Pilot study on in vitro Silver Nanoparticles permeation through meningeal membrane

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Abstract:

Silver nanoparticles (AgNPs) are used as a common ingredient in antiseptic sprays and mists; they can easily come into contact with the upper-airway mucosa. The intranasal pathway represents the only direct connection between the external environment and brain structures, which are generally considered to be well protected. Drugs absorption through this route has been widely studied, but toxicological knowledge is scant. The olfactory bundles are surrounded by meningeal sheets in their path from the nasal mucosa to the olfactory bulb. This study investigated the transmeningeal absorption of 19 nm AgNPs, using excised porcine meninges mounted on Franz diffusion cells in vitro. Two donor solutions were used: one containing AgNPs (0.5 g/L), and another containing only the water-soluble silver species derived from the ultrafiltration of the first one. Each experiment was carried out separately for 2 hours. Results showed silver flux permeation through the meninges, with similar values in both experiments (0.78 ± 0.71 ng cm^{-2} h^{-1} and 0.73 ± 0.43 ng cm^{-2} h^{-1}, for AgNPs and Ag ions respectively, mean and SD). This study demonstrates that the meningeal barrier is permeable to silver and silver ions, when it is exposed to this metal in nanoparticular form; this might lead to neurotoxic and neurodegenerative effects, as recently shown by other studies. Silver nanoparticles are used by workers and consumers, and potential penetration through meningeal membrane needs to be considered and prevented when it is possible an inhalation exposure.

Key terms: Franz cells, in vitro, intransal absorption, neurotoxicology.

1. Background:

Silver nanoparticles (AgNPs) are the main ingredient in many antibacterial sprays, which are commonly used on a wide variety of surfaces, in occupational and private settings. Despite European and American regulations,[1] their use is often not supervised; in addition, there are no specific rules regarding their safe use in other regions of the world. Kim and his coworkers investigated some of these commercial products; a relevant risk of nanoparticles inhalation was found in some cases, as the margin of exposure was higher than the no-risk concern level of 1000.[2] Furthermore, the use of spray containing AgNPs is even being recommended as a remedy for respiratory disorders such as asthma, which means that many patients could be exposed to considerable amounts of AgNPs through inhalation.[3] Although silver is generally well tolerated and non-toxic for humans, there are isolated reports of neurological, kidney and liver disorders as a result of its absorption in large quantities.[4, 5]

Inhalation exposure to NPs poses a well-known problem because of their possible translocation trough the alveolar-capillary barrier into the respiratory system, which can lead to a systemic uptake of xenobiotics. On the other hand, a possible direct absorption via the intranasal route - circumventing the very tight blood brain barrier (BBB) - is still an overlooked issue.

The central nervous system (CNS) is highly protected from xenobiotics penetration, due to the presence of barrier structures; however, there is a direct connection between the external environment and the front portions of the CNS via the olfactory nerve. The latter connects the olfactory bulb to the nasal cavity, passing through the openings in the cribriform plate of the ethmoid bone, which is covered by the dura mater. Along this route, the branches of the olfactory nerve are surrounded by extensions of the dura mater, which descend into the nose through these apertures.[6]

This biological pathway has been extensively studied in recent decades, in association with drug administration in cognitive, neurodegenerative and psychiatric diseases, and also in some functional disorders of the CNS.[7-12] Studies have shown that nanoparticular drug administration conveys a 1.6 to 3.3 times higher substance concentration in all the compartments of the CNS (cerebral spinal fluid, olfactory bulb, olfactory tract, brain and cerebellum), compared to traditional drug administration.[13]

To this day, the potential absorption of metal NPs by means of this mechanism has been poorly investgated; nevertheless, it represents a matter of concern, since the CNS may be exposed to the neurotoxic effects of xenobiotics in professional and/or environmental scenarios.[14-16].

Absorption through this path can take place by intraneuronal passage (i.e. inside the sensory neurons) and also by extracellular passage, which consists of the transcellular path (through the sustentacolar supporting cells) and of the paracellular path (through the intercellular cleft).[17]

The aim of this study is to assess the potential permeability of the meningeal membranes that are involved in the extracellular pathway, whose penetration properties to silver nanoparticles (AgNPs, the most commonly used NPs) are still unknown.

The aim of this study is to assess the potential permeability of silver NPs through meningeal membranes that are the first barrier to CNS.

