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Anti-Inflammatory Potentials of Silkworm extracts

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ABSTRACT

Silkworm is recently highlighted as a biological resource. Our study aimed to verify the anti-inflammatory action of silkworm-extract, and to understand its applicability for development of oral hygiene goods. Immortalized human oral keratinocytes(IHOK) and immortalized human normal gingival fibroblasts(hTERT-hNOF) were used for this study. To investigate the cytotoxic effect of Silkworm extracts, MTT assays were carried out. To identify anti-inflammatory potentials of Silkworm extracts, it was measured that Reactive oxygen species(ROS) generation and the secretion of inflammatory cytokines(IL-6 and IL-8). All data was carried out by multiple repeats and analyzed by Mann-whitney U test(SPSS ver. 21.0). Silkworm extracts (up to 2%) had little effect on cell viability (≥80% cell growth) in oral epithelial cells and fibroblasts. Next, we identified the antioxidant effect of Silkworm extracts. As results, LPS-induced ROS was significantly reduced by Silkworm extracts in two cell lines. Lastly, inflammatory cytokines(IL-6 and IL-8) was weakly decreased by Silkworm extract in fibroblasts, not in oral epithelial cells. In conclusions, Silkworm extracts have the potentials anti-inflammatory effects, including ROS reduction and the decreased secreted level of inflammatory cytokines in some cells. This study suggests that Silkworm extracts could have the possibilities as preventative and therapeutic products for oral inflammation, including the periodontal diseases.

Keywords: Antioxidants; Cytokines; Inflammation; Periodontitis;Silkworm

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INTRODUCTION

As a representative oral disease, the periodontitis is caused by bacterial activities in the dental plaque accumulated on the surface of teeth, contiguous with supragingival and subgingival plaque. As the major antigens of bacteria in the dental plaque, the lipolysaccharide(LPS) and endotoxin cause an inflammatory response in gingival tissue. And in order to remove this inflammatory response, an immune response is caused by immunocytes within the gingival tissue. In this process of inflammatory-immune response, various inflammatory cytokines and substrate breaker-enzymes such as matrix metalloproteases(MMPs) are revealed, and due to their activities, the extracellular matrix(ECM) inside the gingival tissue is destroyed. If this response gets intensified, the gingival tissue gets severe damage to the inside, which is eventually led to diseases such as gingivitis and periodontitis (Graves *et al*., 2001; Kim, H., 2018). The removal of causative organisms is the most effective method for the prevention and treatment of periodontitis, and one thing that could be preferentially considered is antibiotics. However, the antibiotics cannot be used for a long time as it could give antibiotic resistance to bacteria. To complement this weakness, there have been active researches on antibacterial activity toward the causative organisms of periodontitis by using natural substances that have been used as medicinal herbs or for food in folk remedy or traditional Korean medicine (Przybyłek I*et al*., 2019). As a larva belonging to Bombycidae, the silkworm(*Bombyxmori L.)* has been used for medicinal purpose to treat the malnutrition, tuberculosis, stroke, and diabetes in folk remedy for a long time, and there have been some reports on the antioxidation, antithrombosis, antibiosis, anti-inflammation, and protection of liver (Kim K J *et al*., 2018; Lee, J., 2017). As the importance of silkworm is recently highlighted as a new biological resource, there are active researches that search the biological activity with the use of silkworm, and then separate the actions having effects on the metabolism (Kim I G*et al*., 2018; Lee A*et al*., 2017). Thus, this study aimed to verify the anti-inflammatory action of silkworm extract, and also to understand its applicability to the development of oral hygiene goods for the prevention and treatment aid of periodontal diseases in the future.

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MATERIALS& METHODS

Cell culture

The experiment was carried out by using two kinds of cells such as IHOK cell which was the immortalized oral epithelial cell that was transfected by HPV16 E6/E7, and the immortalized hTERT-hNOF in which human's normal gingival fibroblasts was transfected by hTERT(Illeperuma R P *et al*., 2012; Lee S K *et al*., 2006).Both cells were cultured in F-medium including DMEM and F12 in the proportion of 3:1, and FBS(10%), and penicillin/streptomycin(1%). The environment of cell culture medium maintained the temperature(37℃) and CO2(5%).

