Toxic effects of repeated oral exposure of silver nanoparticles on small intestine mucosa of mice

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Abstract
As the use of silver nanoparticles (AgNPs) is increasing fast in industry, food, medicines, etc., exposure to AgNPs is increasing in quantity day by day. So, it is imperative to know the adverse effects of AgNPs in man. In this study, we selected mice as an animal model and observed the effect of AgNPs on small intestinal mucosa. AgNPs ranging from 3 to 20 nm were administered orally at a dose of 5, 10, 15 and 20 mg/kg body weight to the Swiss-albino male mice for 21 d. There was a significant decrease (p < 0.05) in the body weight of mice in all the AgNPs-treated groups. Mice treated at a dose of 10 mg/kg showed the maximum weight loss. Effects were noted by using light microscopy as well as transmission electron microscopy. It was found that AgNPs damage the epithelial cell microvilli as well as intestinal glands. It may be hypothesized that loss of microvilli reduced absorptive capacity of intestinal epithelium and hence weight loss.

Introduction
Since ancient civilizations, man has used silver in medicine, eating utensils, ornaments, coins, clothes and as a disinfectant for water and for treating human wounds (Castellano et al., 2007; Richard et al., 2002). Now a day’s, new forms of silver having particle size less than 100 nm, i.e. silver nanoparticles (AgNPs) comes into focus due to its anti-bacterial activity (Alt et al., 2004; Martinez-Gutierrez et al., 2010; Nersisyan et al., 2003; Panacek et al., 2006; Shrivastava et al., 2007; Sondi & Salopek-Sondi, 2004; Xing et al., 2011), anti-viral and anti-tumor activity (Huang et al., 2011) as well as anti-platelet property (Shrivastava et al., 2009). AgNPs are use in daily life such as in medicine, toothpastes, paints, washing machines, food materials, water purification and shampoos. Due to these widespread uses, AgNPs released into the environment are bound to spread either in the air, water or soil. Already, It has been shown that AgNPs can enter the human body through several ports, and there have been several excellent reviews regarding intestinal uptake of particles (Florence & Hussain, 2001; Hussain et al., 2001; Jeong et al., 2010). There are reasons to be sure that exposure of gastrointestinal tract to the AgNPs must be taking place consciously or unconsciously. Nanomaterials can be ingested directly via water, food, cosmetics, drugs, drug delivery devices, etc. (Oberdörster et al., 2005; Peters et al., 1997).

Beside its widespread use, toxicity study of AgNPs needs further investigation. Present work was conducted to know the effect of AgNPs on small intestine using light microscopy and transmission electron microscope (TEM).

Methods
Animal model for in vivo study
A total of 50, 8–10 weeks-old Swiss albino male mice were obtained from animal house of the Department of Anatomy, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India. After being checked for infections of any kind for 1 week, all 50 mice were weighed. Animals with an average weight of 23 ± 5 g were used for the study. Commercial mice food (Pashu Aahar Kendra, Varanasi, India) along with water was given ad libitum. Mice were housed individually in plastic cages with a bed lined with absorbent material, i.e. rice husk providing nesting material. The cages were placed in a conventional room and bedding changed daily. The room was air conditioned at 23 °C and 40–60% humidity with a light/dark cycle of 12 h each. All the work performed on animals was in accordance with and approved by the Animal Ethics Committee of Institute of Medical Sciences, Banaras Hindu University (Varanasi, India). The animals were treated with utmost humane care and all aseptic precautions were observed.

Keywords
Microvilli, mitotic figure, silver nanoparticles, transmission electron microscope

History
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Synthesis of AgNPs

AgNPs were synthesized essentially as described by Shrivastava et al. (2007). Briefly, AgNO\textsubscript{3} (0.017 g) was dissolved in deionized water (100 ml) along with sodium hydroxide (0.01 M) to form a solution of stable soluble complex of silver ions. During this process, liquid ammonia (30\%) was added drop wise. Further, a mixture of D-glucose (0.01 M) and hydrazine (0.01 M) were also added to the solution of silver ions with continuous stirring. This ensures the complete reduction of the silver ions at a final concentration of 0.005 M. The pH of the solution was adjusted to 7.4 with citric acid (1 M). The brown solutions AgNPs were stored in closed glass vials at 4°C for future experiments. Before each experiment, the solution that contains nanoparticles was sonicated (Labsonic 2000, B. Braun, Apeldoorn, The Netherlands) for about 2 min and passed through filters of 0.2 \mu m pore size (Sartorius) in order to remove solid insoluble, larger nanoparticles or particle agglomerates, if any.

