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In Vivo Antitumor Activity of Metal Silver and Silver Nanoparticles in the L5178Y-R Murine Lymphoma Model

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Authors' contributions

This work was carried out in collaboration between all authors. Author RGF designed the study, was the thesis advisor of author JHLG and wrote the final version of the manuscript. Author JHLG performed the experiments and statistical analysis for his Master of Sciences thesis and wrote the first draft of the manuscript. Author PTG managed the analysis of the study. Author EMC supported the study as a technician and data managing. Authors RTG and CRP managed the analysis of the study. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: To evaluate the antitumor potential of metal silver and polyvinilpyrrolidone nanoparticle-encapsulated silver on L5178Y-R murine lymphoma cell growth and survival of tumor-bearing mice.
Study Design: *In vitro* and *in vivo* (pre-clinical) study.
Place and Duration of Study: Universidad Autónoma de Nuevo León, Facultad de Ciencias Biológicas, Departamento de Microbiología e Inmunología, San Nicolás de los Garza, N.L., México, from January 2009 to December 2011.
Methodology: Concentration-response cell viability assay was performed *in vitro* and mice survival studies were done using a L5178Y-R tumor-bearing mouse model. The PROBIT regression analysis was performed to determine the *in vitro* LC_{50}. *In vivo* survival distributions were calculated by Kaplan-Meier and Cutler-Ederer analysis, and

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survival curves comparisons and hypothesis testing was done using the log-rank method.
Results: Metal silver induced up to 100% L5178Y-R cells cytotoxicity, with an LC_{50} of 1.8 X 10^{-8} M, whereas silver nanoparticles caused up to 78% cytotoxicity, with an LC_{50} of 14.4 X 10^{-8} M. In addition, Intramuscular administration of metal silver and silver nanoparticles administered at the time of tumor injection significantly (*P* = .05) increased mice survival, where 70% and 60% of mice survived at day 35 respectively, as compared with such treatments administered 7 days after tumor induction (55% and 25% survival respectively); vincristine treatment caused 50% mice survival and tumor-bearing control mice had 20% survival. These results open further approaches on treating several types of cancer using free and nanoparticle-encapsulated silver-based therapies.

Keywords: Silver; silver nanoparticles; lymphoma; antitumor activity; preclinical; mouse.

1. INTRODUCTION

Cancer is one of the major death causes. In 2008, it was responsible for 7.6 millions of deaths worldwide, particularly in the economically developing countries, and it continuously increases because of the aging and growth of the world population and cancer-causing behaviors [1]. The T-cell acute lymphoblastic leukemia(T-ALL) presents several drawbacks related to currently used therapies, for example, increased risk to septicemias and fungal infections [2], the unlikelihood of reaching disease-free remission [3], the high risk of severe and continuous neutropenias after subsequent rounds of chemotherapy [4], and the inability to sometimes achieve a successful stem-cell transplantation along with the question whether all patients with proper blood-related donor should receive an allogenic transplant or whether this should be reserved only for high-risk patients [5]. All together, it is essential to search for new chemotherapeutic approaches to treat T-ALL, as well as other types of cancer. Silver colloids and silver nanoparticles have previously demonstrated bactericidal, bacteriostatic [6] and antiviral activity [7]. In addition, *in vitro* evaluations have confirmed cytotoxic activity against some tumor cell lines [7]. However, no investigations have been conducted to demonstrate their effectiveness to increase tumor-bearing mice survival. The aim of the present study was to evaluate the antitumoral activity of polyvinylpyrrolidone (PVP)-coated silver nanoparticles and metal silver against the murine lymphoma cell line L5178Y-R *in vitro*, as well as to evaluate their effectiveness in a tumor-bearing mouse model. It was observed that Metal silver and silver nanoparticles induced up to 100% and 78% L5178Y-R cells *in vitro* cytotoxicity, respectively; in addition, *in vivo* treatment of tumor-bearing mice with metal silver and silver nanoparticles administered at the time of tumor injection significantly increased mice survival, as compared with controls.

