Bactericidal silver nanoparticles present an antiangiogenic effect in the Chorioallantoic Membrane Model (CAM)

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Despite the antimicrobial effect of silver is known since ancient times, its use is still controversial due to toxic effects. Cytotoxicity and induction of apoptosis induced by silver nanoparticles have also been described as well as proliferative effects and induction of oxidative stress. The particle led to subtle obstructive effects in the chick embryo Chorioallantoic Membrane (CAM) microcirculation. These effects occurred without loss of embryo viability and were associated with the partial preservation of the capillary diameters and connectivity. The result of cytotoxicity in cell culture of human fibroblasts, The percentage of cell viability on treatment with Ag 22 ppm showed decreased significantly compared to the untreated control group, however the appearance of cell culture of human fibroblast cells showed normal morphology with loss of the fibroblastoid type, there is retraction of cytoplasmic processes and rounding in Ag concentration of 100 treatment and 50%, while in other concentrations there is a stimulus for growth and proliferation.

Keywords: angiogenesis, silver nanoparticles, cytotoxicity, apoptosis

1. Introduction

The antimicrobial effect of silver [1] is known since ancient times and several studies corroborate its beneficial effects in humans [2-3], its use is still controversial due to toxic effects [4-5]. Both silver ions and silver nanoparticles present inhibitory and lethal effects on bacterial species such as E. coli [6-7], S. aureus and yeast species [8]. Recently, the application of bactericidal silver nanoparticles as a biocompatible sanitizing agent was reviewed by the comparison between ions and nanoparticles of silver and includes a critical analysis on the new developments in association with antibiotics and biological synthesis of nanoparticles in models of fungi [9]. Cytotoxicity and induction of apoptosis induced by silver nanoparticles have also been described [10] as well as antiproliferative [11] effects and induction of oxidative stress [5].

A relationship between the structure of silver nanoparticles and their antimicrobial activity on gram-negative bacterium E. coli was reported [12]. It was found that the higher the concentration of the nanoparticles, the greater is the reduction in bacterial growth, which is consistent with the antimicrobial activity associated with silver. Besides these results, the recent literature disclose the consensus on that the shape and/or size of the nanoparticles influence this action [13]. This fact reinforces the requirement for stringent characterization and standardization of silver based materials, in order to study their applicability in the medical field.

This work was designed to specifically test in vitro previously described biological effects of a particular composition of bactericidal silver based product. There were investigated the effects of the commercially available product containing silver nanoparticles in pure water upon inflammatory and angiogenesis pathways in the chorioallantoic membrane, as well as upon particular relevant aspects of the embryo genotoxicity.

2. Materials and Methods

2.1 Silver nanoparticles

Silver nanoparticles (nanopra, lot number 1208) gently provided by Khemia in plastic ambar colored spray flasks were characterized with the dynamic light scattering method (DLS). The informed electrical conductivity of the product was 22ppm. The hydrodynamic ray for the particles was compared to results of membrane filtration cutoff. The actual silver concentration (12.5 ± 0.3 mg L\(^{-1}\)) was measured with an ICP OES Thermo iCAP 6300 Duo apparatus at 328,068 nm, axial view with restricted nebulization chamber and Burgner type nebulizator, at the Analytical Chemistry Facility, Institute of Chemistry from The University of São Paulo both in the product itself and in the low (9,13 ± 0,09 mg L\(^{-1}\)) obtained after the filtration through a AMICON 30K, disposable centrifuge tube. The silver content of the high diameter
particle fraction (3.38 mg L\(^{-1}\)) was estimated by difference. The nanoparticle size (20 ± 0.3 nm) was measured in 500kcps setup with a Zetasizer apparatus (Malvern Instruments). Measurements were done monthly in sealed cuvets (3mL aliquots), throughout a six month period, in order to assert the product stability. Silver nanoparticles were employed as furnished, in pure water diluted solutions or after a 30 kDa molecular weight filtration step, with the filter retained fraction.

