**Quantitative characterization of the effect of biogenic silver-based nanoparticles on breast cancer cells by high content analysis**

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**Abstract**

This study aimed to evaluate the antitumor potential and cytotoxicity induction mechanisms of green synthesized AgCl-NPs and Ag/AgCl-NPs through image-based high content analysis method. The antitumor potential of AgCl-NPs and Ag/AgCl-NPs was evaluated in breast cancer BT-474 and MDA-MB-436 cell lines treated with 0-40 μg/mL AgCl-NPs or 0-12.5 μg/mL Ag/AgCl-NPs. Normal human retinal pigment epithelial 1 (RPE-1) cells were used for comparison. The growth rate of the RPE-1 cells treated with AgCl-NPs or Ag/AgCl-NPs was little affected, and no significant changes in cell viability were observed. In these cells, the nanoparticle treatments did not induce lysosomal damage, changes in reactive oxygen species (ROS) production or a reduction in the mitochondrial membrane potential. Moreover, the percentage of apoptotic cells was minimally affected, reaching a maximum of 3.51% of the population. The level of BT-474 and MDA-MB-436 cell proliferation was markedly decreased, and cell viability was reduced by 64.19 and 46.19% after treatment with AgCl-NPs and reduced by 98.36 and 82.29% after treatment with Ag/AgCl-NPs. The cells also showed a significant increase in ROS production and loss of mitochondrial membrane potential, which culminated in an increase in the percentage of apoptotic cells. BT-474 cells also presented lysosomal damage when treated with the highest concentrations of both nanoparticle types, and actin polymerization was observed after exposure to Ag/AgCl-NPs. Together, the results obtained show overall cytotoxic effects of both AgCl-NPs and Ag/AgCl-NPs towards breast cancer cells with negligible effects against healthy cells, which suggests their promising anticancer and biomedical applications.

**Introduction**

Breast cancer, together with colon and lung cancer, are the top three most common cancer types [1], with breast cancer being the most common among women and ranking second in mortality rate [2]. The incidence and mortality of breast cancer have increased in Africa, South America and Asia, most likely due to changes in lifestyle and delayed diagnosis and initiation of therapy [1]. In the United States and Europe, the rate of death from breast cancer has decreased in recent years, a reduction that has been attributed to early diagnosis [3; 4]. However, in the United States, the incidence of *in situ* and metastatic cancer continues to increase slightly, especially among women older than 50 years [4]. An estimated one in every 8 or 10 women will develop cancer in their lifetime [1]. Pregnancy later in life, nulliparity, obesity and use of hormones during menopause are considered risk factors for breast cancer [4].

Breast cancer is highly heterogeneous and exhibits several phenotypic and genotypic changes that result in clinical changes. Based on the presence or absence of cell surface receptors, breast cancer is clinically divided into three types: HR+ (positive for hormone receptors), HER2+ (positive for human epidermal growth factor receptor 2) or triple-negative [5]. HR+ breast cancer has receptors for estrogen (ER) and progesterone (PR) and is the most common breast tumor, representing approximately 85% of cases, and can be classified as luminal A (HER2-) or luminal B (HER2+) breast cancer [5]. HER2+ breast cancer expresses HER2 and accounts for 15 to 20% of cases [6]. Between 12 and 24% of cases are triple-negative breast cancer (TNBC), which is characterized by the absence of estrogen and progesterone receptors and non over expression of HER2 [7].

All three breast cancer types may develop metastases, and there is still no cure for metastatic breast cancer, which presents a 5-year survival rate of only 25% [8]. Among the breast tumor types, TNBC most frequently exhibits local recurrence and distant metastases [9].

