Reduced cytotoxicity of silver ions to mammalian cells at high concentration due to the formation of silver chloride

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Abstract

Silver-containing antimicrobial agents are used in various medical products. However, their toxicity to mammalian cells has not been sufficiently evaluated. Numerous studies have unveiled evidence of significant antimicrobial properties associated with Ag ions. In cell culture media or human body fluids, the free Ag^{+} has rich opportunities to complex with Cl^{-}. Surprisingly, studies on the toxicity of solid form $AgCl_{(s)}$ to mammalian cells are quite limited. In this study, we evaluated the cytotoxicity of Ag ions and silver chloride colloids on red blood cells and human mesenchymal stem cells (hMSCs). The adverse effects of silver chloride on red blood cells and hMSC were viewed by SEM and LIVE/DEAD viability staining, respectively. Among different tested chemical forms of silver, AgCl was identified to be the least cytotoxic. Moreover, a decline in the cytotoxicity of AgCl at significantly high concentrations was observed. We attributed the reduced cytotoxicity to aggregated AgCl which limited the bioavailability of free Ag^{+} ions.

Keywords: silver; silver chloride; cytotoxicity; haemolysis; stem cells; speciation

1. Introduction

Currently, silver-containing antimicrobial agents have gained popularity in the fields of wound-dressing, urinary catheters and cardiovascular implants. Ag nanoparticles are of particular interest because of the enhanced nanomaterial synthesis and characterization techniques. The widespread use of Ag, however, has raised issues concerning Ag toxicity to aquatic species and humans. Ag is the second extremely harmful metal after mercury to freshwater fish and invertebrates, with median lethal concentration (LC_{50}) values of between 6.5 and 65 µg Ag/L (Wood, 1996), mainly due to the liberated silver ions. The Ag^+ toxicity is associated with the perturbation of Na^+- K^+ transportation and ionoregulation ability of fish thus leading to cardiovascular collapse (Kennedy, 2010). Most recent Ag toxicity studies were focused on nanosilvers (Asharani, 2008). Apart from releasing active Ag^+, several studies unravelled that nanosilvers exerted toxic effects on cultured cells by increasing lactate dehydrogenase (LDH) leakage, inhibiting mitochondrial function (Hussain, 2005), producing oxidative stress (Carlson, 2008), damaging DNA (Asharani, 2009) and so on. In cell culture media or human body fluid, the free Ag^+ has rich opportunities to complex with Cl^-. AgCl formation has been confirmed to protect rainbow trout against silver toxicity (Bielmyer, 2008). Surprisingly, few published studies have reported dissolved silver chloride ($AgCl_n$) toxicology (Rodgers, 1997). The study on potential toxicity of solid form $AgCl_{(s)}$ to mammalian cells is even more limited. Considering the water solubility of $AgCl_{(s)}$ is only 0.013 mM (Santoro, 2007) at room temperature, it is essential to understand the toxic involvement of this Ag speciation.

Since silver-impregnated medical devices are often exposed to the bloodstream, haemolysis evaluation is one of the fundamental tests in determining the safety of such devices. On the other hand, human mesenchymal stem cells (hMSCs) represent a promising cell-based therapy for wound healing and skin regeneration, as they are openly exposed to wound dressing materials. Therefore, the present study was performed to assess the in vitro acute toxicity of silver nitrate in comparison with silver chloride colloids to red blood cells and bone marrow derived hMSCs. Most of the earlier studies evaluated the Ag cytotoxicity at relatively low doses. For example, Sopjani et al. (2009) has reported that at concentrations of 100 – 500 nM Ag^+ triggered eryptosis by interfering with the energy balance. Employing comet assay and chromosomal aberration tests, Hackenberg (2011) demonstrated hMSCs DNA damage by 1 to 100 µM Ag nanoparticles. However, Ag susceptibility studies have produced different minimal inhibition concentrations (MIC) data due to complex solubility issues that affect the bioavailability of Ag ions. Kim et al. (2007) determined the MIC of Ag nanoparticles to be 3.5 ng/mL for *Staphylococcus aureus*, while other Ag microbiocidal studies have revealed the MICs against *Escherichia coli* and *Staphylococcus aureus* could range from 74 to 740 µM (Chopra, 2007). Hence, acute cytotoxicity assessment of silver species at high concentrations becomes necessary to guarantee a safe therapeutic-window. We attempted to investigate toxic effects of Ag ions from 0.0075 to 5.0 mM. We assumed that Ag ions would react with chloride ions and therefore reduce the cytotoxicity. In particular, the formation of silver chloride and its functional impairment to the above mentioned mammal cells were investigated. The particles were characterized by dynamic light scattering. The adverse effects of silver chloride on red blood cells and hMSCs were viewed by SEM and LIVE/DEAD viability staining, respectively.