2. Material and methods

2.1. Chemicals

All the chemicals used in this study were analytical-grade. Sodium chloride, sodium hydrogen phosphate, potassium dihydrogen phosphate, nitric acid (69% v/v), hydrochloric acid (37% v/v) were purchased from Sigma Aldrich (Milan, Italy), while ammonium hydroxide (25%) was

purchased from J.T. Baker (Milan Italy). Water reagent grade was produced with a Millipore purification pack system (milliQ water).

The physiological solution used as receptor fluid was prepared by dissolving 2.38 g of Na_2HPO_4, 0.19 g of KH_2PO_4 and 9 g of NaCl into 1 L of milliQ water (final pH = 7.35).

2.2 Silver nanoparticles characterization

Polyvinylpirrolidone-stabilized AgNPs (content of silver: 25% w/w, polymer 75%) were supplied by NanoAmor Materials Inc. (Houston, TX, USA). AgNPs from the same batch were characterized and used in previous permeation studies through ex vivo human skin[18] and through porcine oromucosal membrane.[19]

Size and morphology of the AgNPs metal cores, while dispersed in physiological solution, were obtained by means of Transmission Electron Microscopy (EM208; Philips, Eindhoven, The Netherlands, operating at 200 kV) with an high definition acquisition system, based on a side-mounted TEM camera (OSIS Morada) and an iTEM software platform (Olympus Soft Imaging Solutions GmbH, Münster, Germany). The polymer coating composition was confirmed using a FT-IR Spectrum 100 (Perkin Elmer, Waltham, MA, USA; FT-IR, Fourier transform-infrared) fitted with an attenuated total reflection (ATR) (Ge/Ge) accessory.[18]

The average values of the AgNPs size and polydispersity, defined as a relative width of the size distribution, were determined from Dynamic Light Scattering (DLS) measurements, using a Zeta sizer Nano Z (Malvern Instruments Ltd.) analyzer, applying a 633 nm laser with a 173° orientation with respect to the sample.[19]

Zeta potential measurements were carried out using a ZetasizerNano ZS (Malvern). The zeta potential was calculated using Henry's equation.[19]

2.3. Donor phases preparation

Two different donor phases were prepared immediately before the beginning of the experiments, in order to distinguish between the permeation of AgNPs and that of silver ions released from the NPs.

The first donor phase consisted of the AgNPs dispersion, which was prepared using 100 mg (with a 1:4 metal/polymer ratio) of AgNPs dispersed in 50 ml of physiological solution by sonication, in order to obtain a silver concentration of 0.50 g L^{-1}. Using the ultrafiltration technique, 5% of silver in ionized form was revealed in the nanoparticles water suspension. The presence of silver ions did not change significantly during the experiment, which lasted 2 hours.

The second donor phase was prepared applying ultrafiltration to the first one, thus selecting the water-soluble silver species only. To separate the AgNPs from the aqueous solution, 4 ml of the AgNPs solution were ultrafiltered in a centrifuge at 5000 rpm for 30 min, using Amicon Ultra-4 centrifugal filters (10 KDa MWCO). Filtration was reiterated on five different aliquots, that were mixed for a total of 20 mL, in pursuance of an adequate volume for silver quantification analysis and permeation experiments.

In order to define the percentage of silver ions inside the AgNPs solution, the donor phases were analyzed by means of Inductively Coupled Plasma – Optical Emission Spectrometry (ICP–OES).

2.4. Preparation of meningeal membranes

Thanks to the high percentage of genomic and morpho-physiological similarities between pig and human,[20-21] the pig model is commonly used in biomedical research studies, as well as in some cases of animal to human xenotransplantation.

Porcine meninges were collected from a slaughterhouse in Trieste, IT. The membranes were excised from the animal skull immediately after the slaughter. The pigs were up to 1 year old. The head was sawed in half along the cranium caudal line, in order to achieve access to the ventral surface of the skull region. The meningeal surface adherent to the skull was detached from the underlying bone with surgical forceps. For each animal two pieces (~5 cm diameter) of meninges (one from each side of the skull) were obtained. During the transportation to the laboratory, the tissue was stored at 4°C; subsequently, it was stored in a refrigerator compartment at −80°C, for a maximum time of 1 week.

Ex vivo studies have demonstrated that this cryopreservation temperature can adequately preserve neuronal cells viability and specific features.[22-23]

The tissues were removed from the refrigerator and soaked in a physiological solution at room temperature for about 30 minutes before starting the permeation experiments.

