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Time-dependent Toxic Effect and Distribution of Silver Nanoparticles Compared to Silver Nitrate after Intratracheal Instillation in Rats

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Abstract Silver nanoparticles (AgNPs) are widely used because of their anti-bacterial and anti-inflammatory properties; however, the adverse health effects of these nanoparticles, especially to the lungs, have been less studied. We thus investigated the inflammatory response of polyvinylpyrrolidone (PVP)-coated AgNPs and silver nitrate ($AgNO_3$) after 24 h, 14 days and 28 days of single intratracheal instillation in rats. The bronchoalveolar lavage fluid (BALF) samples were collected and analyzed; a significant influx of neutrophils into the lung was found in both treated groups after 24 h with a presence of AgNPs in the alveolar macrophages after 24 h, 14 days and 28 days of instillation. Pro-inflammatory cytokines and enzymatic activities showed a significant increase after 24 h in both treated groups with a higher significance in the $AgNO_3$-treated group than the AgNPs-treated group. After 28 days, these increases were completely recovered in the $AgNO_3$-treated group but were still present in the AgNPs-treated group. The gross examination of lung tissues revealed a clear focal inflammation in the AgNPs-treated group after 28 days. More than 29% and 9% of the initial dose of AgNPs were recovered in lung tissues after 1 day and 28 days, respectively. Comparatively, the $AgNO_3$-treated group recovered only 16.5% and 1%, suggesting that the silver ions are easily absorbed into the circulation and distributed to different tissues more than the nanoparticles. Our results indicated that the PVP-AgNPs caused a subchronic pulmonary inflammation compared to the acute one induced by the ionic form, which can be recovered easily.

Keywords: *silver nanoparticles, pulmonary inflammation, silver distribution, toxicity*

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1. Introduction

Silver (Ag) has been reported to have antimicrobial effects. Therefore, silver nanoparticles (AgNPs) are incorporated in several medical products such as wound dressing [1] and catheters coatings [2]; this is supported by their tiny size (1 to 100 nm in diameter of one dimension at least) [3] that helps create a very high surface area [4]. In addition, they are also used in other consumer products such as deodorant sprays [5], textiles [6], cosmetics, and electronics [7]. However, the consumption of these products exhibits a potential health risk (i.e., silver toxicity) in humans and livestock; this should be evaluated to obtain more data about silver toxicity.

Lungs are considered as the target organ for aerosolized AgNPs in spray products due to their large surface area [8]. It has been found that the deposited particles in the lungs interact with the epithelial cells and lung macrophages leading to pulmonary disorders [9]. Previous studies have demonstrated the toxic effect of AgNPs on lung cells. Polyvinylpyrrolidone (PVP)-AgNPs revealed a cytotoxic and anti-proliferative effect on human lung carcinoma cell line by ROS-dependent and independent pathways [10]. Another study showed the size-dependent cytotoxic effect concluding that only 10 nm AgNPs affected the viability of normal human bronchial epithelial cell line (BEAS-2B) by intracellular ion release effect [11]. Moreover, AgNPs and Ag ions could induce oxidative stress followed by genotoxic and cytotoxic effects [12].

Bronchoalveolar lavage (BAL) is a suitable way to detect the respiratory tract toxicity of inhaled materials by providing information on both biochemical and cytological biomarkers of the inflammatory response induced by these materials [13]. Studies have shown that the intratracheal instillation of 250 µg PVP-AgNPs leads to a cytotoxic and inflammatory response in the lungs of rats after 24 h as indicated by bronchoalveolar lavage fluid

(BALF) analysis. The BALF analysis revealed elevated neutrophilic number, lactate dehydrogenase (LDH), and protein and cytokines levels [14]. Another study on AgNPs intratracheal instillation demonstrated the concentration-dependent manner of pulmonary responses of mice in the acute stage that involves IL-1β and TNF-α in the pathogenesis of the induced toxicity [15]. To examine the mechanism of AgNPs toxicity and investigate whether it is particle-specific or mediated by released silver ions, some researchers studied the difference between the acute toxicity of Ag ions and particles. Some reports recommended that AgNPs toxicity is mediated by the released silver ions ([14,16,17]). One of these studies concluded that the ionic form caused more potent cellular inflammatory response than the nanoparticulate form after 1 day of intratracheal instillation in mice and also in J774.1 murine macrophages cells [16].

Many studies have investigated the toxic effect of AgNPs, but most of them have focused on acute toxic effects. Only a few studies have focused on the subchronic and chronic effects of nanoparticles, which are important for assessing the health risks of AgNPs when used in advanced technologies. This assessment is particularly important when daily exposed to very few concentrations of these nanoparticles for a long period via antimicrobial and deodorant sprays. Moreover, no studies have compared the pulmonary toxicity induced by Ag ions and nanoparticles after a subchronic exposure with the acute stage of toxicity. Therefore, the aim of this study was to differentiate between the time-dependent (acute and subchronic) toxic effects of PVP-AgNPs and compare it to that of ions after single intratracheal instillation in rats.