Characterization of AgNPs

The size, morphology and dispersal of AgNPs were characterized using a TEM model Technai 12 G2, FEI of the Department of Anatomy, BHU, Varanasi. For TEM, the sample were prepared by placing a drop of AgNPs on copper grids (TAAB, England, UK) coated with 2% Collodion in Amyl acetate (TAAB, England, UK) and subsequently dried in air, before transferring it to the microscope operated at an accelerated voltage of 120 kV.

Treatment of animals with AgNPs

Six to eight week old mice were identified by an “Ear Notch Code” and subsequently divided into five groups (n = 5 in each group). Each group was treated with or without AgNP solution as follows: first group (Control) received deionised water as vehicle; second group (Group A) received 5 mg/kg body weight (b.w.)/d AgNPs solution; third group (Group B) received 10 mg/kg b.w./d AgNPs solution; fourth group (Group C) received 15 mg/kg b.w./d AgNPs solution and fifth group (Group D) received 20 mg/kg b.w./d AgNPs solution. All mice were exposed to oral gabbage administration of vehicle and AgNP solution for 21 d. Body weight was recorded weekly i.e. on day 0, 7, 14 and 21. Each mouse from the experimental sets of study was carefully observed for any signs of toxicity or ill health throughout the experiment and was sacrificed on day 21 of AgNP treatment.

Collection of organ from mice

Body weight of all animals was recorded before killing. All animals were sacrificed by the cervical dislocation. Abdominal cavities of mice were cut open by a midline abdominal incision to collect small intestine.

Histopathology of small intestine

Small intestine was fixed in aqueous Bouin’s fluid (Bouin, 1897) for histological studies. Thereafter, the organs were embedded in paraffin, stained with hematoxylin and eosin, and examined under light microscopy. Observations were made on a binocular microscope. The results were recorded using a digital camera system attached to microscope.

TEM of AgNPs treated cells

Different intestinal samples, either with or without nanoparticle pretreatment, were fixed in Karnovsky’s fixative followed by post-fixation in osmium tetroxide. The tissues were dehydrated through an ascending series of acetone concentrations, cleared in toluene and embedded in resin (Araldite CY212) to prepare the blocks for TEM. Ultra-thin sections (70 nm thick) were cut with an ultra-microtome (Leica EM UC6, Leica Microsystems, Wetzlar, Germany). Samples were mounted on Formvar-coated grids followed by staining with uranyl acetate and lead citrate, and examined under a Technai-12 (FEI, Eindhoven, The Netherlands) electron microscope equipped with SIS Mega View III CCD camera (FEI, Eindhoven, The Netherlands) at 120 KV. Measurements were done using AnalySIS software (SIS, Muenster, Germany).

Statistical analysis

The experiment was terminated at the end of day 21 of AgNP’s treatment and repeated three times to check the reproducibility of the results obtained in the first experiment. All the data were analyzed by one-way analysis of variance followed by post-hoc test (Student Newman Keul’s test). The level of significance was tested at \(p < 0.05\) and confidence limit 95\% using SPSS software (Version 16.0; DST Centre for Interdisciplinary Mathematical Sciences, Faculty of Science, Banaras Hindu University, Varanasi, India).

Results

Characterization of AgNPs

TEM imaging of the AgNPs were performed to assess the range of primary particle size, obtain the size distribution and observe the general morphology of the particles. The size of particles ranged from 3 to 20 nm (average diameter of AgNP in the solution = 10.15 nm) (Figure 1) and the shape of the particles, in general, was either oval or circular (Figure 2).

Body weight of mice and food consumption

The most important finding observed during the course of treatment was decrease in the body weight of mice. A significant decrease in the body weight of mice was observed in all the treated groups compared to the control on day 14 and 21 during the experiment (Figure 3). Among treated groups, there was no significant change in the body weight for the first 7 d, i.e. from day 0. However, after day 7, a significant decrease in the body weight of mice was observed on day 14 and 21 of the experiment. It is noteworthy that, despite decrease in the body weight of mice in all AgNPs treated groups, no significant difference was observed in the food consumption (food supplied after due measurement) of mice between the treated and the control groups.

The maximum decrease in the body weight was observed in the group B which received AgNPs at the dose of 10 mg/kg body weight for 21 d. Hence, this dose was considered as an
optimum dose and further description is on mice treated with AgNPs at a dose of 10 mg/kg of body weight of mice for 21 d.

