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Avaliação da resposta tecidual e da citotoxicidade de soluções coloidais de nanopartículas de prata

Tese apresentada à Faculdade de Odontologia de Araçatuba – Universidade Estadual Paulista "Júlio de Mesquita Filho"- UNESP, para obtenção do Título de DOUTOR EM ODONTOLOGIA (Área de concentração em Prótese Dentária).

Orientadora: Profª. Drª. Debora Barros Barbosa

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Takamiya AS. **Avaliação da resposta tecidual e da citotoxicidade de soluções coloidais de nanopartículas de prata** [tese]. Araçatuba: Faculdade de Odontologia da Universidade Estadual Paulista; 2013.

RESUMO

O objetivo deste estudo foi investigar o efeito de diferentes soluções coloidais de nanopartículas de prata sobre a viabilidade celular de fibroblastos (linhagem L929) e sobre a resposta inflamatória de tecido subcutâneo de ratos. Nanopartículas de prata (SNP) com tamanho médio de 5 nm foram sintetizadas através da redução do nitrato de prata pelo citrato de sódio e estabilizadas com amônia (SNP-A) ou polivinilpirrolidona (SNP-P). Para avaliar a viabilidade celular, células L929 foram expostas SNP e agentes estabilizantes (amônia (NH_3) e polivinilpirrolidona (PVP)) (0,1 – 100 μg/mL), e após 6, 24 e 48 h foi realizado o ensaio de citotoxicidade celular pelo método do MTT. A resposta tecidual foi realizada com tubos de polietileno contendo SNP (1.0 μg/mL; 540 μg/mL) e agentes estabilizantes (NH_3 0.13 x 10^{-3} mol/L e PVP 0.19 g/L) implantados no tecido conjuntivo dorsal de ratos Wistar por 7, 15, 30, 60 e 90 dias. Os espécimes foram corados com hematoxilina e eosina e foram realizadas avaliações qualitativa e quantitativa. SNP inibiram a viabilidade celular no teste *in vitro* de maneira concentração-dependente. SNP-A foram mais tóxicas para L929 que as partículas estabilizadas com PVP. O exame histológico mostrou que SNP 540 μg/mL induziram reação tecidual significantemente mais intensa em 30 e 60 dias comparado aos grupos controles (solução fisiológica 0,9% e fibrina) nos mesmos períodos. As respostas inflamatórias causadas por SNP 1,0 μg/mL, NH_3 0,13 x 10^{-3} mol/L e PVP 0,19 g/L foram similares aos controles em todos os períodos experimentais. Foi possível concluir que a exposição à SNP reduziu a viabilidade de células L929 de maneira concentração-dependente. O tipo de agente estabilizante interferiu na citotoxicidade sendo SNP-A mais tóxica para L929. Ambos os tipos de soluções coloidais de nanopartículas de prata (SNP-A e SNP-P) a 540 μg/mL induziram significante resposta inflamatória no tecido subcutâneo de rato.

Palavras-chave: Nanopartícula. Prata. Toxicidade. Biocompatibilidade.

Takamiya AS. **Evaluation of tissue reaction and cytotoxicity of colloidal silver nanoparticles** [Thesis]. Araçatuba: UNESP - São Paulo State University; 2013.

ABSTRACT

The aim of this study was to investigate the effect of different colloidal silver nanoparticles on cell viability of mouse fibroblasts (cell line L929) and on the subcutaneous connective tissue reaction of rats. Silver nanoparticles (SNP) of average size 5 nm were synthesized by the reduction of silver nitrate through sodium citrate and were stabilized with ammonia (SNP-A) or polyvinylpyrrolidone (SNP-P). To evaluate the cell viability, L929 cell were exposure to silver nanoparticles (0.1-100 μg/mL), and after 6, 24 and 48h MTT assay was performed. The tissue reaction was carried out with polyethylene tubes containing silver nanoparticles (1.0 μg/mL; 540 μg/mL) implanted in the dorsal connective tissue of Wistar rats for 7, 15, 30, 60, and 90 days. The specimens were stained with hematoxylin and eosin and qualitative and quantitative evaluations of the reaction were carried out. Silver nanoparticles inhibited the cell viability in the *in vitro* test in a concentration-dependent manner. SNP-A were more toxic to L929 than particles stabilized with polyvinylpyrrolidone (PVP). Histological examination showed that SNP at 540 μg/mL induced significant tissue reaction on 30 and 60 days after implantation compared to the controls groups (fibrin and saline 0.9%) at the same periods. The inflammatory responses caused by SNP at 1.0 μg/ml, NH_3 at 0.13 x 10^{-3} mol/L and PVP at 0.19 g/L solutions were similar to the controls groups in all experimental periods. It was possible to conclude that SNP exposure decreased the viability of L929 cells in a concentration-dependent manner. The type of stabilizing agent interfered on the cytotoxicity of SNP being SNP-A more toxic to L929. Also, both colloidal silver nanoparticles (SNP-A and SNP-P) at 540 μg/mL induced significant inflammatory response in rat's subcutaneous tissue.

Key words: Nanoparticle. Silver. Toxicity. Biocompatibility.

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LISTA DE ABREVIATURAS

Ag = Silver

Ag^{+} = Silver ion

Ag^{0} = Metalic silver

$AgNO_3$ = Silver nitrate

ATP = Adenosine triphosphate

cells/mL = Cells per milliliter

Cm = Centimeter

CO_2 = Carbon dioxide

DMEM = Dulbecco's modified Eagle's medium

DNA = Deoxyribonucleic acid

EDTA = Ethylenediamine tetraacetic acid

G = Gramme

g/L = Gramme per liter

g/mL = Gramme per millilitre

GSH = Glutathione

ISO = International Organization for Standardization

mg/kg = Milligram per kilogram

mg/mL = Milligram per milliliter

mL = Milliliter

Mm = Millimeter

mol/L = Mol per liter

MTT = [3-(4,5-dimetil-2-tiazolil)-2,5-difenil-2H-brometo de

	tetrazolium]
$Na_3C_6H_5O_7$	= Sodium citrate
NH_3	= Ammonia
Nm	= Nanometer
ºC	= Degrees Celsius
OD	= Optical density
pH	= Hydrogen potential
ppm	= Parts per million
PVP	= Polyvinylpyrrolidone
ROS	= Reactive oxygen species
SNP	= Silver nanoparticles
SNP-A	= Silver nanoparticles stabilized with ammonia
SNP-P	= Silver nanoparticles stabilized with Polyvinylpyrrolidone
TEM	= Transmission electron microscopy
units/mL	= Units per millilitre
UV/Visible	= Ultraviolet visible
µg/ml	= Microgram per millilitre
µL	= Microliter
µM	= Micromolar

SUMÁRIO

1. Introduction*

Nanotechnology has emerged as a new science that involves the manipulation and creation of materials engineered at nanoscale (Hussain, Hess et al. 2005; Ahamed, Karns et al. 2008; Arora, Rajwade et al. 2012; Chairuangkitti, Lawanprasert et al. 2013). Application of this new technology has been widely spread among different areas of life being used on textiles, biosensors, opacifiers, bioelectronics, water filtration system and in the biomedical field especially for therapeutic proposals in prostheses, catheters and drug delivery (Arora et al. 2009; Ahamed et al. 2010; Castle et al. 2011). Currently, nanomaterials have been classified according to the number of external dimensions, one, two or three with less than 100 nm, in nanoplates, nanofibers or nanoparticles, respectively (Hoyt 2008; Ahamed et al. 2010; Arora et al. 2012; Chairuangkitti et al. 2013). The small size of nanomaterials frequently results in unique physical and chemical characteristics different from the original bulk substance (Asharani et al. 2009; AshaRani et al. 2009; Monteiro et al. 2009; Eom and Choi 2010). When the materials reach the nano size they became exhibit higher surface area to volume ratio which makes them potentially more reactive (Foldbjerg et al. 2011; Beer et al. 2012).

Among all the nanomaterials, clusters of silver atoms that are defined as silver nanoparticles (SNP) are by far the one with the highest degree of commercialization (Ahamed et al. 2008). Since its introduction in 1990s as a colloidal form in ointments to prevent microbial colonization on burns, the use of SNP have been increasing exponentially, being currently applied in

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* O artigo segue as normas do periódico *Toxicology Letters*, disponível no anexo C

contraceptive devices, surgical instruments, bandages and bone prostheses (Arora et al. 2009; Foldbjerg et al. 2009). This rapid spread of SNP use in medical devices was mainly due to its proven antimicrobial activity against about 650 different disease-causing microorganisms including multidrug resistant strains (Braydich-Stolle et al. 2010; Monteiro et al. 2011; Monteiro et al. 2012). Other reason that have encouraged the use of SNP for therapeutic proposals was the information that in this form silver would be considered less toxic to cells and tissues than otherwise (Arora et al. 2008).

However, it has been stated that the same properties that make SNP interesting in various applications may have adverse effects on human health, since that several materials that are considered biochemically inert and biocompatible in the bulk form can became toxic to different cell types in the nano scale (Wei et al. 2010). Chen et al. (2007) found that SNP can induce toxic effects much more intense than silver in the microparticulate form when implanted intramuscularly in rats (Chen et al. 2007). According to Li et al. (2012) the toxicity increases with the decrease in size of the material, smaller particles can more easily contact the cellular membrane and be internalized by the cell increasing the chance of inducing cytotoxic effects (Li et al. 2012).

Apart from particles size, the potential cytotoxic of SNP have been shown dependent on other factors such as shape, surface properties, or even partially attributed the presence of stabilizing agents (El Badawy et al. 2011). Among the stabilizing agents used in the chemical reaction method to synthesize SNP, ammonia (NH_3) and polyvinylpyrrolidone (PVP) are one of the most frequently employed to control particle growth and prevent aggregation, however their

toxicity profile have not been clearly investigated (Gorup et al. 2011; Lin et al. 2012; Monteiro et al. 2012). From several studies concerning to the SNP toxicity some proposed mechanisms of action of SNP can be found in the currently literature, and they are mainly attributed to dissolution or release of silver ion from SNP, disruption of cell membrane integrity, penetration of SNP into the cell inducing oxidative stress, reactive oxygen species (ROS) generation, interaction with protein and/or enzymes inhibiting essential cell activities or DNA damage and apoptotic cell death (El Badawy et al. 2011; Suresh et al. 2012).

Despite the effort of several investigators and the large number of research regarding SNP toxicity, the exact mechanism of action of these particles and the possible risks to human health or environmental impact in cellular, molecular or whole organism level in eukaryotes have not yet completely understood (Ahamed et al. 2008). Based on this, the European Scientific Committee on Emerging and Newly Identified Health Risks recently stated that the possible risks related to SNP exposure should be strongly assessed case-by-case to define toxicological profile of these particles (Beer et al. 2012).

Thus, considering this actual panel of nanotoxicology, it was our intention in the current study to determine the effect of SNP synthesised with two different stabilizing agents (NH_3 and PVP) on cell viability of mouse fibroblasts (L929). Furthermore, we aimed to analyse the reaction of subcutaneous connective tissue of Wistar rats to SNP and its stabilizing agents.

2. Materials and methods

2.1. Synthesis and Characterization of Silver Colloidal Nanoparticles

Silver colloidal nanoparticles of size average 5nm stabilized with NH_3 (Merck KGaA, Darmstadt; Deutschland, Germany) or PVP (Sigma-Aldrich, St Louis, Missouri, USA) were prepared by the chemical reaction method based on the use of reducing agent, sodium citrate ($Na_3C_6H_5O_7$), to reduce Ag^+ from silver nitrate ($AgNO_3$). (Merck KGaA, Darmstadt; Deutschland, Germany) (Gorup et al. 2011). Briefly, in a tri-neck flasks containing 150 mL of an aqueous solution of $AgNO_3$ at 5.0 x 10^{-3} mol/L were added 7.5 mL of a solution of $Na_3C_6H_5O_7$ at 0.3 mol/L under the temperature of 90ºC for ~ 6 min until the solution turned amber yellow, indicating qualitatively the formation of SNP. After that, 7.5 mL of solution of NH_3 at 1.4 mol/L were added to the yellow solution to stabilize the SNP. This new colloidal solution was kept stirring and heating for two more minutes, and then was cooled at room temperature. In another flask, 1 mL of a PVP solution at 102 g/L was added to the yellow solution. UV/Visible absorption spectroscopy (Spectrophotometer Shimadzu MultSpec-1501; Shimadzu Corporation, Tokyo, Honshu, Japan) confirmed the colloidal SNP formation, and Transmission Electron Microscopy (Electron Microscope FEG-VP Supra 35; Carl Zeiss, Jena, Thuringen, Germany) was used in order to characterize the synthesized SNP.

2.2.Cell culture

L929 mouse fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM supplemented with 10% fetal bovine serum, streptomycin (50 g/mL), 1% antibiotic/antimycotic cocktail (300 units/mL penicillin, 300 µg/mL streptomycin, 5 µg/mL amphotericin B), and L-glutamine 0.3 g/L. All of them were purchased from GIBCO BRL (Gaithersburg, Maryland, USA) Cells were maintained under standard cell culture conditions at 37ºC in a humidified atmosphere of 95% air and 5% CO_2. Cells were routinely passaged using 0.25% trypsin/0.1% EDTA (Sigma-Aldrich, St Louis, Missouri, USA).

2.3. MTT assay

MTT assay was used to measure the cell viability as previously described by Mosman (1983) (Mosmann 1983). Briefly, L929 fibroblasts were seeded into 24-well plates (1 x 10^5 cells/mL of medium/ well). After that, the cells were incubated at 37ºC, in a humidified air atmosphere of 5% CO_2 for 24 h. The cells exposure to SNP was initiated at ~ 100% of cell confluence, and the assay was done in DMEM medium containing 10% fetal bovine serum and antibiotics as described above. Colloidal silver nanoparticles stabilized with NH_3 (SNP-A) and PVP (SNP-P), and solutions of its respective stabilizing agents, NH_3 (70 x 10^{-3} mol/L) and PVP (102 g/L) were added to fibroblast culture with doses ranged from 0 to 100 µg/mL. Three wells were used for each dose and substance tested. Tween 20 and culture medium were used as controls. The exposures of cell cultures were stopped by the discarding of the exposure medium after 6, 24 and 48h. Viable cells were stained with formazan dye (3-[4,5-dimethylthiazol-2-yl]-

2,5-diphenyltetrazolium bromide) (MTT) (Sigma Chemical Co, St Louis, Missouri, USA). MTT was dissolved in PBS at 5 mg/mL, filtered in order to sterilize and remove small amounts of insoluble residues and after the times of cells exposure to SNP and stabilizing agents, stock MTT solution (20 µL per 180 µL of culture medium) was added to all wells of the assay, and plates were incubated at 37ºC for 4 h. The MTT solution was then discarded and 200 µL of isopropylic alcohol were added to each well and mixed for 30 min to dissolve the dark blue crystals. The blue solution was then transferred to a 96-well plate and the optical density (OD) was read in a micro plate reader (Spectra Max 190; Molecular Devices, Sunnyvale, California USA) at 570 nm wavelength. Viability was calculated as the ratio of mean of OD obtained for each condition compared to the controls conditions. Data were analysed statistically by ANOVA and Bonferroni correction. Statistically significant differences were considered if $p < .05$.

All laboratory assays were carried out at least three times.

2.4.Histological study

Animals

Sixty 4-6-month-old male Wistar Albino rats, weighing between 250-280 g, were used. The animals were housed in temperature controlled rooms, and received water and food *ad libitum*. Their care was according to Research Ethics Committee of Araçatuba School of Dentistry, which approved the project prior to the experiments (protocol number FOA-0050-2010).

Polyethylene tubes

Two hundred and forty polyethylene tubes (Abbott Lab of Brazil, Sao Paulo, Sao Paulo, Brazil) with 1.0 mm internal diameter, 1.6 mm external diameter, and 10.0 mm length were used. Fibrin sponge (Technew, Rio de Janeiro, Rio de Janeiro, Brazil) was introduced in the tubes; then it was embedded with 0.1 mL of SNP-A or SNP-P at 540 μg/mL, SNP-A or SNP-P at 1.0 μg/mL, PVP solution at 0.19 g/L or NH_3 solution at 0.13 x 10^{-3} mol/L. In the control groups, the tubes received only the fibrin sponge or the fibrin sponge embedded with 0.1 mL of saline 0.9%.

Protocol in histological study

The backs of the animals were shaved under anaesthesia with xylazine (10 mg/kg) and ketamine (25 mg/kg) and disinfected with 5% iodine solution. The shaved area received a 2 cm wide incision in a head-to-tail orientation with a 15 blade (Bard-Parker, Franklin Lakes, New Jersey, USA). The skin was reflected, creating 2 pockets in each side of the incision, one in the caudal portion and other in the cranial portion. The tubes were implanted into the spaces created and the skin was closed with 4.0 silk suture.

After 7, 15, 30, 60, and 90 days from the implantation time, the animals were killed by overdose of anesthetic solution, and the tubes with surrounding tissues were removed and fixed in 10% buffered formalin at pH 7.0 (International Organization for Standardization. 2007; Federation Dentaire International Commission on Dental Materials 1980; American National Standards Institute 1979). The tubes were then bisected transversely, and both halves were cut again

longitudinally with the use of a sharp blade. This was done to allow the surfaces to be readily kept in contact with the processing solutions. The specimens were processed for glycol methacrylate (Leica Mikrosysteme Vertrieb GMBH DSA, Wetzlar, Hessen, Germany) embedding, serially sectioned into 3 mm cuts, and stained with hematoxylin-eosin (Gomes-Filho et al. 2001).

