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(UN)SAFETY OF SILVER NANOPARTICLES TO PROKARYOTIC CELLS AND *IN VITRO* INTERACTION WITH *Bothrops jararacussu* SNAKE VENOM: EFFECTS ON NERVE-MUSCLE SYNAPSES

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Resumo:

Nanoparticle conjugated venom-toxins from venomous animals for possible therapeutic clues against emerging or neglecting diseases is a promising strategy. In this study, silver nanoparticles (AgNPs 50 nm, 0.081 mg mL^{-1}) were studied against the neuromuscular blockade and myotoxic effects induced by *B. jararacussu* venom (60 µg mL^{-1}) and against prokaryotic cells. Neurotoxicity was evaluated on *ex vivo* mouse phrenic nerve-diaphragm using traditional myographic technique, able to obtain functional contractile responses and to check the neurotransmission. Myotoxicity on mammalian cells was carried out in muscles resulting from pharmacological assays using routine histological techniques and light microscopy. Toxicity to prokaryotyic cells was evaluated on *Salmonella typhimurium* TA100 without metabolic activation, a preliminary test which preceding mutagenic test. The *in vitro* preincubation model between AgNPs and venom was enough to abolish toxic effects of *B. jararacussu* venom, but mammalian cells were highly sensitive to AgNPs more than prokaryotic cells, by acting of dose-independently and dose-dependently ways, respectively. These results were discussed and allowed us to conclude that all nanoparticles obtained should be accompanied of toxicity assays to address the appropriate use of nanoparticles, which here is indicated to combat toxic effects of *B. jararacussu* venom and prokaryotic cells, whereas internal administration to mammalian remains to be studied.

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Palavras-chave: Myotoxicity; neurotoxicity; snake venom.

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Introdução:

Compounds such as silver or gold nanoparticles are known to have antibacterial properties (Mathews et al., 2010), which makes it interesting to address against ophidian venoms, since bacteria cohabit the oral cavity of snakes (Jho et al., 2011) and can cause infections in the bite site (Jorge; Ribeiro, 1997).

Bothrops snakes, popularly known as pit vipers, are responsible for most cases of snakebite in Brazil cause clinical manifestations such as edema, ecchymosis, blisters, necrosis, secondary infection with abscesses, functional disorders or even the amputation of the affected member, as local effects (Brasil, 2001). Systemically, disturbances in coagulation and bleeding are common events. Among the *Bothrops* species, *B. jararacussu* is one of the most feared due its size and venom amount inoculated at the bite site (Milani et al., 1997). *Bothrops* venoms commonly cause a neuromuscular blockade *in vitro* by questionable mechanisms, but consensually attributed to their myotoxic components such as Lys49 phospholipase A_2 homologues, by a catalytically-independent way (Gallaci; Cavalcante, 2010).

Bioactive compounds from venoms also have been used as therapeutic agents (Pal et al., 2002). Nano-silver is one of hydrophylic particles studied in the role of drug delivery vehicles by conjugation with several therapeutically potent venoms, toxins, peptides, proteins and antigen (Biswas et al., 2012). However, it is also known that the physicochemical characteristics of silver nanoparticles may greatly alter their toxicological potential (Santos et al., 2014; Guo et al., 2016). Thus, ideally, any type of nanoparticles obtained should be submitted a toxicity assays for checking safety to use therapeutically.

In this study, the pharmacological effects resulting from the interaction between silver nanoparticles and crude venom of *B. jararacussu* at neuromuscular junction were evaluated in a preincubation model through mice phrenic nerve-diaphragm preparations. Muscles exposed to pharmacological experiments were analysed by light microscopy in order to obtain the myotoxicity index as an indicator in mammalian tissue at celular level and preliminary toxicity assay on *Salmonella* strain (TA100) as an indicator of toxicity to prokariotic cells.

Metodologia:

*Silver nanoparticles (50 nm): N*anoparticles of 0.081mg mL^{-1}, zeta potential -20 mV and pH ~ 6.0 were obtained and certified by Santos et al. (2016).