HR+ breast cancers are usually treated with hormonal therapies, namely, ER antagonists and modulators or aromatase inhibitors, which prevent estrogen synthesis. In general, these therapeutic strategies have good results; however, between 20 and 30% of patients exhibit resistance [5]. HER2+ breast cancers are typically treated with HER2 antagonists [5]. TNBC has the worst prognosis because it does not present the specific therapeutic targets available in the other two breast cancer types and is thus treated with general chemotherapeutic agents and/or radiotherapy [7; 10]. Although patients with TNBC receiving radiotherapy combined with other treatments have a better prognosis than patients not receiving this therapy, TNBC tends to be radioresistant, which is believed to be due to the expression of ERp29, HER1 and mir-27 [11].

Due to the resistance to antitumor treatments [12], alternative treatments have been sought. In this context, metal nanoparticles, such as silver-based nanoparticles, have been shown to be effective in the treatment of cancer *in vivo* and *in vitro* [13; 14].

Nanoparticles are particulate materials that have dimensions up to 100 nm and this nanoscale size directly influences their physicochemical properties [15]. Nanoparticles can be produced by bottom-up or top-down approaches. In the top-down process the synthesis is done by reducing a macro or microscale material to the nanoscale and in bottom-up process smaller entities starting from the atomic level group together to form the nanoparticles [16]. Nanoparticles can be produced by biological methods in which the synthesis comes under bottom-up process where metallic reduction is done by microorganisms or plant extracts [16]. Biological methods for nanoparticle production are environmentally friendly, conducted at pH, temperature and physiological pressures and do not produce toxic waste [17; 18].

Silver nanoparticles (AgNPs) biosynthesized by *Spirulina platensis* have been shown to dose-dependently reduce the viability of Hep-2 cells, a human tumor cell line derived from laryngeal carcinoma [19]. AgNPs also reduced the viability of the THP-1 acute myeloid leukemia [20], MCF-7 breast adenocarcinoma [21] and A549 lung cancer [22] cell lines. In the PC3 prostate cancer cell line, treatment with AgNPs for 24 h induced DNA damage and reduced cell proliferation and nuclear size [23].

Silver chloride (AgCl-NPs) and silver/silver chloride (Ag/AgCl-NPs) nanoparticles have properties similar to those of AgNPs, such as the ability to release Ag+ ions in solution [24]. Both have demonstrated antibacterial [25; 26] and antifungal [27; 28] effects. Ag/AgCl-NPs produced biologically using *Citrus hystrix* extract were found to have antiproliferative action against colorectal tumor lines HCT116 and Caco-2, whereas nontumoral human fibroblasts did not undergo any significant changes in growth [29]. The antiproliferative effect of Ag/AgCl-NPs against tumor cells was also demonstrated in GBM02 (glioblastoma multiforme) cells, against which they showed more pronounced effects than in healthy astrocytes [30]. However, the mechanisms by which these nanoparticles induce cytotoxicity in healthy and tumor cells have not been evaluated. AgCl-NPs have not yet been evaluated as an antitumor agent. Therefore, the aim of the present study was to evaluate the mechanisms of cytotoxicity induction of AgCl-NPs and Ag/AgCl-NPs and their antitumor effects against luminal B and TNBC breast cancer cells.

**Materials and methods**

**Silver chloride nanoparticles**

Microalgae of the species *Chlorella vulgaris* (UTEX 2714) were obtained from the microalgae bank of the University of Texas (UTEX), USA, and cultured in ASM-1 medium [31] at 25 °C with a photoperiod of 12 h:12 h (dark:light) and a light intensity of 123.47 ± 8.23 μmol of photons/m2s measured using a Heinz Walz GmbH light meter equipped with the sensor s/n: SQSA0404. The silver chloride nanoparticles (AgCl-NPs) were synthesized using the *C. vulgaris* culture supernatant as described in Ferreira *et al*. [32]. These nanoparticles have a spherical shape, diameters ranging from 1.6 to 34.4 nm and a mean diameter of 9.8 ± 5.7 nm.