2. Materials and Methods

2.1. Characterization of AgNPs

PVP-coated AgNPs (Sigma-Aldrich, Cat. No. 576832) have a particle size of less than 100 nm. To characterize the morphology and size of AgNPs, transmission electron microscope (TEM; JEM-2000FX, JEOL) and electronic light scattering detector (ELS; zeta potential and particle size analyzer ELSZ-2000, Otsuka Electronics, Japan) were used. AgNPs were sterilized by autoclaving (121 °C for 20 min) and suspended in sterile deionized water containing 1% BSA by ultrasonication.

2.2. Animals

Specific pathogen-free male Sprague-Dawley rats (4 weeks old) were purchased from Clea Japan (Tokyo, Japan). The rats were housed in a clean room with controlled conditions (23±1 °C, 12 h light and 12 h dark cycle) and were fed on standard laboratory pelleted diet and water ad libitum. The animal study was approved by the Ethics Committee for Animal Care and Experimentation of the National Institute for Environmental Studies (Japan).

2.3. Intratracheal Instillation

At 5 weeks of age, the rats were divided into three main groups (control, AgNPs-treated group and $AgNO_3$-treated group) and anesthetized with 2.5% halothane in a carrier gas (nitrous oxide:oxygen=1:2). They were intratracheally instilled with 400 µl aliquot BSA 1% solution (control group), AgNPs suspension (AgNPs-treated group) and $AgNO_3$ solution ($AgNO_3$-treated group) at a dose of 25, 50 and 100 µg Ag/rat. For this purpose, the anesthetized rats were fixed in supine position by their incisors to a string on a board slanted at 60° angle to the bench. Under a good light source, a flexible cannula was inserted into the tracheal orifice, and the solution was gently instilled in the upper third of the trachea followed by 400 µl air by using an insulin syringe. $AgNO_3$ was purchased from Wako, Japan (CAT no: 196-00831).

2.4. Bronchoalveolar Lavage

After instillation for 24 h, 14 days, and 28 days, 4 rats from each group were anesthetized with Na pentobarbital (40 mg/kg body weight) and euthanized by exsanguination via the abdominal aorta. The lungs were collapsed by puncturing the thoracic cavity. A cannula was inserted into the upper third of the trachea, and the lungs were lavaged 3 times by infusion and aspiration of saline (0.035 ml/g body weight) using a syringe.

2.5. BALF Analysis

2.5.1. Cell Count

BALF was centrifuged (4 °C, 1000 rpm, 5 min), and the first BALF supernatants were used for measuring cytokines concentration and enzymatic activity. The cellular pellets were resuspended in BALF supernatant, stained with crystal violet and used for the total cell count using a hemocytometer. BAL smears were prepared by cytocentrifugation and stained with Diff-Quik® (International Reagents, Kobe, Japan) for differential cell count.

2.5.2. Measurement of Enzymatic Activity in BALF

LDH activity was determined spectrophotometrically at a wavelength of 580 nm using LDH cytotoxic test (Wako, Japan). Alkaline phosphatase (ALP) activity was measured spectrophotometrically at a wavelength of 405 nm following Lab AssayTM ALP (Wako, Japan). β-Glucuronidase (βG) activity was measured spectrophotometrically at a wavelength of 540 nm according to the Enzymatic Assay of ß-Glucuronidase (EC 3.2.1.31, Sigma-Aldrich).

2.5.3. Measurement of Cytokines Concentrations in BALF

IL-1β concentration was measured using an enzyme-linked immunosorbent assay kit (ELISA; DuoSet, R&D Systems, Japan), and IL-18 concentration was measured using ELISA kit (Invitrogen). The absorbance was measured at a wavelength 450 nm for both concentrations as mentioned in the manufacturer's protocols.

2.6. Ag Concentrations Measurement

The whole lung, liver, kidneys, and testis tissues were wet-digested using nitric acid and hydrogen peroxide (0.75 and 0.25 ml, respectively for 0.2 g tissue) at 135 °C for 48 h. The digested samples were diluted with deionized water to measure Ag concentrations using

inductively coupled plasma-mass spectrometry (ICP-MS; 7500c, Agilent Technologies, Tokyo) at m/z 107.

2.7. Histopathological Studies

Specimens from the lungs were preserved in 10% neutral buffer formalin. Sections of 5-micron thickness were prepared from all specimens, stained with hematoxyline and eosin (H&E) and examined microscopically.

2.8. Statistical Analysis

Data were subjected to statistical analysis using a statistical software program (GraphPad InStat version 3.00 for Windows 95, GraphPad Software, USA). The differences between the mean values of different groups were determined using one-way ANOVA with Duncan multiple comparison tests. A P value less than 0.05 was considered to be statistically significant. Data were presented as mean ± standard deviation (SD).