2. Material and Methods

2.1. Chemicals

Silver nitrate was purchased from Sigma-Aldrich. De-ionized (DI) water was distilled by a Milli-Q water purification system. 1 x Phosphate-buffered saline (PBS) was ordered from Invitrogen.

2.2. AgCl colloids preparation

34 mg silver nitrate was dissolved in 10 mL DI water to get a 20 mM stock solution. Silver chloride was formed immediately after mixing $AgNO_3$ with PBS. The particle growth and precipitation was monitored by DLS.

2.3. Haemolysis

Rabbit and rat red blood cell (RBC) samples were obtained from apparently healthy animals that were housed in the Experimental Animal Unit of Jilin University. The cells were diluted in PBS (4 vol %) for haemolysis study. $AgNO_3$ stock solution (20 mM) was used as a source of silver ions. The stock solution was diluted by adding DI water to obtain free silver ion concentrations which ranged from 0.08 to 5 mM. From DLS characterization results, we know the AgCl colloidal solution was stable only for about 1 h. To distinguish the cytotoxicity of free Ag^+ ions and AgCl colloids, we always freshly prepared AgCl colloids by vigorously mixing 1 mL of silver nitrate solution and 1 mL of PBS solution. After 30 min, the cell suspension was mixed with Ag^+ and colloidal AgCl and incubated for 1 h at 37°C in the minishaker. A control solution that contained only PBS was used as a reference for 0% hemolysis. Triton X-100 at a concentration of 0.5% served as a positive control. The released haemoglobin concentration was measured at 576 nm.

$$Hemolysis(\%) = \frac{OD_{576oligomer} - OD_{576blank}}{OD_{576Triton-X100} - OD_{576blank}} \times 100$$

3.

2.4. Human mesenchymal stem cell culture, staining and cytotoxicity study

Human bone marrow-derived MSCs (Cambrex, Walkersville, MD) were cultured in Mesenchymal Stem Cell Growth Medium with 1% penicillin/streptomycin. The cells were subcultured three times after reaching 90% confluence at 37°C/5% CO_2. Passage 5 hMSCs were seeded at the density of $1x10^4$/well (in 96-well plate) in either fresh media containing (1) 0.0075 to 10 mM silver nitrate, (2) 0.0075 to 10 mM AgCl colloids aged for 30 min.

To visualize the cell attachment and morphology after incubation with silver compounds, in the form of either silver nitrate or silver chloride colloids, the cells were stained with the LIVE/DEAD® cell viability/cytotoxicity kit (Molecular Probes) for 30 min, and examined using an Olympus BX61 microscope.

The cell viability was examined using 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. Cells were allowed to attach in 24-well plates in 1 mL of medium with different concentrations of silver nitrate or silver chloride colloids. After overnight incubation at 37 °C in 5% CO_2, the light absorbance was measured at 490 nm with a microplate reader. The cell viability was expressed as the ratio of the number of viable cells with Ag treatment to that without treatment. Experiments were repeated in triplicates, and consistent results were obtained.

2.5. Red blood cell preparation for SEM

The shape changes of red blood cells incubated with AgCl colloids were observed using a field emission SEM (JEOL JSM-6701F). The cells were harvested by centrifugation at 1500

rpm for 5 min. They were washed with PBS three times and then fixed in PBS containing 2.5% glutaraldehyde for 1 h. The cells were further washed with DI water, followed by dehydration using a series of ethanol washes. Several drops of the suspension were placed on a carbon tape and left to dry under room temperature. The samples were coated with platinum prior to SEM analyses.