2. MATERIALS AND METHODS

2.1 Reagents and Culture Medium

L-glutamine and penicillin-streptomycin solutions were obtained from Life Technologies (Grand Island, NY). RPMI 1640 medium, fetal bovine serum (FBS), sodium dodecyl sulfate (SDS), N, N-dimethylformamide (DMF), phosphate-buffered saline (PBS), and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). Vincristine was obtained from Vintec (Columbia, S.A. de C.V., Mexico, DF). The extraction buffer was prepared by dissolving 20% (wt/vol) SDS at 37º C in

a solution of 50% each DMF and demineralized water, and the pH was adjusted to 4.7. Silver nanoparticles were purchased from Nanostructured & Amorphous Materials, Inc. (Houston, TX). These were spherical 0.2 wt% polyvinylpyrrolidone-face coated, 30-50 nm average particle size nanoparticles, containing silver powder (99.9% Ag), with a specific surface area of 5-10 m^2/g and cubic cystallographic structure, according to supplier **(**Nanostructured & Amorphous Materials, Inc.); 65-75% Ag purity and 10.49 g/cm^3(lit.) density colloidal silver was obtained from Sigma-Aldrich (catalog # 85131). Silver powder and pvp-silver nanoparticles were dissolved or suspended, respectively, at desired concentrations, in complete RPMI 1640 medium for *in vitro* cytotoxicity assays, or in saline solution for intramuscular administration.

2.2 Animals

Six-week old female BALB/c mice (22-28g) were purchased from Harlan Sprague-Dawley Inc. (Indianapolis, IN). They were kept in a pathogen- and stress-free environment at 24ºC, under a light-dark cycle (light phase, 06:00-18:00 h), and given water and food *ad libitum*.

2.3 Tumor Cell Line

The tumor cell line L5178Y-R (mouse DBA/2 lymphoma) was purchased from The American Type Culture Collection. (Rockville, MD), and was maintained in culture flasks with RPMI 1640 medium supplemented with 10% FBS, 1% L-glutamine, and 0.5% penicillin-streptomycin solution (referred as complete RPMI 1640 medium) at 37ºC, in a humidified atmosphere of 5% CO_2 in air. Cellular density was kept between 10^5 and 10^6 cells/ml.

2.4 Cell Preparation and Culture

In order to determine the direct *in vitro* effect of metal silver and silver nanoparticles on L5178Y-R tumor cell, lymphoma cell cultures were collected and the cellular suspensions were washed three times in RPMI 1640, and re-suspended and adjusted to 5 x 10^4 cells/ml with complete RPMI medium.

2.5 Effect of Metal Silver or Silver Nanoparticles on L5178Y-R Lymphoma Cell Cytotoxicity

One hundred microliters of L5178Y-R cell suspensions were added to flat-bottomed 96-well plates (Becton Dickinson, Cockeysville, MD), containing triplicate cultures (100 μl) of complete RPMI (unstimulated control), metal silver or silver nanoparticles at various concentrations. After incubation for 44 h at 37ºC with 5% CO_2, MTT (0.5 mg/ml, final concentration) was added, and cultures were additionally incubated for 4 h. Next, cell cultures were incubated for 16 h with extraction buffer (100 μl) and optical densities, resulting from dissolved formazan crystals, were then read in a microplate reader (DTX 880 Multimode detector, Becton Dickinson, Austria) at 570 nm [8]. The percentage of lymphoma cytotoxicity was calculated as follows:

$$\% \text{ cytotoxicity} = 100 - \frac{A_{570} \text{ in metal silver- or silver nanoparticles-treated cells}}{A_{570} \text{ in untreated cells}} \times 100$$

Considering 65-75% Ag purity in metal silver solution and 99.9% Ag purity in nanoparticles suspension, treatments were performed at equimolar concentrations and results expressed in % cytotoxicity at concentrations ranging from 0.9 to 57.9 X 10^{-8} M. For this, 116 X 10^{-8} M stock silver solutions (metal) and suspensions (nanoparticles) were prepared by using 357μg/ml and 250 μg/ml, respectively, and 1:2 serial dilutions were made to provide for the assay treatment concentrations.