Integrity of the membranes was checked before and after each experiment, using the following method: intact membranes were mounted on Franz Cells, and the donor chambers were filled with MilliQ water. The receiving chamber was monitored for presence of the solution for a period of 30 minutes, and if integrity was confirmed, the permeability experiment was commenced.[24]

After the experiments the procedure was repeated, and if a leak of saline solution appeared in the receiving chamber, the membranes were discarded.

2.5. In vitro diffusion system

Meningeal permeation studies were performed using static Franz diffusion cells.[25] The receiver compartments had a mean volume of 14.0 ml and were maintained at 37°C, in order to reproduce physiological conditions throughout the experiment, by means of thermostated water circulation in the jacket surrounding the cells. Salts concentration in the receiver fluids was approximately the same that can be found in blood. The solution in each cell was continuously stirred using a Teflon coated magnetic stirrer. Each excised sheet of meninge was clamped between the donor and the receptor compartment in such a way that the dura mater faced the donor compartment; the mean exposed area of the meningeal membranes was 3.29 cm^2. The experiments were performed as follows:

Exp. 1: At time 0, the exposure chambers of 3 Franz diffusion cells were filled with 1 mL of physiological solution and 0.5 mL of AgNPs suspension (0.75 mg cm^{-2}), in order to provide an infinite dose: the concentration in each cell has been confirmed at the end of the experiments by means of ICP-AES analysis.

At selected intervals (20, 40, 60, 80, 100, 120, min) 1 mL of the receiving bathing solution was withdrawn and collected for the analysis, and immediately replaced with an equal volume of fresh made physiological solution. In order to avoid the precipitation of silver chloride (AgCl), 100 µL of NH_4OH 1 N was added to each sample collected. The experiment lasted 2 hours. At the end the pieces of meninges were removed, washed abundantly with milliQ water, and subsequently stored in the freezer together with meningeal bathing and donor solutions for the following analysis.

Exp. 2: The exposure chambers of 3 Franz diffusion cells were filled with 1 mL of physiological solution and 0.5 mL of the Ag ultrafiltered solution to verify the differences between ion and nanoparticle permeation. The remaining test conditions were the same as in Exp. 1.

Blanks: For each experiment, two cells were added as blank. The blank cells were treated as the other cells with the exception that the exposure chambers were filled with physiological solution only.

2.6. Meningeal digestion after the experiment

All the exposed meningeal samples were collected and stored individually in the freezer at −25°C for subsequent digestion and analysis. At the time of the analysis, the membranes were dried for 2 h at room temperature, weighted, cut into sections and put into glass tubes with 5 mL of HNO_3 69% v/v for digestion. The resulting solutions were heated at 80°C for 8 h, and finally a volume of 10.0 mL was restored with milliQ water. The analysis was performed by means of ICP-OES.

2.7. Quantitative analysis

An Inductive Coupled Plasma Mass Spectrometer (ICP-MS 7500CE Agilent instrument with integrated autosampler) was used to determin the total silver concentration in the receiver phases. A seven-point standard curve was used for ICP-MS measurements (range 0 – 10 g L^{-1}, ion mass 107 u.m.a.). The limit of detection for silver was 0.005 g L^{-1} for ICP-MS, and the precision of the measurements - expressed as repeatability (RSD %) - was always lower than 5%.

The total silver concentration in the donor phases and in the solutions (obtained from tissue mineralization) was measured by means of Inductively Coupled Plasma - Optical Emission Spectrometry (ICP–OES), using an Optima 8000 ICP-OES Spectrometer (PerkinElmer, USA) equipped with an S10 Autosampler (PerkinElmer, USA). The measurements were carried out using a calibration curve obtained by dilution (range: 0 – 10 mg L^{-1}) of silver ICP standard solution for ICP–OES analysis (Sigma–Aldrich, Italy). The limit of detection (LOD) at the operative wavelength of 328.068 nm was 0.010 mg L^{-1}. The precision of the measurements, expressed as repeatability (RSD %), was always lower than 5%.

2.8. Data analysis

Data analysis was performed with Excel 2007® for Windows and Stata Software (version 11.0, StataCorp LP, College Station, TX, USA). All data were reported in terms of mean or median as a measure of central tendency, and standard deviation (SD) or quartiles as a measure of dispersion. The differences among independent data were assessed by means of the Mann–Whitney test. A p value of <0.05 was considered as the limit for statistical significance.