Preparation of Silkworm extract

In case of silkworm extract, after mixing the silkworm powder(60g) and ethanol(600ml), it was stored at room temperature for 24 hours, and then filtered with the use of 0.45μm-filter. The filtered extract was concentrated by using a decompression concentrator.

Cytotoxicity test(MTT assay)

To verify the cytotoxic effects of silkworm extract, The MTT was performed with those two kinds of cells(IHOK as oral epithelial cell, and hTERT-hNOFs as gingival fibroblasts. After seeding both IHOKs and hTERT-hNOFs(4×10^3) in the 96 well plates, each concentration(0, 0.5%, 1%, 2%, 4%) of silkworm extract was treated. And 24 hours later, the 1X MTT solution(DuchefaBiochemie) was applied to each well for four hours, and then treated with DMSO. Using the Microplate reader(Bio-rad), the absorbance was measured in the wavelength(540 nm).

Measurement of ROS generation

The formation of reactive oxygen species(ROS) oxygen was measured by using a flow cytometry. This experiment aimed to verify if the treatment of silkworm extract to each cell would have an ability to reduce the formation of active oxygen caused by inflammatory response. First, each concentration of silkworm extract was pretreated to each cell for 30 minutes. After treating an inflammation inducer, LPS(10 ng/ml) for an hour, the H_2DCFDA(Thermo fisher scientific) as fluorescent probe as much as 10uM was applied for 20 minutes for the measurement of active oxygen. After that, the active oxygen was measured by using the flow cytometry(Beckman coulter). As a positive control, the H2O2 was used.

Sandwich Enzyme Linked Immunosorbent Assay(ELISA)

In order to observe changes in inflammatory cytokines(IL-6, IL-8) by the treatment of

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silkworm extract, the Sandwich Enzyme Linked Immunosorbent Assay(ELISA) was performed. After pre-treating each concentration of silkworm extract to the basic culture medium without FBS in each cell for 30 minutes, the inflammation inducer, LPS(10ng/ml) was treated for 24 hours. By collecting the conditioned medium according to each condition, the secretion degree of inflammatory cytokines was observed. After combining the Capture Ab(IL-6 2 μg/ml, IL-8 8 μg/ml) of each cytokine with the 96 well plates, the blocking was treated for an hour. After that, the conditioned medium was applied for two hours. After that, with the use of detection antibody of each cytokine, streptavidin-HRP, and TMB solution(Invitrogen), the antigen-antibody reaction was verified, and the absorbance was measured in the wavelength range(450 nm).

Statistical Analysis

All the data is the results performed many times, and the data values were analyzed by using the Mann-whitney U test(SPSS software version 21.0).

RESULTS AND DISCUSSION

We performed the cytotoxic experiment of silkworm extract in two-kinds of cell(oral epithelial cell, gingival fibroblasts) that would be preferentially used for experiment. In the results, in case of oral epithelial cells, the cells viability was increased by 1.19 times and 1.04 times by the 0.5% and 1% treatment of silkworm extract respectively. In case of 2% and 4% treatment of silkworm extract, the cell viability was decreased by 0.91 times and 0.64 times in Figure 1(Left graph), respectively. In the gingival fibroblasts, the cell viability was decreased in case of 0.5% treatment of silkworm extract, which showed no statistical differences. In case of 1%-4% treatment of silkworm extract, the cell growth was overall increased in Figure 1(Right graph]. Based on such results of cytotoxic experiment, the optimum concentration was selected for the further researches on the anti-inflammatory effects of silkworm extract. Based on the standard of Tests for In Vitro Cytotoxicity (ISO10993-5, 2009), the further research was performed by selecting the concentration(0-2%) of silkworm extract with no cytotoxicity(maintaining the cell viability for 80% or more). The inflammatory response has a correlation with oxidative stress, and the oxidative stress causes the overproduction of reactive oxygen species(ROS)(Hussain T *et al*., 2016). And the oxidative stress could be suppressed by the antioxidant system. Thus, we verified the antioxidant effects of silkworm extract as anti-inflammatory effect. In the results, in case of the experimental group treated with LPS as an inflammation inducer, the formation of ROS was 2.68 times increased in the oral epithelial cell in Figure 2, and it was 1.64

