

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

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ABSTRACT

Background: Silver, especially oxidized silver, has been used as a medicine considered to have bactericidal properties. In the present day, ionic silver (Ag^+) is also used in the manufacture of cosmetics, socks, food containers, detergents, sprays and other products to prevent the spread of germs. Unfortunately, ionic silver is assumed to be toxic not only to bacteria, but also to humans and the environment. Therefore, it is essential to identify the optimum dosage of ionic silver considered safe by investigating the effects of ionic silver concentration on cell death through activation of mutant p-53 expression by caspase 3 in the oral epithelium. **Purpose:** This research aimed to analyze the effects of concentrated liquid ionic silver (Ag^+) on caspase-3 and mutant p53 expression in the oral mucosal epithelium. **Methods:** This research constituted a laboratory-based experimental study with posttest-only design. The research subjects consisted of 28 Wistar rats, 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 rat was then treated orally with 0.5ml of liquid ionic silver at fixed concentrations twice a day for seven days. The Wistar rats were then terminated and their tissue samples processed by means of histopathological and immunohistochemical staining examination. The monoclonal Caspase-3 and mutant p53 expressions in each group were evaluated with the data being tabulated and analyzed statistically. **Results:** Mutant p53 expression was also found in the control group. Moreover, the higher the concentration of liquid ionic silver, the greater the elevated Caspase-3 and mutant p53 expressions. **Conclusion:** The 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 long been considered a remarkable substance, having been used to prevent microorganism-related infections as long ago as the fifteenth century. Historically, silver has also been effective in destroying almost all strains of microorganisms. Research into silver, first conducted at the beginning of 4000 BC,^{1,2} confirmed it to be the third most widely used metal after gold and copper in ancient times. In recent years, silver has not only been widely used in a variety of medical procedures, but also for sanitation of public facilities such as baths, toilets, hand sanitizers,

topical therapies outside the body, wound bandages, catheters as well as for oral antimicrobial medicine.^{1,2}

Ionic silver can immobilize enzymes during oxygen metabolism in viruses, fungi, bacteria and single-celled pathogenic microbes. Within minutes, the pathogenic microbes can weaken and die before being removed from the body by the immune, lymphatic and elimination systems. Unlike antibiotic therapy, which is generally harmful to animal enzymes, ionic silver tends to maintain the integrity of tissue cells. Therefore, ionic silver is considered safe for humans, reptiles, plants and other living creatures.³ Liquid

ionic silver (Aquasil®) at a concentration of 15ppm is even widely marketed as a mouthwash.

According to Murphy and Evans (2012), ionic silver can be employed during wound healing treatment.⁴ However, *in vivo* research suggests that ionic silver at concentrations of 10 ppm and 32 ppm cannot cause toxic effects to the 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 to leave the number of erythrocytes, granulocytes or granulocytes unaffected. Silver is actually contained in the blood vessels, largely in the form of ions. However, it remains unclear whether the ions circulate through the digestive system or through their attachment to blood components. Moreover, ionic silver is not found in urine and has no effects on hydrogen peroxide production.

On the other hand, other previous research revealed that when ionic silver diffuses into the body through the respiratory system, the ions will bind to pulmonary epithelial cells and macrophages with the result that cell function will be limited.³ According to research conducted by Heydarnejad (2014), ionic silver also caused toxicity when its dose was increased to 10 ppm on day 7.⁵ Researchers at Herald University (2014), even argue that nano-silver can trigger the formation of free radicals in cells, a condition subsequently leading to changes in the shape and quantity of proteins. Other researchers even posit that the excess production of free radicals in cells causes cancer and nerve disorders, such as Alzheimer's disease and Parkinson's disease.⁶

It has also been accepted that the higher the dose of ionic silver administered, the more oxidative stress increases resulting in metabolic disturbances in mitochondria. In addition, the more reactive oxygen species (ROS) generated affects the cell cycle,^{1,2} leading to either apoptosis (programmed cell death) or necrosis (cell destruction). If such disruption occurs, in addition to producing apoptosis and necrosis in the cell cycle, cells can also undergo mutation.⁵ Caspase-3 is the first signaling protein in the apoptosis system which subsequently triggers the activation of p53 in cells targeted to initiate the apoptosis process. Conversely, mutant p53 expression is a symptom of function deviation from p53 often found in cancerous tissues, but sometimes also encountered in non-cancer patients.