2.6.Statistical analysis

All the experiments were replicated three times. The results of dose-response haemolysis are presented as means ± standard deviations (S.D.) of three separate experiments with the aid of Windows Excel computer software.

3. Results

3.1. Silver chloride particle characterization

We prepared colloidal AgCl at total Ag concentrations ranging from 0.02 to 2.0 mM. AgCl colloids seemed easily agglomerated or aggregated in PBS at high concentrations. When 0.31 to 2.0 mM $AgNO_3$ was reacted with PBS, the colour of solution changed from transparent to light blue, grey and colourless within 4 h. Visible precipitates settled to the bottom were further revealed by light microscope (Fig. 1). The individual primary grains formed are roughly circular, close inspection reveals particles of ~ 5 μm diameter. The aggregates gradually assembled into a network of branching, treelike shapes with dimensions exceeding hundreds of micrometer range. Similarly, Choi et al. (2011) revealed that AgCl tends to form larger particles. The settled precipitates definitely exceeded the nanometric range in dimensions. No obvious precipitation was observed within 1 h when $[AgNO_3] \leq 0.16$ mM.

3.2. Haemolytic properties of Ag^+ and AgCl colloid

In Fig. 2, the haemolysis percentage of rat blood cells caused by Ag^+ in DI and pre-formed AgCl was compared after 1 h incubation at 37ºC. As expected, haemolysis induced by Ag^+ indicated a dose-dependent increase from 0.02 mM to 0.16 mM, the haemolytic percentages of rat red blood cells incubated with 0.02, 0.04, 0.08 and 0.16 mM $AgNO_3$ were 50%, 80%, 90% and 100%, respectively; however, haemolysis dropped significantly at 0.31 mM (80%) and even lower at 0.625 mM (50%). At concentrations above 0.625 mM, the measured haemolytic percentages were maintained at 40%. Although colloidal AgCl was not haemolytic at 0.04 mM, similar to the case of Ag^+, it showed the maximum haemolysis at 0.16 mM and a declined haemolytic property from 0.31 mM. Unlike rat RBCs, rabbit RBSs were extremely fragile to silver toxicity. Ag^+ in DI water caused 100% rabbit RBCs haemolysis from 0.02 to 2.5 mM. Even the formation of AgCl colloids (from 0.08 to 2.5 mM) did not protect the rabbit RBCs from being haemolyzed.

3.3. Red blood cell morphology changes

In agreement with quantity results of haemoglobin release, morphological changes of rat red blood cells observed by light microscope displayed that, while control cells kept a typical biconcave shape, exposure of rat RBCs to Ag^+ in DI caused serious haemolysis and showing many ghost cells. The number of ghost cells increased with increasing concentration of Ag^+ from 0.16 to 0.62 mM (Fig. 3). We hypothesized that haemoglobin wrapped inside these cell fragments contributed to the lower haemoglobin readings. Exposure of RBCs to AgCl colloids did not exhibit apparent morphological changes at 0.02 mM. Haemolysis started at 0.04 mM and less RBCs were left at concentrations from 0.08 to 0.31 mM. Surprisingly, the light microscope images showed more swollen cells and less ghost cells as the AgCl colloids concentration increased to 0.62 mM. These echinocyte II/III cells were further characterized

by SEM. The SEM micrographs (Fig. 4) showed loss of biconcave structure and ruffled membrane on the surface of RBCs.

3.4. hMSCs morphology alterations and cytotoxicity evaluation

After overnight incubation, a dose-dependent (from 0.0075 to 0.125 mM) cell death induced by Ag was confirmed by MTS test. At a concentration of 0.125 mM, silver nitrate killed 86% cells as evidenced by necrosis cells lifting of 96-well plate surface. Incubation with the high concentrations of Ag, in contrary, left cells still attached on tissue culture plate surface, which indicated the absence of cellular necrosis. Treated cells appeared to have shrunk with a few cellular extensions as compared to control cells. The restricted cell spreading patterns could be due to damage to cytoskeletal functions by AgCl colloids. Similar results were observed by Asharani et al. in human glioblastoma cells (U251) treated with 200 μg/mL Ag-starch nanoparticles. The cells were observed even at 10 mM, with white patches on the cell body.

