Local Silver Nanoparticles Administration Promotes Inflammation and Hyperalgesia in Rats

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Silver has no known function in the organism. However, nanosilver has the highest degree of commercialization of all nanomaterials used in healthcare. The aim of this study was to assess the potential deleterious effect of local nanosilver administration in an animal model. Wistar rats received a subcutaneous injection (hind paw) of either 500ppm nanosilver (group S1), 20ppm nanosilver (group S2) or saline (control group). Animals were tested by means of plantar test, analgesy-meter and plethysmometer. 24 h after the administration, λ-carrageenan was injected (same site), which lead to localized inflammation. The above-mentioned assessments were performed repeatedly until 24 h after λ-carrageenan administration. 24 h after colloidal silver administration, both S1 and S2 groups had a significantly higher sensibility to mechanical stimuli. 48 h after colloidal silver administration, the S1 group had a significantly higher sensibility to thermal stimuli. Paw edema was more pronounced in the treatment groups in the first 30 h after the nanosilver injection. Local subcutaneous nanosilver administration leads to an increased inflammatory response and to hyperalgesia. Considering the constant increase in nanosilver's biomedical use, the current paper sends a clear warning for the need of urgent more in-depth research on the matter.

Keywords: nanosilver , λ-carrageenan, paw edema, hyperalgesia

Silver has been used for medical purposes since ancient times - Hippocrates believed silver powder had beneficial healing and anti-disease properties [1]. Aqueous solutions that contained colloidal silver for oral administrations appeared in the early part of the 20th century [2]. These solutions, marketed as *health maintainers or immuno-boosters* [3] were quite popular in the 90's. In the 00's, silver nanoparticle suspensions (particles with one or more dimensions on the order of 100 nm or less) [4] entered the market with similar medical recommendations. Silver suspensions have been investigated as treatment of various infections, emphysema, bronchitis [5], chronic rhinosinusitis [6] psoriasis, atopic dermatitis [7] or cystic fibrosis [8]. They have also been used as anti-inflammatory agents in cystitis, prostatitis, colitis, gastritis, tonsillitis, appendicitis and sinusitis [1].

It is common belief that silver is relatively non-toxic to humans. Most studies report only that prolonged exposure to silver may lead to a condition called argyria (irreversible pigmentation of the skin and/or eyes), but the level of exposure required is very high and this disease is quite rare nowadays [9]. Occupational exposure studies consider working with metallic silver as a minimal health risk [10].

However, silver is not an essential element and has no known function in the organism. In 1999, FDA issued a statement to address the widespread use of oral silver suspensions in which it highlighted the absence of scientific evidences to support their use [11]. As such, the product was considered *misbranded* under the law without appropriate FDA approval as a new drug. Despite FDA's warning, this metal remained available and is nowadays accessible both in brick and mortar stores and in the on-line market as a homeopathic remedy or a dietary supplement [12]. The market-available silver suspensions mostly come from unknown or unreliable sources and there is no actual control of the product's quality. Some on-line shops also sell generators that produce colloidal silver at home, another important factor that contributes to the lack of control for this product. In the current setting, it is quite difficult to assess the amount of silver one individual consumes and the risk of chronic treatment with silver suspensions.

It is estimated that of all nanomaterials in the medical and healthcare sector, nanosilver has the highest degree of commercialization [3], with approximately 320 t of nanosilver produced worldwide per year [2]. Together with the uncontrolled on-line market for oral silver suspensions, this metal is also used in the manufacturing of silver-embedded medical equipments [13] (surgical tools, catheters, bandages, needles and stethoscopes) and in implantology [14].

In 2005, the in vitro toxicity of several nanoparticles was assessed [15] and the authors concluded that silver has the highest concentration-dependent toxicity. In 2014, a Scientific Committee employed by the European Commission issued a document regarding nanosilver and its safety [16]. The experts concluded that nanosilver's toxic effects are still unknown because too little information is available.

After implant surgery, the post-operative period is characterized by a prolonged systemic and localized inflammatory response [17]. Consequently, silver particles have prolonged interaction with both normal and inflammatory tissue after implant surgery takes place. Some studies suggest silver particles promote inflammation in normal tissue [18], but the effect of the metal on an pre-existing inflammatory environment is unknown.

