Cytotoxicity and Antitumor Activity of Biogenic Silver Nanoparticles Against Non-Muscle Invasive Bladder Cancer

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Abstract. Bladder cancer is the fifth most common form of malignancy in the United States, and for most of the last three decades, the treatment and outcomes for patients with this disease have not changed. Nanomedicine aims to provide the means to target chemotherapies directly and selectively to cancerous cells and enhance their therapeutic efficacy. In this scenario, we employed biogenic Silver Nanoparticles (AgNPs) as an anticancer agent against non-muscle invasive bladder cancer (NMIBC). Bladder cancer was chemically induced with N-methyl-N-nitrosourea (MNU) on C57BL/6junib female mice and treated by intravesical route with biogenic silver nanoparticles concentrations of 0.5, 0.2, and 0.05 mg/mL. The histopathological analyzes showed the treated with AgNP 0.5 group presented 42.85% of pTa, 28.57% of pTis and 28.57% of pT1, indicating that this treatment was not effective in regressing the neoplastic lesions. MNU + AgNP 0.2 group showed 28.57% of tumor regression, being these animals showed flat hyperplasia (28.57%). Finally, treatment with 0.05 AgNP led to 57.13% of tumor regression, with 14.28% of the animals showing normal urothelium and 42.85% showing flat hyperplasia, considering a benign lesion. Further, to understand the antitumor effect of AgNPs, we evaluated the molecular mechanism of cytotoxicity in human bladder carcinoma 5637 cell. The results showed the dose-time dependent cytotoxicity, and detailed analysis demonstrated the induction of cell death via apoptosis. Besides, we found that AgNP inhibition in cell migration and proliferation. Thus, these findings confirm the antitumor properties of AgNPs and suggest that they may be a cost-effective alternative and promising candidate for the treatment of bladder cancer.

Keywords: Biogenic silver nanoparticle, Bladder cancer, Nanotechnology, Toxicology.

1. Introduction

Nanotechnology has been presented as a very innovative area in the improvement of industrialized products and especially in the development of more effective drugs with less toxic effects. Because it is a new perspective in the search for therapeutic alternatives for some diseases whose cure is still unknown, the interaction between nanomaterials and biological systems has aroused an increasing interest in science. In this scenario, bladder cancer (BC) is a potentially lethal disease requiring aggressive treatment, and if left untreated, less than 15% of patients survive within two years [1, 2]. In addition, few pharmacological and therapeutic alternatives are effective in their more advanced stages.

Bladder cancer (BC) in the USA is the fourth most incidence tumor in men and the ninth in women, showing high morbidity and mortality rates. The European Association of Urology considers BC as the eleventh most common cancer diagnosed worldwide [1]. American Cancer Society reported on 2017 [2] that 79,030 new cases of BC (60,490 men and 18,540 women) and with 16,879 deaths.
More than 70% of BC is superficial (non-muscle invasive bladder cancer): pTis (flat carcinoma in situ) stage, pTa (papillary carcinoma non-invasive) stage and pT1 (tumor invading mucosa or submucosa of the bladder wall) stage. Unfortunately, despite the prognosis associated with NMIBC tumors, almost 50% of patients will experience a recurrence of their disease within 4 years of their initial diagnosis, and 11% will progress to muscle-invasive disease (MIBC). It is known that the primary treatment for high-grade NMIBC is based on surgery by transurethral resection of bladder tumor (TURBT), followed by intravesical immunotherapy with Bacillus Calmette–Guerin (BCG)[3].

This study demonstrates the use of silver biosynthetic nanoparticles in the form of the aqueous dispersion for intravesical instillation in the treatment of non-muscle invasive bladder cancer (NMIBC). These nanoparticles are obtained by reducing the silver by enzymes and substrates present in solution obtained after the filtration of the biomass of the Fusarium oxysporum fungus.

Biogenic Silver Nanoparticles (AgNPs) anticancer property has been analysed in vitro against various types of cancer cells – human hepatoma cells [4], lung cancer [5], breast cancer [6] and cervical carcinoma [7]. They impart toxicity to cancer cells by decreasing mitochondrial function, reactive oxygen species (ROS) production, releasing lactate dehydrogenase (LDH), cell cycle deregulation, induction of apoptotic genes like Bax, formation of micronuclei, chromosome aberration and DNA damage [8].

Thus, this study evaluated the efficacy and toxicity of biogenic Silver Nanoparticles (AgNPs) in the treatment of NMIBC chemically induced in rodents.

