The Effects of Ag^+ Ion in Osteoblast Cell Proliferation (*In Vitro Study*)

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Abstract

Silver ion had been known have an antibacterial power. Silver ion produce antimicrobial activity by bactericide and bacteriostatic power to support healing process. The speed of healing process is measured with the expression of Bone Morphogenetic Protein (BMP)-2. BMP-2 is expressed by active osteoblast and could start the bone healing process. This study is a in vitro study with post-test only control group design.

The sample of this study is human osteoblast cell culture with 6 amounts of repetition in 6 group. The assessment was done with MTT Assay method. The mean osteoblast cell was found in 1.25 ppm concentration. There is a significant difference between groups with p-value 0.000. Increasing silver ion dose in nanoparticle can inhibit proliferation and differentiation of osteoblast cell. Silver ion with fivefold concentration compared to the concentration used for osteoblast proliferation is proven effective in killing bacteria.

Silver ion property as antimicrobial is effective in higher dose, but in 1.25 ppm concentration, it could increase osteoblast cell proliferation. Silver ion with certain dose could trigger proliferation of osteoblast cell, but silver could be toxic if it's exposed to human body so further study is needed in dosage safety but effective concentration.

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Introduction

Silver (Ag +) is an ion that can be used as an antibacterial. Silver's antibacterial benefits have been known and used for a long time, even before antibiotics were discovered. In recent years, there have been many studies showing the potential of silver to kill multi-drug resistant bacteria using a combination strategy thereby increasing the efficiency of the drug.[1] Several studies have evaluated the use of silver ions in some bone infections such as osteomyelitis. Silver ion produces antimicrobial activity, with bactericidal and bacteriostatic activity.[2]

The use of silver in ion / colloid preparations has a stronger antibacterial activity than in nanoparticle preparations even though with the same mechanism of action.[3] Another study also found that examination using an electron microscope showed that both types of bacterial cells (gram positive and negative) which were treated with silver ions experienced lysis and cytoplasmic leakage.[4] For this reason, silver has been shown to have a role in the healing process. The acceleration of the healing process was measured by increasing the expression of Bone Morphogenetic Protein (BMP) -2. BMP-2 expressed by active osteoblasts can represent the initial process of bone healing.[5]

Bone is a part of the endoskeleton that is very dynamic which can trigger the process of bone formation and resorption. Bone is a mineralized connective tissue that exhibits four

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types of cells: osteoblasts, bone lining cells, osteocytes, and osteoclasts. Bones provide important functions in the body, such as the motor system, support and protection of the organs in them, storage of calcium and phosphate, and storage of bone marrow. Bone is a very dynamic organ that is constantly being absorbed by osteoclasts. One of the main keys in the process of bone regeneration is osteoblasts, which originate from mesenchymal stem cells and produce collagen and hydroxyapatite crystals, forming the extracellular bone matrix. Other cells that are also involved in the bone regeneration process are osteoclasts. Osteoclasts originate from hematopoietic stem cells and are able to dissolve organic and inorganic compounds from the bone matrix by releasing hydrogen ions and proteases, such as cathepsin K. Osteoclasts trigger demineralization in bone, in contrast to osteoblasts. Activated mature osteoclasts appear as polarized multinucleated cells. If there is an imbalance in the activation of the number of osteoblasts and osteoclasts, bone damage will occur if there are more osteoclasts than osteoblasts. Therefore, there is a need for therapy that can trigger osteoblast proliferation in bone regeneration.

To date, no studies have proven whether silver ion water is effective in bone healing in some cases of mandibular defects. Based on this background, studies are needed that show Ag^+ ions play a role in the induction of osteoblasts.

LITERATURE REVIEW

Osteoblast cell

In the process of intramembranous ossification, the mesenchymal cells differentiate directly into ostoblasts and produce a surrounding osteoid matrix, which then calcifies immediately. The enzyme alkaline phosphatase (ALP) plays a role in the mineralization process through crystallization of calcium salts. The bone matrix produced by early osteoblasts is referred to as woven bone. It appears as an irregular collagen structure due to the rapid production of the matrix from osteoblasts.

Osteocalcin (OCN) is an osteoblast-specific protein, containing three γ-caboxiglutamic acid residues which have calcium binding properties. OCN is often associated with the growth of hydroxyapatite crystals. The initiation of OCN formation occurred on the 9th and 11th days, and peaked its expression on the 15th day. The amount of OCN in plasma is influenced by the formation of new bone, and its concentration can be an indicator of osteoblast activity.