Snake venom: Crude venom from *Bothrops jararacussu* specimens was obtained and certified by Ph.D. José Carlos Cogo, from University Brazil, SP, Brazil.

Animals: Male Swiss white mice (26–32 g) were provided by Animais de Laboratorio (Anilab, Paulinia, Brazil) and housed in a Smaflex® system, at 25 ± 3 °C on a 12 h light/dark cycle, with food (Presence®, Paulinia, SP, Brazil) and water ad libitum.

Mouse phrenic nerve-diaphragm muscle (PND) preparation: The preparation was obtained according to Bülbring (1946) and the myographic recording was performed as described elsewhere (Farrapo et al., 2011). PND could stabilize for at least 20 min before the start of experiments. A concentration-response curve was obtained using 5, 25, 50, 100, 200 and 500 µL of AgNPs 50 nm (0.081 mg mL^{-1}). *B. jararacussu* venom (Bjssu, 60 µg mL^{-1}, n=4) alone or preincubated for 30 min with selected concentration of AgNps 5 µL (n=4) before addition into the bath was compared to the Tyrode control solution (n = 4).

Quantitative histological study: The resulting PND preparations from pharmacological assays were analysed by according to Ferraz et al. (2014). A score of tissue damage parameters was applied by 3 examiners: edema, intense myonecrosis characterized by atrophy of the muscle fibers, hyaline aspect, sarcolemmal disruption and lysis of the myofibrils. The cells damage was expressed as a myotoxicity index (MI), i.e., the percentage of the damaged muscle cells number divided by the total number of cells in three non-overlapping, non-adjacent areas of each preparation**.**

Toxicity assay of AgNPs to prokaryotic cells (Salmonella typhimurium) without metabolic activation: A preincubation method (Maron; Ames, 1983) to evaluate AgNPs (0.081 mg mL^{-1}) was carried out using TA100 *S. typhimurium* strain (kindly provided by B.N. Ames, Berkeley, CA, USA), as a need to know the enough concentration able to kill prokaryotic cells. Experiments were carried out in triplicate. For TA100, the standard mutagen used as positive control in experiments without S9 mix was sodium azide (1.25 µg/plate). Dimethylsulfoxide (DMSO) was used as negative (solvent) control (50 µL/plate). Toxicity was evaluated either as a reduction in the number of His+ revertants or as an alteration in the auxotrophic background (Mortelmans; Zeiger, 2000; Resende et al., 2012; Yoshida et al., 2016).

Statistical analysis: All results of pharmacological and histological analysis were used for statistical comparison of the data using Student's *t*-test and the confidence level was set as 5 % (α = 0.05), whereas for toxicity tests a Salanal statistical software package (U.S. Environmental Protection Agency. The data (revertants/plate) were assessed by analysis of variance (ANOVA), followed by linear regression.

Resultados e Discussão:

Nowadays, nanotechnology is synonymous of breakthrough and has been applied in medicine, cosmetics, electronics, and food industries. Among them, medicinal nanoparticles have been extensively produced, but studies also have been reported toxic effects on living beings. Silver nanoparticles, in detriment to beneficial effects as antimicrobial attributed to Ag^{+}, can cause neurotoxicity (Xu et al., 2015); anomalies in zebrafish embryos exposed to nanoparticles, influence on acetylcholinesterase activity and in immune system differentiation (Myrzakhanova et al., 2013); hepatotoxicity and oxidative stress in rats (Patlolla et al., 2015).

Figure 1A shows the pharmacological effects of nanoparticles (0.081 mg mL^{-1}) added into the bath showed be a volume-independent event (5, 25, 50, 100, 200, and 500 µL). Figure 1B shows that an amount as small as 5 µL was able to cause a reduction of more than 20 % on muscular response (*, $p<0.05$ compared to Tyrode control, dotted line) which, in turn, did not discriminate statistically of volumes until 200 µL ($p>0.05$). An amount of 500 µL AgNPs did discriminate from 5 µL. The minor volume was selected for further neutralization assays since induced homogeneous responses.