2. Materials and methods

2.1. Cell Culture

Human urinary bladder grade II (5637, BCRJ code: 0026) were obtained from Cell Bank of Rio de Janeiro (BCRJ, Brazil) and cultured as a monolayer in RPMI-1640 medium modified to contain 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, fetal bovine serum to a final concentration of 10% and 1% penicillin and streptomycin. The cell line was cultured in a humidified incubator with 5% CO2 at 37 °C. All experiments were conducted in 1-10 cell passage number. To analyze the viability and oxidative stress cells were seeded 1.0 x 10^4 cells per well in 96-well plates (Corning Inc., USA) in 200 µL RMPI medium.

2.2. MTT assay

To determine the viability 5637 cells were treated with increasing concentrations of AgNP (1-50 µM) diluted in serum-free medium h at 37 °C. After 24 h, the medium was replaced by 100 µL of 0.5 mg/mL (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium (MTT) solution (Sigma-Aldrich, USA) diluted in serum-free RMPI medium. After 2 hours of incubation at 37 °C, the medium replaced by 100 µL of DMSO to dissolve the formazan crystals. Finally, the plates were shaken for 10 minutes, and the absorbance was determined by the microplate reader Cytation 5 (Biotek Instruments, USA) at λ = 570 nm.

2.3. Live and Dead cell assay

5637 cells were treated with increasing concentrations of AgNP (1-50 µM) diluted in a serum-free RMPI medium at 37 °C. After 24 h, the medium was replaced by solution Live and Dead in Fluorobrite RMPI (Thermo Fisher Scientific, USA) with 1 µM of Calcein-AM (Thermo Fisher Scientific, USA), 1 µM of Hoechst 33342 (Sigma-Aldrich, USA) and 1 µM of Propidium Iodide (PI) (Abcam, UK). After 30 minutes of incubation at 37 °C, images were acquired in GFP, DAPI and PI filters using the Cytation™ 5 Cell Imaging Multi-Mode Reader (BioTek Instruments, Winooaki, VT). Gen5 software (Biotek, Winooaki, VT, USA) was used to count the live and dead cells. Next, Calcein viability was analyzed by fluorescence measure (~492/517 nm). Cell viability was normalized to untreated control.
2.4. NMIBC Induction Protocol and Treatment

Thirty-five female mice of the C57BL/6 lineage obtained from the Multidisciplinary Center for Biological Investigation (CEMIB) at the University of Campinas (CEMIB/ UNICAMP) were used. The Ethics Committee for Animal Experimentation (CEUA)/ UNICAMP approved the animal procedures (protocol number 4017-1). For induction of CBNMI, 28 animals were anesthetized with 2% Xylazine Hydrochloride (5mg/ Kg iv; König, São Paulo, Brazil) and 10% Ketamine Hydrochloride (60mg / kg, Fort Dodge, Iowa) and maintained in this state for 45 minutes to prevent spontaneous urination and instillation a dose of 1.5 mg / kg of N-methyl-N-nitrosourea (MNU-Sigma, St. Louis, MO, USA) dissolved in 0.1 mL of citrate (1M pH 6.0) every 15 days (weeks 0, 2, 4), totaling 3 doses [9]. The other 7 animals that did not receive MNU were considered as Control Group. Subsequently, the animals were divided into 4 groups (7 animals per group): MNU group (Cancer): received an intravesical dose of 0.1 mL of 0.9% physiological solution for 3 consecutive weeks; MNU + AgNP 0.5 group: received an intravesical dose of 0.5 mg / mL AgNP for 3 consecutive weeks; MNU + AgNP 0.2 group: received an intravesical dose of 0.2 mg / mL AgNP (10 µg/g body weight) for 3 consecutive weeks; and Group MNU + AgNP 0.05: received an intravesical dose of 0.05 mg / mL AgNP (2 µg/g body weight) for 3 consecutive weeks. Intravesical doses in the different experimental groups were instilled via a flexible 20-gauge catheter (Abocath, São Paulo, Brazil). Animals from all experimental groups received water and the same solid diet ad libitum (Nuvilab, Colombo, PR, Brazil). After the treatment period, the animals were euthanized, and the urinary bladders were collected and submitted to histopathological analyzes.

2.5. NMIBC: Histopathological Analysis

Samples of urinary bladders (n = 5 per group) were processed as previously described [4]. Subsequently, 5-µm thick sections were cut on a rotary microtome (Slee CUT5062 RM 2165; Slee Mainz, Mainz, Germany), stained with hematoxylin -eosin and photographed with a Leica DM2500 photomicroscope (Leica, Munich, Germany). A senior uropathologist analyzed the urinary bladder lesions based on the criteria of the Health/World International Society of Urological Pathology Organization.