2.6 *In vivo* Tumor-bearing Mice Survival by Metal Silver and Silver Nanoparticles

We used the L5178Y-R lymphoma mouse model to test metal silver and silver nanoparticles antitumor activity. The lymphoma was maintained and administered to Balb/c mice as previously reported [8]. Every experiment consisted of 9 groups of 5 mice; L5178Y-R cells ($5x10^6$ cells/mL) suspended in 0.2 mL of PBS were intramuscularly (i.m.) injected in the right thigh on 6 groups. Treatment groups consisted of i.m. administration of metal silver or silver nanoparticles (20 mg/kg) at day 0 (at the time of tumor injection), i.m. administration of metal silver or silver nanoparticles (20 mg/kg) at day 7 of tumor injection; other groups were untreated tumor-bearing mice, i.m. vincristine (0.55 mg/kg) treated tumor-bearing mice (every third day; positive control), i.m. administration of metal silver or silver nanoparticles (20 mg/kg) alone (toxicity control) and untreated mice (negative control). Treatments were applied every second day (except for vincristine) to complete 30 days. Overall survival was registered during the 35 day-study.

2.7 Statistical Analysis

The results were expressed as mean ± SEM of the response of 3 replicate determinations per treatment (*in vitro* study) or 5 mice per experimental group (9 groups as explained above) from three independent experiments. Level of significance was assessed by Dunnet's *t* test; to calculate the LC_{50} *in vitro*, a PROBIT regression model was used. Survival curves were calculated by the Kaplan-Meier and the Cutler-Ederer methods, and the statistical significance of differences in the survival curves between the groups was evaluated by log-rank test.

3. RESULTS AND DISCUSSION

3.1 *In vitro* Cytotoxicity Tests

Metal silver induced from 44% to 100% significant ($P<.01$) L5178Y-R cells cytotoxicity, at testing molar concentrations ranging from 0.9 to 57.9 X 10^{-8} M respectively, with an LC_{50} of 1.8 X 10^{-8} M, whereas silver nanoparticles caused from 11% to 78% significant ($P\leq.05$) cytotoxicity at the same concentration range respectively, with an LC_{50} of 14.4 X 10^{-8} M (Fig. 1). Metal silver cytotoxic effect was significantly ($P < 0.01$) higher than that of silver nanoparticles. Metal silver was not toxic for normal murine thymic lymphocytes at concentrations as high as 28.9 X 10^{-8} M, but was observed to stimulate Con A-treated lymphoproliferation, whereas silver nanoparticles induced thymus lymphocyte proliferation up to 7.2 X 10^{-8} M, but caused toxicity from 14.4 X 10^{-8} M (data not shown). It is known that pvp-coated silver nanoparticles are targeted to several body tissues, in particular, towards tumor cells [9,10].