Usage and potential of silver as antimicrobial agent

Silver have many usage, including antimicrobial activity. Silver are known to be biologically active when dispersed in ionic form, when dissolved in water. For a long time, silver has been consistently used to limit the spread of disease through its daily use. The use of silver can be found in silver coated medical devices, implants, syringes, medical instruments coated with silver nanoparticles, vascular portal devices, orthopedic implants designed to release silver ions continuously, biliary duct implant wires coated with silver to inhibit biofilm formation, topical gels for local skin infections, in wound dressings, silver hydrosol to reduce the risk of infection in dental procedures, denture materials, deodorants, and many more.[1]

There are three mechanisms of action of silver ion which are known in their role as antimicrobial. First, silver cations can form porous and puncture bacterial cell walls by interacting with peptidoglycan components. The second mechanism, silver ions can enter bacterial cells, both by inhibiting cellular respiration and interfering with the metabolic pathways of the bacterial cell, resulting in the formation of reactive oxygen species (ROS). The last mechanism, when silver is in the cell, this compound is able to disrupt DNA and its replication cycle.[1,6] The increase in bacterial resistance to most antibiotics in recent years has been the main reason for reassessing the use of silver as a treatment for bacterial infections, including research using colloidal silver and antibiotics.[1] The potential of silver compounds as antimicrobials requires an understanding of its pharmacokinetics and pharmacodynamics. Pharmacokinetic analysis is carried out routinely on drugs and chemical compounds before they are used for medical purposes.[7]

Pharmacokinetics of this ion is dose-dependent and exposure route-dependent. The half-life (T1 / 2) of the silver compound is 99 hours (1 mg/kg) for intravenous exposure and 30 hours (10 mg / kg) for oral exposure in mice. Lee in his study evaluated the pharmacokinetics of silver compounds on a single injection dose (0.5 mg/kg) and found a half-life of 11.7 to 16.3 days. This suggests that the pharmacokinetics of silver compounds are also animal species-

dependent.[7,8] This is associated with longer transit times in the systemic circulation of larger animals than in smaller mice.

Xue conducted research on silver compound agglomerates in male and female rats after intravenous injection. Blood silver levels peaked at 10 minutes after administration in both samples. The half-life was found to be longer in female species, namely 29.9 hours compared to 15.6 hours. The area under curve (AUC), mean residence time (MRT), and volume distribution (Vd) were found to be higher in females, but the clearance of silver compounds was found to be lower in females. This indicates a sex-dependent condition of the pharmacokinetic profile of silver.[9]

Several studies have evaluated the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of silver ion. The best antibacterial optimal concentration of metal ions requires at least more than 90% antibacterial rate and more than 80% cell proliferation rate at L929. Based on the bactericidal rate test conducted by Ning in 2016, the MIC of silver ions against *S. aureus* and *E. coli* was found to be 10-6 - 10-7 M where silver ions have certain antimicrobial activity without being accompanied by cytotoxicity. At this level, the proliferation rate of L929 cells (fibroblasts) increases with decreasing silver ion concentration. Meanwhile, in the evaluation of minimum bactericidal concentration (MBC) it was found that a silver ion concentration of 0.25 μM could kill 99% of *S. aureus* and *E. coli* within 24 hours after exposure. In other studies, Agarwal has reported that silver nitrate solutions with concentrations of 0.05 mM to 5 mM can kill up to 90% of bacteria.[10,11]

Pharmacodynamic of Silver

Pharmacodynamic profile of a substance includes absorption, distribution, metabolism, and elimination of a drug or compound. The absorption of silver compounds was found to vary in different groups of species. Research conducted by Park regarding AUCoral / AUCiv showed the bioavailability of silver nanoparticles of 1.2% and 4.2% at a dose of 10 mg/kg and 1 mg/kg, indicating low bioavailability of silver compounds.[12] After oral exposure to silver ion (9 mg/kg) and silver nanoparticles (90 mg/kg) in mice, silver content was found to be 7-10 times higher in the silver ion group than in silver nanoparticles, indicating a higher silver uptake in the silver ion group. administration in the form of ions. These data indicate that after oral silver nanoparticles exposure, only a small portion of silver particles can be absorbed by the body.[7,12,13]

The distribution of silver mainly occurs in the liver as the primary organ, followed by the spleen and kidneys, either on oral, intravenous, subcutaneous or per inhalation exposure. Silver deposition in the liver has been found in several types of cells, including Kupffer cells, hepatocytes, and sinusoid endothelial cells.[7,14] In the kidney, silver deposition is found in all parts of the kidney, including the cortex, medulla, inner medulla, and glomerular cortex. Sex differences in silver accumulation were also found with repeated exposure to silver ions in mice, with a twofold increase in females versus males. A high silver distribution was also found in the spleen after repeated exposure to intravenous silver ions.[7] Other studies have also shown that silver ions are able to distribute to the brain, regardless of the route of exposure. van der Zande found 90% detectable silver in brain tissue after 28 days of oral exposure from silver, and persisted up to 2 months post exposure.[13]

