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Research article

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Biodistribution of gold nanoparticles in mouse lung following intratracheal instillation

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Abstract

Background: The fate of gold nanoparticles, 2, 40 and 100 nm, administered intratracheally to adult female mice was examined. The nanoparticles were traced by autometallography (AMG) at both ultrastructural and light microscopic levels. Also, the gold content was quantified by inductively coupled plasma mass spectrometry (ICP-MS) and neutron activation analysis (NAA). The liver is the major site of deposition of circulating gold nanoparticles. Therefore the degree of translocation was determined by the hepatic deposition of gold. Mice were instilled with 5 intratracheal doses of gold nanoparticles distributed over a period of 3 weeks and were killed 24 h after the last dose. One group of mice were given a single intratracheal dose and were killed after 1 h.

Results: The instilled nanoparticles were found in lung macrophages already 1 h after a single instillation. In mice instilled treated repeatedly during 3 weeks, the load was substantial. Ultrastructurally, AMG silver enhanced gold nanoparticles were found in lysosome-/endosome-like organelles of the macrophages and analysis with AMG, ICP-MS and NAA of the liver revealed an almost total lack of translocation of nanoparticles. In mice given repeated instillations of 2 nm gold nanoparticles, 1.4‰ (by ICP-MS) to 1.9‰ (by NAA) of the instilled gold was detected in the liver. With the 40 nm gold, no gold was detected in the liver (detection level 2 ng, 0.1‰) except for one mouse in which 3‰ of the instilled gold was found in the liver. No gold was detected in any liver of mice instilled with 100 nm gold (detection level 2 ng, 0.1‰) except in a single animal with 0.39‰ of the dose in the liver.

Conclusion: We found that that: (1) inert gold nanoparticles, administered intratracheally are phagocytosed by lung macrophages; (2) only a tiny fraction of the gold particles is translocated into systemic circulation. (3) The translocation rate was greatest with the 2 nm gold particles.

Background

Epidemiological studies have reported associations between episodes of increased air pollution with particulate matter and adverse health effects in susceptible individuals [1-6]. It has been suggested, that the nanosized particles are the most dangerous fraction [7]. Air-borne nanoparticles (ultrafine particles) have been found to penetrate into the systemic circulation following inhalation [8,9]. Additionally, there is experimental evidence that respiratory exposure to nanoparticles may promote thrombosis [10,11]. It has been suggested that lung injury increases the degree of the nanoparticle translocation [12]. For a better understanding of the potential hazards of inhaled nanoparticles it is important to establish whether nanoparticles are translocated, i.e. transported through the alveolar barrier to the systemic circulation. It is known, that nanoparticles, once in the lungs, can be recognized and phagocytosed by lung macrophages [13]. The degree of translocation seems to be dependent on particle size, chemical composition, shape, electrical charge etc [14]. Smaller particles have been shown to more easily penetrate through the alveolar barrier than larger ones [14,15]. Once in the systemic circulation, the nanoparticles can reach extrapulmonary organs like liver, spleen, etc [8,9,16]. Translocation of inhaled ultrafine particles into the brain via the olfactory system has also been reported [16-18].

As a model we chose gold nanoparticles which are considered nontoxic [19] and can be traced histochemically by AMG, even ultrastructurally. Such gold nanoparticles are already in use for diagnostics, therapy and research [20-24]. Takenaka and coworkers treated rats with an aerosol of gold nanoparticles, 5-8 nm in diameter, produced by a spark generator. They observed accumulation of such particles in the pulmonary macrophages and epithelial cells and a low degree of systemic translocation [25]. Semmler-Behnke and coworkers demonstrated that intratracheally instilled 18 nm gold nanoparticles are retained in the rat lungs whereas 1.4 nm nanoparticles are being translocated in significant amounts to the secondary organs [26]. We hypothesized that 40 and 100 nm gold nanoparticles would be retained in lung macrophages and translocated to a minor degree while according to present ideas; 2 nm particles would be translocated and subsequently accumulate in the liver. We have previously reported that 40 nm colloidal gold nanoparticles are easily detected in liver after intravenous injection [27] and that the liver accumulates and retains most of the dose for a long period of time [28]. We therefore assume that if gold nanoparticles are translocated through alveolar epithelium they will be accumulated primarily in the liver.