3. Results

3.1. Characterization of AgNPs dispersion

Transmission electron microscopy (TEM) measurements revealed that the primary size of the AgNPs used in donor solution was quite uniform and as small as 19 ± 5 nm (number of measured nanoparticles: 100). Concentration levels used in the permeation experiments showed no aggregates (Fig. 1). The quantitative analysis of the filtered aliquots revealed that 5% of the donor solution was ionized and a dose of 3.8 μg cm^{-2} of silver was applied as donor phase in Exp. 2.

Size distributions obtained by DLS revealed a z-average size (d, nm) equal to 57.1 and a polidispersity index (PdI) of 0.28, while Zeta potential was equal to -11.4 ± 0.2 mV. The resulting

value is larger than the primary size revealed by TEM, because the hydrodynamic diameter includes PVP and coordinated molecules, and polymer-protected metal NPs can form agglomerates consisting of various metal cores wrapped up in the same polymer chain.[19, 26]

3.2. Ag permeation through the meningeal membrane

Permeation of silver through the meninges was demonstrated by this experiments. Figure 2 shows the trend of silver permeation in the receiver phases, which is increasing as a function of time; similar final values were found both in the cells that were exposed to AgNPs and in those that were exposed to the ultrafiltered solution (0.043 ± 0.034 μg cm^{-2} h^{-1} and 0.040 ± 0.020 μg cm^{-2} h^{-1}, respectively, $p > 0.005$). Even flux permeation and lag time values were similar, during the second hour of the experiment (flow: 0.78 ± 0.71 ng cm^{-2} h^{-1} and 0.73 ± 0.43 ng cm^{-2} h^{-1}, respectively, lag time: 63.8 ± 5.1 min and 64.1 ± 4.8 min). Silver amounts inside the meninges were similar as well (0.27 ± 0.05 μg cm^{-2} in the AgNPs exposed mucosa and 0:25 ± 0:06 μg cm^{-2} in the ultrafiltered solution exposed mucosa, $p> 0.005$), as shown in Figure 3. Silver concentrations in the receiving solution and in the digested tissue samples of the blank cells were always less that the LOD; they are not reported in the figures.

4. Discussion

AgNPs are a common ingredient of antiseptic mists and sprays, and their use is also recommended for the treatment of respiratory disorders; hence contact with the human olfactory mucosa and upper airways is quite common. In our study we demonstrated that meningeal membrane is permeable to silver when exposed to AgNPs. Meningeal membrane, which envelopes the CNS and the olfactory bundles in the tract from the olfactory bulb to the nasal cavity mucosa, is involved in the extracellular absorption pathway.[6, 17, 27] To verify if AgNPs can pass as NPs or as ions, two

solutions were tested: the first contained 19 nm AgNPs, the second was derived from ultrafiltration of the first one where NPs had been removed. The final solution contained only the silver that had dissolved from NPs. As the concentrations we found beyond the membranes were similar in both experiments, we infer that permeation is probably due to the portion of ionized metal. This result is in line with previous studies that investigated AgNPs behavior through other biological membranes, such as the skin and the oral mucosa.[19, 28]

However, we should consider that in vitro studies using Franz cells can underestimated real conditions, since active processes cannot be studied. Animal studies have shown that a translocation of NPs from the olfactory mucosa to the olfactory bulb is possible for silver,[29] carbon,[30-31] graphite,[30] manganese,[32] gold,[33] and titanium dioxide.[34-35] NPs can reach the CNS using the intracellular pathway or can release ions that can permeate the meningeal membrane.

n most cases, the AA argue for a slow absorption through the intraneural pathway inside the olfactory bundles, since the size of the NPs that were tested in those studies (30 - 70 nm) was too large for the particles to pass through the tight junctions of the basement membrane in the olfactory mucosa. On the other hand, the injection of radioactive gold nanoparticles under the olfactory mucosa caused higher values of radioactivity inside the cerebrospinal fluid (CSF) that surrounds the olfactory bulb and the corpus callosum.[36] The AA interpreted this result as possible evidence of a direct perineural link between the olfactory mucosa and the CSF.[37] In medical literature, this route of translocation is considered faster when compared to the intraneural one.[38]

Although our study was not designed to distinguish between the different intranasal pathways, we believe that penetration of the 19 nm AgNPs through the tight junctions is unlikely. The permeation of silver through the meningeal membrane, that we demonstrated, is however an interesting fact, considering the anatomical structure of the olfactory bundles. Small groups of these neurons are indeed wrapped by a sole common Schwann cell, which creates some extracellular cavities - also known as "Mesaxons" - between one neuron and another. These cavities serve as a potential

reservoir of ions,[39-40] that can diffuse into the extracellular fluid, determining a potential delayed absorption of the metal from the perineural space.