**Histopathology of small intestine**

Histological observation under light microscope revealed that in control mice the villi of small intestine were lined by simple columnar epithelium containing the tall absorptive cells with striated border due to microvilli, sometimes called terminal bar (Figure 4A). A few goblet cells were also observed between the columnar epithelial cells. Lamina propria was present in the core of each villus (Figure 4A). Intestinal glands were observed in the lamina propria with mitotic figures (Figure 5A). After oral administration of AgNPs, the following changes were observed in the small intestine.

(a) The striated border (terminal bar) was not demarkable as the microvilli were lost (damaged) (Figure 4B).

(b) Increased number of inflammatory cells in the lamina propria of each villus leading to the widening of lamina propria zone (Figure 4B).

(c) Increased number of mitotic figure in the intestinal glands (Figure 5B and C). This increased number of mitotic figure indicates that AgNPs damages the intestinal epithelial cell and to replace these damaged absorptive cells, stem cells in intestinal glands starts proliferating and show high mitotic activity.

**TEM of small intestine**

In control mice, striated border of enterocytes shows layer of densely packed microvilli (Figure 6A) which are nothing but the cylindrical protrusion of the apical cytoplasm to amplify area for absorption covered by plasmalemma (cell wall). However, after oral administration of AgNPs in mice, the
microvilli were totally disrupted. A few distorted one were still present in the area with shrunken lumen, which showed bead-like swelling probably because of AgNPs inside lumen. Small fragments of microvilli could be seen scattered in the area, i.e. lumen of intestine. AgNPs were present in various strata of small intestine including broken microvilli (Figure 6B).

**Discussion**

In the present work, we have attempted to investigate the adverse effects of repeated sustained oral exposure of AgNPs on the mucosa of the small intestine in mice. Intestine is lined by simple columnar epithelium. On the luminal aspect of each epithelial cell, microvilli were projected which are nothing but the cytoplasmic extension of enterocytes. These microvilli give the border a striated appearance in light microscopy, which is also known as ‘‘terminal bar’’.

In the present study, we observed that after administration of AgNPs, this terminal bar got lost which clearly indicated the damage to the microvilli. TEM also revealed that these microvilli were completely disrupted and broken, which indicates that AgNPs can cross the protective barrier of the small intestine i.e. the intestinal mucins and glycoproteins and cause impairment to the microvilli.

From the above histological finding, we can explain the significant decrease in body weight of mice in AgNPs treated mice. The main function of small intestine is absorption. Due to the loss of microvilli of enterocytes, the absorptive surface area of the intestine was markedly reduced which led to decrease in the absorption of nutrient materials resulting in a reduction in the body weight of mice.

There are certain evidences that after subcutaneous injection, AgNPs are translocated to the blood circulation and distributed throughout the main organs, especially in the kidney, liver, spleen, brain and lung in the form of particles (Tang et al., 2009). Tang et al. postulates that AgNPs crossed the BBB and by transcytosis of capillary endothelial cells these particles get accumulated in the brain micro-vessels vascular endothelial cells of rat (Tang et al., 2010). They also proposed that AgNPs induce degenerative changes in the some endothelial cells, which lead to the loosening of tight junction between the endothelial cells and AgNPs cross through these crevices. In our study, we observed the structural changes in the enterocytes, i.e. loss of microvilli, which may give passage to AgNPs for entering into the intestinal wall and then hence into the portal circulation and systemic circulation.

Ultra-structural studies have shown that activated platelets, when exposed to AgNPs in a concentration-dependent manner, lost their characteristic well-developed hyaloplasmic processes, pseudopods (Shrivastava et al., 2009). In addition, AgNPs were also found to be accumulated within platelet granules. After 28 d of repeated oral administration of AgNPs, a dose-dependent increased accumulation of AgNPs was also observed in the lamina propria in both the small and large intestine of Sprague–Dawley rats (Jeong et al., 2010). Jeong et al. (2010) also reported frequent cell shedding at the tip of the villi in intestine. Dose-dependent increase in the pigmentation of the villi observed after 90 days oral exposure of AgNPs, which was apparent treatment related effects (Kim et al., 2010). More recently, Loeschner et al. (2011) also reported presence of silver granules in the intestinal system of rats after 28 d repeated oral exposure of AgNPs or to silver acetate to rats (Loeschner et al., 2011). Similarly, in the present study, AgNPs were present in the various strata of small intestine including broken microvilli. A few distorted
microvilli were still present in the area with shrunken lumen, which showed bead-like swelling probably because of AgNPs inside lumen. Furthermore, in AgNPs treated mice, the microvilli on the intestinal absorptive cells were found to be badly damaged and broken (Figure 4B). It is possible that the nanoparticles are taken up across the intestinal barrier since particles with diameters less than 1 \( \mu \text{m} \) are particularly susceptible to absorption by the intestinal lymphatic system (Florence et al., 1997).