The objective of the present study was to evaluate the extent of gold nanoparticle translocation from the lungs

into the systemic circulation. As a model for evaluation of whether nanoparticles penetrate lung epithelium we instilled gold nanoparticles, sized 2, 40 and 100 nm in mice intratracheally, and examined the lungs and the liver by optical and electron microscopy. We wanted to establish the significance of size in translocation and to identify where and in which cells the nanoparticles accumulated using LM and EM autometallography (AMG). AMG enables tracing of gold nanoparticles in morphologically intact tissue by silver enhancement of the individual nanoparticles [20,29]. To add the quantitative dimension the tissues were further analyzed by ICP-MS and neutron activation (NAA).

Experimental

Gold nanoparticles

Gold nanoparticles from Fitzgerald Industries Inc, USA had been produced by citrate reduction. They were negatively charged, monodisperse and spherical. The 2 nm contained 15×10^{13} particles/ml (12.1 μg), the 40 nm 9×10^{10} /ml (58.2 μg) and the 100 nm 6×10^9 /ml (60.1 μg).

The animal model

The study was performed in accordance with Danish law and guidelines for animal welfare. A total of 44 adult female C57BL mice were used. The mice were 8-12 weeks old with a body of weight of 18-21 grams. The mice were housed in plastic cages under the following conditions: 12 h light/dark cycle, 22 \pm 2°C and 50 \pm 10% relative humidity. Food (Altromin No. 1314, Altromin Spezialfutterwerke, Germany) and tap water were available *ad libitum*.

Single instillations

Eight mice were divided into 3 experimental groups and 1 control group, 2 mice in each. Two mice were instilled intratracheally with 50 μl 2, 40 or 100 nm gold nanoparticles and were killed after 1 h. Two control mice were instilled with distilled water and treated identically. These 8 animals were used for histochemical analysis with the AMG method. In addition, 4 mice were used to NAA analysis. Two of them were given 50 μl 40 nm gold nanoparticles and the other 2 were instilled with distilled water and served as controls. These animals were sacrificed after 1 day and analyzed with NAA.

Repeated instillations

Twenty mice were divided into 3 experimental groups and 1 control group, 5 mice in each. Five mice were given 50 μl of a 2 nm gold suspension, similarly 5 mice were given 40 nm gold and 5 mice 100 nm gold. The 5 mice served as controls and were exposed to distilled water only. These 20 mice were intratracheally instilled 5 times during a period of 3 weeks and sacrificed 24 hours after the last instillation. Tissues from these 20 mice were used both for

histochemical analysis with AMG and quantitative analysis with the ICP-MS method. In a second experiment 12 mice were used to NAA analysis. Six mice were given 2 nm gold nanoparticles and the other 6 animals served as controls. The livers of these animals were analyzed with NAA. The neutron activation dose was greater in this experiment resulting in a lower detection level (see NNA section).

Transcardial perfusion

The mice were anesthetized with sodium pentobarbital (50 mg/kg body weight) and transcardially perfused at 120 mm Hg with 3% buffered glutaraldehyde for 5 min (1 min of rapid flow followed by 4 min of reduced flow 5-10 ml/min). The lungs and liver were taken out immediately after perfusion and placed in the 3% buffered glutaraldehyde solution. In the "repeated instillations" groups, the median lobe of the liver was used for histological analysis. The lobe was divided at the gall bladder bifurcation. The right part of the lobe was used for paraffin sections whereas the left part was used for Epon sections and electron microscopy. The animals that were used for quantitative analysis with neutron activation were sacrificed by cervical dislocation and were not transcardially perfused.

Tissue processing

The tissue selected for paraffin sectioning was dehydrated and paraffin embedded. The tissue was then cut into 8 μ m thick sections. In all mice the same part of the liver was used for paraffin embedding, i.e., the right part of the median lobe, divided at the gall bladder bifurcation. This block of the liver measures approximately 10 \times 5 \times 5 mm. Seven to ten sections were cut from each mouse organ. The sectioning was performed with random start, and every 125th section was selected for analysis. These randomly selected sections were then AMG developed for 60 min and counterstained with toluidine blue. Tissue to be embedded in Epon blocks was cut in approximately 2 \times 2 \times 2 mm blocks with a razorblade or scalpel and rinsed in 0.10 M phosphate buffer (pH 7.4) for 2 \times 5 min, then dehydrated in graded ethanol solutions and embedded in Epon. Semithin sections (3 μ m) were cut with an ultramicrotome (Leica EM UC6, Leica Microsystems GmbH, Germany) and placed on glass slides before being AMG developed. After light microscopic analyses, selected sections were re-embedded on top of a blank Epon block and trimmed to include only the regions of interest. Thereafter, ultrathin sections were cut and counterstained with lead citrate and uranyl acetate before electron microscopic analysis on a Philips Morgagni 268D, FEI, USA.