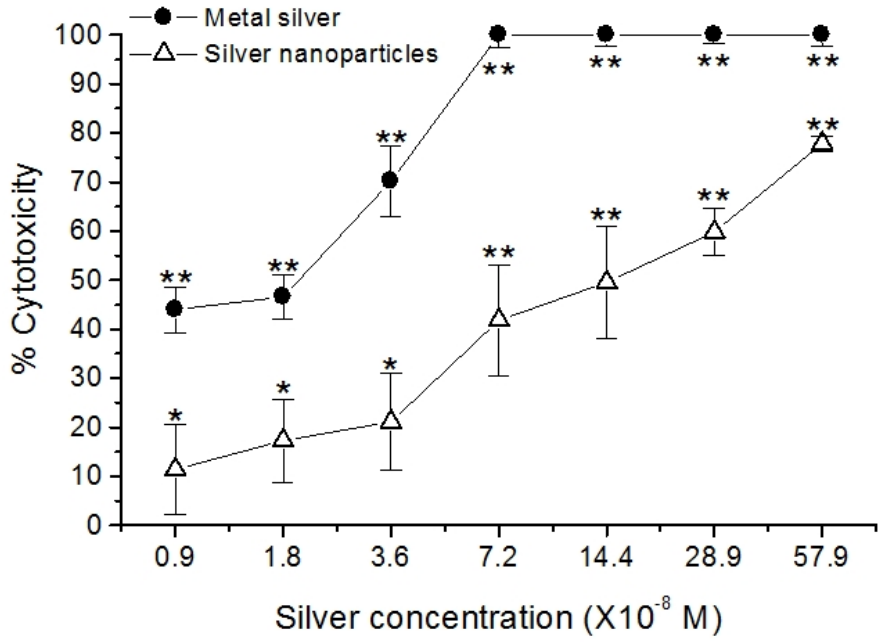

Fig. 1. Cytotoxicity induced by metal silver and silver nanoparticles on L5178Y lymphoma cells. L5178Y-R lymphoma cells ($5x10^4$ cells/ml) were incubated in the presence or absence of metal silver and silver nanoparticles at concentrations ranging from 0.9 to 57.9 X 10^{-8} M for 44 h, after which viability was measured using MTT, as explained in the text. Optical densities were read in a microplate reader at 570 nm. Optical density for untreated control was 0.65 ± 0.09. Data represent mean ± standard error of triplicates from three independent experiments. *$P < 0.05$; **$P < .01$, as compared with untreated control

The cytotoxic activity of silver particulate nano-compounds has been investigated on animal cell lines being the inhibitory concentration 20 (IC_{20}) 16.7 X 10^{-6} M for L5178Y-R cells [10,11]; it was furthermore demonstrated that nano-compounds can induce primary DNA damage in animal cell cultures, notwithstanding being incapable to induce mutagenicity [10]. In our study, we showed that metal or silver nanoparticles caused necrosis, rather than apoptosis, on L5178Y-R cells (data not shown) and the highest concentration tested was 57.9 X 10^{-8} M (Fig. 1), which may indicate a more potent activity as compared with other reports [10], or it may be due that we did not filter the silver treatments prior to use, which other authors commonly do [10], because we observed significant silver retention on the filter.

It has been reported that silver nanoparticle delivery to mammal cells by the nano-carrier chitosan induces apoptosis at very low concentrations (on HT-29 human colon cancer cells), the LC_{50} was determined as 0.3 X 10^{-8} M, and it was proposed that this concentration was more effective than previously reported data [12]. However, our results indicated LC_{50} of 1.8 X 10^{-8} M and 14.4 X 10^{-8} M by metal silver and pvp-coated silver nanoparticles respectively, as compared with that of chitosan-carried nanoparticles, showing differential activities of silver nanoparticles depending on their composition and on the tumor cell type, but both preparations were significantly toxic for tumor cells. It has also been indicated that the biological activity of silver nanoparticles is mediated mainly by silver which are present at the

initial phase of dispersion or that they are eventually released by the nanoparticles throughout time [13]. In the present study, we observed that metal silver caused 8-fold lower LC50 than silver nanoparticles, which may be due to the availability of the silver to tumor cells, which may be reduced after encapsulation. We also observed that both treatments caused necrosis on L5178Y-R lymphoma cells (data not shown), which agrees with a report related to the use of 7- to 20-nm silver nanoparticles (12 X 10^{-8} M) inducing cell death by necrosis on HT-1080 human fibrosarcoma cells and A431 skin carcinoma cells, and apoptosis at about 0.9 X 10^{-8} M [14].

The cytotoxic response and the type of cell death induced by silver nanoparticles on several cell lines and several types of *in vitro* tissues has been previously investigated; reported data have shown that the nanoparticle size may play a role on inducing toxicity, being 15-nm particles more toxic than 30 nm and 55 nm-nanoparticles, as reported for macrophages, where nanoparticles caused apoptosis [15]. It was also demonstrated that the type of cell death induced on A549 lung cancer cells by silver nanoparticles relies mainly on a dose-dependent fashion (2.3-14 X 10^{-8} M), that induces necrosis/late apoptosis in most cells, whereas increasing the dose induces early apoptosis in a small proportion of A540 cells [16]. We have further confirmed the phenomenon of necrosis observed in our study, by not detecting caspase family proteases after *in vitro* cell death induction (data not shown).