2.2 Bactericidal effect

The test for minimum inhibitory concentration (MIC) was determined by means of broth microdilution following the established standards by the CLSI (CLSI, 2006). The silver material was tested in a range from 0.08 to 51.2 mg / L from a stock solution of approximately 128 mg / L concentration. These serial dilutions were performed in Mueller-Hinton broth (DIFCO, Lawrence, KS, USA). In the initial dilution (51.2 mg / L) 800 uL of stock solution were added to 1200 mL of Mueller-Hinton broth. After homogenization, 1 mL was transferred in eppendorf tubes following, containing 1 mL of Mueller-Hinton broth, to perform the full range of serial dilution, 100 mL of each dilution were deposited into microplate Elisa. The bacterial samples chosen were those of clinical interest, producing several mechanisms of resistance to antibiotics (Table 1).

<table>
<thead>
<tr>
<th>Bacterial sample</th>
<th>Resistance Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>SPM-1 (β-lactam)</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>CMY-2 (Cephalosporin, penicillin, cephamicin)</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>IMP-1 (β-lactam)</td>
</tr>
<tr>
<td>Acinetobacter baumannii</td>
<td>OXA-23 (β-lactam)</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>KPC-2 (β-lactam)</td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>Ribosomal RNA mutation 23S (Linezolid)</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>MecA (β-lactam)</td>
</tr>
<tr>
<td>Escherichia coli ATCC 25922</td>
<td>Sensible</td>
</tr>
<tr>
<td>Staphylococcus aureus ATCC 25923</td>
<td>Sensible</td>
</tr>
<tr>
<td>Klebsiella pneumoniae ATCC 700603</td>
<td>Producing β-lactamase (cephalosporin, penicillin, monobactam)</td>
</tr>
</tbody>
</table>

For each sample a bacterial suspension was made directly from a culture grown 16 hours to reach a turbidity equivalent to 0.5 Macfarland, which was confirmed in a spectrophotometer Spectronic 20 to between 0.8 and 1.0 absorbance and wavelength λ 625 nm. For the broth microdilution test, the bacterial suspension with turbidity already reached the equivalent of 0.5 McFarland was diluted in sterile Mueller-Hinton broth at 1:10, resulting in an inoculum of approximately 107 CFU / mL. Next, 5 mL of this suspension were inoculated into the microplate containing dilution of antimicrobial agent, with a final bacterial concentration of 10⁴ CFU / mL (CLSI 2008) and incubated for 16 to 20 hours. The MIC was defined as the lowest concentration able to inhibit bacterial growth. The minimum bactericidal concentration MBC was performed from the MIC obtained by broth microdilution. Were re-seeded broth free of silver material, all wells with concentrations that showed no visible growth.

2.3 Cell toxicity

The cell studies were conducted with the product as provided or in pure water dilutions. Normal Human Dermal Fibroblasts (FN1) were grown 5 x 10⁴ cells / mL in RPMI 1640 containing 10% fetal calf serum in 96 wells plates. Adherence was obtained after 3-6h at 37 ° C / 5% CO₂. MTT colorimetric assay was performed as described (Denizot and Lang, 1986, modified). Briefly, adhered cells were exposed to silver nanoparticles in serum free RPMI -1640 medium for 24h. After this time, the medium was removed through soaking and then each well was added with 50 μL of MTT (1mg/ml) in phenol red free RPMI – 1640 medium. The plates were shaken for 3h and the medium was removed by inversion. MTT Formazan was dissolved through swirling with 100 μL of DMSO for 30 min and measured in spectrophotometric ELISA reader at 580 nm. Negative controls were processed as samples, excluding the silver nanoparticles.

2.4 Collagen

The cell plates were stained with Picrosiris red cytochemical method. Collagen has an anisotropic molecular structure and appears birefringent having two refractive indexes and can therefore be seen when viewed with polarized light. The stained preparations were analyzed in a Zeiss photomicroscope with 40x and 10x eyepieces. It was used picrosirus red staining, which estimates the association of a large number of molecules which are placed parallel to the collagen.
molecules. The largest the collagen fiber, the higher is the number of aligned stain molecules and the observed color changes from red to yellow. The method of staining with picrosirius, together with the polarization microscopy, is a cytochemical method specific to the perception of structures composed of oriented collagen fibers.