AMG development

The AMG developer consisted of a 60 ml gum arabic solution and 10 ml sodium citrate buffer (25.5 g of citric acid \cdot 1H₂O + 23.5 g sodium citrate \cdot 2H₂O to 100 ml distilled water). Immediately before use 15 ml reducing

agent (0.85 g of hydroquinone dissolved in 15 ml distilled water at 40 °C) and 15 ml of a solution containing silver ions (0.12 g silver lactate in 15 ml distilled water at 40 °C) were added, and the AMG developer was thoroughly stirred [28]. The glass slides were put in a jar filled with the AMG developer and placed in a water bath at 26 °C. During AMG development an electric device shook the jars gently. The entire set-up was covered with a dark hood while the developing took place. After 60 minutes the AMG development was stopped by rinsing the slices in water and afterwards replacing the developer with a 5% sodium thiosulphate solution for 10 min (the AMG stop bath solution). The jars were then placed under gently running water for 20 minutes.

Post AMG treatment

The sections from the different sources were counterstained with a 0.1% aqueous toluidine blue solution (pH 4.0), dehydrated in alcohol to xylene, and ultimately embedded in DEPEX and covered with a cover glass. Black/brown silver grains represented silver-encapsulated gold nanoparticles. All procedures and protocols have previously been described in details [28].

Inductively coupled plasma mass spectroscopy (ICP-MS)

The median lobe, comprising about one third of the liver mass in mice, was used for morphological observations while the ICP-MS analysis was performed on the remaining part of the liver. Water was purified by reverse osmosis (Elix 100, Millipore, Bedford, MA, USA) and a Milli-Q system (Millipore). Polystyrene test tubes (Sarstedt, Numbrecht, Germany) were washed in tenfold diluted nitric acid (65% analytical grade; Merck, Darmstadt, Germany) and stored containing 0.7 M nitric acid purified by sub-boiling distillation in quartz still (Heraeus, Hanau, Germany). Digestion vessels were cleaned before use by adding 7.5 ml hydrochloric acid (30% p.a. plus, Fluka, Buchs, Switzerland) and 2.5 ml nitric acid and performing a wash cycle in the microwave oven (Mars 5, CEM Corporation, Matthews, NC, USA) at 300 W for 30 min.

Each sample was transferred to a freshly cleaned and rinsed microwave digestion vessel. Then 5 ml of sub-boiling distilled nitric acid and 0.5 ml hydrogen peroxide (30% p.a., Riedel-de-Haen, Seelze, Germany) was added, and the samples were digested for 60 min at 600 W. After cooling the vessels were opened, 1 ml hydrochloric acid was added to each vessel, and a further 10 min digestion at 600 W was carried out. Upon cooling the clear digest was transferred to a polystyrene test tube and diluted to 10 ml with high purity water. Prior to analysis the digests, sample preparation blanks, and calibrants were diluted appropriately and thallium was added to a concentration of 25 μ g/l as internal standard. A certified gold standard solution from Ultra Scientific (Kingstown, RI, USA) was

serially diluted to prepare calibrant solutions. Analyses of the diluted sample digests were carried out using inductively coupled plasma - sector field mass spectrometry (Element, Thermo Scientific, Bremen, Germany). The ICP-MS analysis was performed by ALS Scandinavia AB, Luleå, Sweden.

Neutron activation analysis

Neutron activation analysis (NAA) was performed at the Nuclear Research Reactor of the Helmholtz Center Berlin, Germany. The liver samples were freeze-dried and irradiated in an O-ring sealed test tube at a reproducibly achievable position within the reactor tank. For calibration purposes a pre-weighed gold standard was irradiated at the same location for the same time; from its measured radioactivity the Au mass in the liver samples was analyzed. For validation of the NAA method different amounts of gold was added to livers of untreated mice. In the first experiment the detection limit was 3.9 ng. In the second experiment the samples were irradiated with a greater dose of neutrons for obtaining a lower detection limit. The slope of the regression line of added *versus* detected gold was 1.013 and the detection limit was about 1 ng gold.

