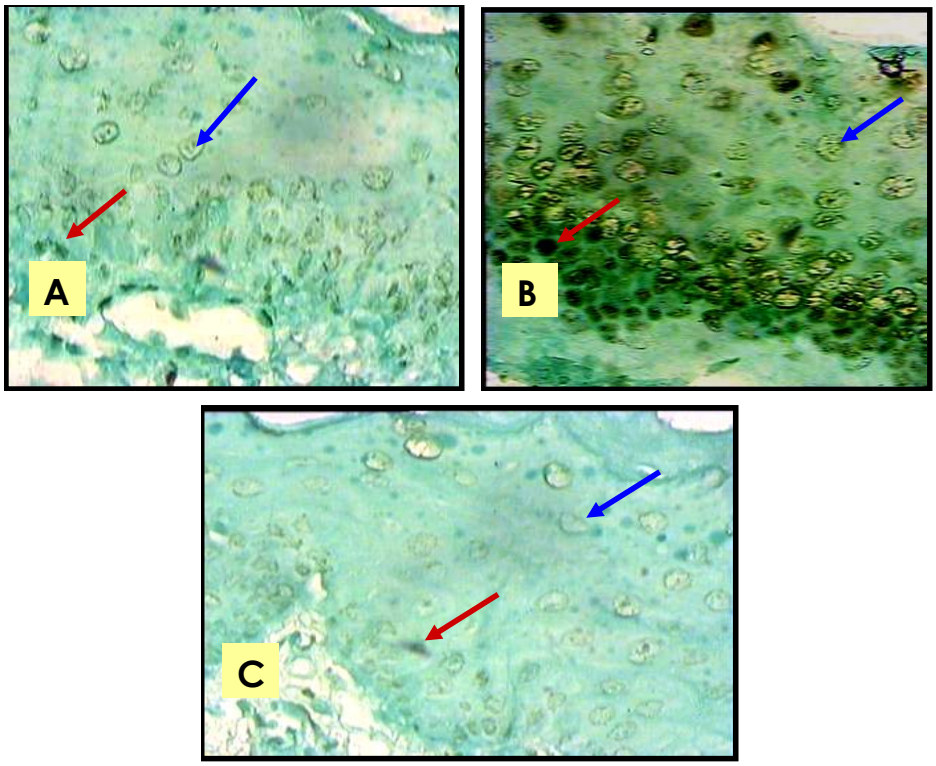


**Figure 1.** Apoptosis expression appearance using Tunnel assay with 400× magnification. A) Control; B) Bacterial toxin Aa serotype B exposure; C) Treatment with 100 nanomolar minocycline. Red arrow indicates positive result which noticed by the presence brown spot in the nucleus of gingival epithelial cells, blue arrow indicates negative result which noticed by the absence of brown spot in the nucleus.