3. Results

3.1. Characterization of AgNPs

In order to characterize the morphology, size and zeta potential of NPs, TEM and ELS detector were used. Figure 1 shows the TEM image of AgNPs suspended in BSA 1%; NPs were slightly aggregated and spherical in shape with the mean size being 50–90 nm. The average hydrodynamic diameter of AgNPs sonicated for 10 min in deionized water was 167.1 nm ± 53.1 and the polydispersity index was 0.202. The zeta potential of AgNPs in water was −53.2 mv as measured by ELSZ-2000.

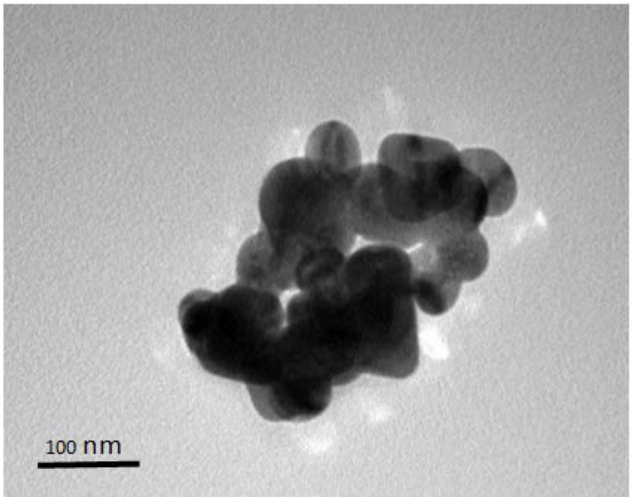

Figure 1. Representative TEM image (250.000X magnification) of PVP-AgNPs. AgNPs were suspended in BSA 1% and sonicated for 30 min. A drop was placed onto a collodion membrane-attached sheet mesh (150-A) and dried at room temperature. AgNPs are spherical and approximately 50–90 nm in diameter

3.2. BALF Analysis

3.2.1. Dose-dependent Effect

To detect the differences between the toxic effects of different doses (low, medium and high) of $AgNO_3$ and the same doses of AgNPs, we established a dose-dependent experiment. Figure 2 (A-F) shows the dose-dependent effect of AgNPs compared to $AgNO_3$ on the inflammatory and cytotoxic markers in BALF samples. As shown in Figure 2 (A), neutrophils ratio was significantly increased in both treated groups compared to the control. However, there was a significant increase in the case of $AgNO_3$-treated group with different doses (25, 50 and 100 µg Ag/rat) compared to the same doses in the AgNPs-treated group. The results indicated that ions were more toxic than particles. The highest percent of neutrophils was induced by the highest doses in both treated groups up to 76.4% in $AgNO_3$ and 64.8% in AgNPs-treated groups.

The enzymatic activities of LDH, ALP and βG are shown in Figure 2 (B, C and D) and cytokines concentrations (IL-1β and IL-18) are shown in Figure 2 (E and F). These parameters showed a significant increase in 25, 50 and 100 µg Ag/rat $AgNO_3$-treated groups compared to the control. The AgNPs-treated group showed significant increase only in the case of 50 and 100 µg Ag/rat, but not in the lowest dose group. In addition, there was a significant increase in $AgNO_3$-treated groups compared to AgNPs–treated groups in case of LDH, βG and IL-1β. These findings indicate that the ions were more toxic than particles, and the lowest dose of AgNPs did not induce any toxic effect compared to the same dose of ions. Moreover, the toxicity was dose-dependent and increased on increasing the dose.

3.2.2. Time-dependent Effect

In order to know the effect of time on these toxic signs and whether it will be recovered or not, we analyzed BALF samples after 24 h, 14 days and 28 days of instillation of 100 µg Ag/rat. Figure 3 (A-F) shows the time-dependent effect of AgNPs compared to $AgNO_3$ on the inflammatory and cytotoxic markers in BALF samples after single intratracheal instillation of 100 µg Ag/rat. As shown in Figure 3 (A), the neutrophils ratio was significantly decreased in BALF samples after 14 days and 28 days of the instillation compared to the percent after 24 h. The results showed that the neutrophils ratio was significantly increased in both treated groups compared to the control. Furthermore, the $AgNO_3$-treated group showed significantly higher neutrophils ratio compared to the AgNPs-treated group after 24 h. The results indicate the toxic effects of Ag ions than the AgNPs during the first 24 h.

The enzymatic activities of LDH, ALP and βG are shown in Figure 3 (B, C and D), and cytokines concentrations (IL-1β and IL-18) are shown in Figure 3 (E and F). In the case of $AgNO_3$-treated group, all these parameters showed a significant increase only after 24 h of the instillation compared to the control and showed no any significant differences after 14 and 28 days. Compared with the control and $AgNO_3$-treated group, the AgNPs-treated group showed a continuous significant increase in activities of ALP and βG and in cytokines concentrations after 14 and 28 days of the instillation. These findings indicate the complete and quick recovery of rats after the acute toxicity caused by silver ions. Moreover, NPs lead to a subchronic toxic effect that persisted up to 28 days after the instillation.