Results

Autometallographic analysis

Single instillations

In all 6 mice instilled intratracheally with a single dose of 2, 40 or 100 nm gold nanoparticles, gold was detected in the macrophages of the lungs after 1 h. Ultrastructurally, the nanoparticles were located inside lysosome-like vesicles. AMG stained sections from control animals were all void of gold (Fig. 1C).

Repeated instillations

20 animals were instilled intratracheally with gold nanoparticles 5 times during a period of 3 weeks. Initially, there were 4 groups consisting of 5 animals in each. These animals were exposed to 2, 40, 100 nm gold nanoparticles and distilled water, respectively. However, only 16 animals survived the experiment: 3 animals exposed to 2 nm gold nanoparticles and 1 animal exposed to 40 nm particles died during the instillation. All the animals exposed to 100 nm gold nanoparticles or distilled water only survived. The distribution of gold in the lungs of the animals treated by multiple instillations was identical to the pattern seen in the single instillation group although the AMG staining intensity and the amount of loaded cells were higher. Nanoparticles of all three sizes were retained in the lungs (Fig. 1A, B, D, E). The instilled gold nanoparticles were located in lysosome-like organelles in lung macrophages (Fig. 1D, E). The nanoparticles were present in macrophages, both those attached to the alveolar wall and inside the interstitium. However, we could not deter-

mine, whether the nanoparticles were located only in the macrophages or both in the macrophages and epithelial cells. Careful microscopic analysis of randomly selected cryosections of the liver revealed no sign of gold nanoparticles in the exposed animals. Only in one animal, exposed to multiple instillations of 40 nm gold nanoparticles, AMG enhanced gold nanoparticles were seen in a few Kupffer cells.