**Silver/silver chloride nanoparticles**

Yeast of the species *Candida lusitaniae* were cultured for 7 days at 30 °C in rich medium (4% glucose, 1% bacterial peptone and 1% yeast extract, pH 6.5). Ag/AgCl-NPs were produced by *C. lusitaniae* as described in Eugenio *et al*. [26]. These nanoparticles have mostly spherical shapes, diameters ranging from 2 to 22 nm and a mean diameter of 6.9 ± 4.5 nm.

**Cells and treatment**

The antitumor effect of the nanoparticles was evaluated in MDA-MB-436 (TNBC) and BT-474 (mammary adenocarcinoma) cells and as a control of cytotoxicity, the non-tumor cell line RPE-1 (retinal pigment epithelium) was used. MDA-MB-436 and BT-474 cells were cultured in RPMI GlutaMAX medium (Gibco, ref .: 6187010) and RPE-1 strain in DMEM high glucose GlutaMAX medium (Gibco, ref .: 61965026). Cells were cultured supplemented with 10% fetal bovine serum and 1% antibiotic solution (penicillin-streptomycin 10000 U/mL, Gibco, ref.:15140122) and incubated at 37 ºC in a 5% CO2 atmosphere. Upon reaching confluence, cells were resuspended in an enzymatic solution of 0.05% trypsin-EDTA (Gibco, ref.: 25300054) for 2 to 5 min at 37 °C. To perform the experiments, the suspended cells were counted (Cellometer® Auto T4, Nexcelom Bioscience) and seeded in 96-well plates at a cell density of 4 x 10³ cells/well. After 24 h, the cells were treated with Ag/AgCl-NPs (0, 0.5, 1, 2.5, 5, 7.5, 10 or 12.5 μg/mL) or AgCl-NPs (0, 2.5, 5, 10, 15, 20, 30 or 40 μg/mL). The experiments with tumor cells were performed with six replicates, while the experiments with nontumor cells were performed in triplicate.

**Cell proliferation**

To evaluate the effect of the nanoparticles on cell proliferation, cells were treated for 0, 24, 48 or 72 h with nanoparticles and stained with Hoechst 33258 1 μM (Thermo Fisher ref.: H1399). The IN Cell Analyzer 2200 high-content analysis (HCA) system (GE Healthcare Life Sciences) was used to automatically acquire fluorescence images from six random fields in each well. Image analysis was performed using IN Cell Analyzer 1000 software (GE Healthcare Life Sciences) with algorithms for automatic detection of objects (nuclei).

**Cell viability**

To evaluate the effect of the nanoparticles on cell viability after 24, 48 and 72 h of treatment, the cells were stained for 30 min at 37 °C with 1 μM Hoechst 33258 (Thermo Fisher ref.: H1399), and cell viability was quantified using the LIVE/DEAD™ viability/cytotoxicity kit for mammalian cells (Thermo Fisher ref.: L3224) according to the manufacturer’s instructions. Images were obtained using the IN Cell Analyzer 2200 HCA system (GE Healthcare Life Sciences). The images were analyzed using IN Cell Analyzer 1000 software (GE Healthcare Life Sciences) with algorithms for automatic object detection and a 2D linear classification filter to automatically determine the percentages of live and dead cells.

**Apoptosis evaluation**

To evaluate whether treatment with silver-based nanoparticles induces apoptosis, cells were plated and treated with nanoparticles as described above and were stained after 24, 48 and 72 h for 30 min at 37 °C with 1 μM Hoechst 33258 (Thermo Fisher ref.: H1399), 0.4 μM ethidium homodimer-1 (EthD-1; Thermo Fisher ref.: E1169) and 8 μM CellEvent™ Caspase-3/7 Green Detection Reagent (Thermo Fisher ref.: C10423). Fluorescence images were acquired using the IN Cell Analyzer 2200 HCA system (GE Healthcare Life Sciences). The images were analyzed using IN Cell Analyzer 1000 software (GE Healthcare Life Sciences) with algorithms for automatic object detection and classification filters (decision tree type) for automatic determination of the percentage of apoptotic cells (excluding dead cells stained with EthD-1).

