Effects of liquid ionic silver concentration on caspase-3 and p53mt expressions in oral mucosal epithelium of wistar rats

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Research Report

Effects of liquid ionic silver concentration on caspase-3 and p53mt expressions in oral mucosal epithelium of wistar rats

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ABSTRACT

***Background**: Silver has been used as a medicine, especially oxidized silver, considered as bactericide. Nowadays ionic silver (Ag^+) is also used for making cosmetics, socks, food containers, detergents, sprays, and other products to stop the spread of germs. Unfortunately, ionic silver is assumed to be toxic not only to bacteria, but also to humans and environment. Therefore, it is essential to know the optimum dosage of ionic silver still considered to be safe by examining the effects of ionic silver concentration on cell death through activation of p-53mt expression mutant by caspase 3 in the oral epithelium. **Purpose**: This research aimed to analyze the effects of liquid ionic silver (Ag^+) concentration on caspase-3 and mutant p53 expressions in the oral mucosal epithelium **Methods:** This research was a laboratory experimental study with posttest only design. There were 28 Wistar rats as research samples, divided into four treatment groups, namely KK (with Aquadest), KP 1 (with 5% liquid ionic silver), KP 2 (with 10% liquid ionic silver), and KP 3 (with 15% liquid ionic silver). Each of those rats then was treated with 0.5 ml of liquid ionic silver at certain concentrations determined twice a day per oral for seven days. Next, all Wistar rats were terminated. Their tissue samples then was processed for histopathological and immunohistochemical staining examinations. Subsequently, monoclonal caspase-3 and mutant p53 expressions in each group were evaluated. The data then were tabulated and analyzed statistically. **Results**: Mutant p53 expression was also found in the control group. Besides, the higher the concentration of liquid ionic silver was, the greater the caspase-3 and mutant p53 expressions elevated **Conclusion**: Concentration of liquid ionic silver plays an important role in elevating caspase-3 and mutant p53 expressions.*

***Keywords**: ionic silver (Ag^+), oral epithelium, Caspase-3, mutant p53 expression,*

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INTRODUCTION

Silver has been considered as a phenomenal substance. Silver even has been used to prevent infections caused by microorganisms since six centuries ago. Historically, silver also has been effective in killing almost all types of microorganisms. A research on silver, the third

metal after gold and copper often used in ancient times, was firstly conducted at the beginning of 4000 BC.[1,2] In recent years, silver is not only used for a treatment, but also widely used for sanitation of public facilities, such as baths, toilets, and hand sanitizers, for topical therapies outside the body, for wound bandage, for catheters, as well as for oral antimicrobial medicine.[1,2]

Ionic silver, moreover, can immobilize enzymes of oxygen metabolism in viruses, fungi, bacteria, and single-celled pathogenic microbes. Thus, within minutes the pathogenic microbes can become weak and dead, and then released from the body by the immune system, lymphatic system, and elimination system. Unlike antibiotic therapy generally damaging an animal's enzymes, ionic silver tends to maintain the integrity of tissue cells in the animal. Therefore, ionic silver is considered safe for humans, reptiles, plants, and other living creatures.[3] Liquid ionic silver (Aquasil®) at a concentration of 15ppm even is sold freely as a mouthwash.[4]

Furthermore, ionic silver, according to Murphy and Evans (2012), can be used for wound healing treatment.[5] An in vivo research, nevertheless, argues that ionic silver at concentrations of 10 ppm and 32 ppm cannot cause toxic effects on lungs, liver, brain, circulatory system, and reproductive system.[3] Ionic silver at concentrations of 10 ppm and 32 ppm in the blood vessels is also known not to affect the number of erythrocytes, granulocytes, or granulocytes. Silver actually has already been contained in blood vessels, mostly in the form of ions. However, it is still not known whether the ions circulate through the digestive system or through their attachment to the blood components. Besides, ionic silver is not found in urine. Ionic silver also has no effects on hydrogen peroxide production.