Reactions in the tissue in contact with the material on the opening of the tube were scored according to previous studies (Costa et al. 2000; Gomes-Filho et al. 2001; Yaltirik et al. 2004) as 0, none or few inflammatory cells and no reaction; 1, less than 25 cells and mild reaction; 2, between 25 and 125 cells and moderate reaction; and 3, 125 and more cells and severe reaction. Fibrous capsules were considered to be thin when thickness was <150 mm and thick at >150 mm. Necrosis was recorded as present or absent. An average of the number of cells for each group was obtained from 10 separate areas. Data were then statistically analysed by Kruskal-Wallis at a significance of 5%.

3. Results

3.1.Silver colloidal nanoparticles characterization

SNP at 540 µg/mL with average diameter of 5 nm were obtained through the reduction of $AgNO_3$ with $Na_3C_6H_5O_7$ followed by stabilization with NH_3 or PVP. These procedures resulted in two different colloidal suspensions of SNP. In the UV/ Visible analysis (Fig. 1), the absorption peak of SNP ranged from 430 to 460 nm, characterizing silver particles in nano size. Well-defined plasmon bands centred at 430 nm were revealed by the absorption spectra of the SNP-A and SNP-P colloidal suspensions (Fig. 1 a and b). The symmetrical shapes of all plasmon bands in Fig. 1 confirmed the colloidal stability and the particle size distribution. These bands have been commonly assigned to nanoparticles having a spherical shape.

TEM observations (Fig. 2) indicated that the nanoparticles were well formed, spherical and dispersed, and with mean diameter of 5 nm (Fig. 2 a and b). Furthermore, it was possible to noted a few agglomerates of particles mainly when stabilized by PVP (Fig. 2 b).

3.2.MTT assay

The MTT assay was used to measure the cell toxicit and by extension the viability of L929 cells after exposition to SNP-A, SNP-P and stabilizing agents (PVP and NH_3) for 6, 24 and 48 h. As observed in the figures 3 and 4, a reduction in the viability of L929 cells challenged by SNP-A occurred in a dose dependent manner and SNP-P occurred in a bell-shaped for all periods analysed. MTT

conversion by cells significantly decreased with SNP-A treatment at 2.5 μg/mL or higher doses after 6, 24 and 48 h compared to the control (cells without treatment) ($p < .01$) (Figure 3). Cell treatment for 6, 24 and 48 h with SNP-P showed similar toxic effects significantly reducing the cell viability compared to the control, although at lower doses ranging from 15 to 100 μg/mL ($p < .01$) (Figure 4). Additionally, between the two types of SNP tested at concentrations ranging from 2.5 to 50 μg/mL, SNP-A was significantly more cytotoxic in the three different times evaluated ($p < .001$). Furthermore, at concentration of 1.0 μg/mL SNP-A significantly reduced cell viability compared to SNP-P only after 24 h of treatment ($p < .01$) and at the lowest concentration (0.1 μg/mL) the significant reduction occurred after 6 h of treatment ($p < .01$).

With regarding to the stabilizing agents, solutions of NH_3 and PVP did not exhibit any cytotoxic effect on L929 cell after 6, 24 and 48 h of exposure (Figures 5 and 6).

3.3.Histological study

The histological analysis was performed after 7, 15, 30, 60 and 90 days, according to ISO 10993-6 which specifies test methods for the assessment of the local effects after implantation of biomaterials intended for use in medical devices or for to be applied topically in clinical indications (International Organization for Standardization. 2007). Data related to the fibrous capsule, necrosis and the comparisons in each period of time are presented in the table 1.

In the control groups (saline 0.9% and fibrin) at 7 and 15th day it was possible to observe in the tissue around the implanted tubes a moderate

inflammatory response with granulation tissue containing mainly macrophages and lymphocytes. Over this area, there were young fibroblasts in a disorganized distribution (Fig. 7 A-B and F-G). Starting on thirty up to ninety day after implantation there was a mild inflammatory infiltrate with reduction in the number of inflammatory cells in a thin fibrous capsule with well-organized collagen fibers (Fig. 7 C-E and H-J). No statistically significant difference was observed between the scores of two control groups in all periods analysed ($p < .05$).

Compared to the control groups, the tissue reaction was significantly more intense for the tubes containing SNP-A and SNP-P at 540 µg/mL, at 30 and 60 days ($p < .01$). Histological analyses at 7, 15, 30 and 60 days after implantation revealed a moderate inflammatory reaction consisting mainly of macrophages and lymphocytes in a fibrous capsule (Fig. 8 A-D and F-I). Only at 90 days, the intensity of inflammation was reduced and the fibrous capsules near the tubes were thin showing few fibroblasts, inflammatory cells and blood vessel similar to the control groups (Fig. 8 E and J). The score analysis between the groups of SNP-A and SNP-P at 540 µg/mL in the same experimental periods did not show any significant difference ($p > .05$).

In sections obtained on days 7 and 15 after the implantation of tubes containing fibrin embedded with SNP-A and SNP-P in a concentration which was non-toxic in *in vitro* assay (1.0 µg/mL), it was possible to observe the presence of a granulation tissue containing macrophages and lymphocytes in a moderate inflammatory reaction (Fig. 7 K-L and P-Q). Mild inflammatory cell infiltration and a reduction in the thickness of the fibrous capsule were evident from day 30

onward, similarly to the control groups (Fig. 7 M-O and R-T). There were no statistically significant differences among the inflammation scores for the different types SNP at 1.0 μg/mL in all periods analysed compared to the controls ($p > .05$). However, comparing the two different concentrations of SNP with the same stabilizing agent, the groups of higher concentration (540 μg/mL) showed significantly more inflammatory cells than the groups of SNP at 1.0 μg/mL on 30 and 60 days ($p < .01$).

On days 7 and 15, moderate inflammatory cell infiltration by lymphocytes and macrophages was found in a thick fibrous capsule for ammonia and PVP solution (Fig. 8 K-L and P-Q). The intensity of inflammation was reduced on days 30, 60, and 90, at which point, a thin fibrous capsule with few inflammatory cells developed near the tubes was observed (Fig. 8 M-O and R-T). Ammonia and PVP solution did not exhibit any statistically significant difference in inflammatory cell number compared to the two controls in all periods analysed ($p > .05$). Furthermore, it was possible to note that comparing the tissue reaction induced for each type of SNP at 1.0 μg/mL with the response induced for its respective stabilizing agent any statistically significant difference was observed for all experimental periods ($p > .05$). SNP-A and SNP-P at 540 μg/mL, 30 and 60 days after implantation, induced tissue reaction statistically more intense compared to its stabilizing agents ($p < .01$).

Discussion

There is a general concern of the scientific community regarding to the potential toxicity of nanoparticles, mainly because, apart from the size difference, nanomaterials also differ in their physicochemical properties from larger-sized materials of the same chemical composition (Hussain et al. 2005; Ahamed et al. 2008; Arora et al. 2009; Arora et al. 2012). It has been accepted that nanoparticles can permeate through cellular membranes, translocate to several tissues from the contact sites, and cause a range of toxic effects (Kim et al. 2010; Park et al. 2010; Kim et al. 2011; Loeschner et al. 2011; Maneewattanapinyo et al. 2011; van der Zande et al. 2012).

Although in terms of toxicological impact of manufactured nanomaterials on human health there is still no conclusive data, the well-known antimicrobial activity of SNP has encouraged their application in medicine coating catheters, implants, and in drugs delivery which would involve intra body applications and so easy access of SNP to the tissues and organs (Arora et al. 2009; Ahamed et al. 2010; Castle et al. 2011; Monteiro et al. 2011; Arora et al. 2012; Monteiro et al. 2012). In all these medical applications, keep the particle size and stability is important to ensure their suitable performance. Furthermore, the stability of nanoparticles plays a significant role in several physical, chemical properties and consequently influences on the cytotoxicity profile (Gorup et al. 2011; Lankoff et al. 2012). According to Gorup et al. (2011) to prevent aggregation and precipitation of metal nanoparticles, surfactants, mercaptans, and polymeric compounds including polyvinylpyrrolidone, polyvinyl alcohol, and some amino acids can be used (Gorup et al. 2011).

Therefore, due to the potential use in medical applications of SNP stabilized by different stabilizing agents, it was clear the necessity of investigating the biocompatibility of these particles by using *in vitro* and *in vivo* mammalian models.

In the present *in vitro* study we used a mouse fibroblast cell line (L929) which is secondary cell line considered easy to maintain and preferred in most toxicological studies for presenting better reproducibility of data (Wei et al. 2010; Gomes-Filho et al. 2011). Cell viability was determined by MTT assay which is some of the most widely used tools in cell biology for measuring the metabolic activity of cells ranging from mammalian to microbial origin (Berridge et al. 2005). This method is based on the ability of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble tetrazolium salt MTT into dark blue formazan crystals. The advantages of this method are its simplicity, rapidity and precision (Berridge et al. 2005; Gomes-Filho et al. 2011).

The results of MTT assay showed that the toxicity of both SNP-A and SNP-P was dose-dependent, with SNP-P being the less toxic to L929. Although the experimental conditions in this study differed from those used in others studies, our results agree with previous investigations that showed that SNP was cytotoxic in a concentration-dependent manner. Wei et al. (2010) evaluated the toxicity of SNP on L929 and reported that they induced manifestations such as severe morphological abnormalities, disruption of the mitochondrial respiratory chain, DNA damage and apoptosis also in a dose-dependent manner (Wei et al. 2010). Another study with HaCaT cells treated with SNP 10 nm showed that the exposure to increasing concentrations of SNP (0.006 – 333 μM) caused a

concentration- and time-dependent decrease of cell viability demonstrated by the MTT assay (Zanette et al. 2011).

From the figures 3 and 4, it was observed that for all the experimental periods SNP-P affected cell viability with significant reduction in the number of viable cell at silver concentrations greater or equal 15 µg/mL. On the other hand, SNP-A induced significant cytotoxic effect from lower concentration (2.5 µg/mL or higher). Several other studies investigating the effect of SNP on different cell lines reported cytotoxic results with similar concentrations, such as mouse fibroblast cell (50 and 100 µg/mL), alveolar macrophages (10–75 µg/mL), human dermal fibroblasts (10-200 µg/mL) or human intestinal cells (5-100 µg/mL) (Hussain et al. 2005; Carlson et al. 2008; Arora et al. 2009; Braydich-Stolle et al. 2010). Although, the exact cytotoxicity mechanisms of SNP still remain unclear, many proposed mechanisms can be found in the literature. These mechanisms include, dissolution of Ag+ from the SNP, association of SNP with cell membranes causing physical damage or subsequent penetration that lead to cell malfunction, depletion of glutathione (GSH), increased reactive oxygen species generation (ROS) and oxidative stress, which cause disruption of the mitochondrial respiratory chain (Miura and Shinohara 2009; Wei et al. 2010; El Badawy et al. 2011; Zanette et al. 2011; Kaur and Tikoo 2013). Excessive accumulation of ROS can also stimulate inflammatory responses. Furthermore, the mitochondrial dysfunction interrupts ATP synthesis, and induces DNA damage and finally cell death may occur (Kaur and Tikoo 2013).

In the present study, SNP-A were significantly more cytotoxic to L929 when compared to SNP-P in almost all concentrations analysed. Here in, silver

colloidal suspensions were prepared with NH_3 and PVP as stabilizers. NH_3 stabilizes metallic SNP by the formation of soluble diammine silver complexes, which trap free silver ions responsible for particle growth (Gorup et al. 2011). The polymers of the PVP group bond on the SNP surfaces through the nitrogen atom in their molecule, resulting in flocculation (Kvítek 2008). Despite the flocculation process, the chain of the polymer molecule keep the SNP stabilized by PVP separated from each other, and the particles are able to interact with the cells because of their high surface energy and mobility (Kvítek 2008).

Soluble diammine silver complex present in the SNP-A might be potentiated its cytotoxic effect on L929. According to El Badawy et al. (2011), the toxicity of SNP maybe partially attributed to the presence of impurities such as silver ion, residual reducing and stabilizing agents from SNP synthesis, since that the removal of the residual impurities had a dramatic impact on the reduction of toxicity of SNP (El Badawy et al. 2011).

SNP-P have a negatively charged surface and are dispersible in physiological media containing serum (Tan et al. 2009; El Badawy et al. 2011).It has been previously reported that they affect various cellular functions such as proliferation of PC-12 cells causing DNA damage, cellular content of glutathione (GSH) in Caco-2 cell line, induce increased generation of ROS and cell death in THP-1 monocytes and show genotoxic effects on lung cancer cells (Foldbjerg et al. 2009; Foldbjerg et al. 2011; Bohmert et al. 2012). However, since the head groups of PVP interact with surfaces of SNP, whereas the tail groups can tune the distances among adsorbed head groups, the presence of the PVP on the SNP-P surface may have interfered with the interaction of the particle with the cell

membrane or with the process of cellular uptake (Tan et al. 2009), then reducing the cytotoxic effect observed in the present study.

To exclude the possibility that PVP and NH_3 in the SNP suspension leads to the observed toxicity of the supernatant, the toxicity of isolated stabilizing agents was investigated in the MTT assay. The results showed no significant effect on the metabolic activitie of L929 for both agents. These findings are in line with other studies that stated that PVP is considered non-toxic and biocompatible to cells and organs (Luther et al. 2011; Beer et al. 2012).

To evaluate the biocompatibility of SNP and stabilizing agent we also used a model of implantation in the subcutaneous tissue of rats which is one of the most appropriate tests to determine the local effects of compounds (Gomes-Filho et al. 2010; Gomes-Filho et al. 2011). In the present study, polyethylene tubes filled with fibrin and fibrin embedded with saline 0.9% induced few inflammatory reactions in the subcutaneous tissue and allowed normal tissue repair, which was similar to the results of previous study (Gomes-Filho et al. 2010).

Moderate inflammatory response, which was reduced with the time showing mild inflammatory at 90 days after implantation, was observed for both types of SNP at 540 µg/mL. Gomes Filho et al. (2010) found similar tissue reaction to SNP at 47 ppm (47 µg/mL) and 2.5% sodium hypochlorite (Gomes-Filho et al. 2010). Xue et al. (2011) showed that after intramuscular administration of SNP the area demonstrated severe inflammatory cells infiltration on day 7, but these changes diminished by day 14 (Xue et al. 2012). In contrast with our results, Chen et al. (2007) reported that after implanting SNP into the rat's back muscle a serious inflammatory response even after 90 and 180

days, with granuloma formation and a large number of macrophages around the implanted particles (Chen et al. 2007).

In the present study we evaluated the tissue reaction to SNP also at 1.0 µg/mL. To select this concentration we take into account the results previously obtained from *in vitro* analysis of the cell viability assays. This concentration corresponded to the higher non-toxic concentration of SNP-A and SNP-P to L929. The tissue reaction induced by implantation of SNP-A and SNP-P at 1.0 µg/mL did not show any significant difference from the scores of the control groups ($p >$ .05). We observed initially the presence of granulation tissue with macrophages and lymphocytes, from the 30th days onward there were a reduction in the intensity of inflammation. Similar results with mild response were obtained for SNP at 23 ppm (23 µg/mL) that were also implanted in subcutaneous tissue of rats (Gomes-Filho et al. 2010).

The histological analyses of the tissue response induced by stabilizing agents NH_3 at 0.13 x 10^{-3} mol/L and PVP at 0.19 g/L did not differ from those presented by the two controls groups, suggesting that stabilizing agents are not responsible for inflammatory response caused by colloidal silver nanoparticles.

Differently from that observed in *in vitro* study which showed significant reduction in the cell viability after treatment with SNP-A compared to SNP-P, comparing the tissue response caused by SNP-A and SNP-P at the same concentration in all experimental periods, any significant difference was noted. This difference might be due to the fact that the *in vitro* model analysed the effect of SNP on a monolayer of cells and in *in vivo* model several types of cells and complex mechanisms of defence were evolved (Lin et al. 2012). According to Lin

et al. (2012) the extension of results obtained from *in vitro* experiments to the prediction of *in vivo* toxicity can be dificult, since that the complex cell–cell and cell–matrix interactions, the diversity of cell types, and hormonal effects present *in vivo* are all missing from cultured cellular systems (Lin, Bu et al. 2012).

We evaluated the effect of SNP 5 nm stabilized by NH_3 and PVP using an *in vitro* model accessing only the cell viability with one cell line, and one *in vivo* model analysing the inflammatory reaction of subcutaneous tissue. Thus, further studies concerning the toxicology of SNP with different cell lines and *in vivo* studies related to distribution and accumulation of SNP in organs after different ways of exposition, as well as investigation of the effects of chronic low-dose SNP exposure to elucidate the exact way whereby these particles interact with systems at molecular, cellular level and as a whole in order to establish their toxicological profile and ensure their safe application are strongly recommended. Finally, it is important to consider that although SNP have shown to be cytotoxic at high concentrations, these results do not discourage their future use, since that the possible applications for these particles are still in development. One of the most challenging objectives which will likely drive the future in nanotechnology is the functionalization of SNP in order that they selectively interact with specific biological targets taking advantage of their ability to traverse biological barriers and go into intracellular space or even interact with the cell surface aiming biomedical applications.