We may speculate that AgNPs somehow interact with the structural elements of the microvillus of the intestinal absorptive cells causing structural changes, and finally destruction of the microvilli. As evident from Figure 2(B), the terminal bar was absent in AgNPs treated intestinal cells and could be made out at light microscopy level with 1000X magnification. This view corroborates with Sondi & Salopek-Sondi (2004).

Sondi & Salopek-Sondi (2004) reported that AgNPs treated \( E. \text{coli} \) show significant changes in bacterial membranes. They also reported the formation of “pits” on the surfaces of the bacterial cell wall and accumulation of AgNPs in the bacterial membrane. A similar effect was also reported in \( E. \text{coli} \) bacteria treated with highly reactive metal oxide nanoparticles (Stoimenov et al., 2002). More recently, Shrivastava et al. (2007) also reported AgNPs managing to enter the gram-negative bacteria by making perforations in the membrane and thus resulting in cell lysis. A bacterial membrane with pits on the surfaces shows a considerable increase in permeability, leaving the bacterial cells incompetent of regulating transport through the plasma membrane and, finally, resulting in cell death (Sondi & Salopek-Sondi, 2004). Sondi & Salopek-Sondi, (2004) hypothesized that the change in membrane permeability is probably due to
progressive release of lipopolysaccharide molecules and membrane proteins. It is well known that the outer membrane of gram-negative bacterial cells is primarily made of tightly packed lipopolysaccharide molecules, which impart an effective permeability barrier (Nikado et al., 1985; Raetz, 1990; Silva et al., 1973).

The above supposition of Sondi & Salopek-Sondi (2004) may possibly explain the destruction of hyaloplasmic processes (pseudopods) of AgNPs-treated platelets (Shrivastava et al., 2009) and microvilli of the intestinal absorptive cells in mice in the present study. These AgNPs also pass through a thick layer of glycolcalyx, which is a polysaccharide, covers the microvilli of the intestinal absorptive cells and platelets too. The thick layer of glycolcalyx imparts protective function to microvilli from any corrosive material. A similar layer of glycolcalyx is also present on the outer surface of the platelets.

At this point, we may speculate that AgNPs somehow interact with this protective barrier and other structural elements of the microvilli of the intestinal absorptive cells causing structural changes, resulting in the membrane permeability and finally destruction of the microvilli. Subsequently, the epithelial cells of the gastro-intestinal tract get destroyed. Therefore, it is conceivable that in order to replace these damaged enterocytes, the neck cells of the crypt of Lieberkuhn start mitosis at a faster rate. Hence, the AgNPs administrated mice show larger number of mitotic figures as compared to the control animals. If the normal cells do not replace the damaged enterocytes, the site may result in the formation of ulcer.

Furthermore, it could also be hypothesized that the AgNPs may possibly interfere with the cell cycle kinetics without inducing cell death. Because small intestine is an organ, which affords a generous population of continuous dividing cells, it may also be speculated that when AgNPs breach the protective barrier of the intestinal cells, somehow these nanoparticles trigger the regulators of the cell cycle and the process of cell division is accelerated and cellular uptake of AgNPs will be reduced over time due to augmented cell division. Several studies have shown that the uptake of the nanoparticles by cells is influenced by different phases of their cell cycle and cell division can dilute the concentration of nanoparticles in the cell population (Errington et al., 2010; Kim et al., 2012; Summers, 2012; Summers et al., 2011). Moreover, it may also be speculated that AgNPs affect the cellular response at the molecular level and may increase the expression of certain genes related to cell cycle pathway and cause abnormal cell proliferation (Kawata et al., 2009).

**Conclusion**

Based on our results, it is postulated that AgNPs somehow interact with the protective layer of the glycolcalyx and other structural elements of the microvilli of the intestinal absorptive cells causing structural changes, resulting in the alteration of membrane permeability and finally destruction of the microvilli. Subsequently, the epithelial cells of gastro-intestinal tract get destroyed and are the reason for the decrease in body weight of mice. We thus conclude that AgNPs destroy the mucosa of small intestine and impede its function.

**Declaration of interest**

Authors have no conflict of interest. Madhu Yashpal is thankful to University Grants Commission, New Delhi, India for financial support in the form of Dr D.S. Kothari Postdoctoral Fellowship [F.4-2/2006 (BSR)/13-455/2011 (BSR)].

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