On the other hand, another previous researrch reveals that when ionic silver diffuse into the body through the respiratory system, the ions will bind to lung epithelial cells and macrophages so that the cell function will be limited.[3] Ionic silver, according to Heydarnejad Research (2014), can also generate toxicity as their dose increase into 10 ppm on day 7.[6] Researchers at Herald University (2014) even argue that nano-silver can trigger the formation of free radicals in cells. This condition then leads to changes in the shape and quantity of proteins. Other researchers even say that the excess production of free radicals in cells causes cancer and nerve disorder, such as Alzheimer and Parkinson.[7]

In addition, it is also known that the higher the dose of ionic silver is used, the greater the oxidative stress increases resulting in metabolic disturbances in mitochondria as well as the more reactive oxygen species (ROS) generates affecting cell cycle,[1,2] leading to either

apoptosis (programmed cell death) or necrosis (cell destruction). If a disturbance occurs, in addition to producing apoptosis and necrosis in the cell cycle, cells can also turn into mutant ones.[6] Caspase-3 is the first signaling system in the apoptosis system which later triggers the activation of p53 in cells targeted to begin the apoptosis process. On the other hand, mutant p53 expression is a sign of a function deviation from p53 often found in cancerous tissues, but sometimes also found in non-cancer patients

Since ionic silver may trigger changes in cells from normal to mutant ones and oral mucosal cells can potentially be exposed to ionic silver directly due to oral consumption, ionic silver is assumed to cause local and systemic effects. As a result, this research aimed to reveal the systemic effects of ionic silver at a concentration of 15 ppm diffusing through digestion on mutant p53 and caspase-3 expressions in oral mucosal epithelium.[3] In this research, the oral mucosal epithelial cells of Wistar rats were used since the cells were equivalent to oral mucosal cells of human.[8]

MATERIALS AND METHODS

Twenty-eight Wistar rats (Rattus norvegicus) aged 3-4 months and weighed 200 grams were used as samples in this research. The Wistar rats were also declared healthy on physical examination by veterinarians. Those rats were obtained from the Laboratory of the Department of Biochemical Sciences, Faculty of Medicine, Universitas Airlangga. Next, the rats were divided into four groups, namely 3 treatment groups given liquid ionic silver, and one control group. The first treatment group was given 5 ppm of liquid ionic silver. The second one was given 10 ppm of liquid ionic silver. And, the third group was given 15 ppm of liquid ionic silver. Meanwhile, the control group was given distilled water. Each of those groups was given 0.5 ml of liquid ionic silver at certain concentrations determined twice a day for 7 days.

After seven days, the rats were terminated and their cheek mucosa was cut to 3x4 mm in size using a scalpel no. 15. Next, the mucosal tissues were soaked in a fixative solution, 10% Acetate Buffered Formalin, and then processed using the Autotechnicon® tool. Afterwards, the fixed specimens were hydrated with ethanol, and then clearing process was performed using xylene twice for 60 minutes with the same material and during the same time. Subsequently, media infiltration (embedding process) was performed using paraffin wax (Tissue Prep, Fisher Sci., 56-570 C melting point). The last process was casting or blocking specimens, in which the mucosal epithelium specimens were planted in paraffin. After that, the tissues were

observed using embedding rings, and the paraffin blocks were placed at 4^0 C for 15 minutes to harden. Hematoxylin and eosin staining then was carried out.

Analysis of caspase-3 and mutant p53 expressions on cheek mucosal specimens in the paraffin blocks was performed with immunohistochemical staining technique using anti-Caspase 3 monoclonal antibody of the Wistar rats (Cleaved Caspase-3 (Asp175), SignalStain® and Cell Signaling Technology® (trademarks of Cell Signaling Technology, Inc.), mouse monoclonal antibody, and anti- mutant p53 (p53 (DO-7): sc-47698, Santa Cruz Biotechnology, Inc.). Next, observation was performed using a light microscope with a magnification of 40x. The operational definition of mutant p53 protein and caspase-3 expressions was indicated by the appearance of brown color in the basement membrane of epithelial cells. The examiners were consisted of three anatomists and histologists. The mutant p53 protein and caspase-3 expressions then were calculated in 20 fields of view as suggested by Soini et al (1998) and Pizem and Cor (2003).[8,9] Those three examiners conducted the examination and calculation of samples separately. The results of each calculation were written on the worksheet. The mean value per field of view was calculated and then analyzed statistically.[9,10] The statistical data analysis was carried out with normality and homogeneity tests (One-Sample Kolmogorov-Smirnov Test), followed by one-way ANOVA test with significance of α: 0.05. Correlation and regression analysis (post hoc Tukey's HSD) then was conducted in each group.