4. Conclusion

In summary, our results demonstrated that SNP exposure decreased the viability of L929 cells in a concentration-dependent manner. The type of stabilizing agent interfered on the cytotoxicity being SNP-A more toxic to L929 than SNP-P. In addition, SNP at 540 μg/mL induced significant inflammatory response in rat's subcutaneous tissue. At 1.0 μg/mL the intensity of the inflammation caused by SNP was reduced, and at this concentration the two types of SNP were considered biocompatible.

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Figures

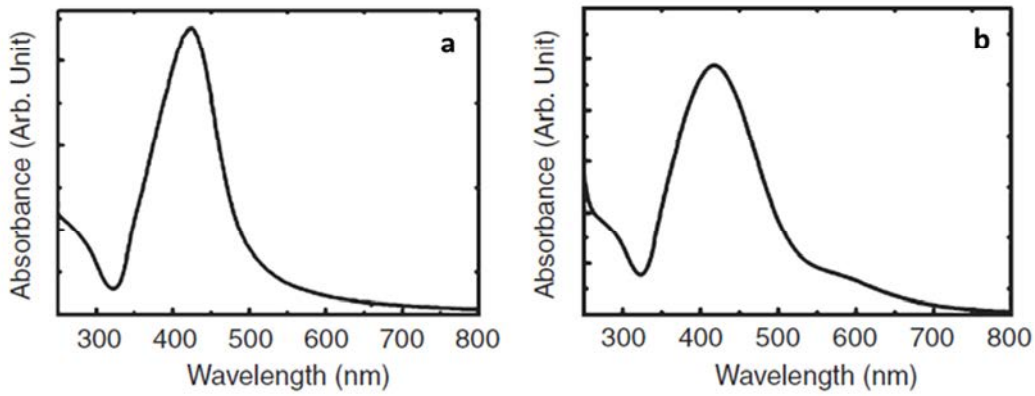

Figure 1. UV-visible spectra of silver nanoparticles (SNP) colloidal suspensions with 5 nm stabilized with ammonia (a) and polyvinylpyrrolidone (b).

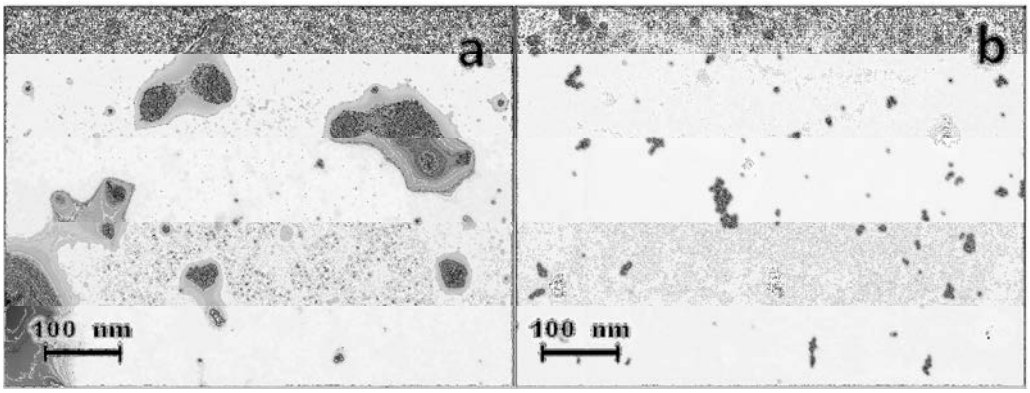

Figure 2. Scanning electron microscopy images of colloidal silver nanoparticles (SNP) with 5 nm stabilized with polyvinylpyrrolidone (a) and ammonia (b).

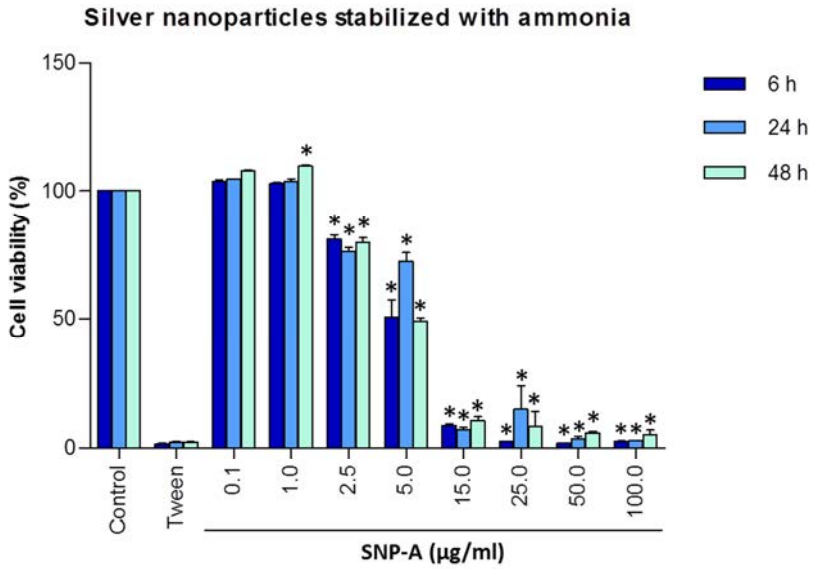

Figure 3. Viability of fibroblasts (L929) after 6, 24 and 48 h of treatment with SNP-A expressed as percentage. Error bars indicate the standard deviations of the means. *$p < .05$, as compared to the control group by using a one-way ANOVA followed by Bonferroni test.

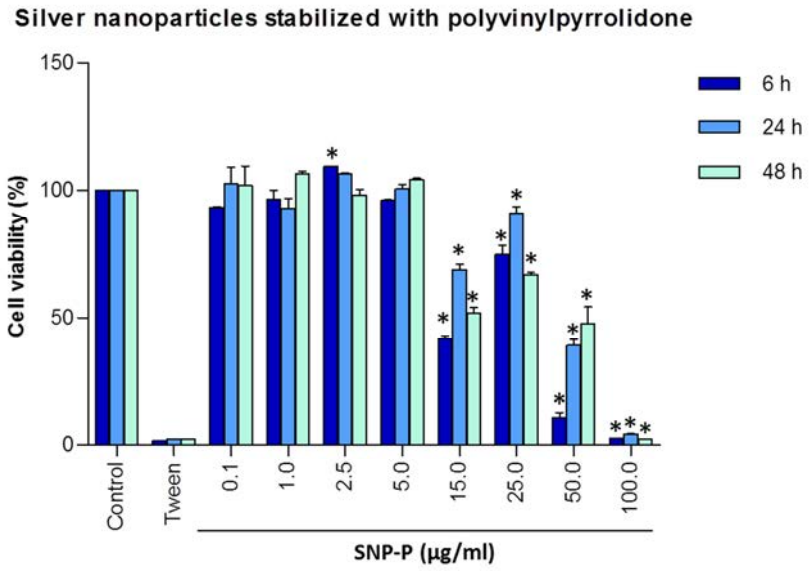

Figure 4. Viability of fibroblasts (L929) after 6, 24 and 48 h of treatment with SNP-P expressed as percentage. Error bars indicate the standard deviations of the means. *$p < .05$, as compared to the control group by using a one-way ANOVA followed by Bonferroni test.

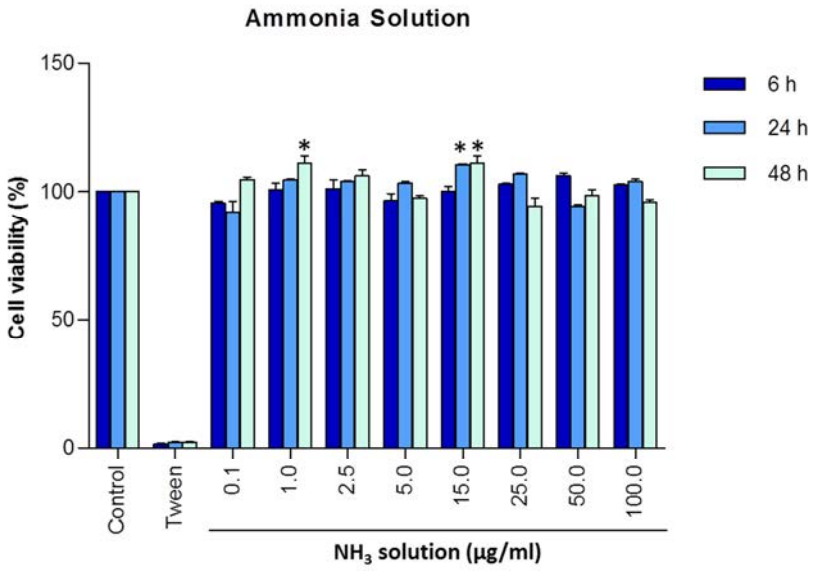

Figure 5. Viability of fibroblasts (L929) after 6, 24 and 48 h of treatment with NH_3 solution expressed as percentage. Error bars indicate the standard deviations of the means. $*p < .05$, as compared to the control group by using a one-way ANOVA followed by Bonferroni test.

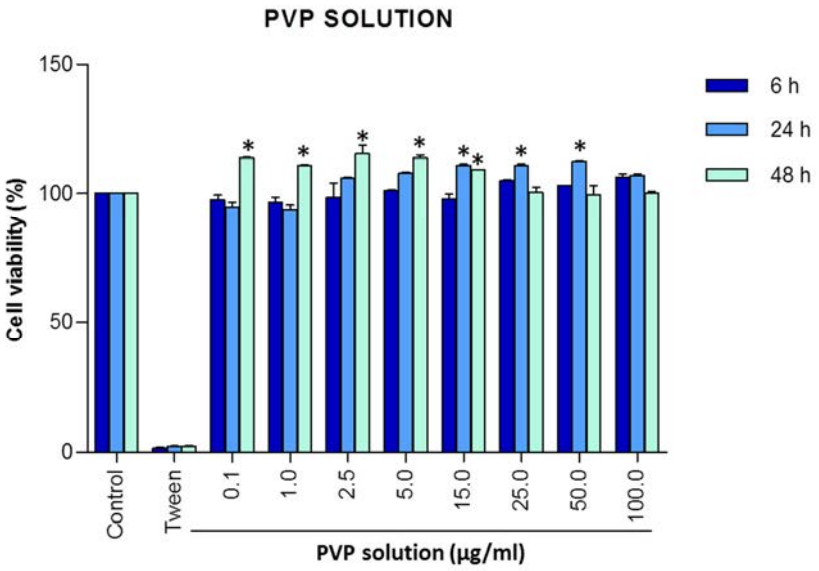

Figure 6. Viability of fibroblasts (L929) after 6, 24 and 48 h of treatment with PVP solution expressed as percentage. Error bars indicate the standard deviations of the means. *$p < .05$, as compared to the control groups by using a one-way ANOVA followed by Bonferroni test.

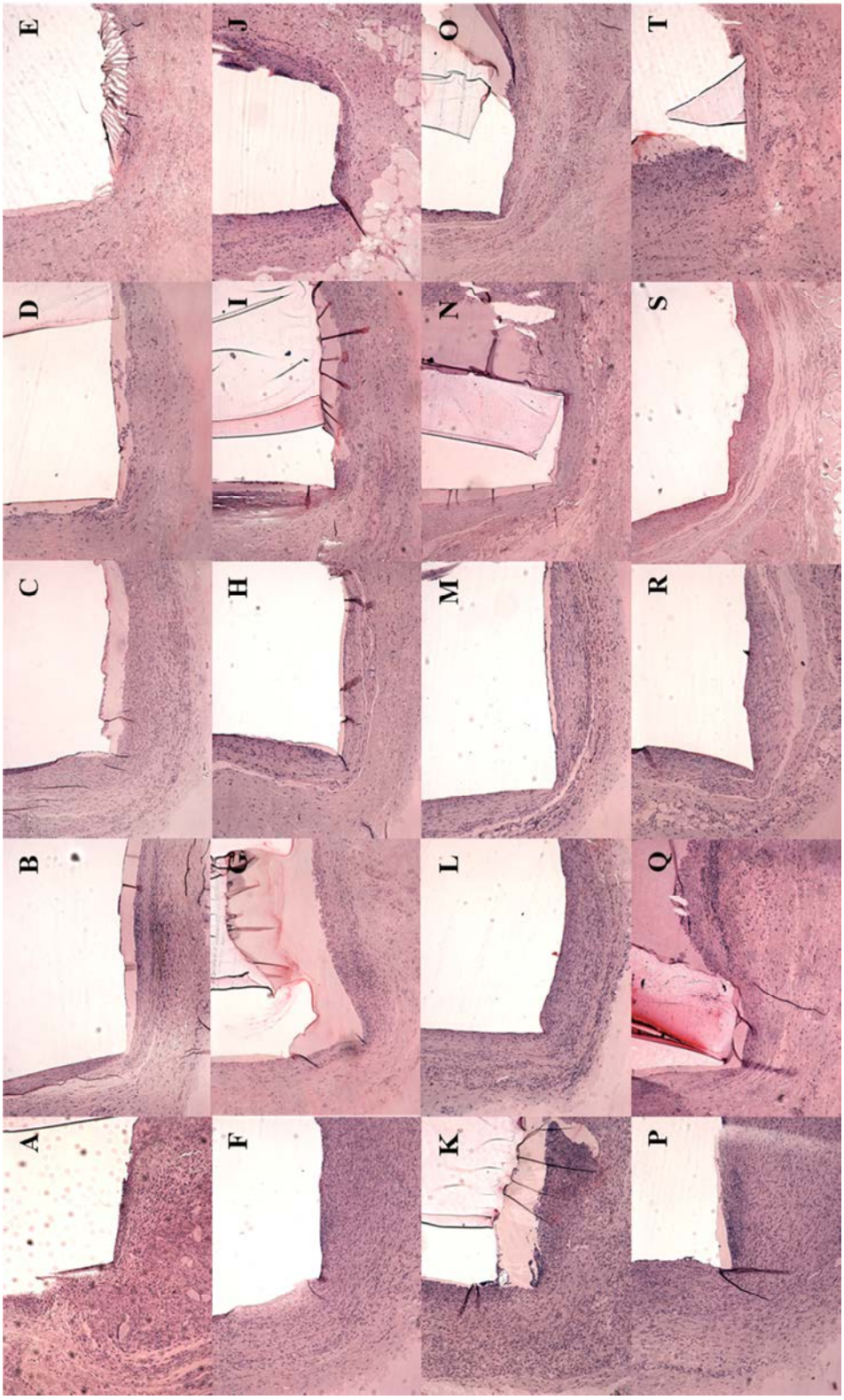

Figure 7. 7 days: (A) control (saline 0.9%)**, (F)** control (fibrin)**, (K)** SNP-A (1.0 µg/mL)**, (P)** SNP-P (1.0 µg/mL) thick fibrous capsule formation and moderate cell inflammatory infiltration consisting of macrophages and lymphocytes (hematoxylin-eosin staining, 10x). **15 days: (B)** control (saline 0.9%)**, (G)** control (fibrin)**, (L)** SNP-A (1.0 µg/mL)**, (Q)** SNP-P (1.0 µg/mL) thick fibrous capsule formation and moderate cell inflammatory infiltration consisting of macrophages and lymphocytes (hematoxylin-eosin staining, 10x). **30 days: (C)** control (saline 0.9%)**, (H)**

control (fibrin)**, (M)** SNP-A (1.0 µg/mL)**, (R)** SNP-P (1.0 µg/mL) thin fibrous capsule formation and mild cell inflammatory infiltration consisting of lymphocytes, macrophages (hematoxylin-eosin staining, 10x). **60 days: (D)** control (saline 0.9%)**, (I)** control (fibrin)**, (N)** SNP-A (1.0 µg/mL)**, (S)** SNP-P (1.0 µg/mL) thin fibrous capsule formation and mild cell inflammatory infiltration consisting of lymphocytes, macrophages (hematoxylin-eosin staining, 10x). **90 days: (E)** control (saline 0.9%)**, (J)** control (fibrin)**, (O)** SNP-A (1.0 µg/mL)**, (T)** SNP-P (1.0 µg/mL) thin fibrous capsule formation and mild cell inflammatory infiltration consisting of lymphocytes, macrophages (hematoxylin-eosin staining, 10x).