To examine the possibility of finding the nanoparticles in the lungs after 28 days of instillation, we examined the

BALF cells microscopically. Figure 4 shows a representative BALF cells image after intratracheal instillation of AgNPs (100 µg/rat) after 28 days of instillation. The black arrows show alveolar macrophages with internalized AgNPs. The results indicate the presence of AgNPs in the activated alveolar macrophages even after 28 days. These results confirmed the previous data showing the persistence of NPs in lungs for a longer time.

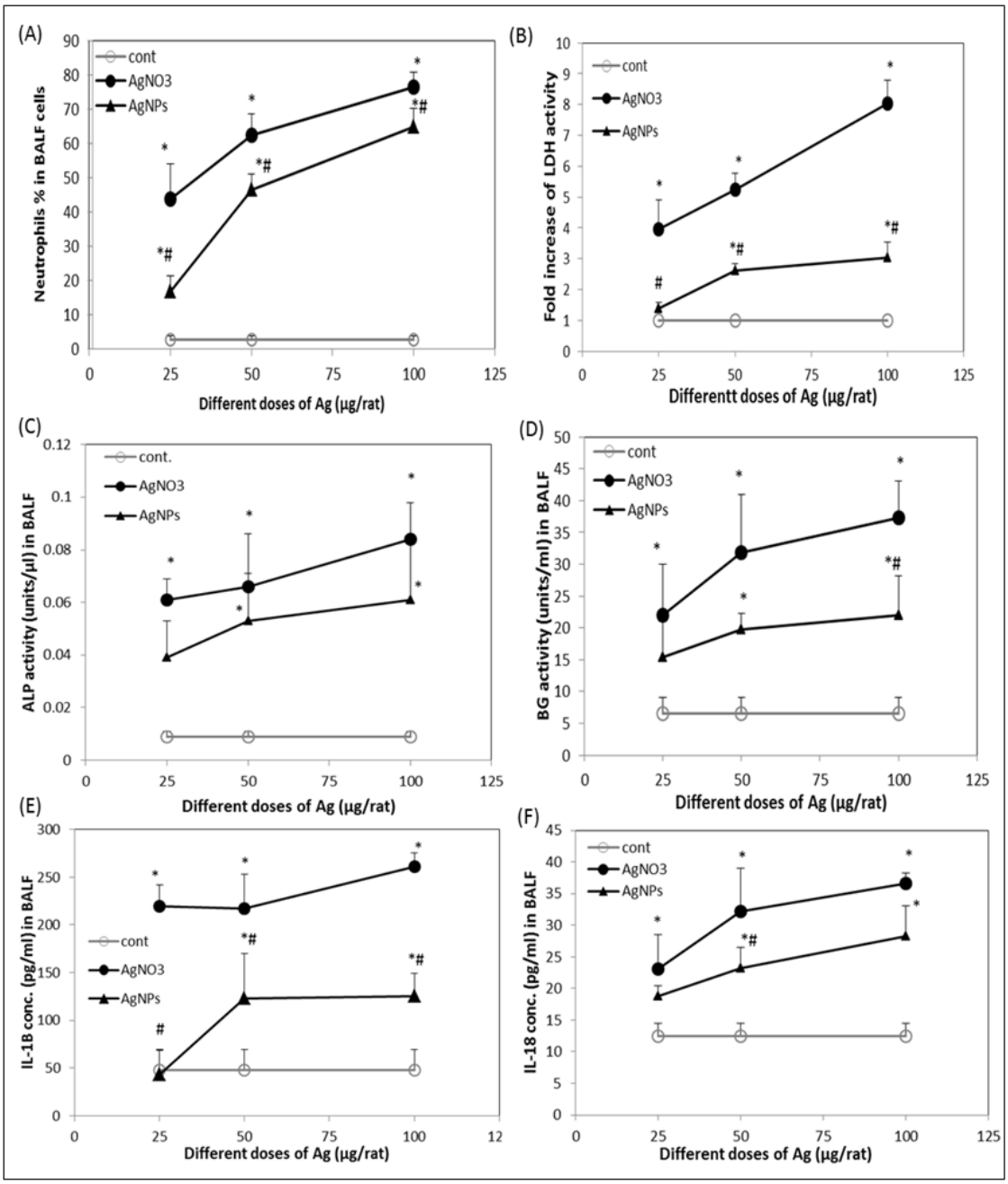

Figure 2. Dose-dependent effect of AgNPs compared to $AgNO_3$ on inflammation and cytotoxic markers in BALF samples. Lungs were lavaged after 24 h of single intratracheal instillations of 25, 50 and 100 µg Ag/rat. (A) Neutrophils ratio (%), (B) LDH activity, (C) ALP activity, (D) βG activity, (E) IL-1β and (F) IL-18 concentrations. The results are shown as mean ± SD., n=4 for each group. (*) represented the significant difference from control group. (#) showed the significant difference from the corresponding $AgNO_3$-treated group