4. Discussion

With the rapidly expanding use of Ag in medical applications, especially for the management of burn patients, human exposure is frequent and so evaluation of their potential cytotoxicity becomes essential. Former studies have identified the toxic outcome of silver in several *in vitro* cell systems and *in vivo* animal models. The current study compares cytotoxic potentials of Ag^+ and AgCl to red blood cells and hMSCs. To assess the impact of AgCl formation on ionic silver cytotoxicity, we separately tested haemolysis of free Ag ions in DI and pre-formed AgCl colloidal in PBS. Free Ag ions at concentration of 0.02 mM caused significant rat blood haemolysis (50%). Contrary to the expectation, silver ions of higher concentration (above 0.31 mM) were less haemolytic than 0.16 mM. Although pre-formed AgCl colloids caused dramatically less haemolysis than Ag^+ at doses 0.02 to 0.16 mM, similar to the case of free ions, AgCl colloids induced the maximum haemolysis at 0.16 mM. At higher doses (0.31- 5.0 mM), the haemolytic difference of Ag^+ and AgCl colloids was not obvious by measuring released haemoglobin amount, but the morphology changes were totally different which will be discussed later. Previously, most studies indicated a dose-dependent increase in lesions related to various levels of silver exposure. To our knowledge this is the first study to disclose the existence of such a turning-point. Although the exact mechanism underlying this critical point is yet to be elucidated, one possible explanation for this would be the variation of the reactive oxygen species (ROS) does not follow a dose-dependent manner. Panda et al. (2011) prepared Ag^+, Ag nanoparticles and colloidal AgCl for sake of genotoxicity and cytotoxicity evaluation on onion bulb cells. In their study, one kind of ROS, $O_2^{\cdot-}$, generated by Ag^+ indicated a significant dose-dependent increase in the range 0-0.37 mM, however, $O_2^{\cdot-}$ content dropped remarkably at the next highest concentration (0.74 mM). Evidence of ROS leading to lipid peroxidation of the RBC membrane and haemolysis has been reported before (Clark, 1983, Asharani, 2010), taken together, our data could suggest a correlation between AgCl colloids and free radicals generation.

Recent reports have established the binding of Ag^+ ions with dissolved organic matter, thiosulfate and chloride might lead to a mitigated toxicity of silver species (Hogstrand, 1996; Russell, 1998). We hypothesize the reduced cytotoxicity of Ag^+ was due to the formation of AgCl colloids.

To assess the impact of Cl^- presence on haemolysis, the formation and growth of AgCl in PBS (total $[Cl^-]$ = 139.6 mM) was studied. $[Cl^-]$ was much higher than the added $[Ag^+]$ which would ensure complete complexation of Ag^+ ions. At low Ag^+ concentration (0.08 and 0.16 mM), no AgCl precipitation was observed after mixing with PBS (Fig. 1) within 1 h. As the

Ag^+ became more concentrated, for example, at [$AgNO_3$] = 0.31 mM, AgCl colloids were observed to become bigger in size as a function of time. The mean diameter of AgCl colloids (at 5, 10, 15, 20, 25 and 30 m) measured by DLS was 79, 105, 137, 179, 222 and 387 nm, respectively. Whereas the reaction time was longer, the presence of sedimenting particles (5080 μm in diameter) were detected by the DLS instrument. The poor data quality was not good enough for DLS characterization after 30 m. These findings suggest that at high concentrations AgCl colloids are not stable at all. Potential change of AgCl colloidal particle size over time, however, is often ignored by some earlier studies. For example, Choi et al. (2008) prepared 0.7 mM AgCl colloidal particles by mixing 1 mL of 14 mM silver nitrate and 1 mL of 28 mM sodium chloride with 18 mL of distilled water. The particle sizes were reported from 0.1 to 2 μm with an average diameter of 0.25μm. The authors could interpret experimental results more precisely by incorporating the aging time of their AgCl colloids before adding them to the tested microorganisms. The particle size measured over the duration of toxicological bioassays may enhance our insights on the nature of the Ag exposure. The stock solution of AgCl prepared by Panda et al. (2011) was also very high at 1.48 mM. Again, the descriptions of AgCl size and aggregation diverged from detailed to very limited. Based on our observation, even at a concentration of 1.25 mM, AgCl colloids rapidly precipitated within 15 min. This information is highly important, since the dispersion status of tiny particles might affect the subsequent toxicity.