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times increased in the gingival fibroblasts shown in Figure 3,compared with the control group. Moreover, In case when treating an inflammation inducer, LPS(10 ng/ml) after treating each concentration(0-2%) of silkworm extract 30 minutes before to verify the antioxidant effects of silkworm extract, the formation of ROS increased by LPS was statistically-significantly decreased in both oral epithelial cell and gingival fibroblasts by the treatment of silkworm extract, as display in Figure 2 and Figure 3. In other words, this study verified that the silkworm extract would decrease the formation of ROS induced by inflammatory response. Next, this study verified the secretion degree of inflammatory cytokines according to the treatment of silkworm extract to each cell. Among the inflammatory cytokines, the secretion degree of IL-6 and IL-8 as the representative inflammatory cytokines was verified. In the results, contrary to the expectation, the oral keratinocytes did not show significant differences in the secretion degree of inflammatory cytokines according to the treatment of silkworm extracts, as shown in Figure 4a. Compared to the control group, the gingival fibroblasts showed the increased secretion of IL-6 and IL-8 in the experimental group treated with LPS. In case of pretreatment of silkworm extract, the secretion of IL-6 and IL-8 was insignificantly decreased, which did not show statistically significant differences in Figure 4b. ROS shows differences in the severity of damage according to the kinds of cells, and there could be various responses in accordance with such changes (Noh M K*et al*., 2013). According to the concentration of silkworm extract, the epithelial cells and fibroblasts show the contrasting responses like increase and decrease of cell growth respectively, which is regarded as differences in the reactivity according to each kind of cell, caused by differences in the formation of ROS responding to oxidative stress. Also, the secretion rate of inflammatory cytokines showed different responses in accordance with the kinds of cells while the secretion of inflammatory cytokines increased by LPS was decreased in the fibroblasts by the treatment of silkworm extract. In case of flavonoid like Apigenin, similarly to this study, the inflammatory cytokines like IL-6 and TNF-alpha were decreased by the treatment of Apigenin, so that the results of this study are similar to the results of researches verifying the anti-inflammatory effects (Zhang X*et al*., 2014). In other words, this study observed the results showing that the silkworm extract showed the anti-inflammatory effects by responding to oxidative stress like decreasing the secretion of inflammatory cytokines in some cells and decreasing ROS. In the future, there should be more in-depth researches to verify the effects of silkworm extract as a natural anti-inflammatory substance by adding the experiments on microorganisms and changes in the revelation of antioxidant enzyme

such as Glutathione transferases(GST).

CONCLUSION

As a research for verifying the anti-inflammatory effects of silkworm extract in oral, this study verified the decrease of ROS by the silkworm extract in oral epithelial cells and fibroblasts. These showed the antioxidant effect by the decrease of oxidative stress, and also the fibroblasts of the two-kinds of cells showed weakly decrease of inflammatory cytokines such as IL-6 and IL-8. In other words, by putting together such results of this study, the possibility of silkworm extract as natural anti-inflammatory extract could be verified. In the future, it would be necessary to have additional researches including experiments on microorganisms to carry out more in-depth researches on the silkworm extract for development of enhancing oral hygiene and preventing oral inflammation.

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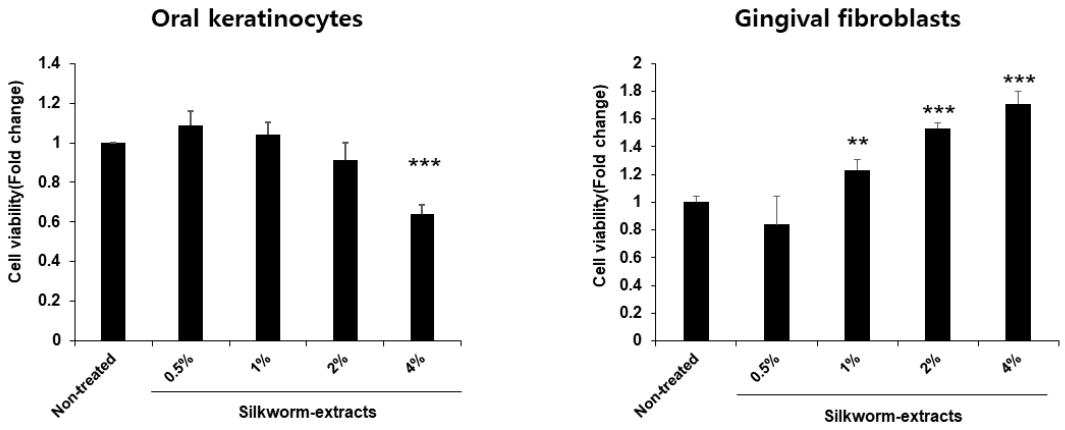