**ROS production**

To evaluate whether treatment with silver-based nanoparticles induced the production of reactive oxygen species (ROS), cells were treated for 24, 48 and 72 h and then stained for 30 min at 37 °C with 1 μM Hoechst 33258 (Thermo Fisher ref.: H1399) and dihydroethidium (DHE) (Thermo Fisher, ref.: D23107). Fluorescence images were acquired using the IN Cell Analyzer 2200 HCA system (GE Healthcare Life Sciences). The images were analyzed using IN Cell Analyzer 1000 software (GE Healthcare Life Sciences) with algorithms for automatic object detection and measurement of the fluorescence intensity of DHE to estimate changes in ROS production.

**Multiparametric cytotoxicity assessment**

To evaluate some of the cytotoxic mechanisms of the nanoparticle treatments, the cells were cultured and treated with silver-based nanoparticles for 48 h and then stained with 1 μM Hoechst 33258 (Thermo Fisher ref.: H1399) combined with 1 μM LysoTracker® Red DND-99 (Molecular Probes ref.: L7528), 50 nM tetramethylrhodamine (TMRM) (Thermo Fisher ref: I34361) or 1.5 μg/mL CellMask™ Deep Red Plasma Membrane Stain (Thermo Fisher ref.: C10046). The effect of the nanoparticle treatments on actin filaments was evaluated in samples fixed with 3.7% formaldehyde, blocked with 10% bovine serum albumin (BSA) and 2% saponin in PBS, permeabilized with 0.5% Triton X-100 and stained with 165 μM rhodamine-phalloidin (Thermo Fisher ref.: R415) and 2 μg/mL DAPI (Thermo Fisher ref.: D1306). Fluorescence images were acquired using the IN Cell Analyzer 2200 HCA system (GE Healthcare Life Sciences) and analyzed using IN Cell Analyzer 1000 software (GE Healthcare Life Sciences) with algorithms for automatic object detection.

**Statistical analysis**

Statistical analyses were performed using a 95% confidence interval in GraphPad Prism 5 software. The statistical tests used were one-way ANOVA with Tukey’s post hoc test or two-way ANOVA with the post hoc Bonferroni test.

**Results**

Two types of silver-based nanoparticles previously described by our group were studied: AgCl-NPs and Ag/AgCl-NPs [26; 32]. The AgCl-NPs were produced using supernatant from culture of the microalga *Chlorella vulgaris* and presented diameters ranging between 1.6 and 34.4 nm, with a mean diameter of 9.8 ± 5.7 nm [32]. Ag/AgCl-NPs were synthesized biologically by the yeast *Candida lusitaniae* and showed diameters ranging from 2 to 22 nm, with a mean diameter of 6.9 ± 4.5 nm [26].