We speculate that three major reasons are associated with the reduced cytotoxicity of Ag^+ observed in our study. First of all, a decreased concentration of Ag^+ is expected due to the formation of AgCl that consumed Ag^+. Secondly, like the ionic Ag^+ may sorb to silver nanoparticles (Liu, 2010), high AgCl colloidal concentrations will provide more surfaces for Ag^+ binding. Thirdly, at higher initial [$AgNO_3$] concentration, primary colloids formed aggregates faster which limits the dissociation of free Ag^+.

In parallel, we performed Ag ions and AgCl colloids haemolysis studies on rabbit red blood cells. At the same concentration, Ag caused different haemolysis between rat and rabbit RBCs. Rabbit RBCs are more sensitive to Ag than rat RBCs. For example, 0.63 and 1.25 mM AgCl caused 80% and 30% rat blood cell lysis, but still caused 100% haemolysis of rabbit blood cells. The reduced haemolysis due to AgCl formation was not observed until [$AgNO_3$] = 5 mM. The difference may be related to the nature of the red blood cell membrane. It is suggested that the phospholipid composition of the red cell membrane is characteristic for each animal species (Kasarov, 1970), therefore leptospirae also caused different red blood cell degradation among tested animals. Contrasting effects of chloride protection on the toxicity of silver are documented in the earlier studies for different test species. For example, Lee et al. (2005) tested the inhibition growth of two green algae, *C. reinhardtii* and *P. subcapitata* exposure to dissolved silver. The two algae differ in their response to the attenuation of silver chloride complexes. In a comparison of Ag^+ toxicity to rainbow trout and European eel, Grosell et al. (2000) found marked fish species-specific differences. The different silver tolerance with the presence of ambient Cl^- was related to the differences in whole body Na^+ turnover rates and differences in chloride homeostatic mechanisms.

SEM analysis of rat red blood cells following 0.6 mM AgCl colloid exposure unveiled the surface characteristics of rat RBCs at an early stage of haemolysis. In addition to the swollen cell body as shown in light microscope images, AgCl exposure resulted in multiple groves on the surface of RBCs. At high magnification, numerous pores on the cell membrane were disclosed. Therefore, the morphologic alterations in rat RBCs can be induced by changes in the cell membrane. Our SEM results are consistent with those AFM studies recently reported

by Asharani et al. (2010). In their study, the authors attributed the swollen echinocytes to the damaged membrane that further challenged the osmotic barrier, thus resulting in increased water uptake by the cells.

Following overnight incubation, it was found that Ag^+ was extremely toxic to hMSCs, they induced significant necrosis at 0.125 mM. However, as shown in Fig. 5, no dose-response was observed in cells treated by AgCl colloids at higher concentrations (0.25 to 10 mM); indeed, 0.5 mM AgCl treated cells displayed less cellular shrinkage than 0.12 mM. The absence of massive cell death indicated AgCl treatment mainly resulted hMSCs apoptosis.

Although our data provided some factors that should be considered in assessing AgCl colloids cytotoxicity, further molecular level studies are required in order to understand the mode of cytotoxic mechanisms.