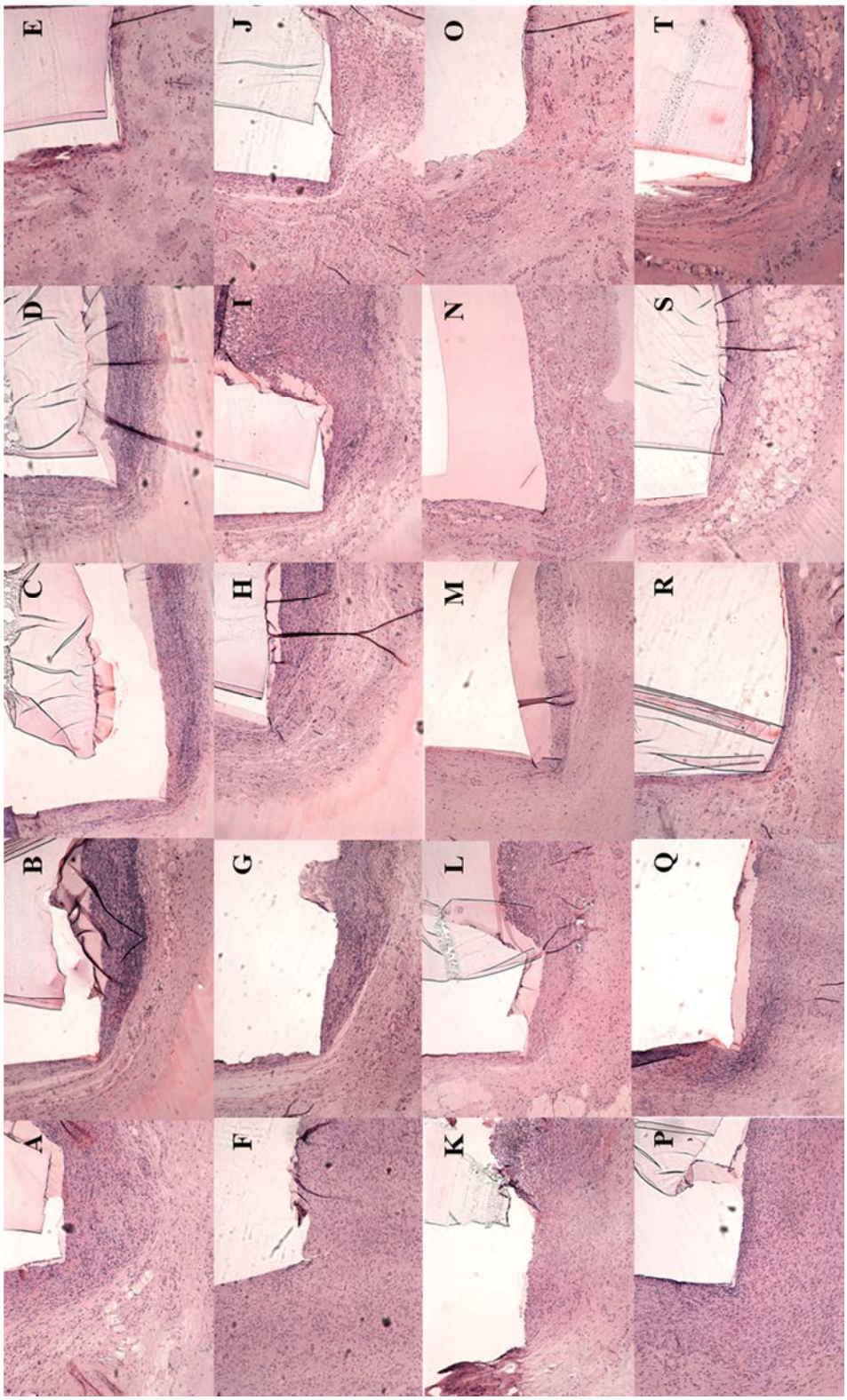

Figure 8. 7 days: (A) SNP-A (540 µg/mL)**, (F)** SNP-P (540 µg/mL)**, (K)** NH_3 solution (0.13 x 10^{-3} mol/L)**, (P)** PVP solution (0.19 g/L) thick fibrous capsule formation and moderate cell inflammatory infiltration consisting of macrophages and lymphocytes (hematoxylin-eosin staining, 10x). **15 days: (B)** SNP-A (540 µg/mL)**, (G)** SNP-P (540 µg/mL)**, (L)** NH_3 solution (0.13 x 10^{-3} mol/L)**, (Q)** PVP solution (0.19 g/L) thick fibrous capsule formation and moderate cell inflammatory infiltration consisting of macrophages and lymphocytes (hematoxylin-eosin staining,

10x). **30 days: (C)** SNP-A (540 µg/mL)**, (H)** SNP-P (540 µg/mL) moderate cell inflammatory infiltration by lymphocytes and macrophages, **(M)** NH_3 solution (0.13 x 10^{-3} mol/L)**, (R)** PVP solution (0.19 g/L) mild cell inflammatory infiltration consisting of lymphocytes and macrophages (hematoxylin-eosin staining, 10x), **60 days: (D)** SNP-A (540 µg/mL)**, (I)** SNP-P (540 µg/mL) moderate cell inflammatory infiltration consisting of lymphocytes and macrophages, **(N)** NH_3 solution (0.13 x 10^{-3} mol/L)**, (S)** PVP solution (0.19 g/L) mild cell inflammatory infiltration consisting of lymphocytes and macrophages (hematoxylin-eosin staining, 10x). **90 days: (A)** SNP-A (540 µg/mL)**, (F)** SNP-P (540 µg/mL)**, (K)** NH_3 solution (0.13 x 10^{-3} mol/L)**, (P)** PVP solution (0.19 g/L) thin fibrous capsule formation and mild cell inflammatory infiltration consisting of lymphocytes, macrophages (hematoxylin-eosin staining, 10x).

Table 1. Percentage of samples in each group categorized according to the inflammatory score, presence of necrosis, and thickness of fibrous capsule.

SUBSTANCE	SCORE					
	0	**1**	**2**	**3**	**Necrosis**	**Capsule**
7 days						
SNP-A (540.0 µg/mL)	0	0	100	0	Absent	Thick
SNP-P (540.0 µg/mL)	0	0	100	0	Absent	Thick
SNP-A (1.0 µg/mL)	0	0	100	0	Absent	Thick
SNP-P (1.0 µg/mL)	0	0	100	0	Absent	Thick
NH_3 solution	0	0	100	0	Absent	Thick
PVP solution	0	0	100	0	Absent	Thick
Fibrin (control)	0	0	100	0	Absent	Thick
Saline 0.9% (control)	0	0	100	0	Absent	Thick
15 days						
SNP-A (540.0 µg/mL)	0	0	100	0	Absent	Thick
SNP-P (540.0 µg/mL)	0	0	100	0	Absent	Thick
SNP-A (1.0 µg/mL)	0	0	100	0	Absent	Thick
SNP-P (1.0 µg/mL)	0	0	100	0	Absent	Thick
NH_3 solution	0	0	100	0	Absent	Thick
PVP solution	0	0	100	0	Absent	Thick
Fibrin (control)	0	0	100	0	Absent	Thick
Saline 0.9% (control)	0	0	100	0	Absent	Thick
30 days						
SNP-A (540.0 µg/mL)	0	0	100	0	Absent	Thin
SNP-P (540.0 µg/mL)	0	0	100	0	Absent	Thin
SNP-A (1.0 µg/mL)	0	100	0	0	Absent	Thin
SNP-P (1.0 µg/mL)	0	100	0	0	Absent	Thin
NH_3 solution	0	100	0	0	Absent	Thin
PVP solution	0	100	0	0	Absent	Thin
Fibrin (control)	0	100	0	0	Absent	Thin
Saline 0.9% (control)	0	100	0	0	Absent	Thin
60 days						

SNP-A (540.0 µg/mL)	0	0	100	0	Absent	Thin
SNP-P (540.0 µg/mL)	0	0	100	0	Absent	Thin
SNP-A (1.0 µg/mL)	0	100	0	0	Absent	Thin
SNP-P (1.0 µg/mL)	0	100	0	0	Absent	Thin
NH_3 solution	0	100	0	0	Absent	Thin
PVP solution	0	100	0	0	Absent	Thin
Fibrin (control)	0	100	0	0	Absent	Thin
Saline 0.9% (control)	0	100	0	0	Absent	Thin
90 days						
SNP-A (540.0 µg/mL)	0	100	0	0	Absent	Thin
SNP-P (540.0 µg/mL)	0	100	0	0	Absent	Thin
SNP-A (1.0 µg/mL)	0	100	0	0	Absent	Thin
SNP-P (1.0 µg/mL)	0	100	0	0	Absent	Thin
NH_3 solution	0	100	0	0	Absent	Thin
PVP solution	0	100	0	0	Absent	Thin
Fibrin (control)	0	100	0	0	Absent	Thin
Saline 0.9% (control)	0	100	0	0	Absent	Thin

Anexo A – Certificado do Comitê de Ética no Uso de Animais (CEUA)

unesp UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO" Campus de Araçatuba

Comitê de Ética no Uso de Animais (CEUA)
Committee for Ethical Use of Animals (CEUA)

CERTIFICADO

Certificamos que o Projeto **"Avaliação da resposta tecidual e da citotoxicidade de soluções coloidais de nanoparticulas de prata"** sob responsabilidade do Pesquisador **JOÃO EDUARDO GOMES FILHO** e colaboração de Aline Satie Takamiya e Débora Barros Barbosa está de acordo com os Princípios Éticos da Experimentação Animal (COBEA) e foi aprovado pelo CEUA, de acordo com o protocolo **FOA-0050-2010**.

CERTIFICATE

We certify that the research **"Evaluation of tissue reaction and cytotoxicity of colloidal silver nanoparticles"**, protocol number **FOA-0050-2010**, under responsibility of **JOÃO EDUARDO GOMES FILHO** and with collaboration of Aline Satie Takamiya and Débora Barros Barbosa agree with Ethical Principles in Animal Research (COBEA) and was approved by CEUA.

Prof. Dr. EDILSON ERVOLINO
Coordenador da CEUA
CEUA Coordinator

Faculdade de Odontologia e Faculdade de Medicina Veterinária
Rua José Bonifácio, 1193, CEP 16015-050 – Vila Mendonça
Tel (18) 3636-3234 Fax (18) 3636-3333 E-mail: ervolino@foa.unesp.br

Anexo B – Resultados

Control Saline 0.9%

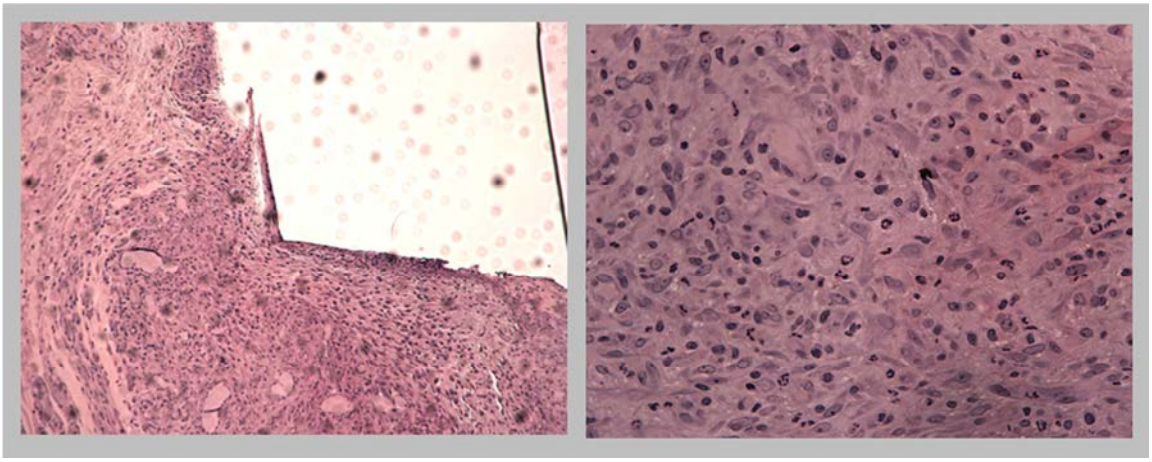

Figure 1. Control (Saline 0.9%) 7 days: moderate cell inflammatory infiltration consisting of lymphocytes and macrophages (hematoxylin-eosin staining, 10x and 40x).

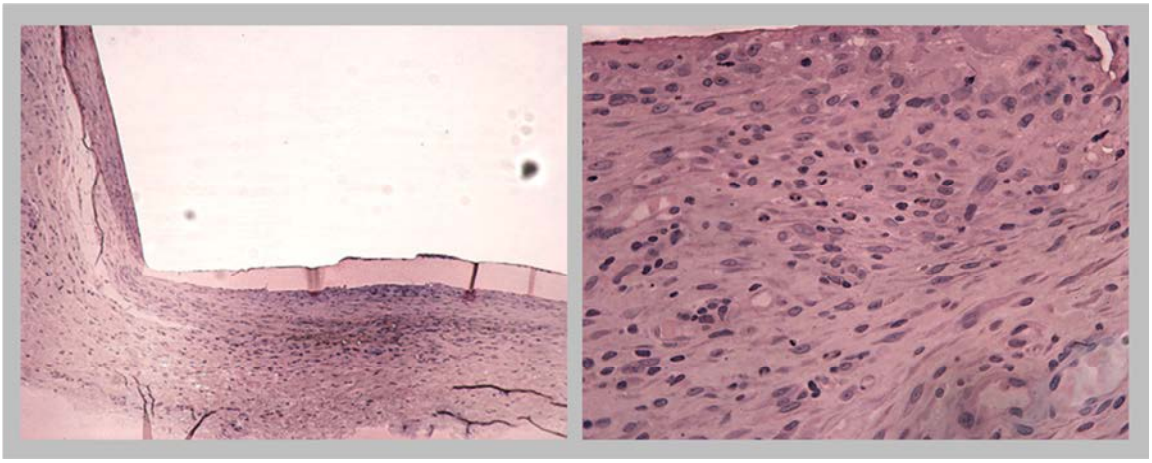

Figure 2. Control (Saline 0.9%) 15 days: fibrous capsule formation and moderate cell inflammatory infiltration consisting of macrophages and lymphocytes (hematoxylin-eosin staining, 10x and 40x).

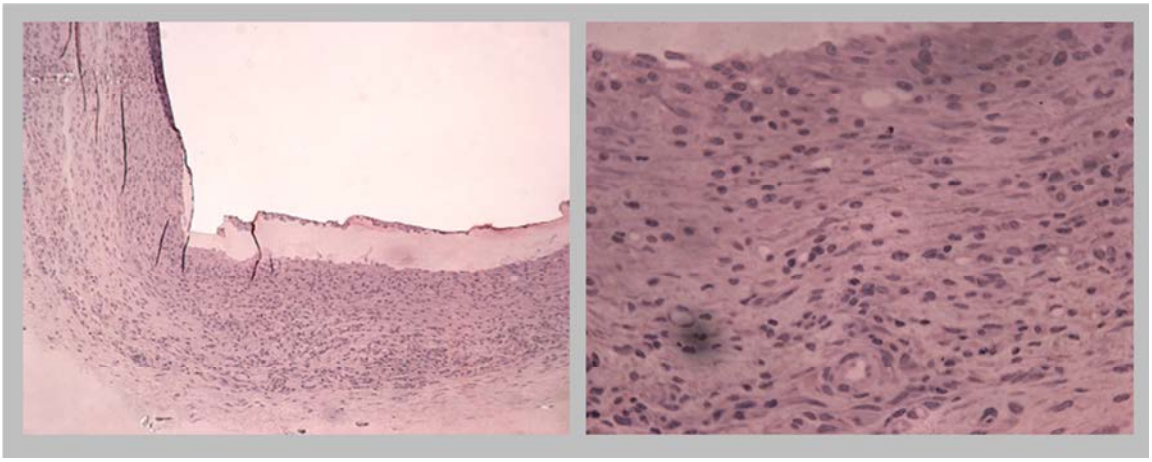

Figure 3. Control (Saline 0.9%) 30 days: fibrous capsule formation and mild cell inflammatory infiltration consisting of lymphocytes, macrophages (hematoxylin-eosin staining, 10x and 40x).

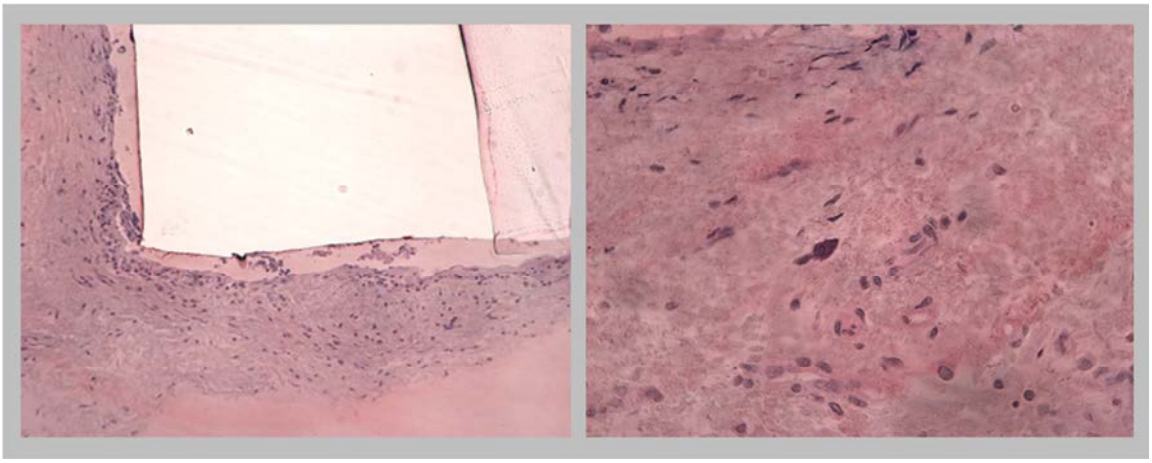

Figure 4. Control (Saline 0.9%) 60 days: thin fibrous capsule formation and few inflammatory cells (hematoxylin-eosin staining, 10x and 40x).