Quantitative analysis with Inductively Coupled Plasma Mass Spectrometry

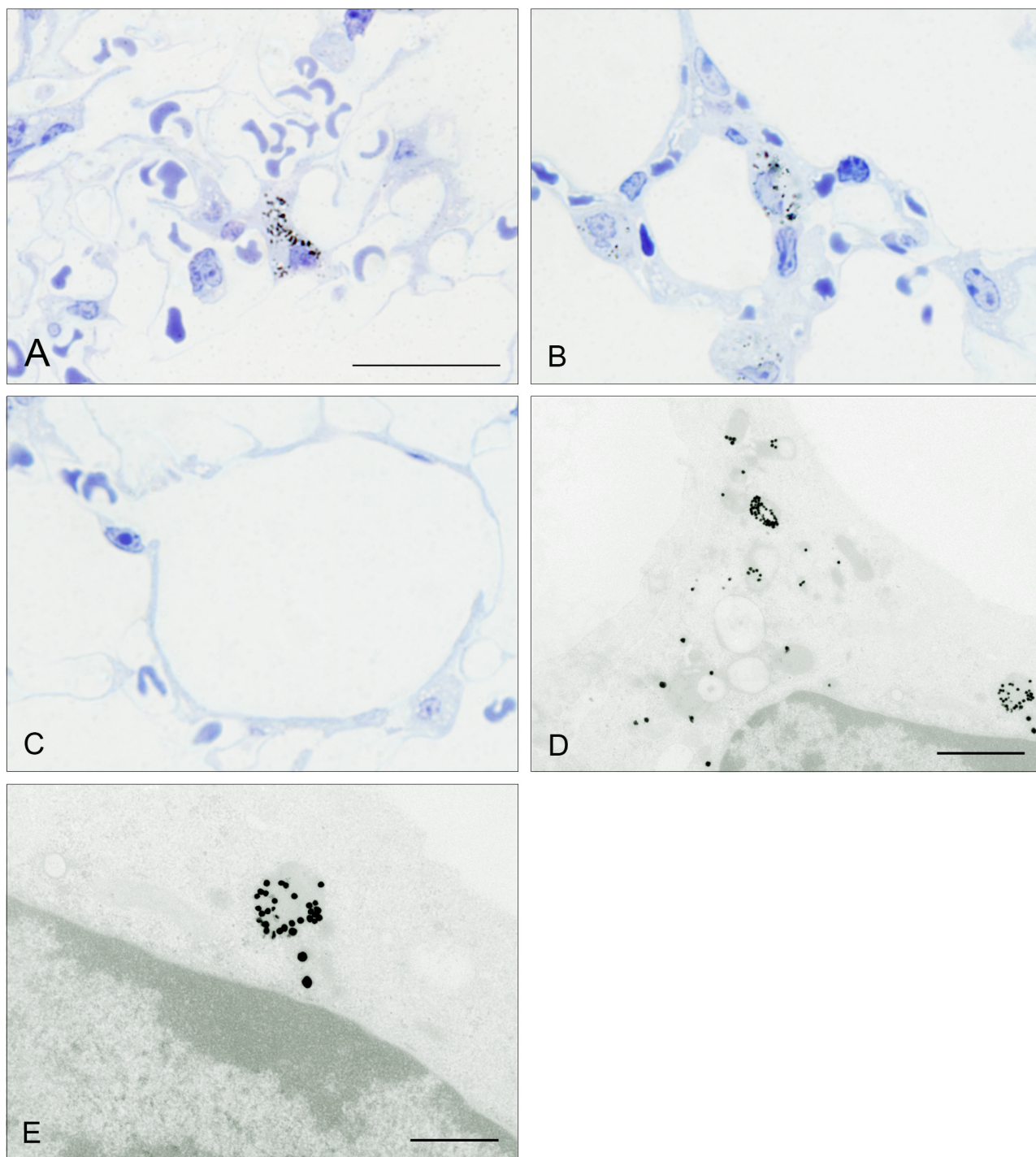
The livers of mice treated with multiple instillations were analyzed with ICP-MS (Table 1). The two mice that survived 5 instillations with 2 nm particles showed traces of gold in the liver, 4 and 4.1 ng gold in each. The total gold mass which was given to these 2 mice during a period of 3 weeks was 3025 ng of pure gold per animal and the amount of gold traced in the liver was 1.32 and 1.36‰ of the total dose given. In 1 animal, receiving 40 nm particles, 43.4 ng of gold was detected in the liver which is 3‰ of the total 14550 ng gold given. The amount of gold in the liver of the remaining three 40 nm gold mice was below detection level. In 1 animal, exposed to 100 nm particles, 5.8 ng of gold was traced in the liver which is 0.39‰ of the total 15 μ gold given. In the remaining 4 mice, given 100 nm gold nanoparticles, the amount of gold in the liver was below the detection limit. No gold was detected in all 5 control mice treated with distilled water.

Neutron activation analysis (NAA)

Two mice of the four mice included in the NAA study were instilled with a dose 2.9 μ g 40 nm gold nanoparticles. No gold was detected in the liver, neither in the experimental animals nor in the controls. The detection limit was 3.9 ng, which is < 2‰ of the instilled amount of gold. In the second experiment 6 mice were instilled intratracheally with 2 nm gold and 6 mice were instilled with distilled water to examine whether the gold instillations were toxic and to quantify the gold with greater sensitivity. The neutron activation dose was greater in this experiment resulting in a lower detection level, i.e. 1 ng. All 6 mice were unaffected by the gold instillations with the 2 nm gold. One day after the 5th instillation the gold content was determined only by NAA. The livers of the mice contained 5.8 ± 1.8 ng (mean \pm standard deviation). Because the mice were instilled with an accumulated dose of 3.03 μ g gold, 1.9 ± 0.6 ‰ of the instilled 2 nm gold particles had accumulated in the liver 1 day after the 5th injection.

Discussion

When nanoparticles are inhaled and deposited in the lung a fraction of them are being phagocytosed by alveolar macrophages [13]. A part of the particles are removed from the lung by the mucociliary escalator, but a part is

**Figure 1**

Light and electron micrographs showing AMG enhanced gold nanoparticles in the lungs of mice following intratracheal instillations. A and B represent LM section taken from the animals exposed to multiple intratracheal instillations with gold, 40 nm and 100 nm nanoparticles, respectively. AMG enhanced gold nanoparticles are located inside lung macrophages resembling cells. C represents a control, void of AMG staining. D and E are EM picture representing the animals exposed to multiples instillations with 40 nm gold nanoparticles. Gold is located inside lysosome/endosome-like vesicles. Scale-bars: 20 μm in A - C; 1 μm in D; 500 nm in E.