Conclusions

In summary, ionic Ag exhibited dramatically higher cytotoxicity than that of pre-formed AgCl colloid. The current study reinforces the importance of Cl^- interaction in acute Ag ions toxicity evaluation. Our results confirm the protection role of Cl^-, on both red blood cells and hMSCs, especially at high doses far beyond previously investigated concentrations. Moreover, AgCl colloids at high concentrations were found to aggregate and to settle within 30 m. The absence of standard aging time will undoubtedly complicate interpretation of Ag acute toxicity data. In order to reduce the discrepancies among different laboratory cytotoxic studies, it would be beneficial to specify detailed experimental conditions.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Acknowledgement

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Figures

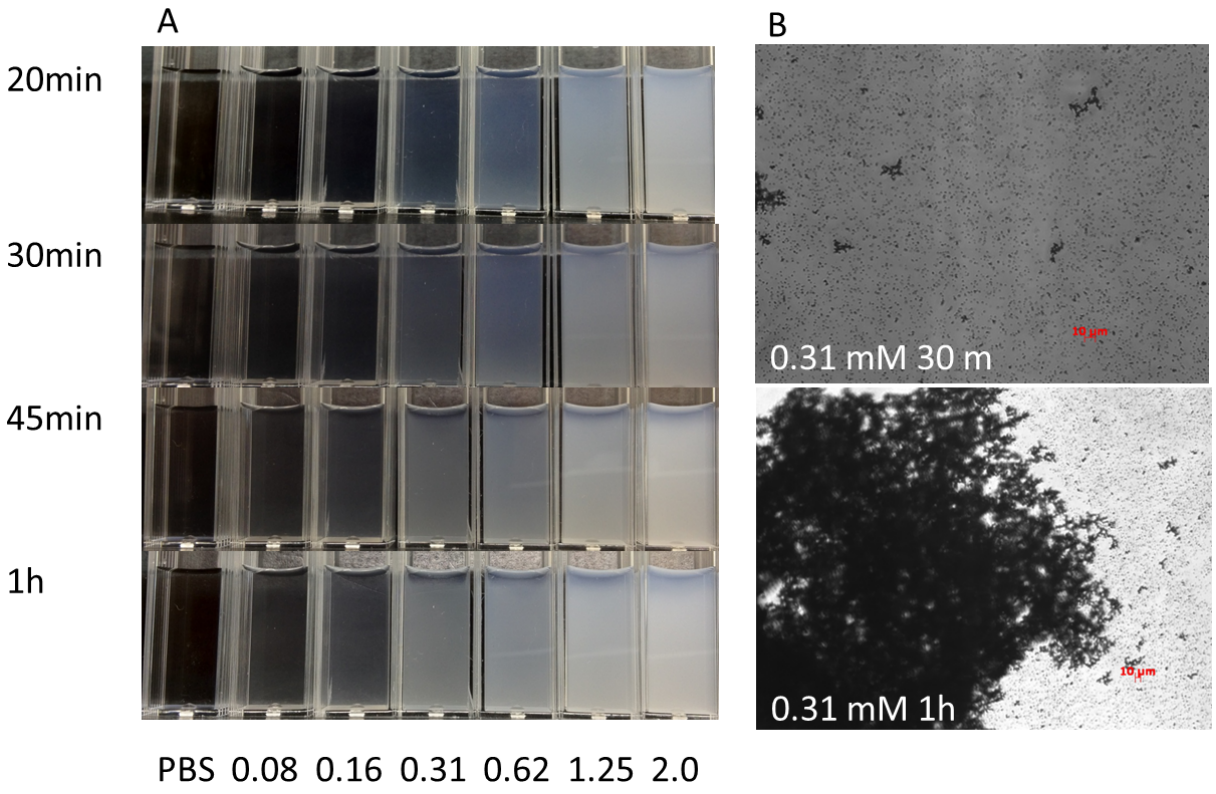

Fig. 1. Unstable AgCl colloids formed after mixing $AgNO_3$ with phosphate-buffered saline. (A) Gradually increased level of AgCl aggregation was obvious with higher concentrations of $AgNO_3$ (0.08 to 2.0 mM) and longer aging time (20 min to 1 h). (B) Light microscopic images of typical AgCl colloids at 30 min and 1 h. Initial [$AgNO_3$] = 0.31 mM.