Since ionic silver may precipitate changes in cells, turning them from normal to mutant, oral mucosal cells can be directly exposed to ionic silver 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 diffused through digestion in mutant p53 and caspase-3 expressions in the oral mucosal epithelium.³ In this research, the oral mucosal epithelial cells of Wistar rats were used since these were considered equivalent to those of humans.⁷

MATERIALS AND METHODS

Twenty-eight Wistar rats (*Rattus norvegicus*) aged 3-4 months, weighing 200 grams and pronounced healthy by veterinarians, were used as samples in this research. These subjects were obtained from the Department of Biochemical Sciences Laboratory, Faculty of Medicine, Universitas Airlangga. They were subsequently divided into four groups, namely three treatment groups given liquid ionic silver and a control group. The first treatment group was given 5 ppm of liquid ionic silver. The second 10 ppm of liquid ionic silver and, the third 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 determined concentrations twice a day for seven days.

After seven days, the subjects were sacrificed and their cheek mucosa were cut to 3x4 mm in size using a no.15 scalpel. The mucosal tissues were soaked in a fixative solution, 10% Acetate Buffered Formalin, and then processed using an Autotechnicon® tool. The fixed specimens were subsequently hydrated with ethanol, before a 60-minute clearing process was performed twice with the same material and for the same duration. Subsequently, media infiltration (embedding process) was performed using paraffin wax (Tissue Prep, Fisher Sci., 56-570 C melting point). The last procedure was that of casting or blocking specimens whereby the mucosal epithelium specimens were planted in paraffin. Thereafter, the tissues were observed using embedding rings and the paraffin blocks were stored at 4⁰ C for 15 minutes to harden. Hematoxylin and eosin staining were then carried out.

An analysis of caspase-3 and mutant p53 expressions in cheek mucosal specimens in paraffin blocks was performed by means of an immunohistochemical staining technique using anti-Caspase 3 monoclonal antibodies of the Wistar rats (Cleaved Caspase-3 (Asp175), SignalStain® and Cell Signaling Technology® (trademarks of Cell Signaling Technology, Inc.), mouse monoclonal antibodies, and anti- mutant p53 (p53 (DO-7): sc-47698, Santa Cruz Biotechnology, Inc.). Observation was subsequently performed using a light microscope at 40x magnification. The operational definition of mutant p53 protein and caspase-3 expressions was indicated by the presence of brown color in the basement membrane of the epithelial cells. The examiners consisted of a trio of anatomists and histologists. The mutant p53 protein and caspase-3 expressions were then calculated in 20 fields of view as recommended by Pizem and Cor (2003).⁸ The three examiners conducted the examination and calculation of samples separately, writing the results of each calculation on the worksheet. The mean value per field of view was calculated before being analyzed statistically^{8,9} with normality and homogeneity tests (One-Sample Kolmogorov-Smirnov Test), followed by a One-way ANOVA test with significance of α : 0.05. Correlation and regression analysis (post hoc Tukey's HSD) was then conducted on each group.

RESULTS

The research was conducted on four groups of male Wistar rats, three groups being given silver ions for seven days and one control group. The procedure was replicated seven times with each group times to identify mutant p53 and caspase-3 expressions. Caspase-3 and mutant p53 expressions were then examined by staining method as shown in Figures 1 and 2. The mean number of caspase-3 and mutant p53 expressions rose with the increase in Ag⁺ concentrations as depicted in Figure 3. The immunohistochemical staining process using *mouse antibody monoclonal anti-caspase 3* was performed on epithelial cells, the results of which are illustrated in Figure 2.