3.3. Ag Concentration Measurements in Different Tissues

Ag concentrations were measured in different tissues to know the distribution and fate of silver inside the body. Table 1 shows the percentage (%) of Ag remained in different tissues after single intratracheal instillation of $AgNO_3$ and AgNPs (100 µg Ag/Rat) as an initial dose. The concentration of Ag in lung tissue of AgNPs-treated group was significantly higher than that in the $AgNO_3$-trated group after 24 h and 14 days. In the AgNPs-treated group, only trace amounts of Ag were detected in the liver, kidney and testis after 24 h. In contrast, 9.75%, 0.59% and 0.15% of the initial dose of silver were recovered in liver, kidney and testis, respectively, in the $AgNO_3$-treated group. After 14 and 28 days, there was no any significant presence of Ag in the tissues of the $AgNO_3$-treated group. In contrast, 15.97% and 9.29% of Ag were recovered in liver tissues of the AgNPs-treated group after 14 and 28 days, respectively. Moreover, there was a significant increase of Ag percentage in kidney and testis of the AgNPs-treated group after 14 and 28 days. These data indicate that ions were easily absorbed into the circulation

and distributed to the different tissues during the first 24 h. In contrast, NPs were slowly absorbed and distributed, and we could not find any NPs in the organs other than the lung except after 14 days of instillation.

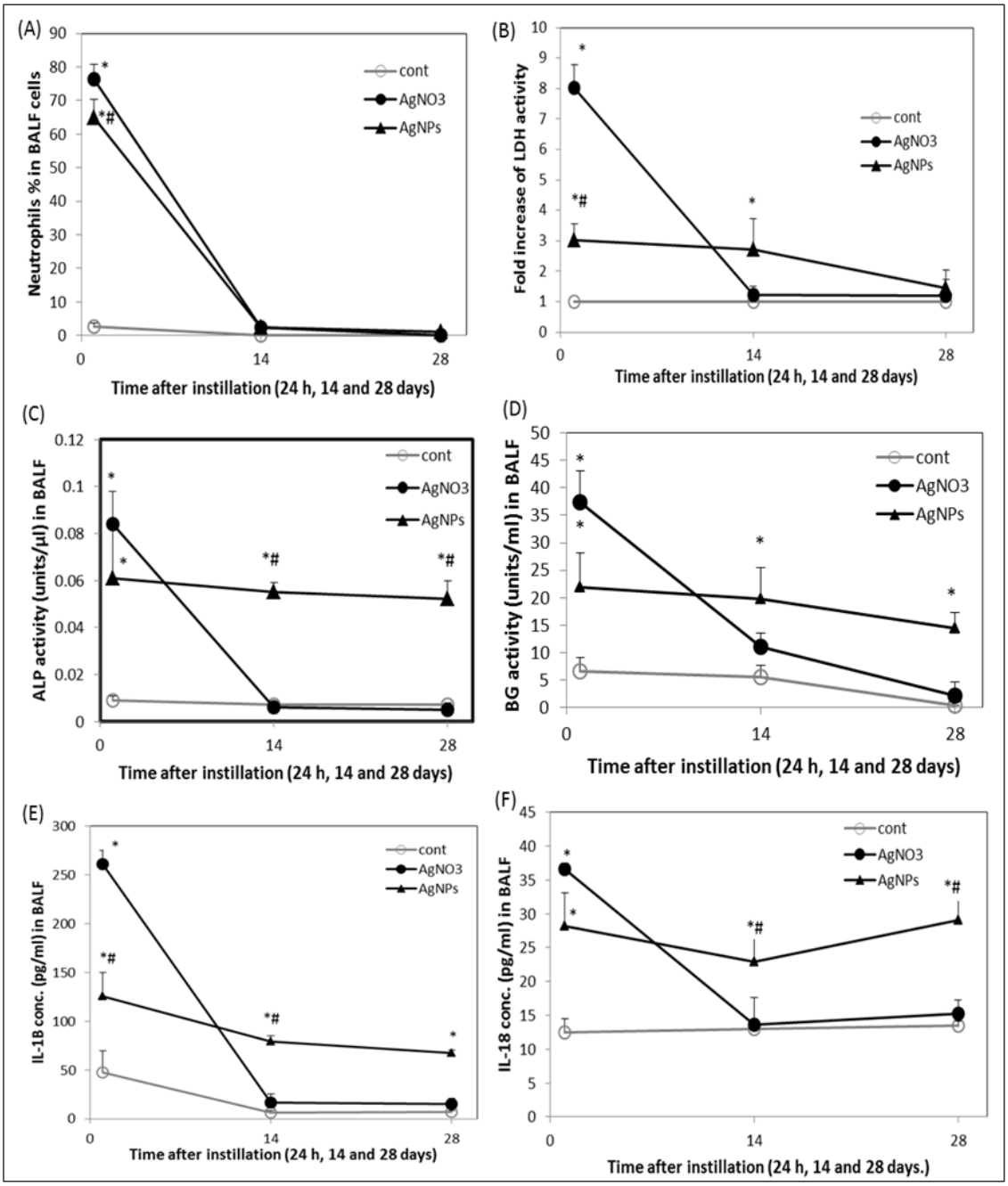

Figure 3. Time-dependent effect of AgNPs compared to $AgNO_3$ on inflammation and cytotoxic markers in BALF samples. BALF samples were collected 24 h, 14 days and 28 days after 100 µg Ag/rat exposure. (A) Neutrophils ratio (%), (B) LDH activity, (C) ALP activity, (D) βG activity, (E) IL-1β and (F) IL-18 concentrations. The results are shown as mean ± SD., n=4 for each group, (*) represented the significant difference from control group, (#) showed the significant difference from the corresponding $AgNO_3$-treated group