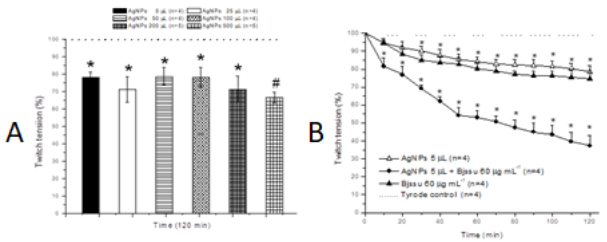

Figure 1. Mouse phrenic nerve-diaphragm preparation (indirect stimuli). (**A**): Volume-response of AgNPs (0.081 mg mL^{-1}), at 120 min. Note that the minor volume (5 µL) did not discriminate from 25, 50, 100 and 200 µL AgNPs, but did discriminate with a volume 100X bigger than 5 µL. (**B**): Neutralizing assays (*B. jararacussu* venom (Bjssu, 60 µg mL^{-1}) precincubated with the selected volume of AgNPs (5 µL of 0.081 mg mL^{-1} solution). The number of experiments (n) is shown in the legend of figure. * $p<0.05$.

Our results were obtained in an *ex vivo* condition with aerated mean and all machinery of contractile responses working, in a robust experimental model (Oshima-Franco et al., 2004). It is known that *B. jararacussu* venom (Bjssu) causes an *in vitro* irreversible neuromuscular blockade. At the end of 120 min around 37.2 ± 5.9 (n=4) of muscle fibers were working face to this venom's batch, whereas the *in vitro* preincubation for 30 min with the minor volume of AgNPs (before addition into the bath) neutralized completely the venom effect. Notice that the set of neutralizing experiments reproduced the same effect of nanoparticles alone with no statistically difference between them ($p>0.05$). The influence of silver nanoparticles on neurotransmission could occur either on phrenic nerve as on diaphragm's muscle fiber. Silver nanoparticles can enter the central nervous system

(Panyala et al., 2008) and induce brain edema and neurotoxicity (Cramer et al., 2014). But, in our experimental model the nerve conduction block induced by silver nanoparticles could induce dysfunction and/or disruption at nodes of Ranvier (Suzuki, 2013) by nodal ionic imbalance due to the bi-directional, nonspecific ion and water pores formed by the insertion of MAC (membrane attack complex) into the nodal axolemma (McGonigal et al., 2010), a subject that needs to be explored.

Edema is a common event seen in bothropic envenoming, and also observed in *ex vivo* preparations by light microscopy.The resulting muscles from pharmacological assays were submitted to light microscopy and analysed under a standardized cell damage score (Ferraz et al., 2014) (Figure 2), by three examiners who quantified the damage caused to muscle cells (myotoxicity index ± S.E.M): Tyrode control (n=4), 8.0 ± 5.0; AgNPs 5 µL (n=4), 56.2 ± 3.8 (*); *B. jararacussu* venom (60 µg mL^{-1} (n=4), 49.6 ± 3.6 (*); Preincubation (n=4), 45.2 ± 4.3 (*), where *, p<0.05 compared to control. Notice that there was no statistically difference among them, except when experimental groups are compared to Tyrode control. Our results show that nanoparticles did damage cells quantitatively either as *B. jarararacussu* venom did alone, but qualitatively with predominant myofibers condensation. If the preincubation for 30 min neutralized the effect of neuromuscular blockade-induced by Bjssu (Figure 2), thus the analysed muscles resulting from neutralizing assays express only the myotoxic effects of nanoparticles. Reactive oxygen species have been proposed as a mechanism to explain toxicity of nanomaterials, including silver nanoparticles, leading to cellular injury and death, by an oxidative catabolism of polyunsaturated fatty acids named lipid hydroperoxidation (Patlolla et al., 2015). Independent of mechanisms at cellular level by which silver nanoparticles act myotoxicity is a real event as shown in this study.