After the data of caspase-3 and mutant p53 expressions had been collated, normality analysis was performed by means of a Kolmogorov-Smirnov test. The results of the normality analysis revealed that the data of mutant p53 (p=0.180) and caspase-3 (p=0.743) expressions was normally distributed. A homogeneity test then was conducted, the results of which showed the p value of the group given 5 ppm of liquid ionic silver to be 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. This indicated that the data obtained was homogeneous. Thus, one-way ANOVA and Tukey HSD tests were carried out, the results of which are shown in Tables 1 and 2.

The results of the one-way ANOVA test on caspase 3 expressions showed α value of 0.05, while those of the

Tukey HSD test then indicated a p value of 1.0 (p> 0.05) at all concentrations. This means that the data obtained was homogeneous. Consequently, a One-way ANOVAs was conducted as illustrated in Table 1.

At the next stage, a post hoc Tukey HSD test was performed whose results indicated 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 numbers of the caspase-3 expressions between the control group and all treatment groups as well as between the treatment groups (p<0.05). Moreover, based on the contents of the above table, the greater the concentration of liquid ionic silver, the higher

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

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

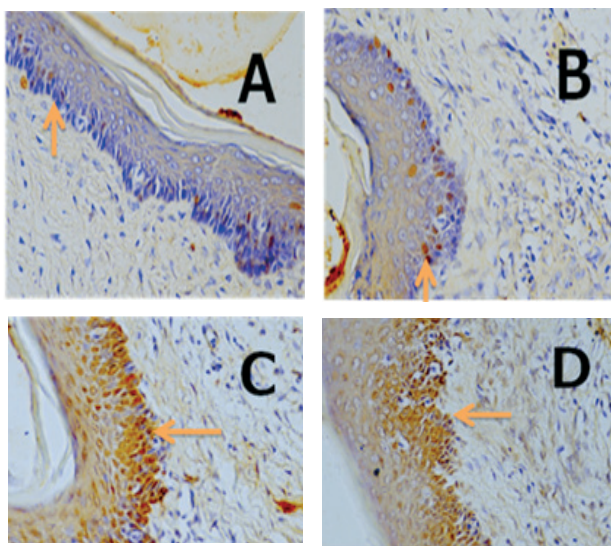


Figure 1. The results of oral immunohistochemistry staining on mutant p53 expression in oral epithelium exposed to Ag⁺ at varying concentrations at a magnification of 40x (orange arrows showing mutant p53 expression).

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).

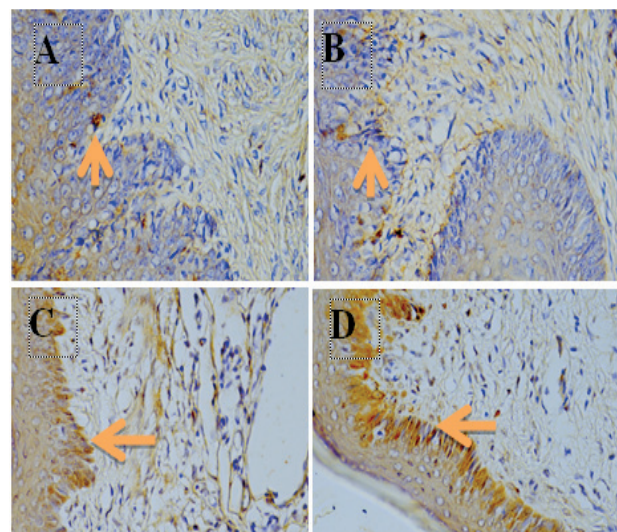


Figure 2. The results of oral immunohistochemistry staining on Caspase 3 expression in oral epithelium exposed to Ag⁺ at varying concentrations with a magnification of 40x (orange arrows showing Caspase 3 expressions)

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).

the mean number of caspase-3 expressions in the mucosal epithelium of Wistar rats. This confirms that the number of cells experiencing apoptosis was increasing.