RESULTS

The research was conducted on four male Wistar rat groups, namely three groups given silver ions for seven days and one control group. Each group was replicated seven times to see mutant p53 and caspase-3 expressions.

Next, caspase-3 and mutant p53 expressions were examined by staining method as shown in Figures 1 and 2. The mean number of caspase-3 and mutant *p53* expressions increased as the increasing of Ag^+ concentrations as depicted in Figure 1.

The immunohistochemical staining process using *mouse antibody monoclonal* anti-caspase 3 was performed on epithelial cells. Results of the immunohistochemical staining process were illustrated in Figure 3.

After the data of caspase-3 and mutant p53 expressions were obtained, normality analysis was performed using Kolmogorov-Smirnov test. Results of the normality analysis revealed that the data of mutant p53 (p=0.180) and caspase-3 (p=0.743) expressions were

normally distributed. Homogeneity test then was conducted. Results of the homogeneity test showed p value of the group given 5 ppm of liquid ionic silver was 1.0, p=1.0 for the group given 10 ppm of liquid ionic silver, and p=0.122 for the group given 15 ppm of liquid ionic silver. It indicates that the data obtained were homogeneous. Thus, one-way ANOVA and Tukey HSD tests were carried out as shown in Tables 2 and 3.

Results of the one-way ANOVA test on caspase 3 expressions showed α value of 0.05. Results of the Tukey HSD test then indicated p value of 1.0 ($p > 0.05$) at all concentrations. It means that the data obtained were homogeneous. Hence, one-way ANOVAs was conducted as illustrated in Table 1.

Next, post hoc Tuskey HSD test was performed. Results of the post hoc Tuskey HSD revealed that the mean number of caspase-3 expressions in the control group was lower than those in the treatment groups. There were even significant differences in the mean number of caspase-3 expressions between the control group and all treatment groups as well as between the treatment groups ($p < 0.05$). Besides, based on the table above, the greater the concentration of liquid ionic silver was, the higher the mean number of caspase-3 expressions in the mucosal epithelium of Wistar rats was. It means that the number of cells experiencing apoptosis was getting higher.

Subsequently, post hoc Tuskey HSD test was carried out. Results of the post hoc Tuskey HSD showed that the mean number of mutant p53 expressions in the control group was lower than those in the treatment groups. In other words, there were significant difference in the mean number of mutant p53 expressions between the control group and all treatment groups with a p value of 0.000 ($p < 0.05$). However, there was no significant difference in the mean number of mutant p53 expressions between Group II (with 10 ppm of silver ions) and Group III (with 15 ppm of silver ions) with a p value of 0.122 ($p > 0.05$). Besides, based on the table above, the greater the concentration of liquid ionic silver was, the higher the mean number of mutant p53 expression in the mucosal epithelium of Wistar rats.

DISCUSSION

This research was an in vivo study on male Wistar rats given liquid ionic silver. In this research, the mucosal epithelial cells of Wistar rats had been exposed to various concentrations (ppm) of liquid ionic silver for 7 days. This research then used mutant p53 protein expressions

to detect abnormalities of p53 beyond wild type. The mutant p53 is a protective genome in which the p53 test contains two types. First, wild p53 is responsible for maintaining damaged cells and directing them to the apoptotic pathway. Second, mutant p53 is a special protein managing cells towards arrest process in the cell cycle at both G1 / S and G2 / M stages. In other words, the mutant p53 plays a role in maintaining the cell cycle so that cell duplication does not occur. The mutant p53 expression can also be considered as a sign that cells will be arrested in the next cell cycle.[11]

Treatment using liquid ionic silver, moreover, can cause changes in cell morphology, cell viability, metabolic activity, and oxidative stress. Liquid ionic silver which diffuses into cells can reduce ATP cell contents causing mitochondrial damage and elevating reactive oxygen species (ROS) production as doses increase. In mitochondria and cell nuclei, liquid ionic silver can trigger mitochondrial and DNA damage. Next, with the involvement of ROS production triggered by silver ions, there will be a disturbance in the mitochondrial respiratory chain and a disruption of ATP synthesis, eventually causing DNA damage.