Table 1. Percentage (%) of Ag remained in different tissues. Numbers indicate % of Ag in the whole organ after single intratracheal instillation of $AgNO_3$ and AgNPs (100 µg Ag/Rat) as an initial dose. Tissues were collected after 24 h, 14 days and 28 days and digested by acids. Then Ag concentrations were measured by ICP-MS at m/z 107. The results are shown as mean ± SD., n=4 for each group. (a) Significantly different from control group. (b) Significantly different from the corresponding AgNO3-treated group

Group Organ	24 h.		14 days		28 days	
	$AgNO_3$	AgNPs	$AgNO_3$	AgNPs	$AgNO_3$	AgNPs
Lung	16.5 ±4.75	29.39[a] ±7.55	4.64 ±1.69	29.09[a] ±16.5	1.08 ±0.47	9.01 ±10.18
Liver	9.75[a] ±2.61	0.03 ±0.01	3.41 ±1.85	15.97[ab] ±4.63	2.09 ±1.69	9.29[a] ±11.1
Kidney	0.59[a] ±0.09	0.004[b] ±0.0004	0.07 ±0.02	0.69[ab] ±0.77	0.06 ±0.04	0.22 ±0.13
Testis	0.15[a] ±0.05	0.0009[b] ±0.0005	0.14[a] ±0.02	0.28[ab] ±0.1	0.06 ±0.01	0.1[a] ±0.03

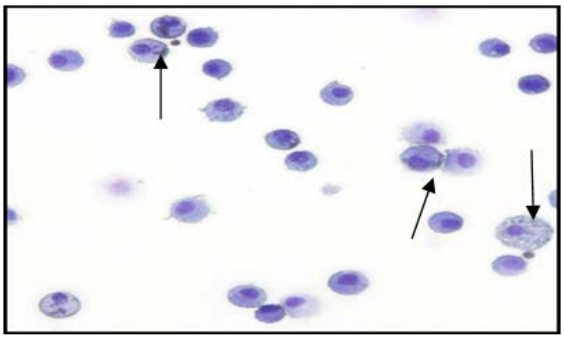

Figure 4. Representative BALF cells image after intratracheal instillation of AgNPs (100 µg/rat) after 28 days of instillation. Black arrows show alveolar macrophages with internalized PVP-AgNPs

3.4. Gross and Histopathological Examination of Lung Tissues

To see the effect on the histology of lung tissues, we examined the tissues macroscopically and microscopically. The gross examinations of lung tissues showed very clear congestion and diffuse inflammation of lung tissues of both treated groups after 24 h of instillation of 100 µg Ag/rat. After 28 days, the AgNPs-treated group showed the persistence of clear focal inflammation of lung tissue as shown in Figure 5 (C). In contrast, $AgNO_3$-treated group lung tissues were completely recovered as shown in Figure 5 (B) compared with the control lung tissues in Figure 5 (A).

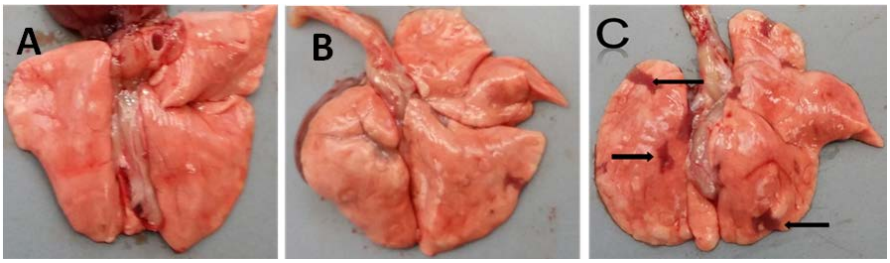

Figure 5. Lung tissues after 28 days of instillation of 100 µg Ag/rat. Panel (C) shows persistence of clear focal inflammation in the AgNPs-treated group (B) recovery of the $AgNO_3$-treated group compared to panel (A), which shows the normal tissue of control group