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One of the main characteristics of inflammation is that pain can result after exposure to normally innocuous stimuli [19] or even in the absence of any external trigger. The extent of post-surgery inflammation influences the severity of pain [20]. This is a consequence of inflammatory mediators' release from inflammatory or damaged cells and from the activation of the arachidonic acid pathway [21]. These mediators will in turn activate nociceptors by either direct or indirect pathways and modulate primary afferent neurons generating peripheral sensitization [22]. As such, inflammatory pain is a distinct type of pain that may be influenced by triggers that have no effect on normal tissue. Clinically, this is expressed as hyperalgesia or allodynia.

The aim of this study was to assess the potential deleterious effect of local nanosilver administration in an animal model. Literature search indicated that this is the first study to assess the effect of silver nanoparticles on pain and inflammation.

Experimental part

Materials and methods

Animals

Adult Wistar male rats (180-200 g) were purchased from the National Institute of Research and Development Victor Babeº, Bucharest. The animals were housed individually at 21 ± 2°C under a 12-h light/ dark cycle with ad libitum access to food and water. About 24 h prior to the beginning of the experiment, each animal was accommodated for 15 min to the testing room.

Ethics Statement

Animal care was in accordance with the *Guide for the Care and Use of Laboratory Animals* published by NIH and with the *Policies on the Use of Animals and Humans in Research* published by the Society for Neuroscience. The International Association for the Study of Pain (IASP) guidelines for the investigation of pain in animals were followed. The design of the experiment was approved by the University of Medicine and Pharmacy Gr. T. Popa ethics committee. All the rats were euthanized at the end of the experiment in accordance with the AVMA Euthanasia Protocol.

Drugs

The following drugs were used in the experiment: colloidal solution with 500 parts per million (ppm) silver nanoparticles purchased from US Research Nanomaterials, Inc, Houston, USA (Silver (Ag) Nanopowder / Nanoparticles (Ag, 99.99%, 30-50 nm, w/~0.2 wt% PVP Coated), colloidal silver 20 ppm (©Nano Silver, 30-50 nm, PVP Coated, Vita Crystal, RO), λ-carrageenan 1% diluted in fresh saline (Sigma- Adrich Germany).

Doses were selected according to literature data. In a dermal toxicity study, Korani et al used doses that ranged from 100 ppm to 10000 ppm nanosilver in a subchronic administration regime [23]. Up to 10 ppm nanosilver were administered daily in pregnant female rats via drinking water in a study to assess the expression of procaspase-3 in newborn rat brain [24]. Another experiment repeatedly injected 60-2000 ppm nanosilver subcutaneously (intralesional administration) in a mouse model of cutaneous leishmaniasis [25].In the present study, one group received nanosilver 500 ppm and the other treatment group received nanosilver 20 ppm single dose.

Study design

Rats were divided in three groups (n=6/group) as follows: Group S1 received 10 microliters colloidal silver 500 ppm, group S2 received 10 microliters colloidal silver 20 ppm and group C received an equivalent volume of saline. All drugs were injected subcutaneously (s.c.) in the intraplantar region of right hind paw. Response latencies were assessed by means of the plantar test and the analgesy-meter; inflammation was assessed by means of the plethysmometer. All animals were evaluated at baseline and 3 and 24 h after colloidal silver/saline administration. After the 24-h assessment, all animals received an s.c. intraplantar injection of 10 microliters λ-carrageenan 1% into the right hind paw. This lead to a localized inflammatory response (acute inflammation). The above-mentioned assessments were performed 3, 6 and 24 h after λ-carrageenan administration.

Tests

Plantar Test

The Plantar Test (Hargreaves method) [26] assesses the animal's response latency to a thermal stimulus. The rats are placed into clear acrylic boxes on a Plexiglas floor and a radiant heat source from the Hargreaves unit (Plantar Test-37370 Ugo Basile) is placed under the hind paw. The time until the animal withdraws or moves its paw (thermal paw withdrawal latency - PWL) is automatically recorded due to a chronometer controlled by an infrared sensor connected to the system. Cut-off is set at 30 s.

Randall-Selito Method

The Analgesy-Meter (7200; Ugo Basile, Italy) assesses the animal's response to increasing paw pressure. The force applied progressively increases by 16 g/s; the animal's paw is placed on a small plinth under a cone-shaped pusher with a rounded tip. When the pressure becomes painful for the animal, it withdraws its paw and the time elapsed until that moment is recorded - mechanic PWL [27].Cut-off is set at 250 g (16 s).

Assessing Inflammation

Colloidal silver's effect on inflammation was assessed with the aid of the Ugo Basile Plethysmometer 7140(©Ugo Basile, Italy). The device is a volume meter that consists of a water filled cell into which the rat paw is dipped and a transducer that records differences in water level caused by volume displacement [28].