Another mechanism of how liquid ionic silver passes into cells is through endocytosis process. In this endocytosis process ionic silver can penetrate to the nucleus and cause DNA damage. As a result, some researchers evaluate the potential use of liquid ionic silver in cancer therapy. However, at certain concentrations in the exposure time of more than seven days, mutant p53 expression can emerge, considered as one of tumor markers.[12]

In this research, mutant p53 expressions also increased as the concentration of liquid ionic silver elevated. Similarly, Zong (2012) argues that the higher the concentration of liquid ionic silver is, the greater the toxic effect will be generated.[10] Changes in the mucosal epithelial cell structure of Wistar rats were highly visible in the stratum spinosum. Stratum spinosum is the thickest layer. In normal cells mutant p53 expression can actually be found. It indicates that the small number of mutant p53 expressions have already existed in Wistar rats.

In this research, in the epithelial cells exposed to 5 ppm of liquid ionic silver, mutant p53 expressions emerged with intact basal structures. In the epithelial cells exposed to 10 ppm of liquid ionic silver, the number of mutant p53 expressions was higher than those cells exposed to 5 ppm of liquid ionic silver with stretching basal structure. And, in the epithelial cells exposed to 15 ppm of liquid ionic silver, the number of mutant p53 expressions was the highest one with loose basal structures passing into the endothelium. Consequently, it can be said that cells exposed to the high concentration of liquid ionic silver will become cancerous. Similarly,

researchers at the University of Herald in 2014 also found the same results related to the appearance of mutant p53 expressions.[7]

Caspase, on the other hand, is an enzyme executing natural cell death called as apoptosis. A large number of caspases play a role in this apoptosis process. The closest caspase triggering the apoptosis process is caspase-3. Thus, caspase 3 was selected to be used as a sign of apoptotic pathways in this research. The results of this research revealed that caspase-3 expressions in each treatment group increased as the concentration of silver ions elevated. Similarly, Alexander (2009) argues that ionic silver liquid therapy is very effective in curing infections in living creatures, in which many microbes are lysis after exposed to liquid ionic silver. It indicates that the higher concentration of liquid ionic silver can lead to greater toxic effects.[1]

In this research, liquid ionic silver was not only effective on microbes, but also caused a change in the epithelial cell structure of Wistar rats. In normal epithelial cells, the number of caspase-3 expressions was small, while in the epithelial cells exposed to 5 ppm of liquid ionic silver caspase-3 expressions emerged with intact basal structures. In the epithelial cells exposed to 10 ppm of liquid ionic silver the number of caspase-3 expressions was higher than those cells exposed to 5 ppm of liquid ionic silver with stretching basal structure. Meanwhile, in the epithelial cells exposed to 15 ppm of liquid ionic silver, the number of caspase-3 expressions was the highest one with loose basal structures passing into the endothelium. Therefore, it can be assumed that cells exposed to the high concentration of liquid ionic silver will become cancerous. Finally, it can be concluded that the increasing of liquid ionic silver concentration is in line with the increasing of mutant p53 and caspase-3 expressions in the buccal mucosa epithelium of Wistar rats.

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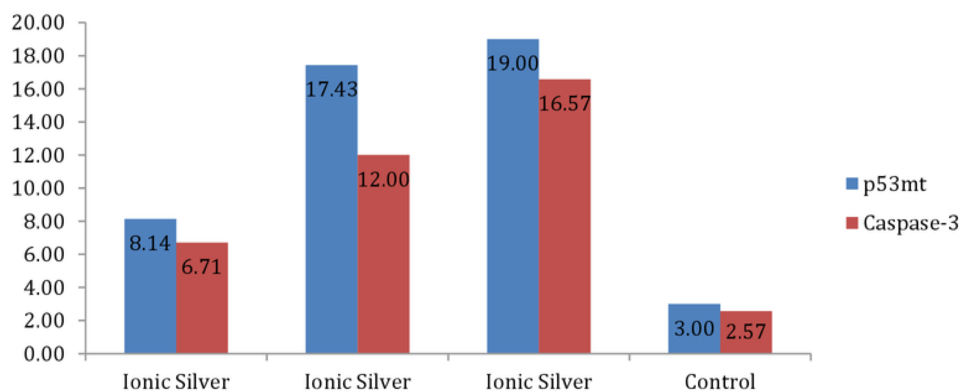