2.6. Statistical Analysis

Histopathological results were compared with a proportion test. The difference between the two proportions was tested using test of proportion with a type-I error of 1%.

3. Results and discussion

3.1. Bladder Cell Carcinoma Viability

To determine the antitumor capacity of biogenic AgNP, the half-maximal inhibitory concentration (IC50) was performed in the bladder carcinoma cell line 5637. For this assay, we used two approaches (MTT assay and calcein/PI assay) these methods are based on different chemical principles, which increases the robustness of the results and help to avoid artifacts [10-12]. Figure 1-A shows that all techniques reported comparable dose–response relationship and similar IC50 values: 10.57 µM for MTT, 9.79 µM for calcein, and 13.72 µM for PI. Next, we assessed the cytotoxicity of biogenic AgNPs on time (Figure 1-B). The results showed that after 6h of treatment the cytotoxicity profile is similar to that observed after 24h of treatment (24h vs 6h *p<0.05). In shorter times (1h and 3h), no significant reduction in viability was obtained when compared to the 24h treatment group (*p<0.05). Therefore, the viability assay has demonstrated that AgNP cytotoxicity is dose and time dependent below conditions evaluated. In addition, the influence of AgNP treatment on cell morphology is illustrated in Figure 1-C. The representative images of Calcein/PI technique show control cells are mostly attached and depicted a cuboidal epithelial-like morphology, upon AgNP treatment; cells are detached and accompanied by morphological changes (cell shrinkage and rounding). We also observed that calcein-negative cells, indicating lost cellular viability, and PI-positive cells, suggesting cell death. A low concentration of AgNPs causes DNA damage and chromosomal aberrations without significant toxicity [8, 13]. Lima et al. observed no genotoxicity effects for different human culture cells treated with up to 10 mg/mL of capped AgNPs with average sizes of 6–80 nm. AgNPs are known to interact with cells and regulate various cellular responses in both passive and active manners [14].
Figure 1. AgNP cytotoxicity in urinary bladder carcinoma 5636 cell. (A) Cells were treated with increasing concentrations of AgNP (1-50 µM) for 24 h. Each value represents the mean ± S.D. of three independent experiments (n = 3), cell viability was normalized to untreated control). Cell viability was measured by MTT formazan absorbance (ex. 570 nm) and Calcein fluorescence (~492/517nm). For cytotoxicity evaluation, counts of PI-positive nuclei were used in relation to 100% labeled nuclei with Hoechst 33342. (B) Cytotoxicity of the AgNP evaluated in time variation. Cells were treated with AgNP IC50 for 1h, 3h, 6h, and 24h. The results were normalized to the untreated group (100% viable cells). Each value represents the mean ± S.D. of three independent experiments. For statistical tests, p <0.05 for ANOVA was used, followed by Tukey's test (24h vs. 1h, 3h, 6h). (C) Representative images of IC50 by Calcein/PI assay obtained by phase contrast and the images below represent the merge of the DAPI, GFP, and PI light cubes. Column: I - Control group; II – IC50 treatment. Phase contrast; Hoechst + Calcein + PI. Scale bar: 200 µm.

3.2. *In vivo Antitumor Activity*

The urinary tract of the animals in the Control group did not present microscopic changes (*Figures 2a, 2b, Table 1*). The normal urothelium was composed of 2-3 layers, being: a layer of basal cells, an intermediate cell layer, and a superficial or apical layer composed of cells in umbrella (*Figures 2a, 2b*). In contrast, the MNU (Cancer) urinary tract presented drastic histopathological changes, such as: urothelial carcinoma with invasion of the lamina propria (pT1) (*Figures 2c, 2d*) and papillary urothelial carcinoma (pTa) (*Figures 2e, 2f*) in 57.15% and 42.85% of the animals,
respectively (Table 1). The pT1 carcinoma was characterized by neoplastic cells grouped into small
groups or cords invading the lamina propria, numerous mitotic figures and pleomorphic cells with
enlarged nuclei. Papillary urothelial carcinoma (pTa) was characterized by extensive papillary lesions,
urothelial cells with disordered arrangement and loss of polarity, intense cellular pleomorphism and
numerous mitosis figures.

The most frequent neoplastic lesions in the MNU + AgNP 0.5 group were pTa (Figure 3a),
pTis (Figure 3b) and pT1 in 42.85%, 28.57% and 28.57% of the animals, respectively (Table 1),
indicating that this treatment was not effective in to regress neoplastic lesions. The pTis carcinoma
was characterized by a disordered proliferation of urothelial cells (hyperplasia) in a flat urothelium,
with marked cellular atypia characterized by bulky nuclei, reduced cytoplasm and multiple and
prominent nucleoli.