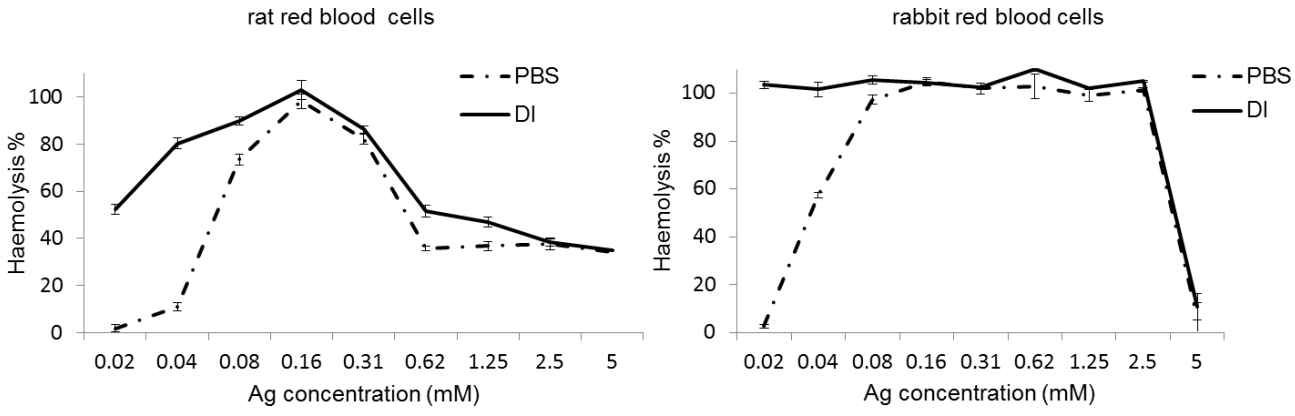

Fig. 2. Heamolysis percentages of rat and rabbit red blood cells caused by Ag^+ ions in de-ionized water and pre-formed AgCl colloids. Data are presented as the mean ± S.D. (n = 3).

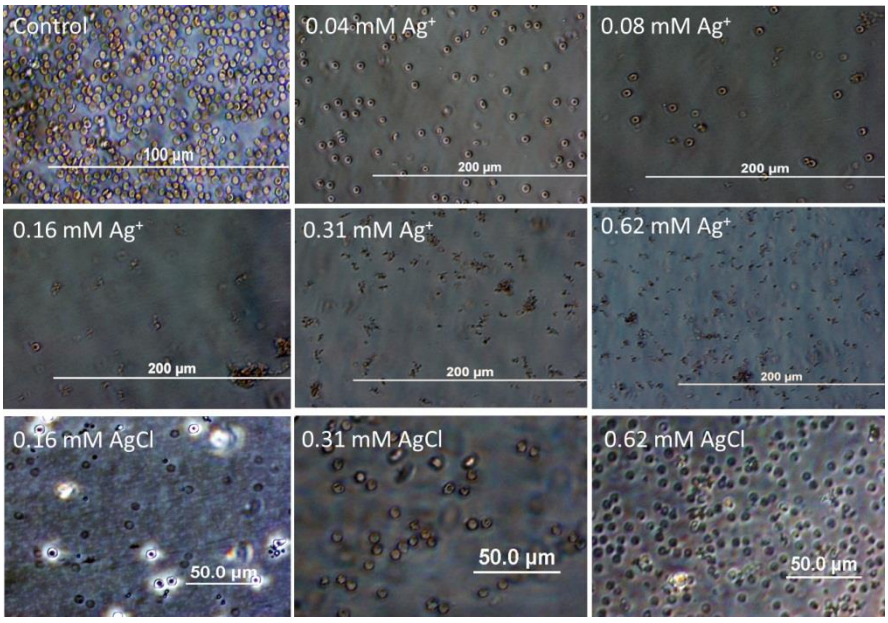

Fig. 3. Morphological observation of rat red blood cells exposure to Ag^{+} ions and AgCl colloids.