Figure 1. p53mt and caspase-3 expressions

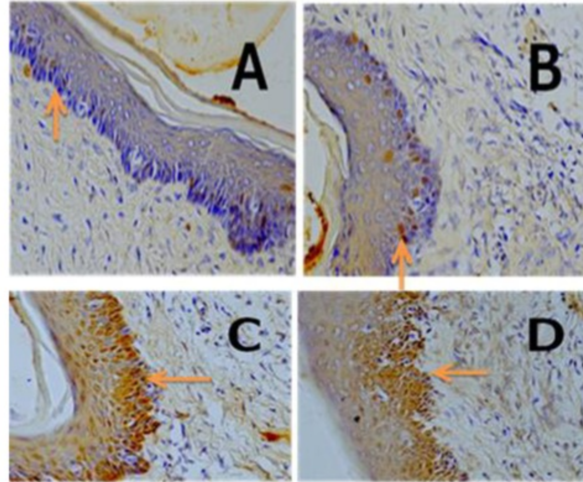

Figure 2. The results of oral immunohistochemistry staining on mutant p53 expressions in oral epithelium exposed to Ag^{+} at varying concentrations with a magnification of 40x (orange arrows showing mutant p53 expressions).

Note: Control group (A), Group given 5 ppm of liquid ionic silver (B), Group given 10 ppm of liquid ionic silver (C), and Group given 15 ppm of liquid ionic silver (D).

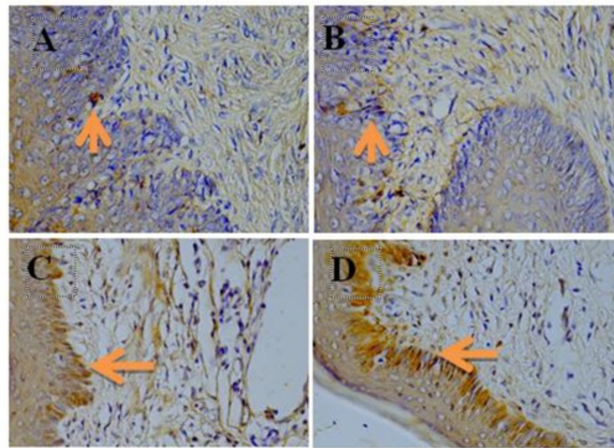

Figure 3. The results of oral immunohistochemistry staining on caspase 3 expressions in oral epithelium exposed to Ag^{+} at varying concentrations with a magnification of 40x (orange arrows showing Caspase 3 expressions)

Note: Control group (A), Group given 5 ppm of liquid ionic silver (B), Group given 10 ppm of liquid ionic silver (C), and Group given 15 ppm of liquid ionic silver (D).

Table 1. Examination results of *Caspase-3* expressions

Groups	Control Group	Treatment Group I (5ppm)	Treatment Group II (10ppm)	Treatment Group III (15ppm)
Control Group		-4.143*	-9.429*	-14.000*
Treatment Group I (5ppm)	-4.143*		-5.286*	-9.857*
Treatment Group II (10ppm)	-9.429*	-5.286*		-4.571*
Treatment Group III (15ppm)	-14.000*	-9.857*	-4.571*	

* = Significant

Table 3. Results of Post Hoc *Tukey HSD* test on mutant p53 expressions

Groups	Control Group	Treatment Group I (5ppm)	Treatment Group II (10ppm)	Treatment Group III (15ppm)
Control Group		-5.143*	-14.429*	-16.000*
Treatment Group I (5ppm)	-5.143*		-9.286*	-10.857*
Treatment Group II (10ppm)	-14.429*	-9.286*		1.571*
Treatment Group III (15ppm)	-16.000*	-10.857*	1.571*	

* = Significant

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