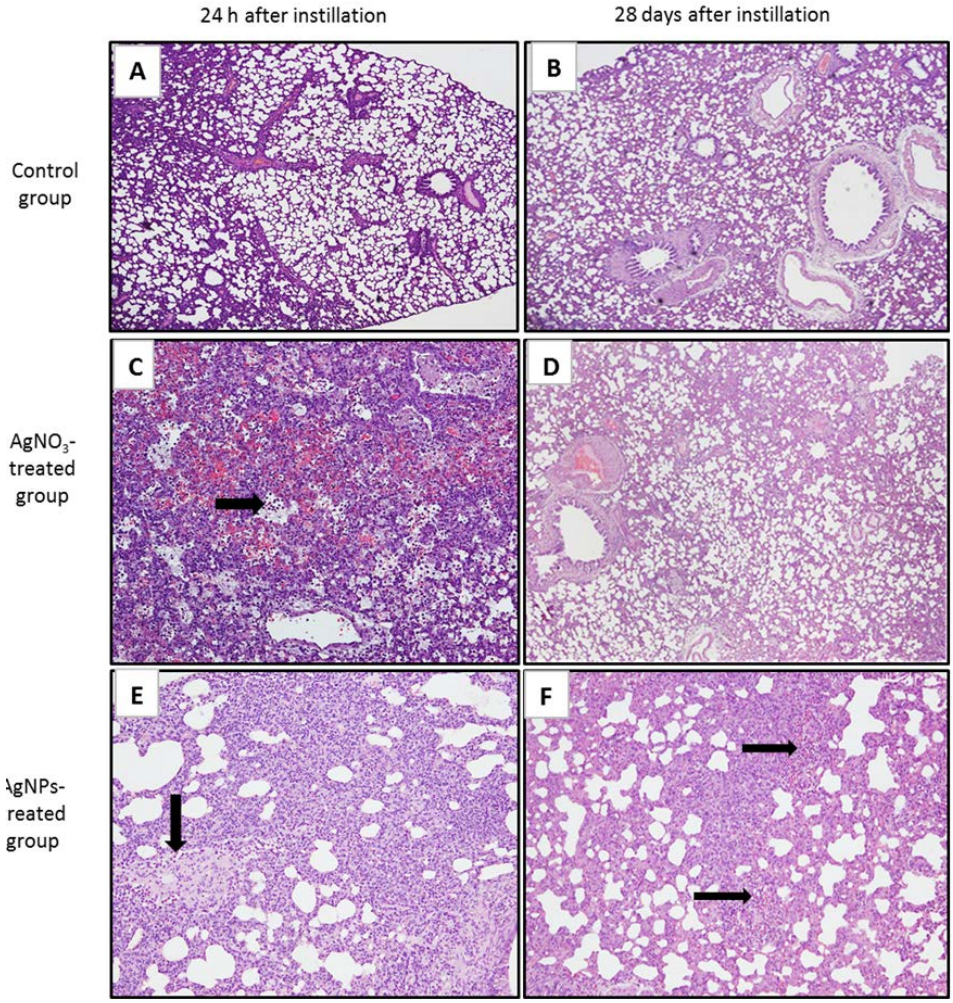

Figure 6. Histopathology of lung tissues (H&E staining). (A and B) show lung tissue of control group after 24 h and 28 days respectively with normal histological architecture, and normal intrapulmonary bronchioles, 4x. (C) Lung tissue of $AgNO_3$-treated group after 24 h shows acute pneumonia where active hyperemia of interstitial capillaries and marked neutrophilic recruitment alveoli (arrow), 10x. (D) Lung tissue of $AgNO_3$-treated group after 28 days shows normal pulmonary alveoli and normal intrapulmonary bronchioles, 4x. (E) Lung tissue of AgNPs-treated group after 24 h shows marked diffuse thickening in interstitial tissue with eosinophilic (arrow) and histiocytic infiltration and fibroblastic proliferation, 10x. (F) Lung tissue of AgNPs-treated group after 28 days shows focal recruitment of eosinophils (arrows) and histiocytic replacing pulmonary tissue, 10x