Although silver is generally considered as well tolerated and non-toxic for humans, recent studies - both in vivo and in vitro - show that it can have neurotoxic effects.[41-43] A in vivo study by Natàlia Garcia-Reyero and coworkers [41] has detected oxidative stress effects, alteration in neurotransmitter production and neurological affections in the CNS of fish exposed to $AgNO_3$ and AgNPs. Skalska and coworkers[44] have shown in vivo that the exposure of adult rats to Ag in ionic and nanoparticular form (10 nm) leads to synaptic degeneration, which is more severe when NPs are used. The brain region that appears to be the most affected is the hippocampus, whose alterations can lead to cognitive impairment.[45-46]

In conclusion, we provide evidence - for the first time - that meninges are permeable to silver, when they are exposed to AgNPs. Within the limitations determined by the use of a meningeal permeation model that was utilized for the first time, these preliminary findings allow us to say that this biological membrane is not completely impermeable to external agents. The intranasal pathway requires further investigative efforts to clarify the permeability properties of the meninges to other NPs. Each metal NP, in fact, has different chemical-physical characteristics, which can vary depending on size, surface chemical properties and external coatings. Interaction with the meningeal membrane requires therefore to be examined case by case, in order to assess the exposure risk with greater precision, and consequently protect workers and consumers who may be exposed to NPs in an adequate way.

Declaration of interest statement: The authors declare no conflict of interest.

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Figures:

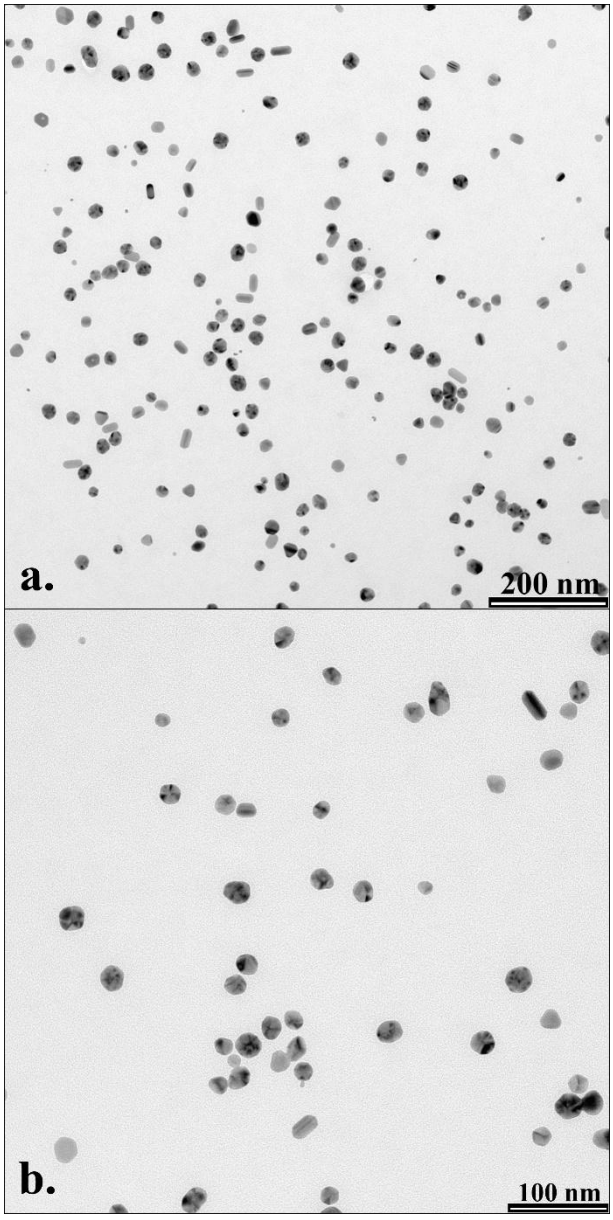
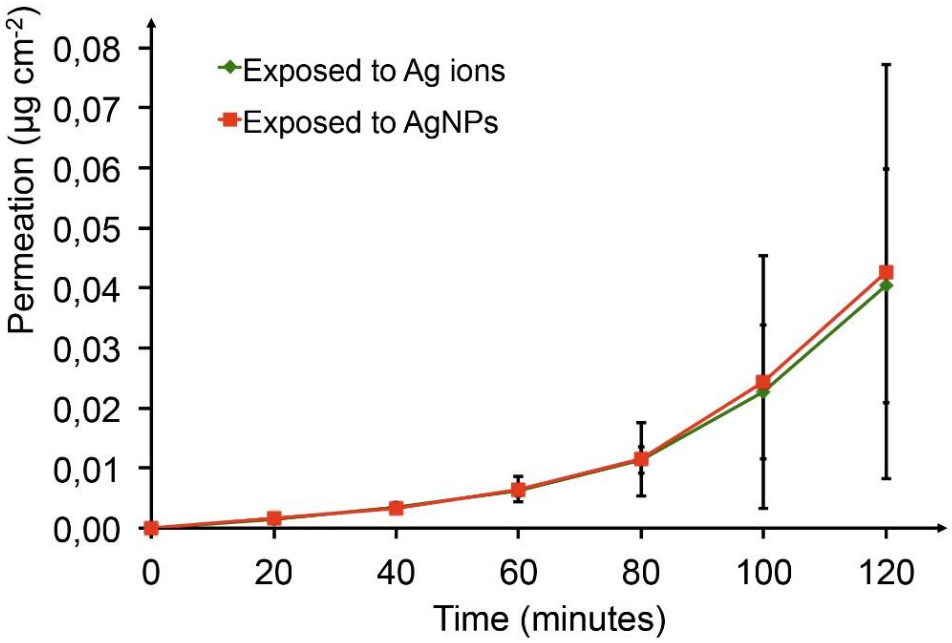