Post-oral silver metabolism is aided by the acidic pH of gastric juices, accelerating the dissolution of the oxidation of nanoparticles and salt compounds into silver ions. The dissolved silver ions then precipitate with chlorine ions so that the concentration is very low. The maximum concentration of dissolved silver in stomach acid is 0.51 mg/L. Higher concentrations result in AgCl bonds which limit the increase in silver bioavailability. If the total silver dose is lower than the number above, AgCl will not be formed as a metabolic compound.[7]

Silver elimination after oral exposure for 28 days showed a low level of urine (<0.1%), while a greater amount was found in feces (49% of the daily dose of silver ion).[7,8]

Silver toxicity

The toxicity of the silver ion is dose-dependent. Until now, there has been no information regarding toxicity in humans related to acute exposure to silver ions and silver nanoparticles either through oral or topical routes that can be identified.[15] Research conducted by Munger regarding repeated exposure to silver ion found that at doses of 2.1-6.8 μg/kg/day, there was no significant change in metabolic, hematological, and urinalysis tests. The results of physical and radiological examinations of multiple

organ also did not show significant result.[16] Another study by Kuehl on silver cytotoxicity showed no difference between the silver exposed and unexposed groups of mice. Viable leukocytes were also evaluated post exposure, and there was a slightly lower leucocyte viability on the first day post exposure and gradually increased to the equivalent of the unexposed group 9 days post exposure.[17] However, when giving silver compounds in doses that exceed the daily dose for a long time, severe cytotoxicity can occur.[18]

One of the most worrying toxicities of silver compounds is the formation of reactive oxygen species (ROS). In his research, Barras explained that silver is a non-redox active metal that cannot directly produce ROS. However, several studies have shown increased production of ROS on exposure to silver ions. There are several possibilities that silver ions play a role in the production of ROS, including (1) perturbation of the electron transfer chain; (2) chemical compound Fenton resulting from the destabilization of Fe-S which causes the release of iron; and (3) inhibition of the anti-ROS role by the formation of thiol-silver bonds.[2]

To date, there are no known studies evaluating the neurotoxicity, reproductive and developmental toxicity and potential immunotoxicity of silver in humans by any route of administration. Regarding the carcinogenic potential, until now no carcinogenic activity of silver has been found in humans. The United States Environmental Protection Agency (USEPA) has explained that silver compounds are not classified as carcinogenic in humans.[15] Meanwhile, several studies have found no acute side effects resulting from silver ingestion. Oral administration of silver nanoparticles is not toxic to mice at an acute dose of up to 5000 mg / kgbb. The study also found no deaths or signs of toxicity during the 14-day post-therapy observation period. In addition, there were no significant differences in haematological parameters.[19]

Repeated exposure to silver nanoparticles at a dose of 5-100 mg / kgbb per day for 5 days showed an increase in ROS concentrations with increasing doses given. Significant increases in levels can also be seen in the liver alanine aminotransferase (ALT) enzymes, aspartate aminotransferase (AST), and alkaline phosphatase (ALP). Histopathological damage can be seen on liver tissue images at doses of 25 mg / kgbb per day or more.[20] Evidence suggests that silver ions can cross the blood-brain barrier and are neurotoxic. Silver nanoparticles (9 mg / kgbb per day) and silver ions (9 mg / kgbb per day) increase dopamine concentrations in the brain.[21]

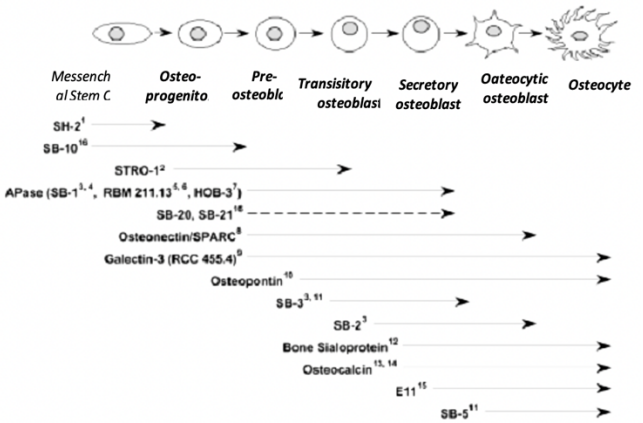

Figure 1. Comprehensive description of osteogenic cell lineages.

Materials and methods

This study is an experimental laboratory in vitro with a post test only control group design research design on human osteoblast cell culture. The research was conducted at the Pharmacology Laboratory of the Faculty of Medicine, and the Research Center of the Faculty of Dental Medicine, Universitas Airlangga. The total sample used was 24 samples divided into 8 groups (cell control group, media control, 10 ppm, 8 ppm, 7 ppm, 5 ppm, 2.5 ppm and 1.25 ppm doses).