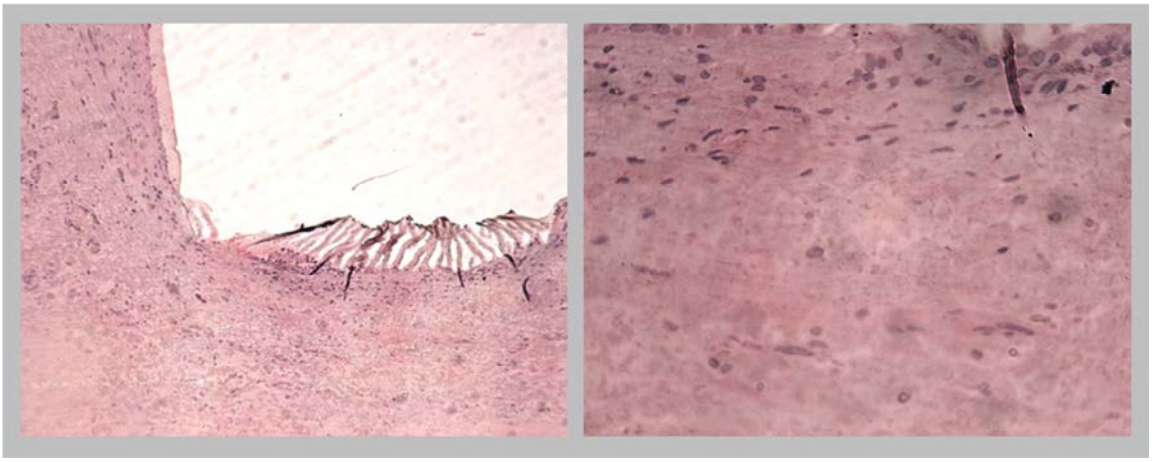

Figure 5. Control (Saline 0.9%) 90 days: thin fibrous capsule formation and few inflammatory cells (hematoxylin-eosin staining, 10x and 40x).

Control Fibrin

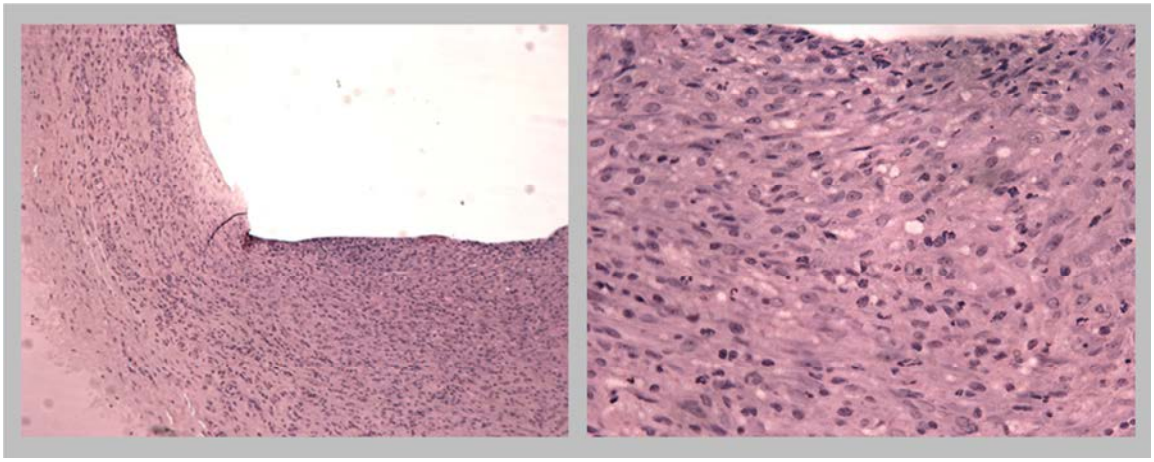

Figure 6. Control (Fibrin) 7 days: moderate cell inflammatory infiltration consisting mainly macrophages and lymphocytes (hematoxylin-eosin staining, 10x and 40x).

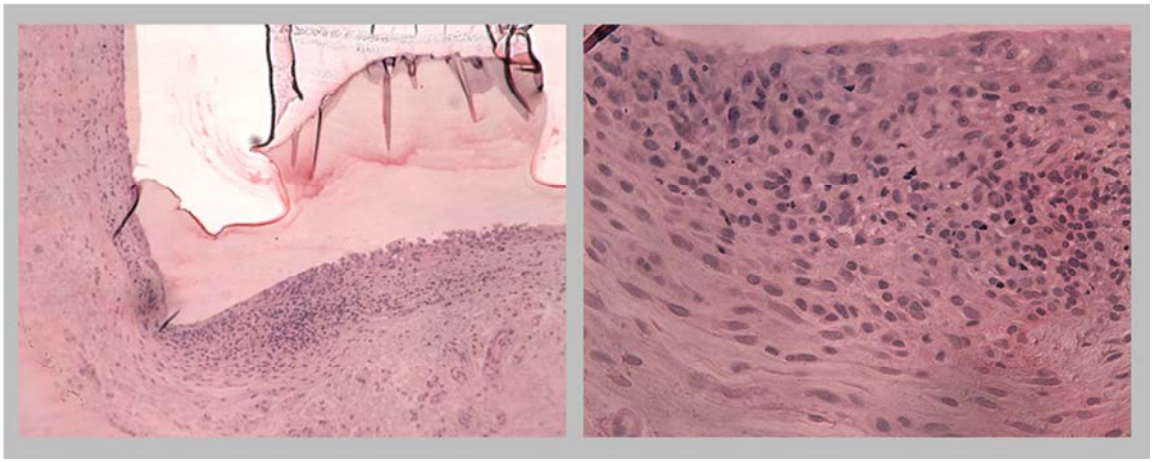

Figure 7. Control (Fibrin) 15 days: fibrous capsule formation and moderate cell inflammatory infiltration consisting mainly of lymphocytes and macrophages (hematoxylin-eosin staining, 10x and 40x).

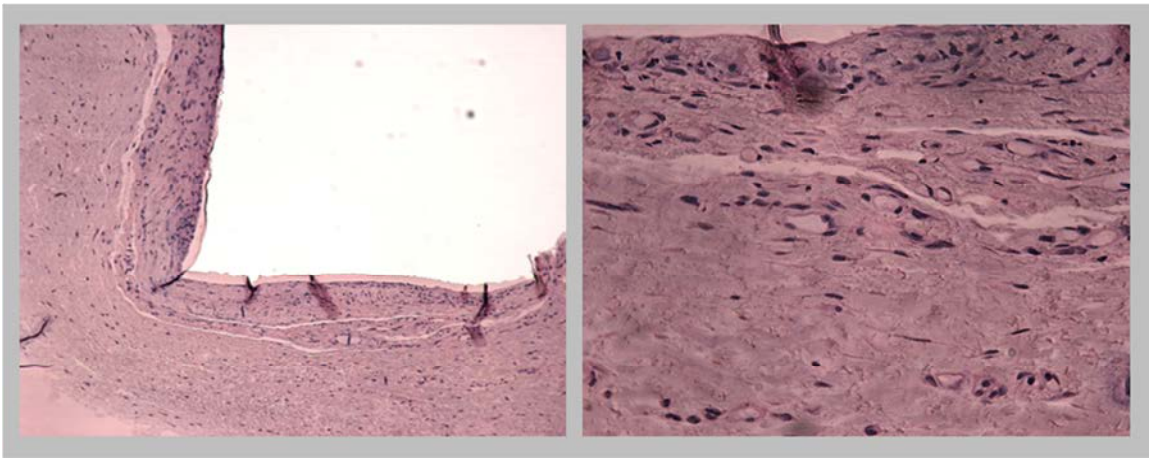

Figure 8. Control (Fibrin) 30 days: thin fibrous capsule formation and few inflammatory cells (hematoxylin-eosin staining, 10x and 40x).

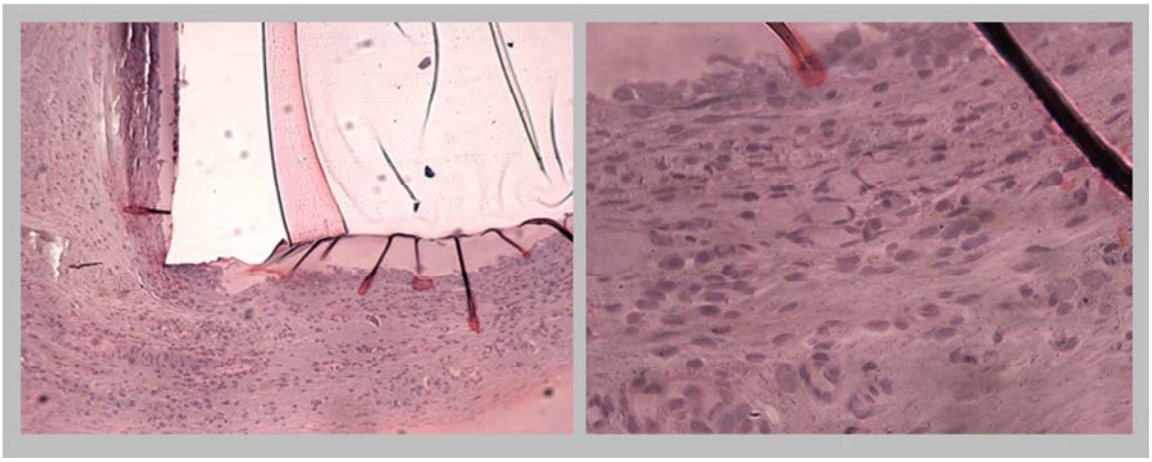

Figure 9. Control (Fibrin) 60 days: thin fibrous capsule formation and few inflammatory cells (hematoxylin-eosin staining, 10x and 40x).

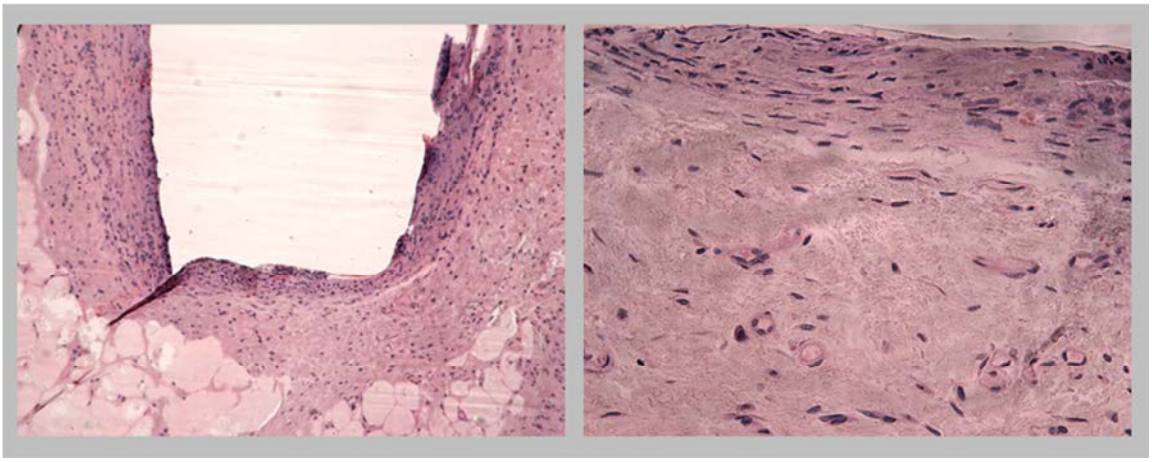

Figure 10. Control (Fibrin) 90 days: thin fibrous capsule formation and mild cell inflammatory infiltration (hematoxylin-eosin staining, 10x and 40x).

Silver nanoparticles stabilized with ammonia (1.0 µg/mL)

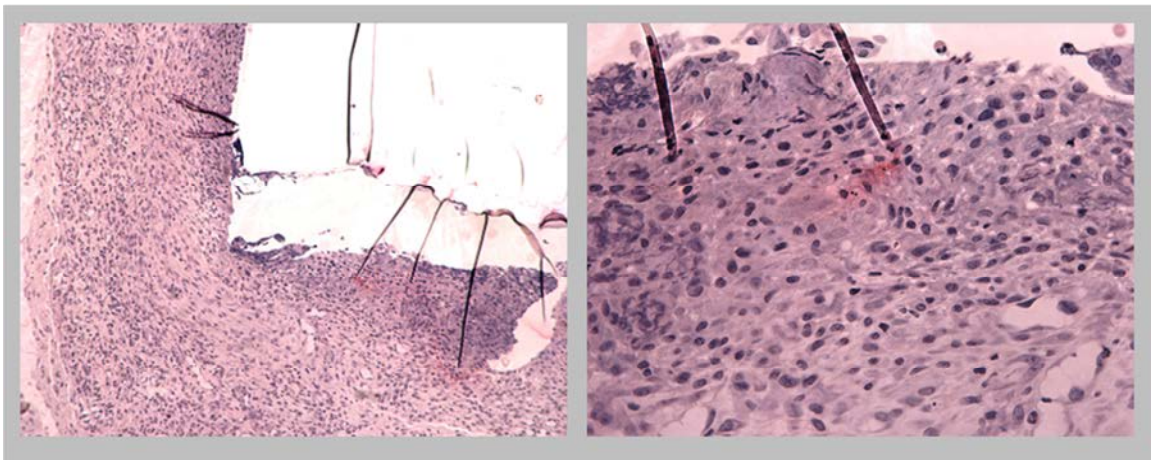

Figure 11. SNP-A (1.0 µg/mL) 7 days: moderate cell inflammatory infiltration consisting of lymphocytes and macrophages (hematoxylin-eosin staining, 10x and 40x).

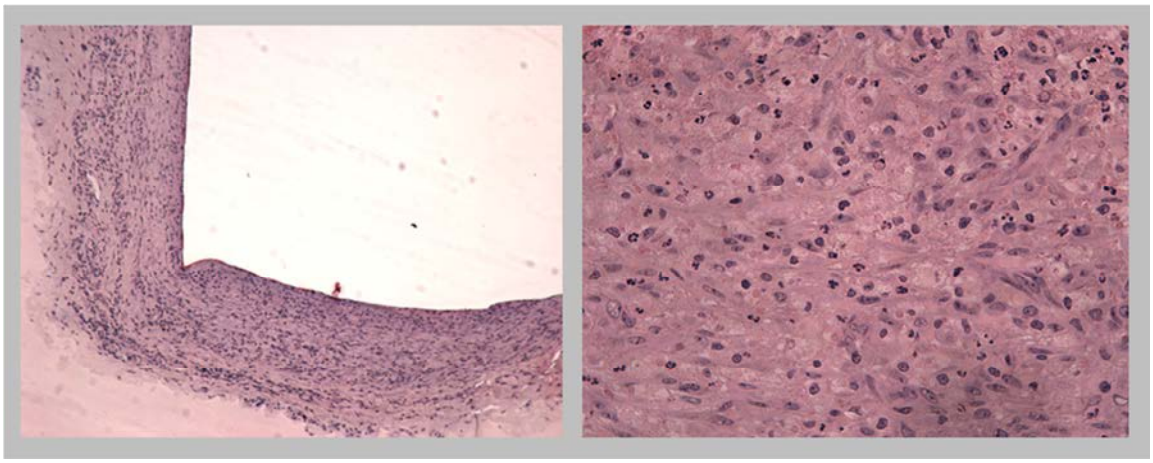

Figure 12. SNP-A (1.0 µg/mL) 15 days: fibrous capsule formation and moderate cell inflammatory infiltration consisting mainly of macrophages and lymphocytes (hematoxylin-eosin staining, 10x and 40x).

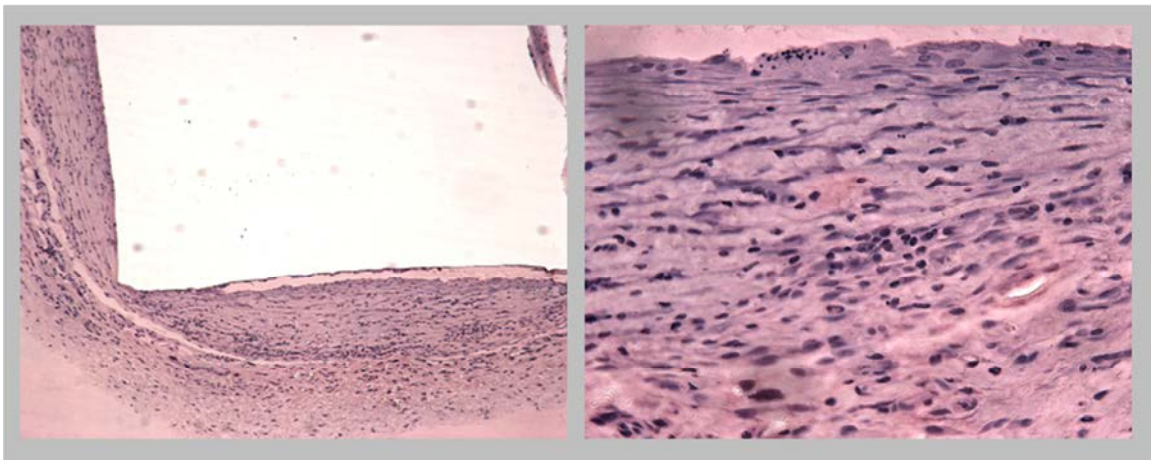

Figure 13. SNP-A (1.0 µg/mL) 30 days: thin fibrous capsule formation and mild cell inflammatory infiltration consisting of lymphocytes and macrophages (hematoxylin-eosin staining, 10x and 40x).