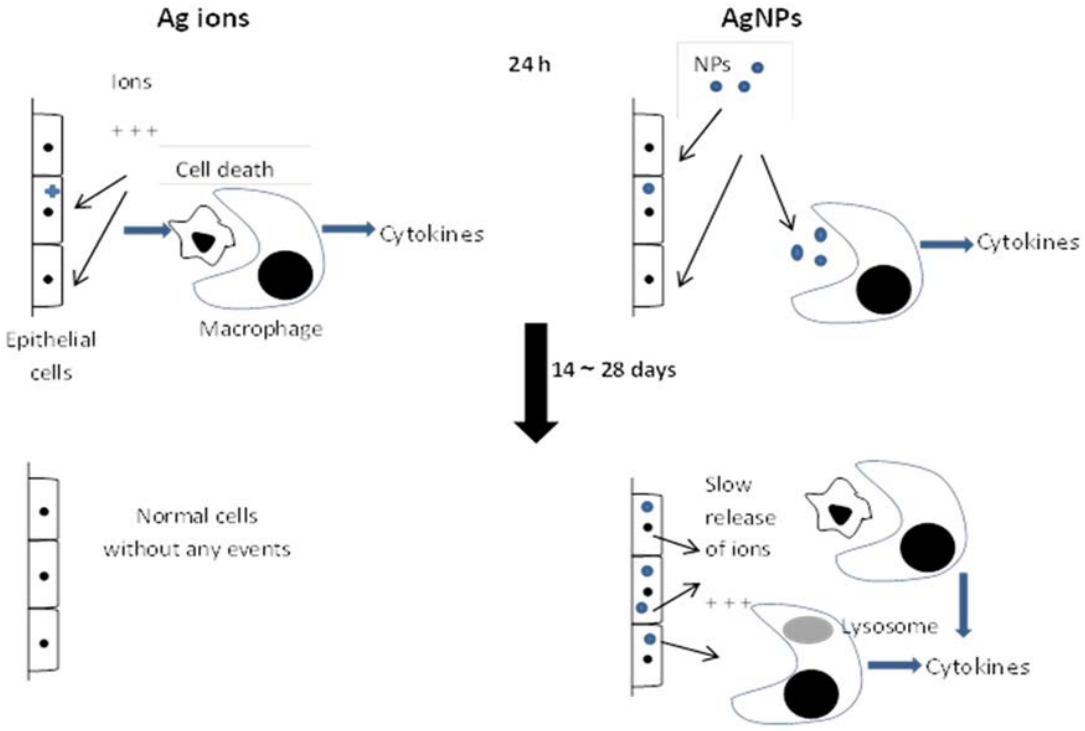

Figure 7. Hypothesis illustrated the events that were induced by AgNPs compared to Ag ions after 24 h, 14 and 28 days

4. Discussion and Conclusion

In this work, we studied the time-dependent pulmonary toxic effect and distribution of AgNPs compared to Ag ions after single intratracheal instillation in rats. First, we exposed rats to different doses of AgNPs and ions (25, 50 and 100 µg Ag/rat) for 24 h to determine the dose appropriate for studying the time-dependent toxic effect. From the enzymatic activities of LDH, ALP and βG, and cytokines concentrations (IL-1β and IL-18) in BALF samples, we found that the ions were more toxic than the particles. The lowest dose of AgNPs did not induce any toxic effect compared to the same dose of ions. In addition, the toxicity was dose-dependent and increased when the dose of both ions and particles were increased. Therefore, we decided to use the highest dose (100 µg Ag/rat) to establish the time-dependent experiment.

We found that the ions were more toxic than nanoparticles after 24 h of instillation. However, after 14 and 28 days, AgNPs were still found to be toxic, causing a significant increase in inflammatory biomarkers compared to control and Ag ions-treated group, which was almost totally recovered after 14 days. The presence of internalized AgNPs in the alveolar macrophages (Figure 4) with the gross and histopathological examinations of lung tissues confirmed the same results as shown in Figures 5 (C) and 6. It is well known that the target organs of AgNPs are lung and liver as confirmed by a subchronic inhalation study in rats in which AgNPs also accumulated in kidney tissues showing some changes in the histological structure [18]. Moreover, a previous study has concluded that AgNPs led to a deleterious effect on the testes that increased progressively with time after oral administration of 20 µg/kg B/W for 90 days [19]. In another recent study, the tissue distribution of Ag measured by ICP_MS was in the same line with the other mentioned data showing that 29.39%, 29.09%, and 9.01% of the initial dose of silver were recovered in lung tissues of AgNPs-treated group after 24 h, 14 days, and 28 days respectively while it was only 1.08%, 4.6%, and 16.5%, respectively, in the case of AgNO3-treated group. Furthermore, there were traces of Ag in liver, kidney and testis of the AgNO3-treated group after 14 and 28 days while it was significantly increased in the AgNPs-treated group.

The findings of our study concluded that ions were easily absorbed into the blood circulation and distributed to the different tissues during the first 24 h causing acute toxicity, which was recovered easily within less time. In contrast, PVP-AgNPs were slowly absorbed and distributed leading to a subchronic toxicity after single intratracheal instillation in rats.

To explain the reason that delayed the toxicity of AgNPs after that of Ag ions, as shown in Figure 7, we hypothesized that during the first 24 h, Ag ions could easily go inside the pulmonary epithelial cells leading to cell death. This was then engulfed by activated macrophages, which subsequently released the cytokines and induced the inflammatory process. This event could be stopped by the cellular defense. While in the case of PVP-coated AgNPs, depending on their size, it might go inside the epithelial cells either without any active process and stayed free in the cytoplasm with very poor release of silver ions because of the cytoplasmic environment, or by endocytosis forming phagolysosomes / endosomes and stayed there to release the silver ions slowly by the lysosomal environment, or others can be trapped by the macrophages and enter the lysosomes and also stayed there for a time for slowly release of silver ions which then induce the cytokines production and inflammatory response. This hypothesis will guide our future work to investigate the mechanism of cell toxicity induced by AgNPs by using in-vitro studies in different pulmonary epithelial cell lines.

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