Table 1: Gold amount in the mouse liver following five intratracheal instillations detected with ICPMS.

Animal number	Particles used for instillations	Total amount/volume given	Amount of gold traced in the liver	Level of the gold in the liver compared with the total amount given
1.	2 nm Au	3025 ng	4 ng	1.3‰
2.	2 nm Au	3025 ng	4.1 ng	1.4‰
3.	40 nm Au	14550 ng	< 2 ng	0
4.	40 nm Au	14550 ng	< 2 ng	0
5.	40 nm Au	14550 ng	< 2 ng	0
6.	40 nm Au	14550 ng	43.4 ng	3‰
7.	100 nm Au	15025 ng	5.8 ng	0.39‰
8.	100 nm Au	15025 ng	< 2 ng	0
9.	100 nm Au	15025 ng	< 2 ng	0
10.	100 nm Au	15025 ng	< 2 ng	0
11.	100 nm Au	15025 ng	< 2 ng	0
12.	H ₂ O	250 µl	< 2 ng	
13.	H ₂ O	250 µl	< 2 ng	
14.	H ₂ O	250 µl	< 2 ng	
15.	H ₂ O	250 µl	< 2 ng	
16.	H ₂ O	250 µl	< 2 ng	

translocated across the alveolar epithelium [30-32]. Translocation of nanoparticles through the alveolar barrier, if it takes place, has been speculated to depend primarily on endocytotic-exocytotic activity and less so by paracellular transport e.g., due to histamine, hydrogen peroxide [13].

We detected 1.32-1.36‰ of the instilled dose of 2 nm gold in the liver by ICP-MS and $1.9 \pm 0.6‰$ by NAA in the second experiment. In 4 mice instilled 40 nm and 4 mice instilled 100 nm gold particles the liver was completely void of gold. However, in the fifth mouse of the 40 nm nanoparticles group gold was detected in the liver by both ICP-MS and by AMG. The gold content of the liver of that particular mouse was very high, which suggests airway damage during the instillation and an uptake of gold nanoparticles through the damaged tissue. One mouse in the 100 nm group had 0.39‰ accumulation of the gold dose in the liver. This is probably also due to damage to the airways during the installation.

Our study supports that only small amounts of gold nanoparticles are translocated from lungs [24,25] However, Semmler-Behnke reported significant translocation of smaller (1.4 nm) gold nanoparticles to the liver (0.7%). Similarly, we found that translocation of 2 nm particles takes place (only 1.3-1.9‰). However, another group reported significant systemic translocation following inhalation of gold nanoparticles with average diameter of 20 nm. Yu and coworkers detected 4.84% of the lung concentration in the blood 15 days after exposure [16]. The discrepancy between Yu and coworker's findings and ours could be caused by differences in the surface characteristics of the gold nanoparticles used. It should be noticed,

however, that they used rats, not mice, in their study. Also they exposed animals via inhalation, not instillation.

We found that gold could be traced in macrophages in the lungs in all 6 mice 1 hour after they were instilled intratracheally with a single dose of 50 µl gold nanoparticles. This proves that the uptake of gold into the lysosome-like organelles of macrophages is a rapid process. To establish the degree of systemic translocation, the livers of the animals were analyzed with AMG and ICP-MS. We have previously found that the liver is the main organ for accumulation of gold nanoparticles, and it is therefore a good probe for the translocation rate [18]. Our data suggest that very small amounts of gold particles 2 nm or less in size are translocated in normal healthy lungs and whereas there is less translocation of 40 nm and the 100 nm gold.

The present study shows that: (1) intratracheally instilled colloidal gold nanoparticles are being phagocytized by macrophages of the lungs; (2) translocation of the intratracheally instilled gold nanoparticles to systemic circulation is below detection limits of the used techniques or very low. (3) The translocation of 2 nm gold nanoparticles is greater than of 40 and 100 nm ones.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors contributed to execution of the presented work. ES designed the study, acquired and interpreted the data and drafted the manuscript. NRJ acquired and interpreted data. GD was involved in the interpretation of the

results and drafted the manuscript. MS, UV and AL were involved in the interpretation of the results and reviewed the manuscript. WK was involved in the acquisition of data, interpretation of the results and reviewed the manuscript. HW designed the study, interpreted data and drafted the manuscript.

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