The present study evaluated the cytotoxic and antitumor potential of AgCl-NPs and Ag/AgCl-NPs against human cells and explored the mechanisms by which cells are affected by silver-based nanoparticles. To evaluate cytotoxicity, the nontumor retinal pigment epithelial 1 (RPE-1) cell line was used, whereas the antitumor potential of the nanoparticles was evaluated using two breast cancer cell lines, BT-474 and MDA-MB-436.To determine whether AgCl-NPs and Ag/AgCl-NPs can affect cell growth and viability, the RPE-1, BT-474 and MDA-MB-436 cell lines were cultured for 24, 48 and 72 h with 0, 2.5, 5, 10, 15, 20, 30 and 40 μg/mL AgCl-NPs or 0, 0.5, 1, 2.5, 5, 7.5, 10 and 12.5 μg/mL Ag/AgCl-NPs. The results showed that the growth of RPE-1 cells treated with AgCl-NPs at concentrations below 30 μg/ml (Figure 1A) or with Ag/AgCl-NPs at concentrations below 5 μg/ml (Figure 1B) was not affected. However, these cells exhibited reduced cell growth when treated with 40 μg/mL AgCl-NPs for 72 h or with Ag/AgCl-NPs at concentrations above 7.5 μg/mL for 48 or 72 h, reaching reductions of 28.87 and 52.55%, respectively. The growth of the BT-474 cell line decreased when exposed to concentrations higher than 10 μg/mL AgCl-NPs, reaching a reduction of 20.54% when treated for 72 h with 40 μg/mL nanoparticles (Figure 1C). When treated with Ag/AgCl-NPs at 2.5 μg/mL for 48 h and 12.5 μg/mL for 72 h, these cells showed 17.98 and 19.51% lower cell growth, respectively (Figure 1D). MDA-MB-436 tumor cells treated for 24 or 48 h with 2.5 to 40 μg/mL AgCl-NPs exhibited significantly lower growth than control group cells (Figure 1E). However, at 72 h of treatment, only concentrations higher than 5 μg/mL induced significant reductions in cell growth. Cells treated with 40 μg/mL AgCl-NPs, for example, exhibited a 63.91% reduction in cell proliferation. In treatments with Ag/AgCl-NPs, these cells showed inhibited cell proliferation when exposed to concentrations between 0.5 and 12.5 μg/mL, and the highest concentration induced a reduction of 70.27% in the number of cells (Figure 1F).

The viability of the nontumor RPE-1 cell line exposed to AgCl-NPs was not altered (Figure 2A). When exposed for 24 h to Ag/AgCl-NP concentrations up to 10 μg/mL, RPE-1 cells did not show significant changes in cell viability; however, when cultured with 12.5 μg/mL Ag/AgCl-NPs, the cell viability was 5.6% lower than that of control group cells (Figure 2B). After 48 and 72 h of treatment, RPE-1 cells exposed to Ag/AgCl-NP concentrations between 2.5 and 12.5 μg/mL underwent reductions in cell viability, reaching a maximum reduction of 6.25% when treated with 12.5 μg/mL nanoparticles for 72 h. BT-474 cells treated with AgCl-NPs only showed reduced viability compared with the control group when exposed to 30 and 40 μg/mL nanoparticles, reaching reductions of 10.5 and 64.19% within 72 h, respectively (Figure 2C). When treated with Ag/AgCl-NPs, BT-474 cells showed reduced cell viability after exposure to 10 and 12.5 μg/mL nanoparticles for 24 and 48 h (Figure 2D). After 72 h of culture, cells treated with 7.5, 10 and 12.5 μg mL Ag/AgCl-NPs showed reductions in cell viability of 13.24, 76.26 and 98.36%, respectively. After 24 and 48 h of treatment with AgCl-NPs, the viability of the MDA-MB-436 cell line was significantly reduced when the cells were exposed to concentrations above 5 μg/ml nanoparticles (Figure 2E). At 72 h, concentrations above 2.5 μg/mL significantly affected the viability of MDA-MB-436 cells, and cells treated with 40 μg/mL AgCl-NPs showed a 46.19% reduction in viability compared with control group cells. When cultured with Ag/AgCl-NPs for 24, 48 or 72 h at concentrations between 0.5 and 12.5 μg/mL, MDA-MB-436 cells underwent significant reductions in cell viability, reaching a reduction of 82.29% when exposed to 12.5 μg/mL Ag/AgCl-NPs (Figure 2F).

To evaluate whether AgCl-NP and Ag/AgCl-NP treatments can induce apoptosis, the treated cells were labeled with CellEvent®, which is a substrate for caspase-3/7 that can bind with DNA and emit green fluorescence after cleavage by caspase. Figure 3 shows the percentage of apoptotic cells in the RPE-1, BT-474 and MDA-MB-436 populations after nanoparticles treatment. RPE-1 nontumor cells treated with less than 30 μg/mL AgCl-NPs showed no significant changes in the apoptosis ratio; however, when treated with 40 μg/mL nanoparticles for 48 or 72 h, these cells showed increases of 2.38% and 5% in the percentage of apoptosis in the population, respectively (Figure 3A). Treatment with Ag/AgCl-NPs induced a minimal increase in the percent apoptosis in the RPE-1 cell population at concentrations above 5 μg/mL nanoparticles, reaching a maximum percentage of 7.3% when the cells were treated for 24 h with 12.5 μg/mL nanoparticles (Figure 3B).