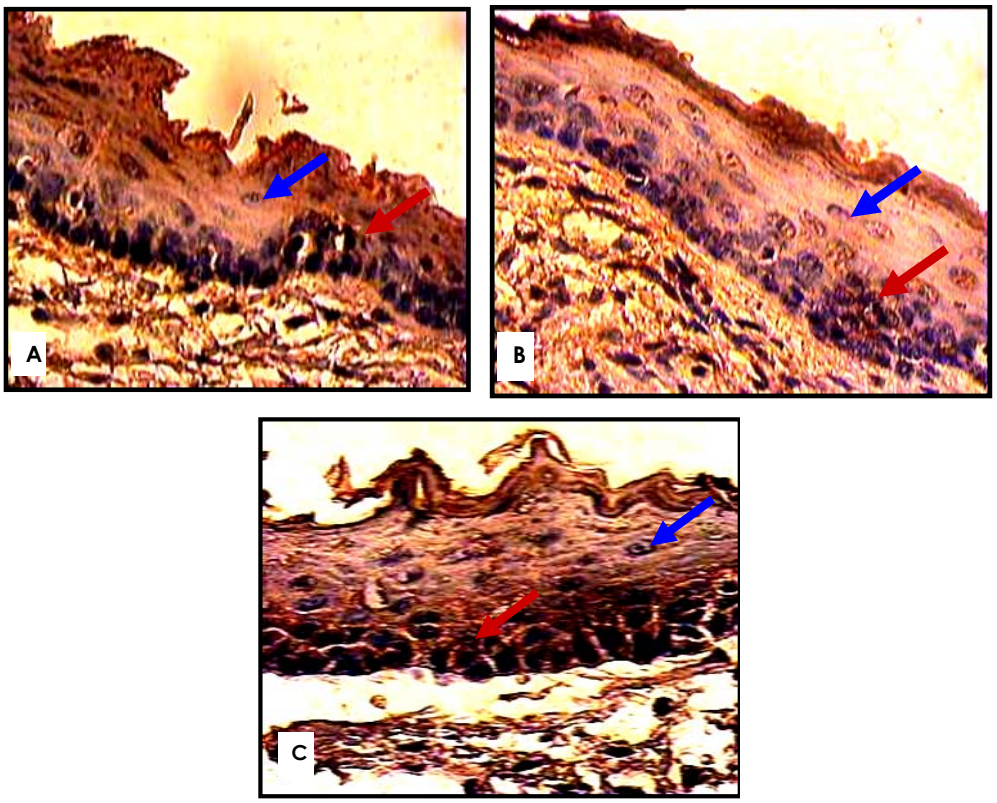


**Figure 2.** Gluthatione expression appearance with 400× magnification. A) Control; B) Bacterial toxin Aa serotype B exposure; C) Treatment with 100 nanomolar minocycline. Red arrow indicates positive result which noticed by the presence brown spot in the nucleus of gingival epithelial cells, blue arrow indicates negative result which noticed by the absence of brown spot in the nucleus.

**Table 1.** Mean, standard deviation, the significance of number of cells which express apoptosis in gingival epithelial cells that exposed to Aa serotype b bacterial toxin after treated with 100 nanomolar solution of minocycline

| Group | N | $\bar{X}$ | SD | Significance |
|---|---|---|---|---|
| Control | 10 | 5.17 | 0.27 | 0.00 |
| Toxin | 10 | 45.17 | 7.68 | 0.00 |
| Toxin + minocycline | 10 | 5.81 | 0.58 | 0.00 |

**Table 2.** Mean, standard deviation, the significance of number of cells which express glutathione in gingival epithelial cells that exposed to Aa serotype b bacterial toxin after treated with 100 nanomolar solution of minocycline

| Group | N | $\bar{X}$ | SD | Significance |
|---|---|---|---|---|
| Control | 10 | 60.51 | 1.12 | 0.00 |
| Toxin | 10 | 29.11 | 2.30 | 0.00 |
| Toxin + minocycline | 10 | 62.57 | 2.09 | 0.00 |

Normality test is done using Kolmogorov Smirnov test. Normal distribution group continued by parametric test of One-Way ANOVA and Tukey HSD test to know the difference in the group with 5% significant rate. Table 1 and table 2 shows that significant difference in number of cells which express apoptosis and gluthatione in gingival epithelial cell that exposed Aa serotype b toxin, after treated with 100 nM solution minocycline and control ($p < 0.05$).

In Figure 1, it is showed the apoptosis examination using Tunnel assay method on mice gingival epithelium biopsy with 400× magnification.

In Figure 2, it is showed the GSH expression on mice gingival epithelium biopsy with 400× magnification.

## DISCUSSION

In an attempt to organize the appropriate treatment strategy, what is needed is the comprehensive understanding of etiopathogenesis mechanism of periodontitis, so that the progressivity process of the disease could be inhibited. In the past, aggressive periodontitis treatment is only centered on the local factor elimination and antibacterial administration. Recent therapy progress is headed towards the increase of the capacity of tissues reparation by increasing cellular survival through the effect of cytoprotection by balancing oxidant and antioxidant.[20] This research was an experimental research to reveal the effect of minocycline antioxidant on gingival epithelium on Balb/c mice which were exposed to bacterial toxin Actinobacillus actinomycetemcomitans serotype b. Bacterial toxin Aa serotype b is cytotoxic towards teeth support tissues, especially gingival epithelial cell 10 times more than macrophage.[5,21] Number of cells which express apoptosis in gingival epithelial cell that exposed Aa serotype b toxin is higher than that were treated with 100 nM solution minocycline and control. Aa serotype b toxin induce free radical increased in intracellular epithel. It will cause oxidative stress that can increased expression Bax, caspase-3, enzyme Poly ADP Ribose Polymerase-1 (PARP-1) and DNA fragmentation. Imbalance condition between free radical formation and antioxidant cause cell damaging with apoptosis pathway.