Statistical Analysis

Statistical analysis and graphic design was performed with the aid of GraphPad 3.0 software (GraphPad Software, La Jolla, CA). Descriptive statistics and analysis of variance (Mixed ANOVA with Tukey post-hoc test) were performed. The data obtained was expressed as the mean value ± standard error. For all analyses, the a priori significance level was set at $p<0.05$.

Results and discussions

Plantar test

There were no significant differences between groups at baseline and three hours after treatment. Twenty-four hours after colloidal silver administration, group averages were 14.73±1.17 for group S1, 16.68±1.3 s for group S2 and 16.08±1.9 s for group C. However, this difference did not reach statistical significance. After λ-carrageenan (CG) was injected, there was an important decrease in thermal PWLs in all groups, with an average of 3.5±0.4 s in the S1 group, 6.50±1.1 s in the S2 group and 6.2±1.3 s in the C

group 3 hours after CG. Twenty-four hours after CG, mean PWLs were significantly lower in the S1 group when compared to both S2 and control groups ($p<0.0001$). At this time point, average values were 6.61±0.6 s in the S1 group, 15.10±1.0 s in the S2 group and 15.46±1.1 s in the C group (fig. 1).

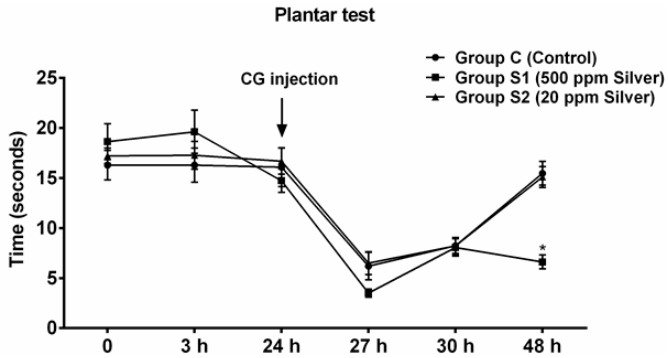

Fig. 1. Average thermal PWLs throughout the experiment.
* = $p<0.05$ (Tukey post-hoc)

Randall-Selito test

There were no significant differences between groups at baseline and three hours after treatment. However, 24 h after colloidal silver administration both treatment groups had a significantly higher sensibility to mechanical stimuli, with averages of 4.16±0.2 s for group S1, 4.75±0.2 s for group S2 and 5.63±0.4 s for group C ($p=0.02$ for S1 vs. C and $p=0.03$ for S2 vs. C) (fig. 2). After CG injection, in all three groups a decrease in mechanical PWLs was recorded - (1 s latency for all rats three hours after CG). Twenty-four hours after CG, control averages increased to 2 s, whereas averages in the treatment groups were 1.60±0.2 s (S1) and 2.25±0.3 s (S2). After CG administration, there were no statistically significant differences between groups (fig. 2).

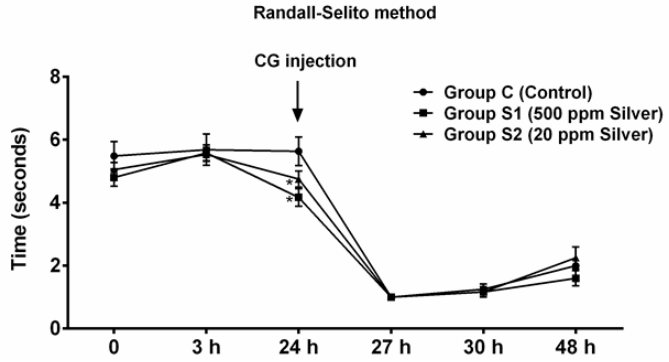

Fig. 2. Average mechanical PWLs throughout the experiment
*=$p<0.05$ (Tukey post-hoc)

Plethysmometry

There were no significant differences between groups at baseline. Three hours after colloidal silver injection, paw volume was 1.31±0.03 in group S1, 1.27±0.03 in group S2 and 0.91±0.03 in group C. This difference was statistically significant, with $p < 0.0001$ for S1 vs. C and $p = 0.0002$ for S2 vs. C (fig. 3). Colloidal silver's effect was persistent - 24 h after treatment S1 and S2 groups had an increased paw volume when compared with C group, with $p = 0.02$ for both S1 vs. C and S2 vs. C. Three hours after CG injection, an increase in paw volume was noted for all groups. However, paw edema was more pronounced in the silver-treated groups, with an average of 2.19±0.1 in group S1, 2.15±0.07 in group S2 and 1.92±0.09 in group C ($p < 0.006$ for S1 vs. C and $p = 0.004$ for S2 vs. C) (fig. 3). In the control group, paw edema reached a maximum 6 h after CG injection (mean of 2.24±0.06); at this time point, no