Fig. 1


Permeation (µg cm-2)
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Exposed to Ag ions
Exposed to AgNPs
0
20
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100
120
Time (minutes)

Fig.2

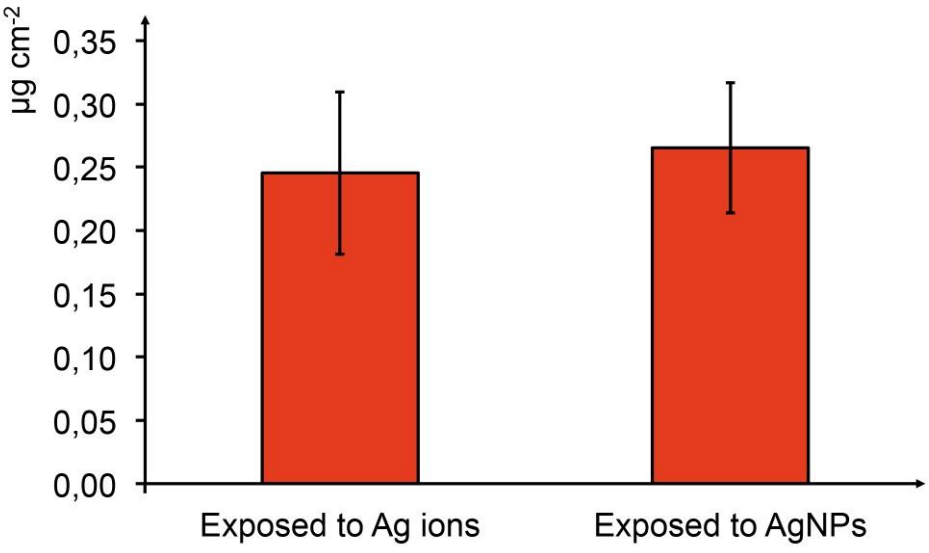
µg cm-2
0,35
0,30
0,25
0,20
0,15
0,10
0,05
0,00
Exposed to Ag ions
Exposed to AgNPs

Fig.3

Figures captions:

Fig. 1 TEM visualization of the AgNPs dispersed in physiological solution: NPs are homogeneous in size and shape and do not form aggregates (A: bar=200nm; B: bar=100nm).

Fig 2. Silver in the receiving phases at any time tested. In cells exposed to AgNPs and to the ultrafiltered solution. Concentration expressed as μg cm^{-2} h^{-1}.

Figure 3. Concentration of silver inside the meningeal tissues, in cells exposed to AgNPs and in cells exposed to Ag ions. Data expressed as μg/cm^2.