2.5 CAM angiogenesis assay

The effect of silver nanoparticles on angiogenesis was studied in the incubated eggs lying on a hatching tray, as described [10-11]. Eight white eggs of Gallus domesticus, Bovans White breed aged approximately 240 days, were obtained from Granja Kunitomo, located on the road Mogi - Salesópolis, Km 12 in Suzano, São Paulo and employed in three independent assays. The eggs average weight of 60.1 g were brought directly from the farm, 26 to 34 h after laying and incubated in an automatic incubator thermostated Zagas brand, up to 40 eggs, provided by the manufacturer for use in laboratory. Before being placed to be incubated, the eggs were carefully cleaned, one by one, with 70% alcohol soaked sterile cotton. The alcohol cleaned eggs were air dried within the laminar flow with the air chamber upward and placed under ultraviolet light for 15 minutes. The control eggs were separated for incubation (n=3). The colloidal silver (nanoprata, Khemia) was employed as provided in spray form in the exposed eggs (n=3). The aspersion over the egg shells was thoroughly made in two steps, in order to expose the entire external surface. The egg was horizontally placed in a ring support and the spray was applied over the upside facing region, from a 15 cm distance, until the superior face is wet. After the product was dried on the surface, the egg was gyrated 180° and the procedure was repeated with the previously unexposed region. The incubation was carried at 37.2 ° C, relative humidity (60%), with the automated periodical turns at two hour intervals, for the controls and the colloidal silver exposed eggs. Viability in the closed shell eggs was observed during the early embryo development with the help of the ovoscope. Incubation proceeded until the 21th day, for survival evaluation of one exposed egg plus its control. The animals were labeled immediately after hatching and kept in one cage together in the lab for five days, fed with triturated corn, water ad libitum. The cage was cleaned twice a day and the general behavior was observed. The CAM sampling was done within the 11th day, for the CAM vasculature analyses. (n=6) In the 11th day, the eggs were placed for at least 20 min at 4°C before being opened. The shells were cut in the air chamber and a few formalin droplets were added over the exposed CAM membrane and left for 5 min. Then, the embryo and the CAM tissues were collected for morphology. The embryos were transferred to plastic tubes and immersed in formalin. The CAM tissues were cleaned with cold water, and fixed distended over glass slides for vasculature analysis with a Zeiss JenaLumar optical microscope. Polarized light helped to increase the capillary web visualization in the nonstained specimens. Photomicrographs were taken with a Cannon powershot A 650 IS with the help of a dedicated chamber adaptor specially built by Eikonal industry.

2.6 Ethical comittee

This work was evaluated and approved by the Institutional Comittee for Animal Use (FCF-CEUA) under number 274/2010.

3. Results

3.1 Bactericidal activity of silver particles

The silver nanoparticles inhibited the growth of all the assayed bacteria in the 3.2 to 25.6 mg.L⁻¹ range, as seen in Table 2. Antimicrobial resistance presented by the different species didn´t correlate with the inhibition, with the exception of *Klebsiella pneumoniae* with different resistance determinants. The KPC-2 *Klebsiella pneumoniae* was particularly resistant to the silver nanoparticles. On the other hand, different susceptibilities to bactericidal effects of silver nanoparticles could be associated with the genera. The Staphylococcus species were resistant, even to the highest concentrations tested, while the Gram negative genera were predominantly susceptible to the bactericidal action of the silver nanoparticles.

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### Table 2 Bacteriostatic and bactericidal activity of silver nanoparticles (Nanoprata, Khemia).