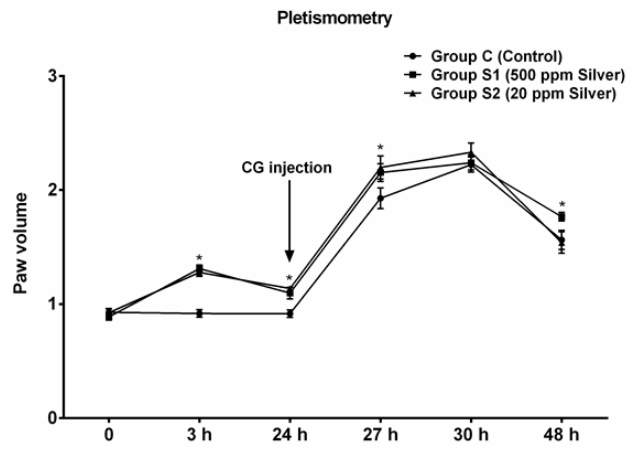

Fig. 3. Paw volume throughout the experiment
*=$p<0.05$ (Tukey post-hoc)

statistically significant differences were noted between groups. Paw edema began to decrease afterwards and 24 hours after CG injection averages were 1.76±0.03 in group S1, 1.54±0.09 in group S2 and 1.56±0.08 in group C. Paw volume of group S1 rats was significantly larger than paw volume in group C ($p=0.01$).

Despite the many advantages of nanotechnology, the use of metals at such a small scale comes with some changes in an element's properties. Certain nanomaterials may exhibit significant toxicity to mammalian cells even if they are biochemically inert and biocompatible in bulk size [29]. Upon reaching nanoscale, like other nanomaterials, silver particles exhibit remarkably unusual physicochemical properties and biological activities [3], which may lead to unpredictable effects and interactions. In vitro, silver nanoparticles are citotoxic for macrophages and generate free radicals [30]; also, a recent review emphasizes nanosilver's effect on cells pointing out that accumulation of nanoparticles of silver within the cell leads to oxidizing stress, genotoxicity, and cytotoxicity through apoptosis [31]. Carlson et al further underlined that the smallest particles have the most toxic effects [18]. In rats, chronic ingestion of silver nanoparticles induced heart and liver dysfunction and promoted systemic oxidation and inflammation [32].

However, scientific papers in the field mainly focus on the effects of inhalation, ingestion or topical use of silver nanoparticles; only few studies explore the effect of direct contact between internal organs/structures and silver nanoparticles and its consequences on local pain and inflammation. In one study performed by Sarhan and Hussein in 2014 [33] that explored the effects of intraperitoneal (i.p.) silver nanoparticles administration, results indicated marked citopathological changes in both renal and hepatic tissues, with increased white blood cell count. In our study, nanosilver induced hyperalgesia, but only in an inflammatory setting. There were no differences in pain behavior throughout the 24-h assessments performed after nanosilver administration. After CG administration, however, tissues previously treated with nanosilver exhibited a more pronounced pain-related behavior and more important inflammation. One study indicated that s.c. administration of silver nanoparticles increased infiltration of endothelial cells, VEGF and NO concentration [34]. Samberg et al. found that human epidermal cells exposed to silver nanoparticles produced an increase in inflammatory cytokines such as IL-1β, IL-6, IL-8 and TNF-α [35]. These findings are in accordance with our results, because both endothelial cell infiltration and increased NO release are associated with both

inflammation and hyperalgesia [36]. To our knowledge, there are no other studies directly measuring the mechanical and thermal sensibility for stimuli in nanosilver infiltrated tissues. As such, potential explanations for our results derive from silver's interaction with local cellular environment, nervous system and inflammation.

One other possible explanation for colloidal silver's hyperalgesic effect is the induction of reactive oxygen species (ROS) - a side-effect of nanosilver reported by in vitro studies [37]. ROS have been more and more implicated in the enhancement of excitatory synaptic transmission [38] and sensitization of dorsal horn neurons [39]; they are considered to be proalgesic mediators that produce elicit pain by stimulating transient receptor potential channels [40]. Also, silver nanoparticles may induce hyperalgesia through cell apoptosis and necrosis [41]; this hypothesis is extremely probable, especially since in the present study nanosilver's hyperalgesic effects were noted 24-48 h after s.c. injection.