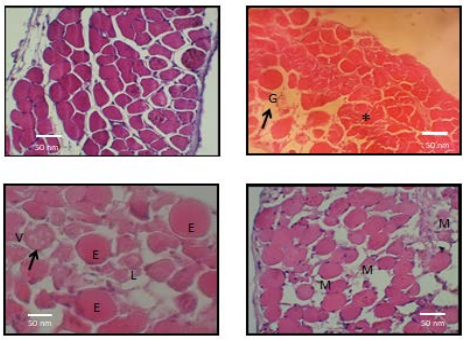

Figure 2. Mammalian diaphragm muscle's cells (H.E. X40, bars=50 nm), transversal sections. Score of applied parameters to damaged cells (Ferraz et al., 2014): edema (E), myofibers condensation (*), vacuoles (V), sarcolemmal disruption or lysis (L), *ghost* cells (G), and extensive myonecrotic areas (M) are shown in comparison to normal appearance of polygonal cells and peripheral nuclei.

Face to extensive myonecrosis-induced by nanoparticles on mammalian cells, and to its ability to neutralize the venom bioactive compounds responsible by causing paralysis in an *ex vivo* system (PND preparation), we focused the prokaryotic cells using a *S. typhimurium* TA100 strain, in a preliminary toxicity assay. Toxicity is characterized by a notable reduction in the background lawn and/or a greater than 50% reduction in the mean number of revertant colonies when compared to the vehicle control. A statistically significant (p<0.05) in mean number of revertant colonies may be used to define toxicity (Concawe, 2012). Table 1 clearly shows that 100 µL containing 0.0081 mg mL^{-1} of AgNPs/plate was enough to abolish the bacteria survival (*p<0.05), showing that to carry out further Ames *Salmonella*/microsome mutagenicity assay, minor volumes than 100 µL will be necessary to establish the highest non-toxic dose, which could be 50 µL for example.

***Table 1 – Revertants/plate, standard deviation and toxicity for TA100 S. typhimurium* after treatment of various doses of AgNPs (0.081 mg mL^{-1}, in triplicate) without metabolic activation (-S9)**

	1	2	3	M ± SD
Control +	1988	1984	1986	1986 ± 2.0
Control -	160	144	146	150 ± 8.7
25 µL AgNPs	153	137	146	145.3 ± 8.0
50 µL AgNPs	112	120	127	119.6 ± 7.5
100 µL AgNPs	0	14	0	4.6 ± 8.0 (*)

1, 2 and 3 = each triplicate. M ± SD = mean and standard deviation. Control+ = Positive control: sodium azide (1.25 µg/ plate). Control - = Negative control: dimethylsulfoxide (DMSO - 50 µL/ plate). *, p<0.05.

As reported in Introduction section, the Ames test results of silver nanoparticles made by Guo et al. (2016) were inconclusive. In our study, AgNPs 50 nm (0.081 mg mL^{-1}) showed be toxic to *S. typhimurium* TA100 strain at a volume of 100 µL, but not at minor volumes as 50 µL. These are promising results to carry out the Ames test in a continuous study, which can establish the safety level of AgNPs, and also to change parameters of getting appropriate silver nanoparticles to medicinal use. For now, this study contributes in the following aspects of AgNps 50 nm (0.081 mg mL^{-1}): 1) it is able to abolish the neuromuscular blockade-induced and myotoxicity-induced by *B. jararacussu* (60 µg mL^{-1}) and it implies other uses to nanoparticles; 2) mammalian cells showed be sensitive to myotoxicity of silver nanoparticles of a dose-independent way, what in turn, limit use; 3) silver nanoparticles showed be toxic to prokaryotic cells (TA100) of a dose-dependent way, which also implies other uses to them.

Conclusões:

The silver nanoparticles assayed in this study with size (50 nm) and concentration (0.081 mg mL^{-1}) characteristics is useful to combat toxic effects of *B. jararacussu* venom, it damages mammalian cells in a dose-independent way, but prokaryotic cells in a dose-dependent way. These results taken together show that an appropriate use of obtained nanoparticles must be thought, but *in vitro*, *ex vivo* and *in vivo* assays are essential to indicate them.

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