<table>
<thead>
<tr>
<th>Bacterial sample</th>
<th>CIM –Sample of Silver (mg.L(^{-1}))</th>
<th>CBM</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas aeruginosa</em> (SPM-1)</td>
<td>6,4</td>
<td>51,2</td>
</tr>
<tr>
<td><em>Escherichia coli</em> (CMY-2)</td>
<td>25,6</td>
<td>51,2</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> (IMP-1)</td>
<td>25,6</td>
<td>51,2</td>
</tr>
<tr>
<td><em>Acinetobacter baumannii</em> (OXA-23)</td>
<td>3,2</td>
<td>6,4</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> (KPC-2)</td>
<td>12,8</td>
<td>ND*</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em> (LNZ)</td>
<td>6,4</td>
<td>ND*</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> (MecA)</td>
<td>25,6</td>
<td>ND*</td>
</tr>
<tr>
<td><em>Escherichia coli</em> 25922</td>
<td>3,2</td>
<td>6,4</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> 25923</td>
<td>12,8</td>
<td>ND*</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> 700603</td>
<td>12,8</td>
<td>25,6</td>
</tr>
</tbody>
</table>

* ND: not determined.

#### 3.2 Cytotoxicity towards human fibroblasts cells

The toxic concentration range of silver nanoparticles to human fibroblast cell line FN1 mitochondria was determined to situate between 2.5 and 22 mg.L\(^{-1}\) (Figure 1). The vehicle used (distilled H\(_2\)O) was not cytotoxic.

![Fig. 1](image1.png)

Fig.1 The cytotoxicity of silver nanoparticles towards human fibroblast cell line FN1 in 24h cultures estimated through mitochondrial function evaluation (MTT test). Controls were exposed to equal amounts of distillate water.

Under silver concentrations equal or above 2.5µg/mL, human fibroblast FN1 cells showed abnormal morphology with loss of the fibroblastoid type, retraction of cytoplasmic processes and rounding. The concentrations lower than 2.5 mg.L\(^{-1}\) didn’t change growth and morphology (Figure 2). The morphologic impairment could be associated with toxicity and with the terminal differentiation of the FN1 cells. The collagen production was increased in the non toxic concentrations of silver, as evidentiated by picrosirius staining (Figure 3).

![Fig. 2](image2.png)

Fig. 2 Human fibroblast FN1 cells morphology after silver nanoparticles 24h exposition in culture. A - Unexposed control, B - 22 mg.L\(^{-1}\), C – 12,5 mg.L\(^{-1}\), D – 6,8 mg.L\(^{-1}\), E – 3,4 mg.L\(^{-1}\) F – 1,7 mg.L\(^{-1}\) G – 0,8 mg.L\(^{-1}\).

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Fig. 3  Effects of 24h exposition to silver nanoparticles upon collagen production by human fibroblast FN1 cells. Picrosirius staining of the 24h exposed culture plates. The yellow color is typical of type I collagen fibers.

3.3 Micro obstructive effects and angiogenesis

The silver nanoparticles treatment didn’t brought gross morphology changes on the CAM vessels. It was possible to observe intact capillary networks (Figure 4). However, there were suggested micro obstructive effects that partially impair the minor vessel replenishment (Figure 4B), low the connectivity of the capillary web and increase the congestion of the higher order vessels (arterioles) (Figure 4 D). The venulae and capillary present with tortuous lining and also with increased diameters (Figure 4 D), in relation to the respective controls (Figure 4 A and C)

Fig. 4  Aspect of the CAM absorptive surface structures in eggs incubated for 10 days. A, C: Control, and B, D: Exposed. The eggshells were cleaned with 70% alcohol, followed by 15 min of UV light. Exposed eggs were additionally sprayed with silver nanoparticles on the eggshell surface before incubation. The membrane roughness (B) and the density of the canaliculi network (D) were reduced in the exposed egg CAMs in relation to its paired controls (A, C).

3.4 Embryo genotoxicity

Macroscopic examination of the fetus in the eleventh day of incubation revealed, preserved crown rump dimensions and general aspect with discrete effects upon muscle mass (Figure 5).
Fig. 5 (A) - Fetal chicken with 11 days of development with silver, (D) - embryo without silver. Observe the fetus with 3.0 cm of crown-rump and its macroscopic characteristics. Members (wings and legs) have formed and individual digits (B-C and E-F).