Some studies have also suggested that nanosilver has neurotoxic effects. Due to their small size, nanoparticles are highly mobile in the human body and systemic distribution can occur after inhalation or oral uptake. Nanoparticles cross the blood-brain barrier, reaching the olfactory bulb and the cerebellum [42] and may have a direct effect on the central nervous system. A study performed by Ganjury et al recently proved that developmental exposure to nanosilver induces neurotoxicity and apoptosis [24]. Neuronal damage, both in the peripheral and in the central setting, could be another possible explanation for silver nanoparticles' hyperalgesic effect.

It is also possible that the pain-related behavior observed after nanosilver administration to be a response to silver's toxicity. Indeed, there have been reports of dermal toxicity [3] after topical application and preferential uptake of nanosilver by several organs and tissues [4], including the musculo-skeletal system. However, we believe this is not applicable to our results because, on the one hand, in our study nanosilver was administered in an acute setting (single dose) and, on the other hand, because the doses used in our study are significantly smaller than the ones used in toxicity studies.

CG administration produced a local edema that persisted until the end of the experiment. Sensibility to thermal and mechanical stimuli increased in all groups shortly after CG injection due to the localized inflammatory response; all groups had similar PWLs 3 and 6 h after CG administration, probably due to the tests' inability to detect differences in pain behavior beneath a certain threshold. However, 24 h after CG injection, thermal sensibility was significantly decreased in the S1 group that had received high-concentration SNPs. In the Randall-Selito test, the rats still had an increased sensitivity for mechanical stimuli and the Analgesy-Meter was probably still unable to detect fine differences.

The present study indicates that silver nanoparticles had a pronounced pro-inflammatory effect that started 3 hours after silver nanoparticle injection and persisted throughout the experiment, with 20-40% increase in paw edema in the silver nanoparticle groups when compared with control (as assessed by plethysmometry). This difference remained significant even after CG injection. Other nanotechnologies have been associated with increased inflammatory response as well - a study performed by Shvedova et al in mice indicated that pharyngeal aspiration of single-walled carbon nanotubes induced a robust inflammatory response with early onset, progressive fibrosis and granulomas. Reference materials also tested - ultrafine carbon black, Si02 or PBS- did not cause thickening of alveolar walls, did not induce formation of granulomas, and resulted in a significantly lower magnitudes of inflammatory responses [29]. However, other studies suggest that silver nanoparticles have anti-inflammatory effects and can attenuate allergic airway inflammation and hyperresponsiveness [43] or decrease inflammation in a postoperative peritoneal adhesion animal model [44]. A study performed by Wright et al. indicated that nanocrystalline silver-coated dressings lead to diminished production of matrix metalloproteinase, decreased inflammation and more rapid wound healing [45]. One other study suggested that nanocrystalline silver's topical effect is so strong it may have therapeutic potential for treatment of several inflammatory skin diseases [46]. Contrary to the above-mentioned studies, our research indicates that nanosilver has a pro-inflammatory effect. This can be partly explained by the route of administration used in our study (local subcutaneous administration), that is different from topical dermal application in terms of concentration and kinetics. Also, most studies involving nanosilver and the cellular microenvironment have contradicting results, most likely because toxicity of nanoparticles depends on many factors including size, shape, chemical composition, surface area and surface charge [47], which may vary greatly across study and/or geographic region.

Limits of the Study

This study has a couple of limitations. First, the number of tests performed is limited: analgesia and inflammation were evaluated by means of behavioral assessment and plethysmometry. Second, administration of nanosilver was local and not systemic, so we could only assess the activation of local mechanisms and processes. The exact mechanisms by which SNPs induce hyperalgesia and inflammation are not entirely explored and assessed. This study should be followed by another one with similar design, but with systemic nanosilver administration, to see if the hyperalgesic and pro-inflammatory effect persist in this setting.

Conclusions

Our results indicate that local subcutaneous silver nanoparticle administration leads to an increased inflammatory response and to hyperalgesia. The current results are insufficient for drawing a definitive conclusion regarding nanosilver's local and systemic toxicity. However, the authors believe that, considering the constant increase in silver nanoparticles' biomedical use, the current paper sends a clear warning for the need of urgent more in-depth research on the matter.

Ethical approval:All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in this study were in accordance with the ethical standards of the institution at which the studies were conducted.

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