Gingival epithelium as the first defense for teeth support tissues need to be protected from the effect of free radical effect triggered by Aa serotype b bacteria. The exposure to crude bacterial toxin Aa serotype b caused the disturbance of energy metabolism in mitochondria and disturb the homeostasis of energy inside the cells through the increase of intracellular calcium.[2] The result of this research showed that by giving minocycline 100 nanomolar, it can decrease

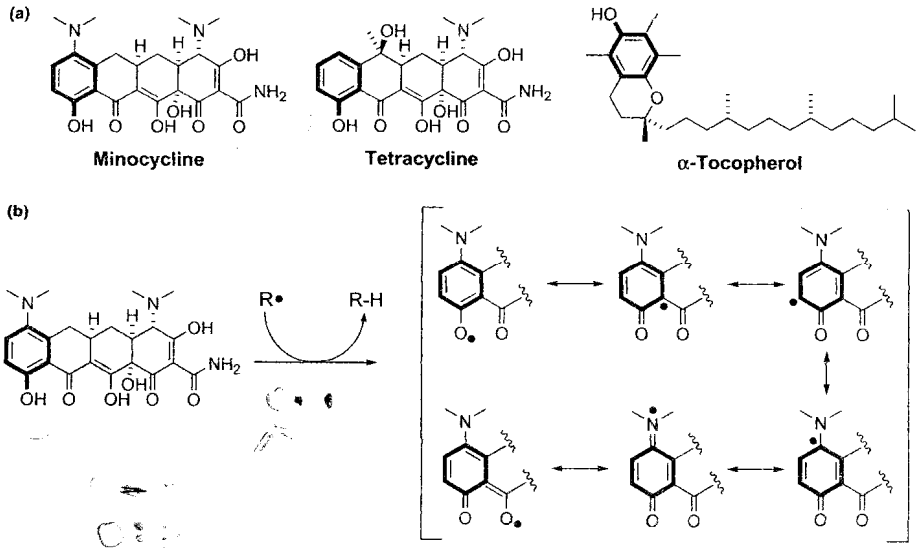


**Figure 3.** Antioxidant mechanism of minocycline.

the apoptosis cells and increase the expression of GSH significantly. GSH is an important antioxidant cell needed for mitochondria to function. GSH is capable of giving protection towards the creation of ROS.[22] The increase in GSH showed the effect of cytoprotection to the cells. GSH is capable of disrupting chain reaction by suppressing dangerous radicals formed during chain reaction. GSH is also capable of preserving the rate of vitamin C in the body due to the ability of GSH in transforming radical ascorbic into ascorbic acid.[1] The decrease in oxidative stress by the application of antioxidant could inhibit the progressivity of a disease. Mitochondria is normally protected from oxidative damage by mitochondrial antioxidant systems, also, mitochondria have antioxidant with low-weight molecules such as alpha-tocopherol and ubiquinol, these molecule are effective for cleaning lipid peroxyl radical and preventing peroxidation of lipid. The defense reaction of cell towards the toxic effect of ROS is the creation of antioxidant, such assuperoxide dismutase (SOD), catalase, gluthatione peroxidase and GSH. Catalase, gluthatione peroxidase enzymes are responsible for the detoxification of $H_2O_2$. Gluthatione peroxidase lowers $H_2O_2$ by oxidizing GSH into GSSG.[23] Some trials on humans by giving high dosage Vitamin E didn't show real improvement, this could be caused by the difficulty of getting through Blood-brain Barrier (BBB). SOD and catalase cannot enter cell membrane so it is less effective for intracellular ROS. Minocycline have the advantage of penetrating through cell membrane and work on mitochondria level, making it very effective as antioxidant.[24] Minocycline have the activity as antioxidant at the level of alpha tocopherol on neuron cell culture. Minocycline works as antioxidant depending on the structure of phenol ring like that of alpha tocopherol (vitamin E).[24] Phenolic antioxidants are effective as antioxidant owing to the free radical chain reaction with phenol ring forming phenol-derived free radical which is relatively stable and non reactive. The main factor that minocycline has a potential effect as phenolic antioxidants are: 1) the level of resonation stabilization from phenol-derived radical; 2) amount and the size of phenol ring substituent that is able to inhibit the reaction with other molecules.

Kraus *et al.*,[24] showed that minocycline was more effective as antioxidant 200–316 times more than tetracycline because the phenol ring on minocycline has dimethylamino substituent which is capable of increasing the resonation stabilization of phenol-derived free radical and had high steric stabilization.

The conclusion of this research was that minocycline in 100 nanomolar proved to be an antioxidant through the lowering of the amount of apoptosis and the increase in expression of GSH.

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