Microscopic examination of the chick fetuses showed abnormal respiratory tissue differentiation. The lungs of the treated animals (Figure 6 and 7) showed complete absence of bronchi associated cartilage tissue, increase of interstitial area and fewer vessels than the normal counterparts. The epithelia presented discrete immaturity, mucus secretion was absent.

Fig. 6 Histological aspect of the chicken embryos’ lungs at incubation day 11. (A, B) – Silver exposed eggs. Lung parenchyma showing reduced cell density and vascular paucity, the bronchioles showed no cartilage. (C and D) – Control unexposed eggs.

The exposure of the eggshell surfaces to silver nanoparticle didn’t prevent the hatching. The outbreak, in the 20th day was apparently normal and the animal presented no gross structural or behavior changes during the first five days after hatching.

At 15 days after hatching the animal came spontaneously to death and the histopathological examination of the lungs revealed an increased volume of these parabronchial muscles, attributable to compensatory pulmonary effort due to the reduced oxygen exchange capacity and chronic hypoxemia [14]. There was found an hemorrhagic pattern of the lungs and the extensive necrosis of the bronchi (Figure 7).
Silver nanoparticles were previously described to be effective and without risk of causing complications in humans, if utilized in appropriate concentrations [8]. This assertion underlines the requirements for defining secure exposure limits for its use, a possibility obviously commanded by the standardization of the product under use. Chemical and physical effects are expected in the interaction of silver nanoparticles with animal cells and tissues. Besides the well-described chemical poisoning related to Argyria mechanisms, particulate material allows the observation and documentation of previously unknown biological effects. In this paper, the silver ion poisoning concentrations of a commercially available silver nanoparticle based product were not achieved, but toxic effects were presented both to human fibroblasts in culture (Figures 1 - 3) and to chicken organisms (Figures 4 - 7) and correlated to a physicochemical description of the assayed particles. The bactericidal and cytotoxic concentrations were found coincident, an achievement that is compatible with the ancient literature reports on widely shared target among living cells (Table 2; Figure 1).

One must be aware about the reason for the absence of reports on human intoxication by these low concentrations of silver nanoparticles, as there are silver based products commercially available in several countries throughout the world. Absorption through intact skin and mucosae may be very low. Silver elimination in hair and skin is also predictable, besides biliary and urinary loss.

Particulate inhalation is generally expected to produce chronic inflammatory effects in vivo, which potentially can be modulated if the inducing material is chemically active in the resulting inflammatory microenvironment. Then, the toxic effects of absorbed silver nanoparticles are not necessarily due only to silver ion binding to macromolecules, preferentially in free thiol groups, as expected when using silver salts. Hussain et al.18 report an evident dose-dependent cytotoxicity in a rat liver cell line after 24 h exposure to silver nanoparticles. Mitochondrial function was shown to decrease significantly and typical apoptosis morphology with cellular shrinkage and irregular shape was observed [17]. Analogous events are related to bacterial cell toxicity, and silver nanoparticles are reported enter the bacterial cells, to undergo a shape-dependent interaction with the bacterial cells, and to exhibit shape and size dependent antimicrobial activity [18].