Osteoblast cell culture was taken from human osteoblast cells then cultured in crayon medium (-86) for ± 5 days then placed in a flesch and then put in 1 ml trypsy edta disk convluent for 1 week in an incubator. Then the cells were centrifuged in 96 wheels and the sediment was added with 1 mm of media. The cell count (per-wheel 5000-10000 cells)is then incubated for 1 hour, and then treated with silver similiar to other groups for 24 hours. Each group was replicated for 6 hours and then treated with dimethyl sulfoxide (DMSO). Incubated for10 minutes and then resaercher performed the MTT Assay test to see the living osteoblasts.

The data collected from the research results were then analyzed with normality test

such as One Sample Kolmogorov Smirnov and Probability Plot, homogeneity test was done with Levene's test, comparative test was done with Kruskal Wallis Test.

Results

Based on the results of the study which can be seen in Figure 2, there was found that the highest number of osteoblasts was at a concentration of 1.25 ppm. At this concentration, showed a higher average cell when compared to the control media. Table 2 shows the results of the analysis of the difference between groups, based on this test, it was found that there were significant differences between groups with a p-value of 0.000, which means p-value ≤ 0.05.
The results are shown in Table 1 and Table 2.

Replication	Control media	Control cell	10 ppm	8 ppm	7 ppm	5 ppm	2.5 ppm	1.25 ppm
1	0.188	0.454	0.207	0.257	0.147	0.194	0.282	0.273
2	0.192	0.493	0.291	0.183	0.153	0.310	0.303	0.336
3	0.213	0.431	0.205	0.200	0.216	0.324	0.310	0.218
4	0.217	0.462	0.193	0.282	0.166	0.166	0.275	0.253
5	0.333	0.419	0.179	0.278	0.182	0.262	0.224	0.336
6	0.331	0.426	0.226	0.177	0.219	0.197	0.292	0.338
Total	1.474	2.685	1.301	1.377	1.083	1.453	1.686	1.784
Mean	0.246	0.448	0.217	0.230	0.181	0.242	0.281	0.297

Table 1. Osteoblast cell count.

Group	Mean	*p-value*
Media Control	0.246	0.000*
Cell control	0.448	
10 ppm	0.217	
8 ppm	0.23	
7 ppm	0.18	
5 ppm	0.242	
2.5 ppm	0.281	
1.25 ppm	0.292	

Table 2. Statistical test result. Note: *p-value<0.05.

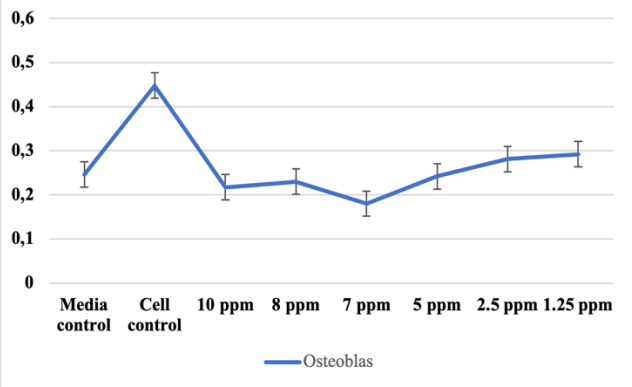

Figure 2. Mean of osteoblast cell count per group.

Discussion

Silver ion has potential as an antibacterial addition, based on several studies showing that silver ion shows changes in the levels of growth markers such as OCN (Osteocalcin), ALP (Alkaline Phospatase) which can trigger regeneration. Regeneration occurs preceded by the proliferation of osteoblasts. In this study, it was shown that there was an increase in the number of osteoblast cells at a concentration of 1.25 ppm. Giving silver ion has a dose of toxicity that can trigger a decrease in the number of osteoblasts due to inhibition of proliferation and differentiation of osteoblasts.[22]

Current research has shown that silver ion has a dose of toxicity that can cause cellular damage. Increasing the dose of silver ion in nanoparticles has been shown to inhibit the proliferation and differentiation of osteoblasts.[23] Silver ion with a concentration of 5 times greater than the concentration used for osteoblast proliferation has been shown to kill bacteria more effectively. The antimicrobial properties of the silver ion have been shown to be effective in larger concentrations. However, until now there have been no studies showing that the concentration is suitable for osteoblast cell viability.[24,25]

Conclusions

Based on this research, silver ion besides having potential as antibacterial, also has potential in the tissue regeneration process. This research has proven that silver ion water in certain doses can trigger osteoblast cell proliferation. However, silver can be toxic in the body, therefore further research is needed regarding safe and effective doses of silver ions in the body.

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Declaration of Interest

The authors report no conflict of interest.

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