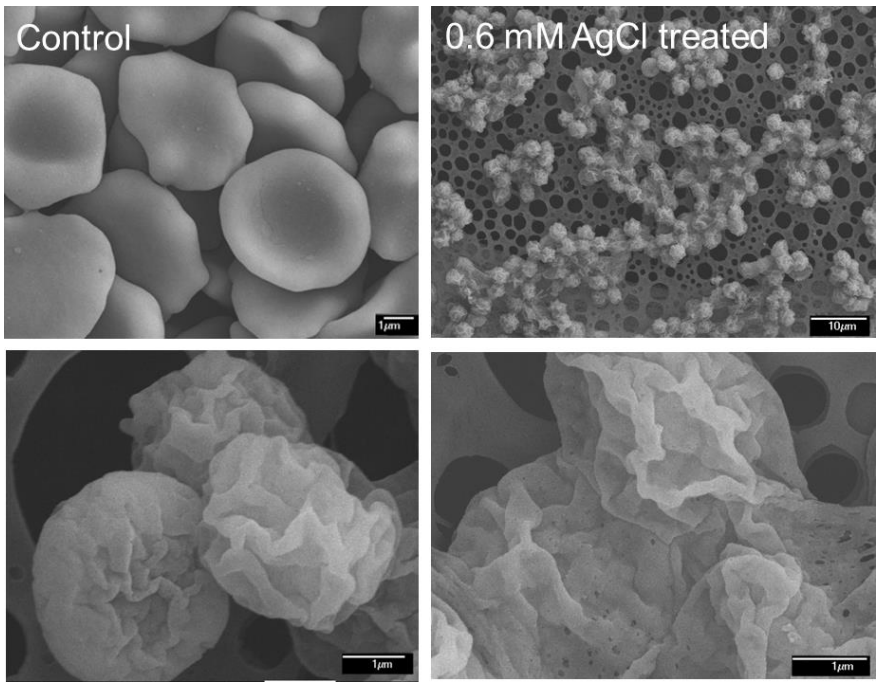

Fig. 4. Scanning electron microscopic images of rat red blood cells before and after treated with 0.62 mM AgCl colloids.

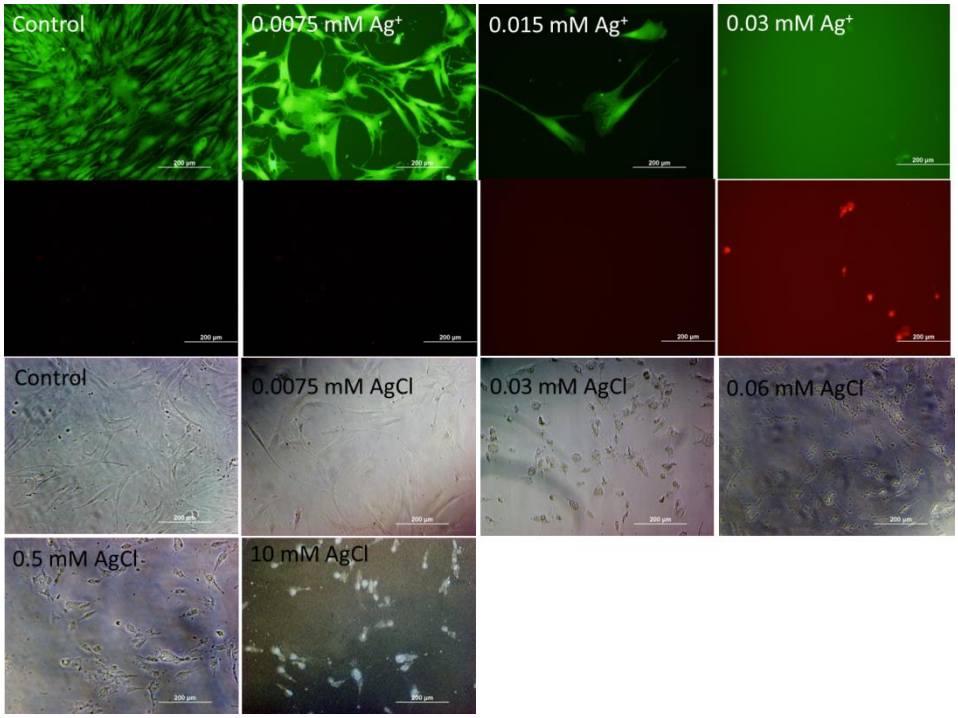

Fig. 5. Ag^+ in de-ionized water caused necrosis of human mesenchymal stem cells (hMSCs**)** while AgCl colloids led to apoptosis of the cells.