Treatments with concentrations below 10 μ/mL AgCl-NPs did not significantly affect the percentage of BT-474 cell apoptosis. At 48 h of treatment, cells exposed to 10, 30 and 40 μg/mL AgCl-NPs showed an increase in the percentage of apoptosis in the population. At 24 and 72 h of treatment, only treatment with 40 μg/mL AgCl-NPs induced an increase in the percentage of apoptosis in the population, reaching 27.86% and 22.54% apoptosis, respectively (Figure 3C). When cultured with Ag/AgCl-NPs, the BT-474 cell line showed an increase in the apoptosis percentage when exposed to nanoparticle concentrations above 5 μg/mL, reaching a maximum percentage of 51.01% after treatment with 12.5 μg/mL Ag/AgCl-NPs for 72 h (Figure 3D).

MDA-MB-436 cells exposed for 48 h to concentrations higher than 2.5 μg/mL AgCl-NPs showed a clear increase in the percentage of apoptosis in the population (Figure 3E). However, at 72 h of treatment, the proportion of apoptotic cells increased significantly only in the groups cultured with concentrations higher than 10 μg/mL AgCl-NPs, culminating in 20.02% apoptotic cells in the group treated with 40 μg/mL. The percentage of apoptotic MDA-MB-436 cells increased significantly after treatment with Ag/AgCl-NPs at concentrations between 0.5 and 12.5 μg/mL for 24, 48 or 72 h, and the highest percentage of apoptosis was achieved after treatment with 12.5 μg/mL nanoparticles for 72 h, leading to 31.05% apoptosis (Figure 3F).

To evaluate whether silver-based nanoparticles induce changes in ROS production after 24, 48 and 72 h of treatment, RPE-1, BT-474 and MDA-MB-436 cells were treated and labeled with DHE, a superoxide indicator that when oxidized is transformed into EthD-1, which intercalates with DNA and emits fluorescence in the red spectral region. The results showed that there were no changes in ROS production in RPE-1 cells after treatment with up to 40 μg/mL AgCl-NPs (Figure 4A) or 12.5 μg/mL Ag/AgCl-NPs (Figure 4B). In contrast, BT-474 cells showed significant increases in ROS production when treated for 24 h with more than 15 μg/mL AgCl-NPs. After 48 and 72 h of treatment, only the groups exposed to 40 μg mL AgCl-NPs showed increased ROS production in BT-474 cells, which showed increases of 104.26% and 173.10% at 48 and 72 h, respectively (Figure 4C). When treated with Ag/AgCl-NPs at concentrations between 7.5 and 12.5 μg/mL, BT-474 cells showed significant increases in ROS production after 24, 48 and 72 h of treatment. For example, at 72 h, compared with the control group, ROS production was 276.52% higher in the group treated with 12.5 μg/mL nanoparticles (Figure 4D). At 24 h of treatment, MDA-MB-436 cells showed an increased ROS concentration when exposed to AgCl-NPs at concentrations above 5 μg/mL. Concentrations above 15 μg/mL AgCl-NPs induced a significant increase in ROS concentration relative to the control group at 48 and 72 h, with the maximum ROS production (200.52% increase compared with the control group) observed in cells treated for 24 h with 40 μg/mL AgCl-NPs (Figure 4E). MDA-MB-436 cells showed significant increases in ROS production when cultured with Ag/AgCl-NPs for 24, 48 and 72 h at concentrations between 0.5 and 12.5 μg/mL, reaching the maximum ROS production when cultured for 72 h with 12.5 μg/mL, which led to an increase of 141.36% compared with control group cells (Figure 4F).