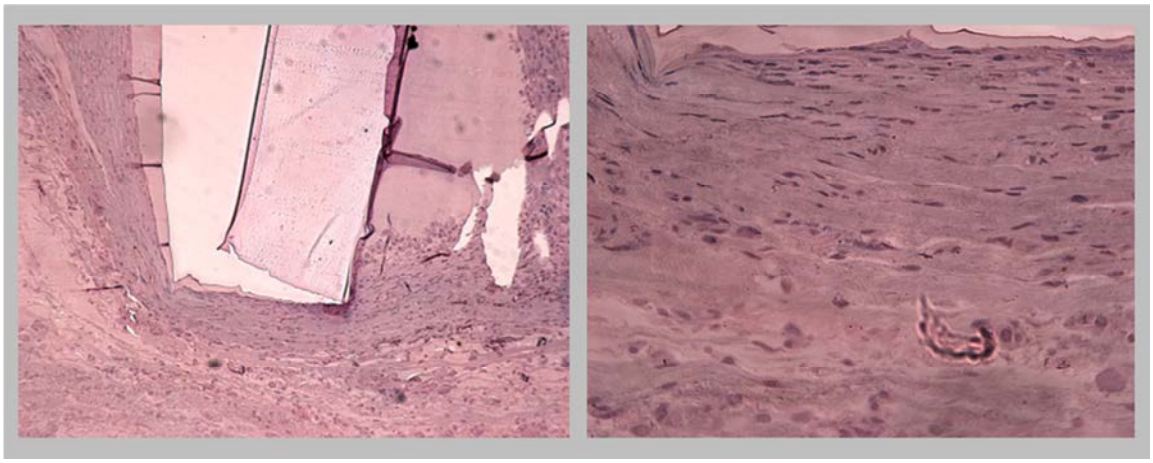

Figure 14. SNP-A (1.0 µg/mL) 60 days: thin fibrous capsule formation with few inflammatory cells (hematoxylin-eosin staining, 10x and 40x).

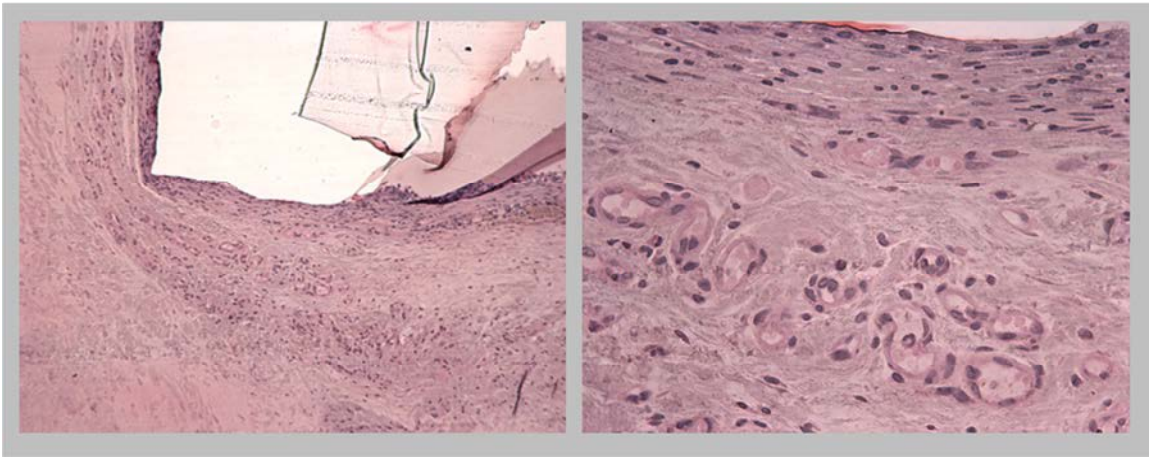

Figure 15. SNP-A (1.0 μg/mL) 90 days: thin fibrous capsule formation and mild cell inflammatory infiltration (hematoxylin-eosin staining, 10x and 40x).

Silver nanoparticles stabilized with polyvinylpyrrolidone (1.0 µg/mL)

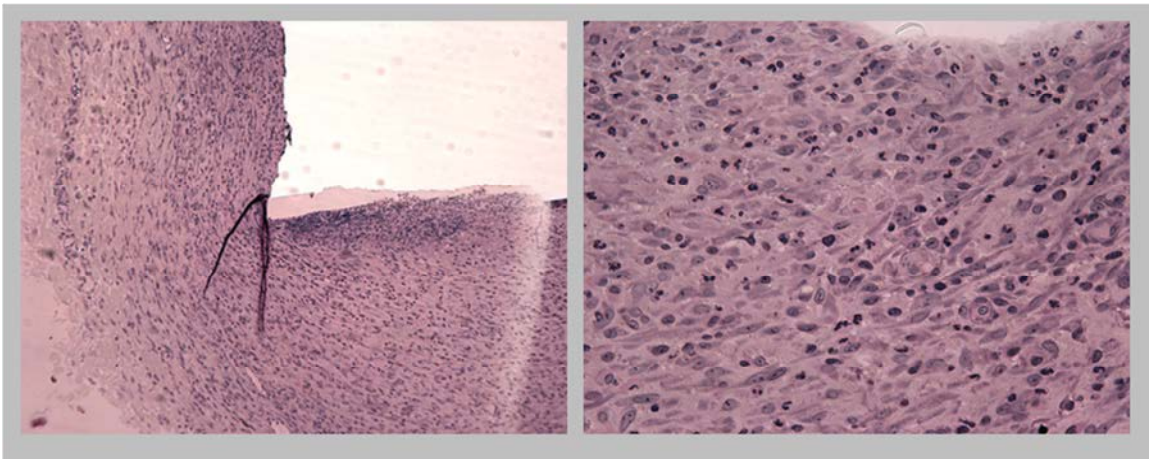

Figure 16. SNP-P (1.0 µg/mL) 7 days: moderate cell inflammatory infiltration consisting mainly of macrophages and lymphocytes (hematoxylin-eosin staining, 10x and 40x).

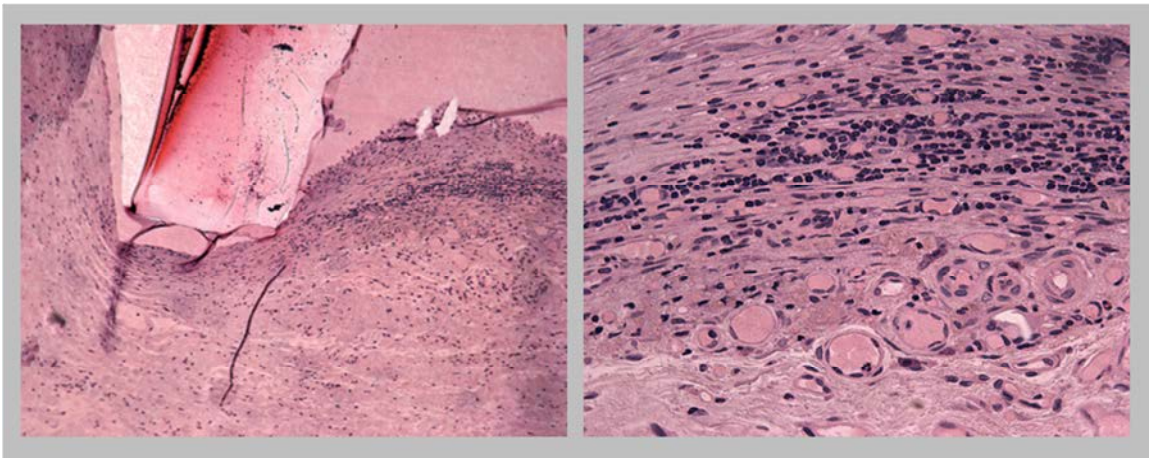

Figure 17. SNP-P (1.0 µg/mL) 15 days: fibrous capsule formation and moderate cell inflammatory infiltration consisting of lymphocytes and macrophages (hematoxylin-eosin staining, 10x and 40x).

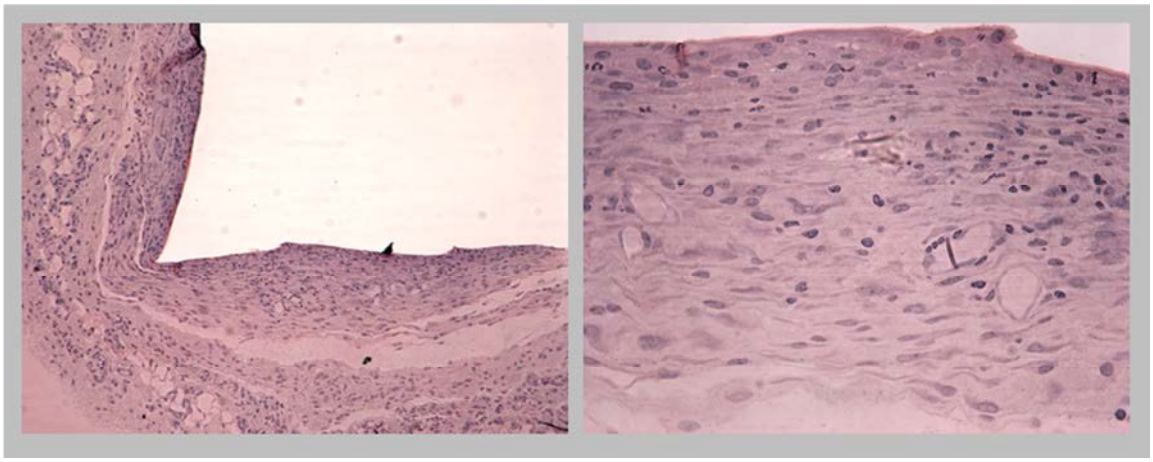

Figure 18. SNP-P (1.0 µg/mL) 30 days: thin fibrous capsule formation and mild cell inflammatory infiltration consisting of few macrophages and lymphocytes (hematoxylin-eosin staining, 10x and 40x).

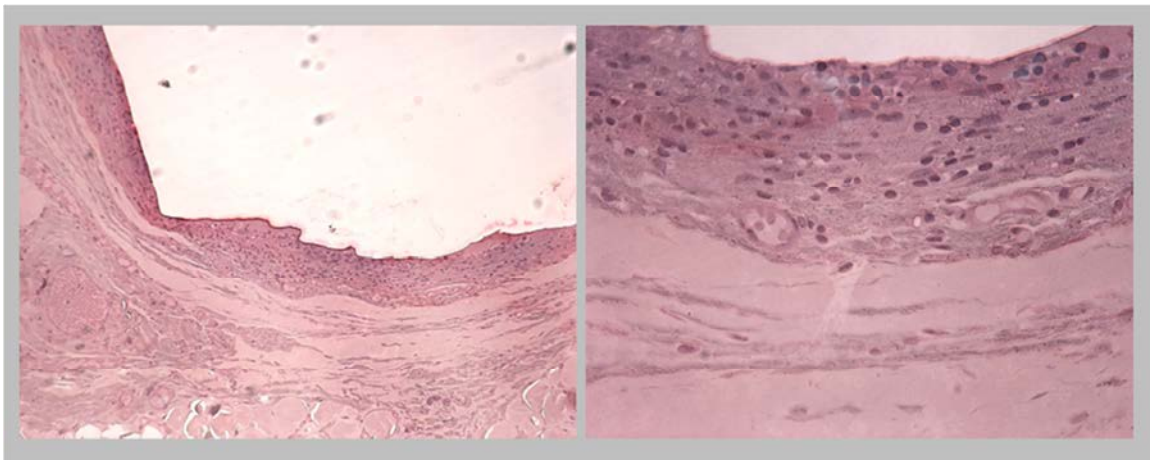

Figure 19. SNP-P (1.0 µg/mL) 60 days: thin fibrous capsule formation and mild cell inflammatory infiltration (hematoxylin-eosin staining, 10x and 40x).

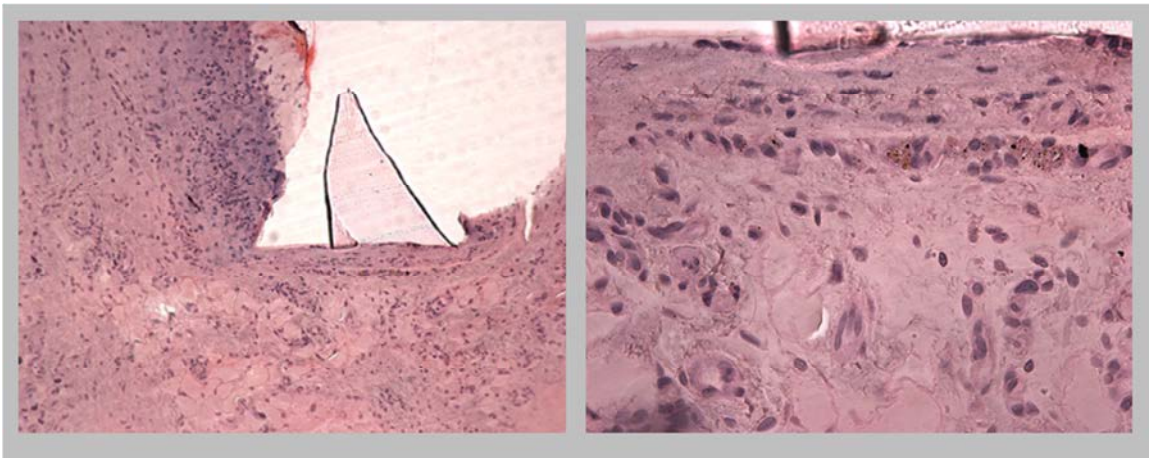

Figure 20. SNP-P (1.0 µg/mL) 90 days thin fibrous capsule formation and mild cell inflammatory infiltration (hematoxylin-eosin staining, 10x and 40x).

Silver nanoparticles stabilized with ammonia (540 µg/mL)

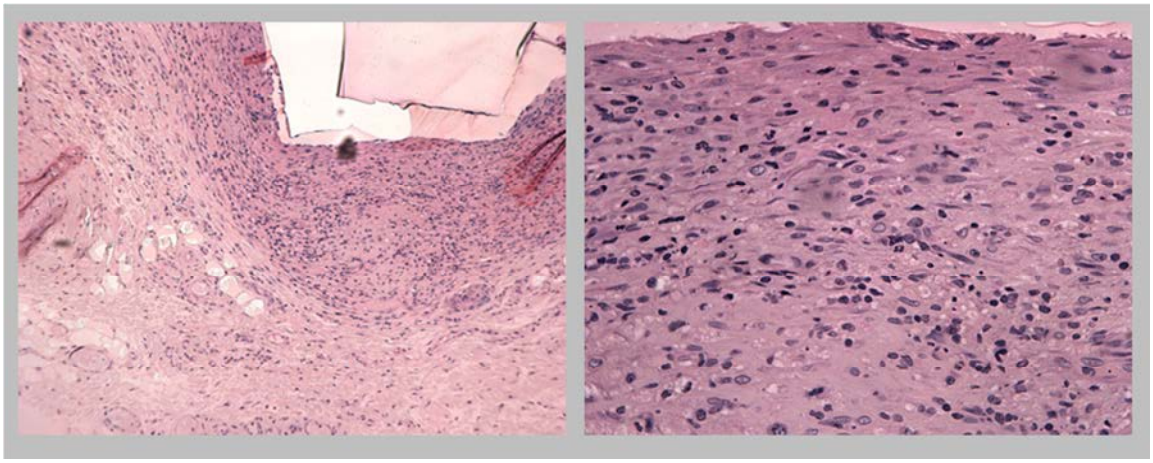

Figure 21. SNP-A (540 µg/mL) 7 days: moderate cell inflammatory infiltration consisting mainly lymphocytes and macrophages (hematoxylin-eosin staining, 10x and 40x).

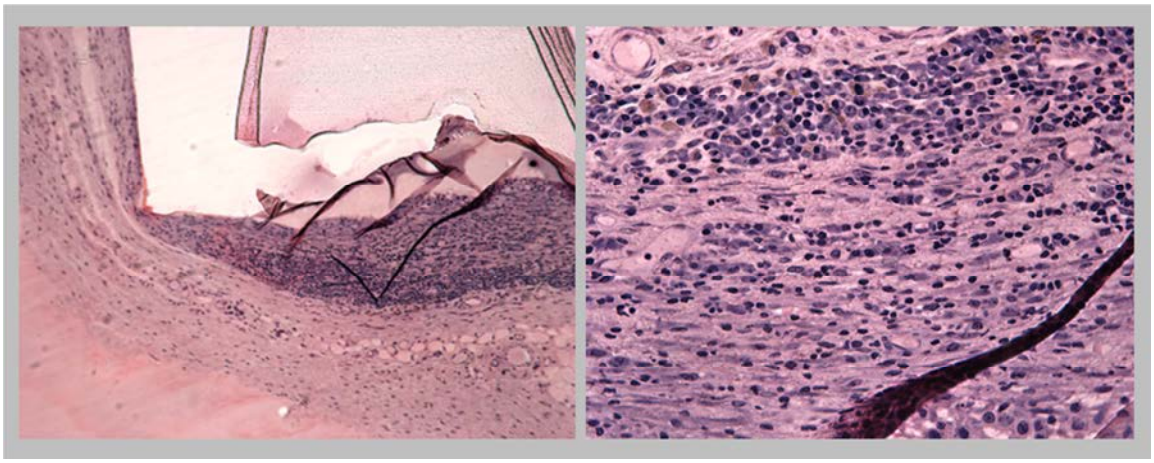

Figure 22. SNP-A (540 µg/mL) 15 days: fibrous capsule formation and moderate cell inflammatory infiltration consisting mainly of macrophages and lymphocytes (hematoxylin-eosin staining, 10x and 40x)

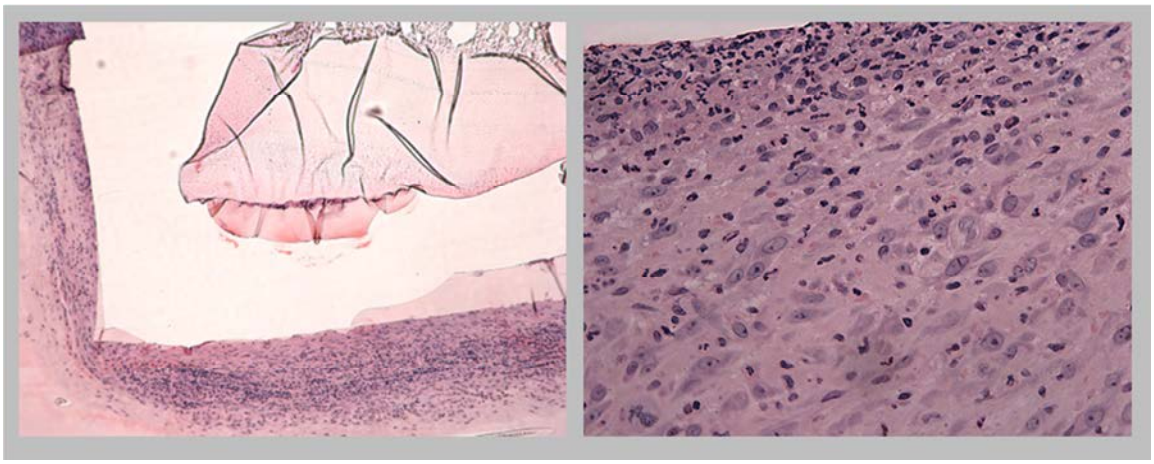

Figure 23. SNP-A (540 µg/mL) 30 days: fibrous capsule formation and moderate cell inflammatory infiltration consisting mainly of macrophages and lymphocytes (hematoxylin-eosin staining, 10x and 40x).