3.2 Effect of Metal Silver and Silver Nanoparticles on *In vivo* Tumor-bearing Mice Survival

Intramuscular administration of metal silver and silver nanoparticles administered at day 0 (at the time of tumor injection) significantly (P =.05) increased the survival of mice, as compared with such treatments or vincristine applied 7 days post tumor injection and untreated control (with tumor). In regard with metal silver and silver nanoparticles administered at day 0, 70% and 60% of mice survived at day 35 respectively, as compared with such treatments applied 7 days post tumor injection where 55% and 25% of mice survived respectively. Vincristine treatment caused 50% mice survival and tumor-bearing control mice had 20% survival after 35 days (Table 1); metal silver and silver nanoparticle treatments alone, and untreated control (without tumor) did not affect mice survival (Table 1).

Table 1. Effect of metal silver and silver nanoparticles on L5178Y-R tumor-bearing mice survival (%)

Treatment	Days						
	5	10	15	20	25	30	35
Vincristine	100	100	100	100	80	53	47
Untreated tumor-bearing	100	100	100	100	80	50	20
Metal silver (7 days post-tumor)	100	100	100	66	58	51	51
Metal silver (at tumor injection day)	93	93	93	93	93	73	66
Silver nanoparticles (7 days post-tumor)	100	100	100	80	60	40	26
Silver nanoparticles (at tumor injection day)	93	86	86	86	80	66	51
Metal silver alone	100	100	100	100	100	100	100
Nanoparticles alone	100	100	100	100	100	100	100
Tumorless non-treated control	100	100	100	100	100	100	100

In vivo studies are scarce, but they have shown antitumor or angiogenic potential in a variety of tumor types, different from our murine L5178Y-R lymphoma model. One of such studies

reported that aqueous dispersions of silver oxide nanoparticles, inhibited tumor growth *in vivo* on a Pliss lymphosarcoma rat model [17]. In addition, Liu et al. [18] demonstrated that a nanopharmaceutical system using TAT-enhanced cell/tissue penetration strategy, developed for multidrug-resistant cancer treatment, possessed strong antitumor activity, particularly using a melanoma murine model. In contrast, Kang et al. [19] showed that PVP-coated silver nanoparticles (average size 2.3nm) caused *in vivo* angiogenesis in B16F10 melanomas in mice.

Previous reports have demonstrated that heparin-conjugated silver nanoparticles possess angiogenesis inhibitory properties which are mediated by the basic fibroblast growth factor [20], and it was also demonstrated that silver nanoparticles of 50 nm in diameter can act as a potent antiangiogenic factor in two *in vivo* models (rat and mouse models), and that this effect induced by the vascular endothelial growth factor is mediated by inhibition of the cell survival signal PI3K/Akt, in a similar fashion as that mediated by the pigment epithelium-derived factor in bovine retinal endothelial cells [21]. However, another study suggested that exposure to pvp-coated silver nanoparticles of 2.3 nm in size can induce angiogenesis in an *in vivo* model and on the SVEC4-10 cell line *in vitro* [19]. In our study, we used pvp-coated silver nanoparticles of a diameter of 30-50 nm, being similar to those used by Gurunathan et al. [21], in addition to metal silver solution. Both treatments, particularly the ones administered at tumor injection day, significantly increased the survival distributions, in comparison with the non-treated control in a murine lymphoma model, which may be mediated by their potential antiangiogenic effect, in combination with the direct cytotoxic response observed *in vitro* (Fig. 1 and Table 1) [21].

Using this study as a base, we also demonstrated for the first time that polyvinylpyrrolidone-coated silver nanoparticles and silver ions effectively increase the survival distributions in a tumor-bearing mouse model *in vivo*.

4. CONCLUSION

Pvp-coated silver nanoparticles and silver ions showed *in vitro* cytotoxicity on the L5178Y-R lymphoma cell line. In addition, we have shown the effectiveness of two silver colloid-based therapies to significantly increase the survival response on a solid tumor-bearing mouse model, particularly, when administered at day of tumor injection, which might be useful for treating solid tumors on initial stages of development, or during therapy to prevent relapses. We also demonstrated that the treatments induced a better survival response in comparison with vincristine administered on day 7 post tumor induction, which is a chemotherapeutic drug commonly used to treat acute leukemia.

CONSENT

Not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication no. 85-23, revised 1985) were followed, as well as specific national laws. All experiments have been examined and approved by the Institutional Ethics Committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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