Mitochondrial toxicity was observed in very low silver concentrations in this work (figure 1), and also associated to apoptosis morphology in FN1 cells (Figure 2). Fibroblast and endothelial cells are key elements on the angiogenesis process, addressed by the CAM modified assay. In the fibroblast cells in culture, the cell death induced by silver nanoparticles followed the differentiation events of collagen production, which were prominent in the sub lethal concentrations (Figure 3). Fibroblast overproduction of matrix proteins and cell differentiation asynchrony can be the main event accounting for the embroyotoxic effects of silver nanoparticles. Its association with hyperplastic alveolar septa and pulmonary congestion occurs in hypoxia associated syndromes [19-20]. The same phenomenon was observed in the chicken embryos exposed to the silver nanoparticles through the eggshells, pointing to the microbstructive effects upon the CAM absorptive surface, as shown in Figure 4. The anticipated hypoxia was investigated for its obligatory angiogenic effects. Angiogenesis is an important physiological process which emerges as a result of extremely regulated and iterative pathways in vivo. The chaotic nature of the biochemical and biophysical components of this process hardly precludes its accurate simulation through cell and molecular models in vitro. In vitro and ex-vivo tissue models help us to address the main individual cell responses to growth factors in the process, as well as part of its interactions, whereas validated in vivo models use mammals in regenerative and inflammatory responses in the study of
angiogenesis. The proangiogenic effects secondary to hypoxia were not observed, nor were found gross modifications of vessel structures. The congestion could also be represented in the CAM micrographs, where there were observed less connectivity in the capillary web and ingurgitation of high order vessels (Figure 4). Sheikpranbabu, et al, 2009 [21] showed that silver nanoparticles of nearly-uniform size, synthesized by Bacillus licheniformis affect endothelial cell permeability. Silver nanoparticles were able to completely block endothelial cell permeability in the solute flux assay and to inhibit vascular endothelial growth factor (VEGF) induced angiogenesis in bovine retinal endothelial cells. The vascular endothelial monolayer forms a semi-selective permeability barrier between blood and the interstitial space to control the movement of blood fluid, proteins, and macromolecules across the vessel wall [21].

Fibroblasts are an important cellular target, firstly disclosed in this work. The complex biology of the main growth factor produced by these cells, the FGFs play a regulatory role in many biological pathways and influence multiple cellular components. Studies of the biological role of the FGF signaling disclose the great redundancy among FGFs, but also the indispensable roles played by FGFR1 and FGFR2 in embryonic development. Paracrine FGFs function in development by influencing the intracellular signaling events of neighbouring cells. In vivo suppression of endothelial FGF signaling is manifested by increased vascular permeability and impaired angiogenic and arteriogenic responses [22]. This is the putative mechanism for the observed effects in the lung parenchyma of the chicken embryo (Figure 6 and 7).

Accordingly, downward maturative asynchrony of the respiratory tissues with complete absence of cartilage differentiation and fibrosis of the fetal bronchi (Figure 6-7) are attributable to inflammatory TGF dependent mechanisms secondary to the hypoxia.

Silver nanoparticles have been shown to have important antiangiogenic properties. The silver nanoparticles showed cytotoxic effects towards Dalton’s lymphoma ascites tumor cells whereas reducing disease progression. These cytotoxic effects of silver were ascribed to active physicochemical interaction of silver atoms with the functional groups of intracellular proteins, as well as with the nitrogen bases and phosphate groups in DNA [23]. Alteration of permeability barrier integrity plays a major role in drug-based therapies, as well as the pathogenesis of cardiovascular diseases, inflammation, acute lung injury syndromes, and carcinogenesis, pointing to important therapeutic targets for silver nanoparticles.

5. Conclusion

Bactericidal effects of the high diameter particle subfraction of the Nanoprata product (silver nanoparticles in pure aqueous solution) were documented with microbiological techniques in the 3.2 to 51.2µg/mL range. The citotoxic effects toward normal human fibroblasts cells were achieved with the non manipulated product in a silver concentration >2.5µg/mL. Concentrations in the 0.6 to 2.5µg/mL induced collagen synthesis by the normal human fibroblasts cells. The silver nanoparticles led to subtle obstructive effects in the chick embryo Chorioallantoic Membrane (CAM) microcirculation and congestion of the medium diameter vessels. Inflammatory mechanisms associated with hypoxia induced maturative asynchrony of respiratory tissues of the chicken embryo. The toxic effects occurred without loss of embryo viability and were associated with the partial preservation of the capillary diameters and connectivity. The hatched animal presented spontaneous death after 15 days, showing ischemic necrosis of the respiratory tissues and compensatory modifications of the bronchial associated stroma. The proposed underlying mechanisms involve the FGF2 signaling through soluble VEGF receptor affecting cartilage, bone and soft tissue differentiation, along with vascular abnormalities.

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