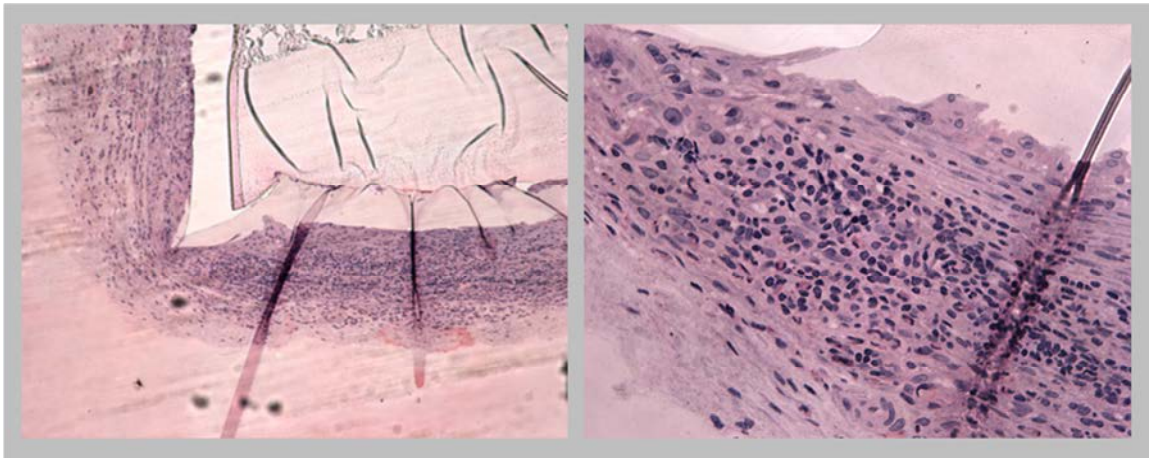

Figure 24. SNP-A (540 µg/mL) 60 days: thick fibrous capsule formation and moderate cell inflammatory infiltration consisting of lymphocytes and macrophages (hematoxylin-eosin staining, 10x and 40x).

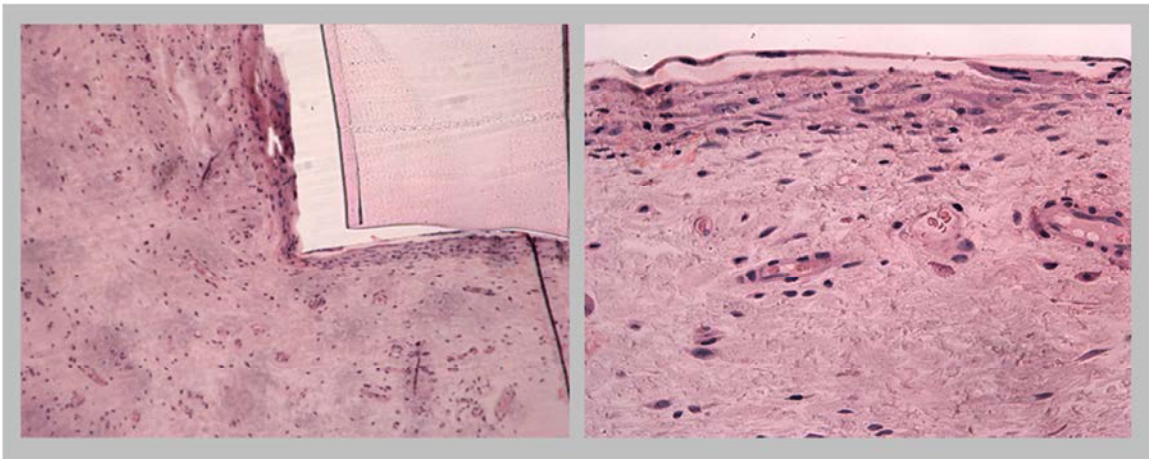

Figure 25. SNP-A (540 µg/mL) 90 days: thin fibrous capsule formation and few inflammatory cells (hematoxylin-eosin staining, 10x and 40x).

Silver nanoparticles stabilized with polyvinylpyrrolidone (540 µg/mL)

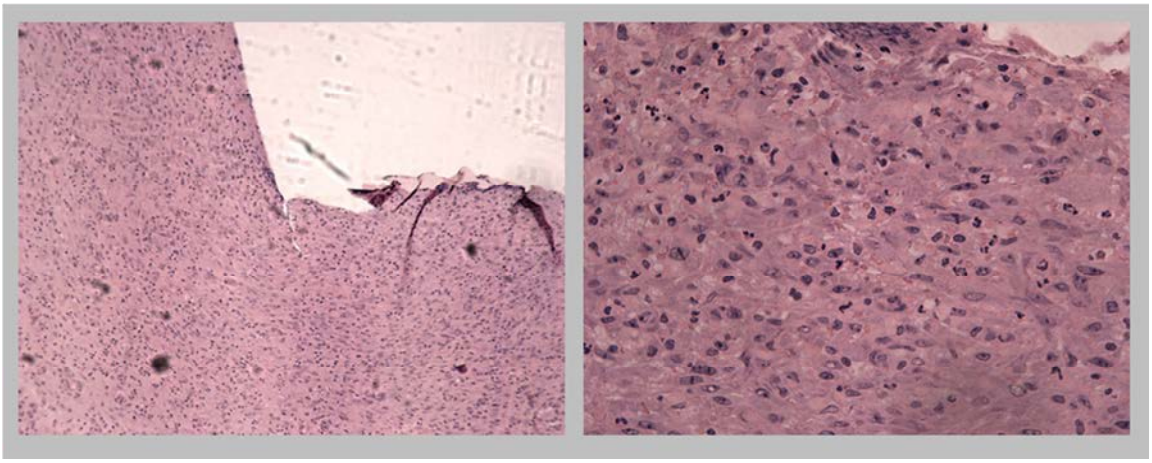

Figure 26. SNP-P (540 µg/mL) 7 days: moderate cell inflammatory infiltration consisting mainly of macrophages and lymphocytes (hematoxylin-eosin staining, 10x and 40x).

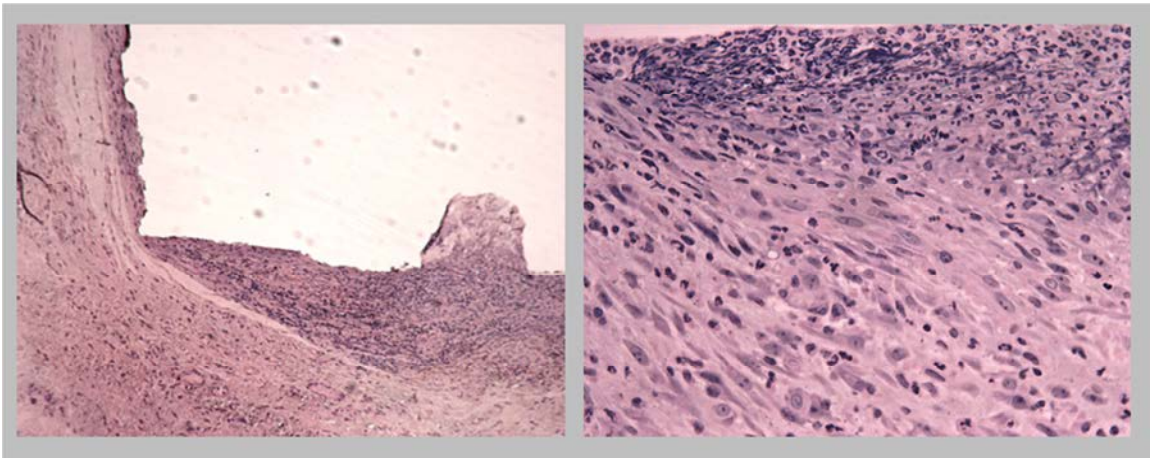

Figure 27. SNP-P (540 µg/mL) 15 days: fibrous capsule formation and moderate cell inflammatory infiltration consisting of lymphocytes and macrophages (hematoxylin-eosin staining, 10x and 40x).

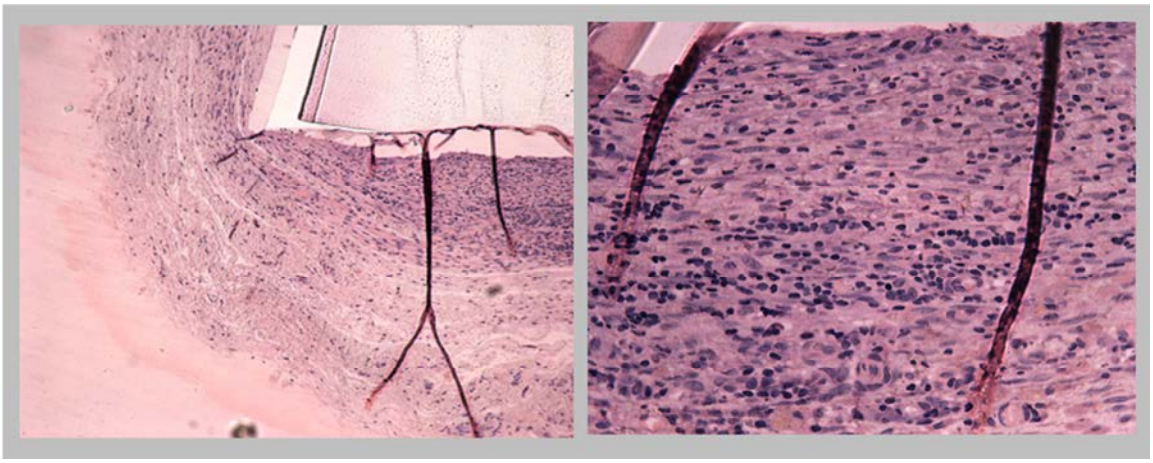

Figure 28. SNP-P (540 µg/mL) 30 days: thick fibrous capsule formation and moderate cell inflammatory infiltration consisting of lymphocytes and macrophages (hematoxylin-eosin staining, 10x and 40x).

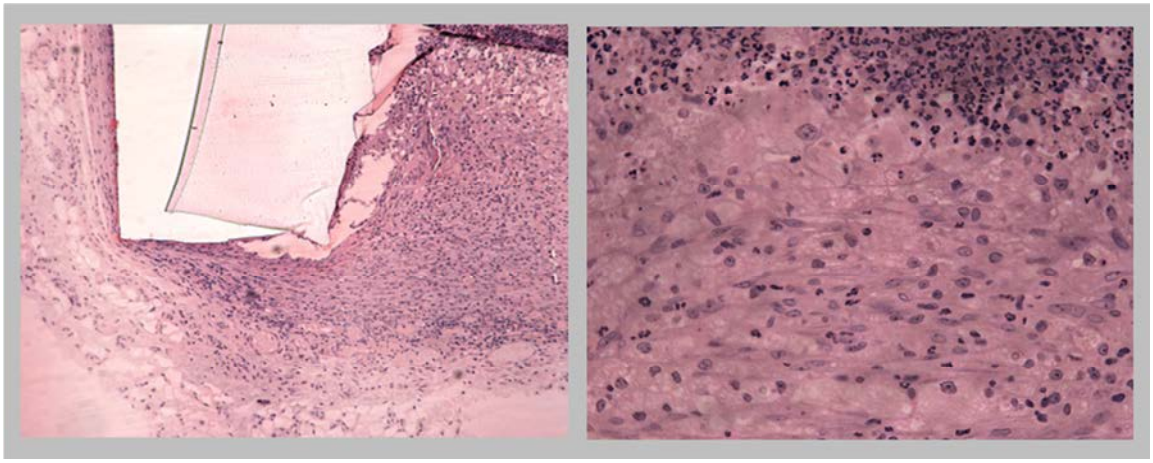

Figure 29. SNP-P (540 µg/mL) 60 days: fibrous capsule formation and moderate cell inflammatory infiltration consisting of lymphocytes and macrophages (hematoxylin-eosin staining, 10x and 40x).

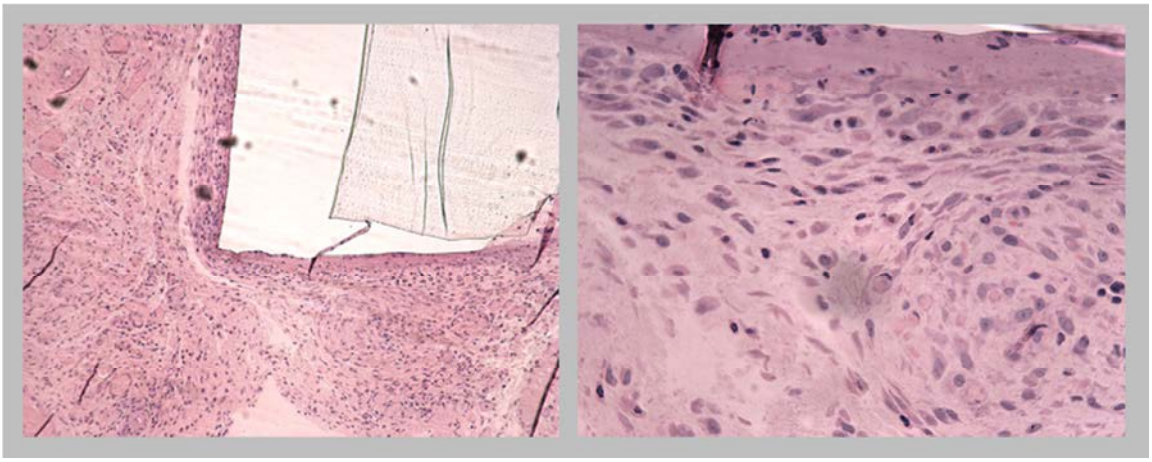

Figure 30. SNP-P (540 µg/mL) 90 days: thin fibrous capsule formation and few inflammatory cells (hematoxylin-eosin staining, 10x and 40x).

Ammonia solution (0.13 x 10^{-3} mol/mL)

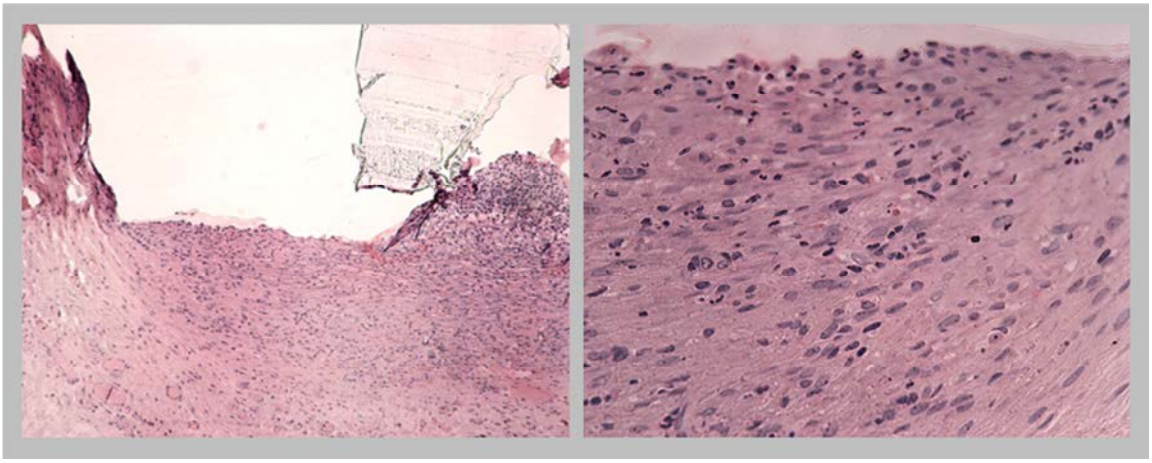

Figure 31. NH_3 solution 7 days: moderate cell inflammatory infiltration consisting mainly of macrophages and lymphocytes (hematoxylin-eosin staining, 10x and 40x).