Figure 1: The cytotoxic effects of Silkworm extracts

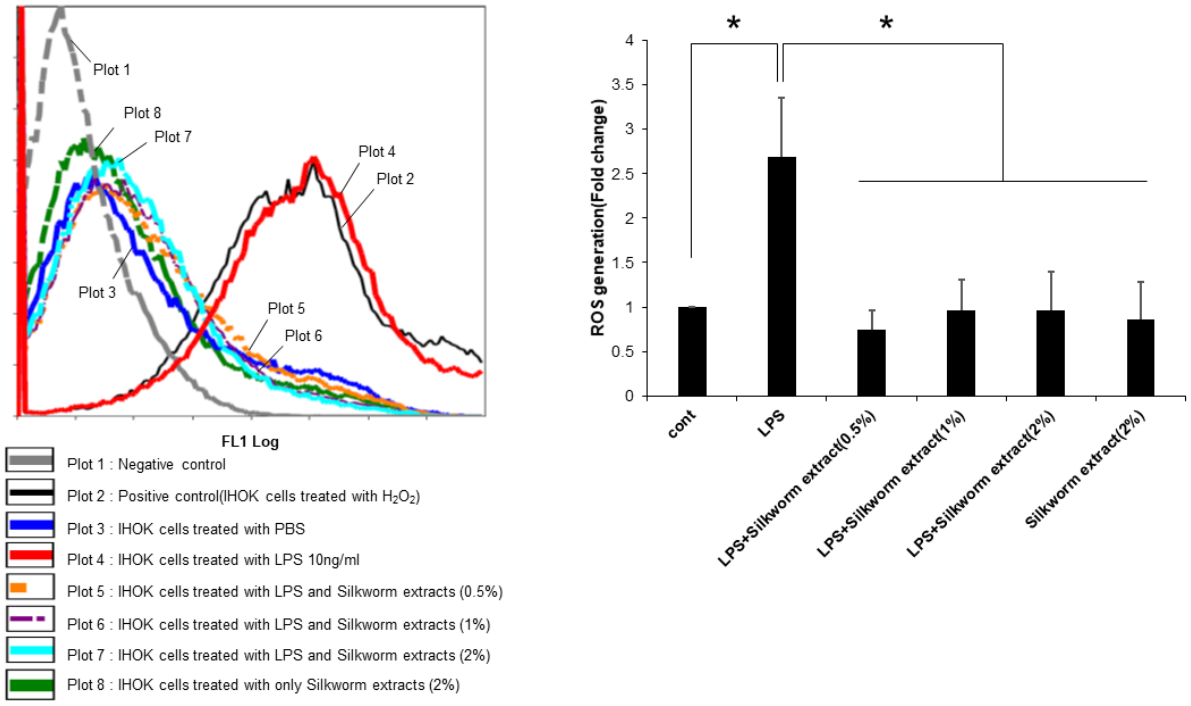

Figure 2: ROS reduction by Silkworm extracts in LPS-treated oral keratinocytes

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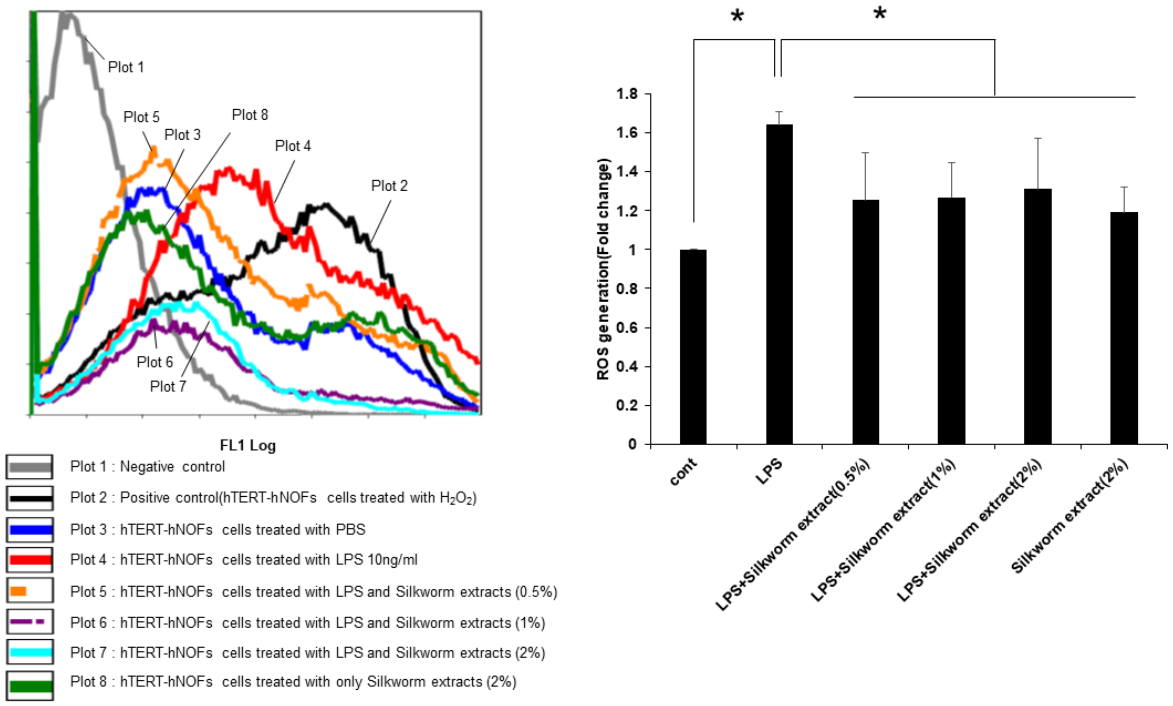