A post hoc Tukey HSD test was subsequently, carried out, the results of which 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 was a 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 ($p > 0.05$). Furthermore, based on the contents of the table above, the greater the concentration of liquid ionic silver, the higher the mean number of mutant p53 expression in the mucosal epithelium of Wistar rats.

DISCUSSION

This research represented an in vivo study during which the mucosal epithelial cells of Wistar rats were exposed to various concentrations (ppm) of liquid ionic silver for seven days. Mutant p53 protein expressions were then used to detect abnormalities of wild-type p53. Mutant p53 is a protective genome which the p53 test confirms the existence of two types. First, wild-type p53 is responsible for supporting damaged cells and directing them to the apoptotic pathway. Second, mutant p53 is a special protein managing cells in the arrest phase of the cell cycle at both the G1 / S and G2 / M stages. In other words, mutant p53 plays a role in maintaining the cell cycle with the result that cell duplication does not occur. The expression of mutant p53 can also be considered as a sign that cells will be arrested in the subsequent cell cycle.¹⁰

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. With the involvement of ROS production initiated by silver ions, disruption to both the mitochondrial respiratory chain and ATP synthesis will occur, eventually causing damage to DNA.

Another process supporting the passage of liquid ionic silver into cells is that of endocytosis through which silver can penetrate the nucleus and harm DNA. As a result, a number of researchers have evaluated the potential use of liquid ionic silver in cancer therapy. However, at certain concentrations and an exposure time in excess of seven days, mutant p53 expression can emerge, being considered a tumor marker.^{1,2}

In this research, mutant p53 expressions also increased as the concentration of liquid ionic silver intensified. Similarly, Wong (2011) argues that the higher the concentration of liquid ionic silver, the greater the toxic effect.¹¹ Changes to the mucosal epithelial cell structure of Wistar rats were highly visible in the stratum spinosum which is the thickest layer. Mutant p53 expression can actually be found in normal cells, indicating that a small number already existed in Wistar rats.

This research found that, 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 in those exposed to 5 ppm of liquid ionic silver with a stretching basal structure. Furthermore, in the epithelial cells exposed to 15 ppm of liquid ionic silver, the number of mutant p53 expressions was highest 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. Researchers at the University of Herald in 2014 also obtained the same results related to the appearance of mutant p53 expressions.⁶

On the other hand, Caspase is an enzyme inducing natural cell death known as apoptosis. Large quantities of caspase play a role in this process. The closest caspase triggering the apoptosis process is caspase-3 which was selected as a sign of apoptotic pathways in this research whose results revealed that caspase-3 expressions in each

Table 2. Results of Post Hoc Tukey HSD test on mutant p53 expression

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

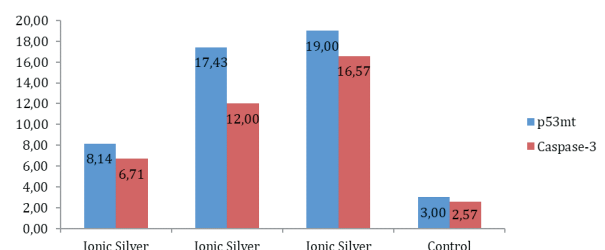


Figure 3. p53mt and Caspase-3 expression

treatment group increased as the concentration of silver ions rose. Similarly, Alexander (2009) argues that ionic silver liquid therapy constitutes an extremely effective cure for infection in living creatures, since considerable numbers of microbes experience lysis after exposure to it. This suggests that higher concentration of liquid ionic silver can produce greater toxic effects.¹

In this research, liquid ionic silver was not only found to be effective on microbes, but also induced a change in the epithelial cell structure of Wistar rats. In normal epithelial cells, the number of caspase-3 expressions was limited, 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 exposed to 5 ppm of liquid ionic silver with a 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 with loose basal structures passing into the endothelium. It can, therefore, be assumed that cells exposed to the high concentration of liquid ionic silver will become cancerous.⁶ Finally, it can be concluded that the increase in liquid ionic silver concentration is in line with that of mutant p53 and caspase-3 expressions in the buccal mucosa epithelium of Wistar rats.

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