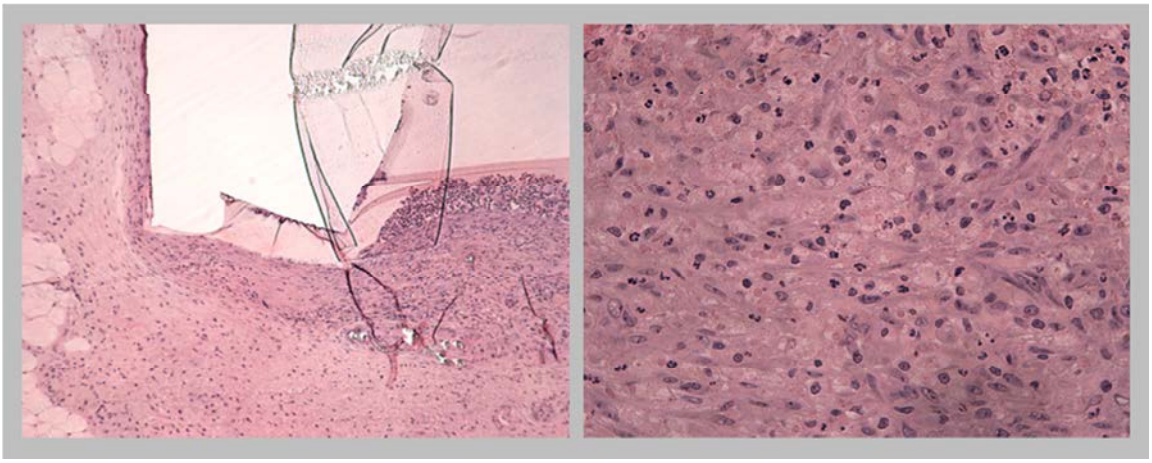

Figure 32. NH_3 solution 15 days: fibrous capsule formation and moderate cell inflammatory infiltration consisting of macrophages and lymphocytes (hematoxylin-eosin staining, 10x and 40x).

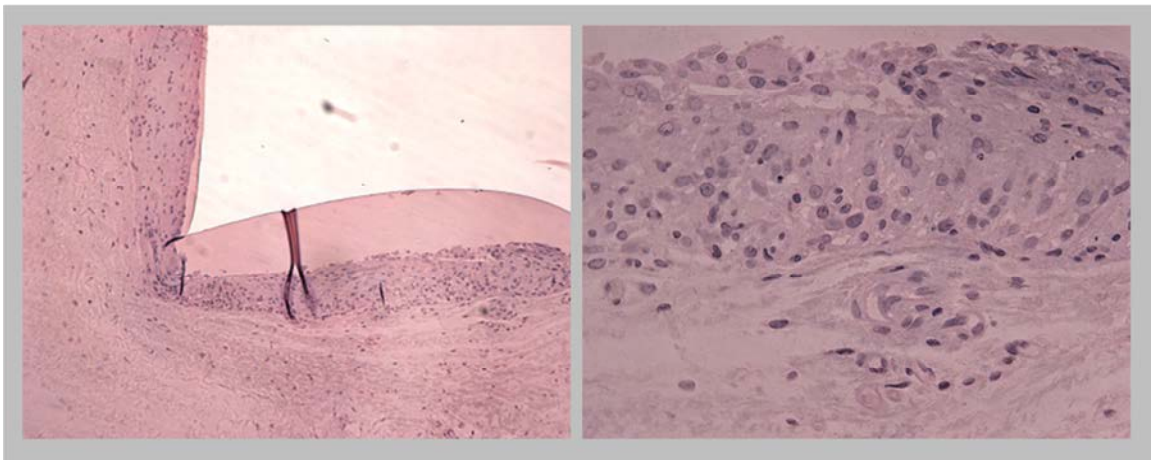

Figure 33. NH_3 solution 30 days: thin fibrous capsule formation and few inflammatory cells (hematoxylin-eosin staining, 10x and 40x).

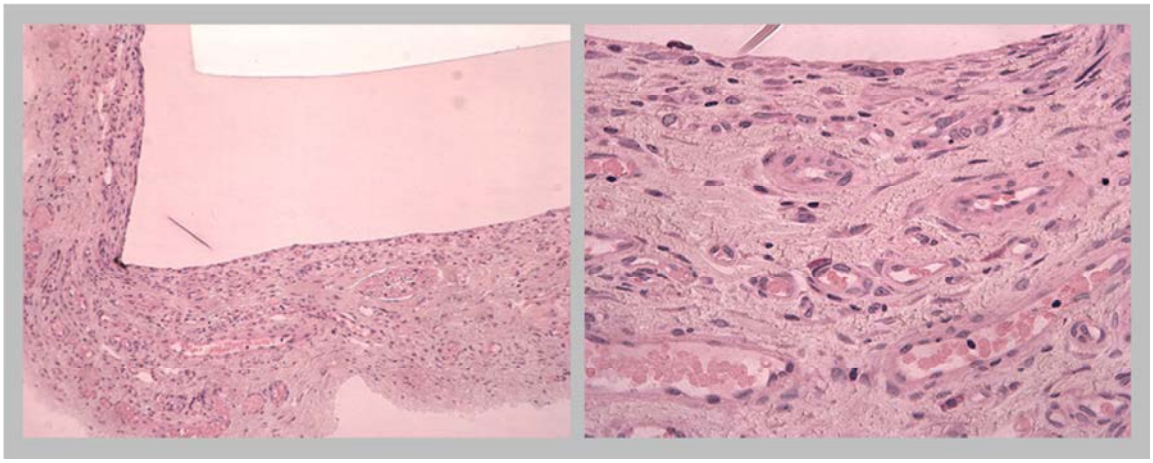

Figure 34. NH_3 solution 60 days: thin fibrous capsule formation and few inflammatory cells (hematoxylin-eosin staining, 10x and 40x).

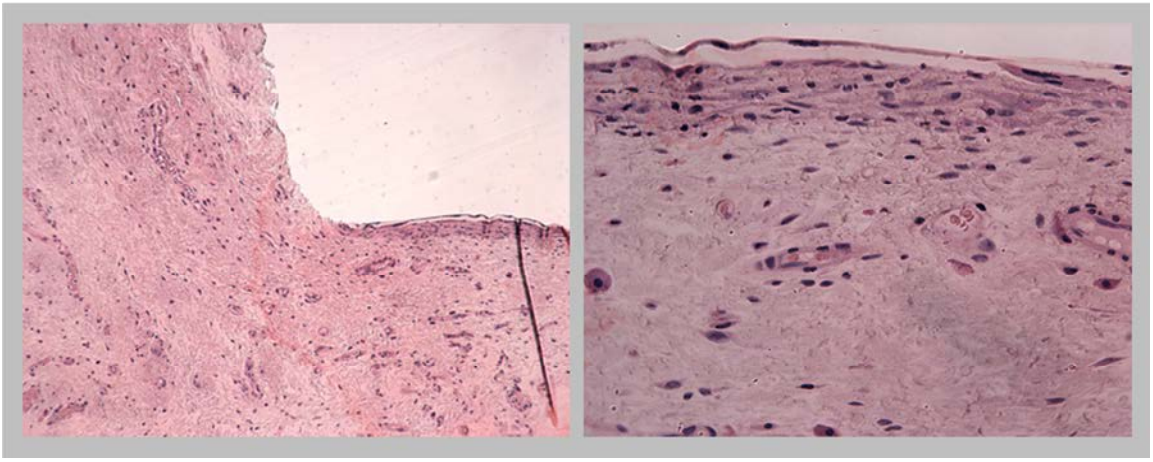

Figure 35. NH_3 solution 90 days: thin fibrous capsule formation and few inflammatory cells (hematoxylin-eosin staining, 10x and 40x).

Polyvinylpyrrolidone solution (0.19 g/L)

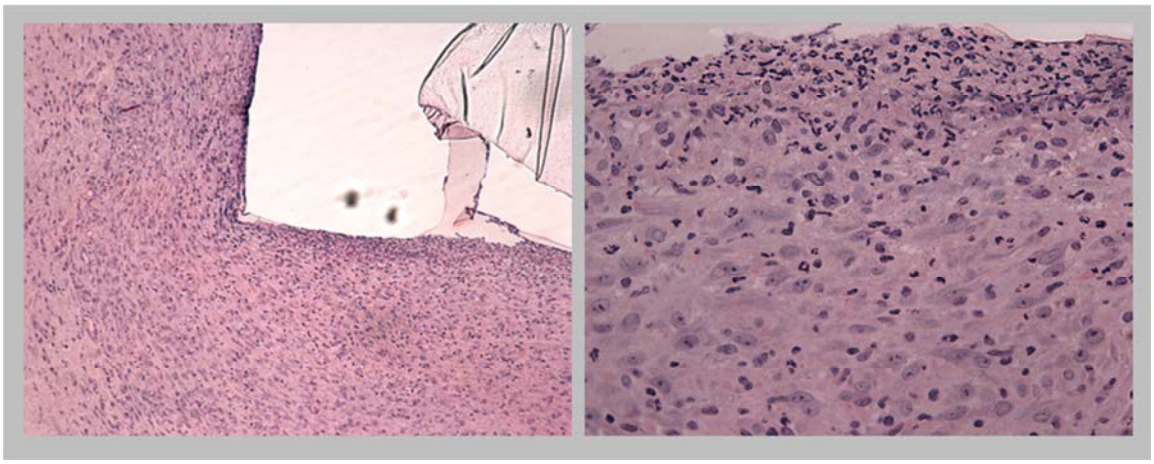

Figure 36. PVP solution 7 days: moderate cell inflammatory infiltration consisting mainly of macrophages and lymphocytes (hematoxylin-eosin staining, 10x and 40x).

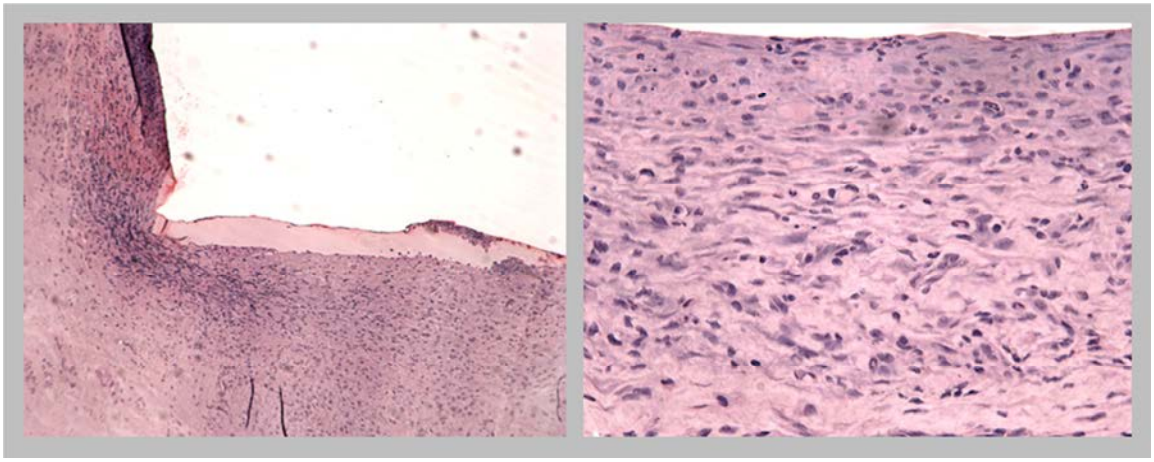

Figure 37. PVP solution 15 days: fibrous capsule formation and moderate cell inflammatory infiltration consisting of lymphocytes and macrophages (hematoxylin-eosin staining, 10x and 40x).

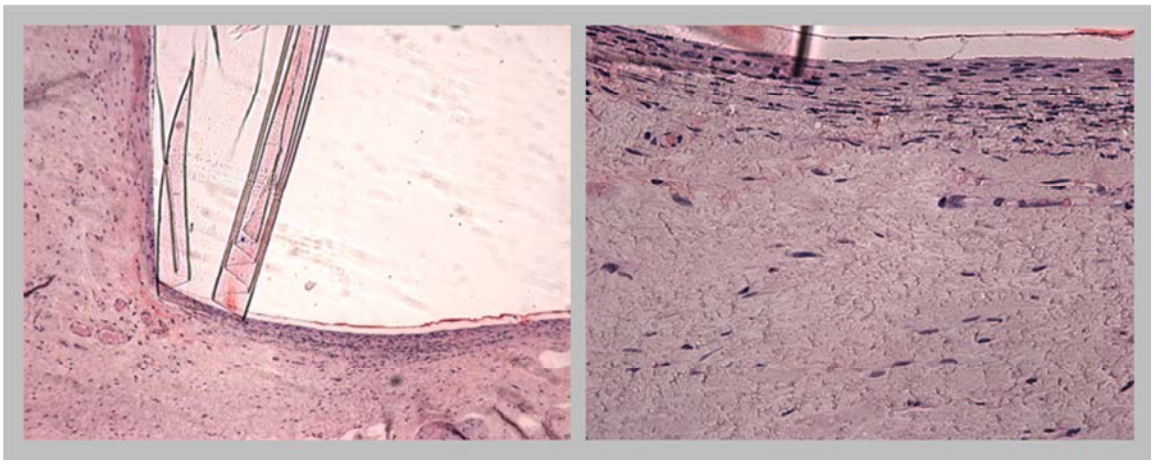

Figure 38. PVP solution 30 days: thin fibrous capsule formation and few inflammatory cells (hematoxylin-eosin staining, 10x and 40x).

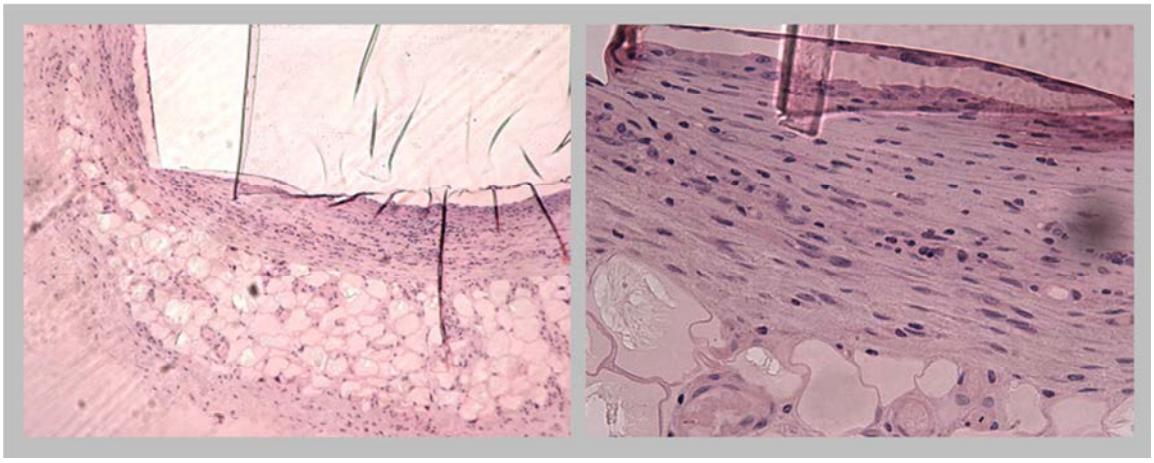

Figure 39. PVP solution 60 days: thin fibrous capsule formation and few inflammatory cells (hematoxylin-eosin staining, 10x and 40x).

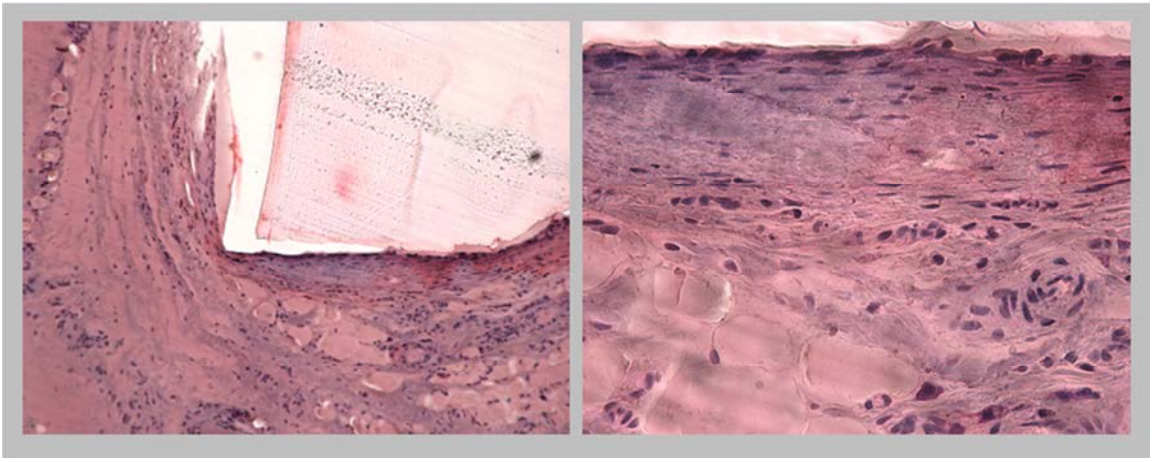

Figure 40. PVP solution 90 days: thin fibrous capsule formation and mild cell inflammatory infiltration (hematoxylin-eosin staining, 10x and 40x).

Anexo C – Normas do periódico *Toxicology Letters*

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INTRODUCTION

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publishes mini-reviews, editorials, commentaries and contemporary issues in toxicology.

Types of paper

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