Histopathological analyses of the animals of the MNU + AgNP 0.2 Group showed 28.57% of
tumor regression, which showed flat hyperplasia (Figure 3c; Table 1). The most frequent neoplastic
lesions in this group were pTis (Figure 3d) and pTa in 57.15% and 14.28% of the animals,
respectively (Table 1). Flat hyperplasia was characterized by thickening of the urothelium and
absence of cytological atypia.

Treatment with AgNP 0.05 presented 57.13% tumor regression (Table 1), with 14.28% of the
animals showing normal urothelium (Figure 3e) and 42.85% presenting flat hyperplasia (Figure 3f),
which is considered a benign lesion. The most frequent neoplastic lesions in this group were pTis and
pTa in 28.57% and 14.28% of the animals, respectively (Table 1).

In 2016 it was shown that AgNPs (at 5 and 10 µg body weight) significantly suppressed the
H1299 tumor growth (lung cancer) in a xenograft severe combined immunodeficient mouse model.
This result is in agreement with our outcomes from bladder cancer (2 or 10 µg/g body weight). The
results demonstrate the anticancer activities of AgNPs, suggesting that these nanometallic strcutures
may act as potential beneficial molecules in cancer chemoprevention/chemotherapy, specifically for
early-stage therapy [15].

Table 1: Percentage of histopathological changes of the urinary bladder of mice from different
experimental groups.

| Groups | Histopathology | Control (n=7) (Cancer) | MNU+AgNP 0.5 mg/mL (n=7) | MNU+AgNP 0.2 mg/mL (n=7) | MNU+AgNP 0.05 mg/mL (n=7) |
|--------|----------------|-----------------------|--------------------------|--------------------------|--------------------------|
| Normal | Normal         | 7(100%)*              | -                        | -                        | 1(14.28%)                |
|        | Flat hyperplasia| -                     | -                        | 2(28.57%)                | 3(42.85%)*               |
|        | Flat Carcinoma in situ (pTis) | - | - | 2(28.57%) | 4(57.15%)* |
|        | Papillary Urothelial Carcinoma (pTa) | - | 3(42.85%)* | 3(42.85%)* | 1(14.28%) |
|        | High-grade urothelial cancer invading the lamina propria (pT1) | - | 4(57.15%)* | 2(28.57%) | - |

Benign Lesions: Flat hyperplasia; Malignant lesions: pTis, pTa, pT1.

The histopathological alterations are expressed as a percentage of the number of mice (n) examined in each group.
*P<0.0001 (proportions test).
Figures 2a - 2f: Photomicrographs of the urinary bladder from the Control (a, b) and MNU (c, d, e, f) groups. (a), (b) Normal urothelium composed of 2-3 layers: a layer of basal cells (closed arrowhead), an intermediate layer of cells (arrow), and a superficial or apical layer composed of umbrella cells (open arrowhead). (c), (d) pT1 tumor: neoplastic cells arranged in small groups (arrows) invading the lamina propria. (e), (f) pTa tumor characterized by extensive papillary lesions, urothelial cells with disordered arrangement and loss of polarity, intense cellular pleomorphism and numerous mitosis figures. a - f: Lp - lamina propria, M - muscular layer, Ur - urothelium.
Figures 3a - 3f: Photomicrographs of urinary bladder from MNU + AgNP 0.5 (a, b), MNU + AgNP 0.2 (c, d) and MNU + AgNP 0.05 (e, f) groups. (a) pTa tumor characterized by extensive papillary lesions, urothelial cells with disordered arrangement and loss of polarity, intense cellular pleomorphism and numerous mitosis figures. (b), (d) pTis tumor characterized by cellular atypia: bulky nuclei with reduced cytoplasm and prominent nucleoli (arrows). (c), (f) Flat hyperplasia (circle) characterized by thickening of the urothelium and absence of cytological atypia. (e) Normal urothelium composed of 2-3 layers: a layer of basal cells, an intermediate layer of cells, and a superficial or apical layer composed of cells in umbrella. a - f: Lp - lamina propria, M - muscular layer, Ur - urothelium.
4. Conclusions
The results showed the dose-time dependent cytotoxicity, and detailed analysis demonstrated the induction of cell death via apoptosis. Besides, we found that AgNP inhibition in cell migration and proliferation. Thus, these findings confirm the antitumor properties of AgNPs, and suggest that they may be a cost-effective alternative and promising candidate for the treatment of bladder cancer.

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