Figure 3: ROS reduction by Silkworm extracts in LPS-treated gingival fibroblasts

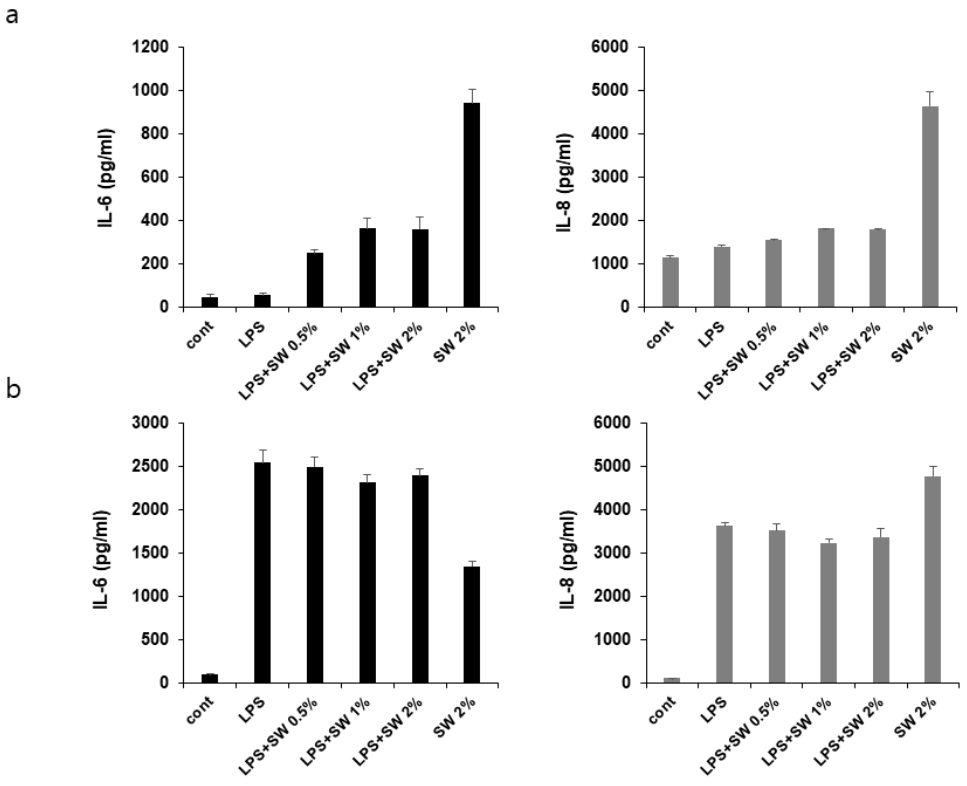

Figure 4: The secreted level of IL-6 and IL-8 by Silkworm extracts in LPS-treated oral keratinocytes and gingival fibroblasts