In addition, multiparametric analyses involving the effect of nanoparticles on mitochondria, lysosomes, actin microfilaments and cell area were carried out (see supplemental material).

**Discussion**

The proliferation and viability of tumor cells were more strongly affected by treatments with nanoparticles than nontumor cells. A similar result was obtained by [33], who demonstrated that after 48 h of treatment with 200 μg/ml AgNPs, the viability of MDA-MB-231 (triple negative breast cancer), MCF-7 (breast cancer), U251 (glioblastoma) and MO59K (glioblastoma) tumor cells was reduced by 57, 68, 42 and 63%, respectively, whereas the viability of normal IMR-90 fibroblasts was not significantly affected. Maity *et* *al*. [34] also showed that AgNPs reduced the viability of MCF-7, Jurkat and EAC (Ehrlich carcinoma) tumor cells in a dose-dependent manner, without significant effects on human or mouse nontumoral lymphocytes. In addition, Jadhav *et al.* [35] observed that nontumor (L929) cells did not undergo significant changes in cell viability when treated with up to 78.62 μg/mL AgNPs, which were synthesized biologically using *Salacia chinensis* extract, while lung (L-132), liver (Hep G2), pancreas (MIA-Pa-Ca-2), oral (KB cells), breast (MDA-MB-231), cervical (HeLa) and prostate (PC-3) cancer cell lines showed significant reductions in cell viability, with IC50 values ranging from 4.002 to 14.37 μg/mL. The same AgNPs were subjected to a hemolysis test, and the results suggested that 78.67 μg/mL induced less than 3% hemolysis, while the safe threshold for biomaterials is 5% according to ISO/TR 7406, demonstrating that these nanoparticles are likely safe for normal erythrocytes [35].

We also found a proapoptotic effect of silver-based nanoparticles which was also observed previously for example, in MCF7, HCC1954 and HCC70 breast tumor cell lines treated with AgNPs, which exhibited up to fivefold increases in the percentage of apoptosis when treated for 24 h with 12.5 μg/mL AgNPs [36]. Yuan *et al.* [37] demonstrated in A2780 ovarian tumor cells that AgNPs induce caspase-mediated apoptosis, possibly through upregulation of the expression of proapoptotic genes (P53, P21, Bax, Bak, cyt-c, caspase-9 and caspase-3) and downregulation of the expression of Bcl-2, an antiapoptotic protein. Treatment with AgNPs also induced an increase in reactive oxygen species (ROS) generation in A2870 cells, which most likely contributed to the increased apoptosis rate [37].

ROS can be generated if electrons escape from the electron transport chain and are received by oxygen molecules, forming superoxide anions (O2-) that can be converted into hydrogen peroxide (H2O2) and reduced to the strongest oxidant in nature: hydroxyl (OH) [38]. Our results showed that silver-based nanoparticle induced no changes in ROS production in RPE-1, while the tumor cells BT-474 and MDA-MB-436 showed significant increases. Cellular ROS generation induces phosphorylation of IKappaB (IKK) protein kinase, which can phosphorylate and degrade IκB proteins that bind to the transcription factor NFκB, thereby keeping it inactive and preventing its translocation to the nucleus. Once phosphorylated, the IκB proteins are degraded, releasing NFκB, which translocates to the nucleus, binds to target promoter regions and activates the transcription of genes responsible for the production of several proinflammatory proteins, such as interleukin IL1α, a recruiter of immune system cells [39; 40]. For example, when treated with AgNPs, HeLa and A549 tumor cell lines showed mitochondrial damage and increased ROS, IL1α and phosphorylated IKKα, IκB and NFκB levels, indicating that AgNPs can activate proinflammatory pathways through ROS generation, which may be beneficial for the treatment of tumors [40]. Yañez-Sánchez *et al.* [41] showed that AgNPs at concentrations higher than 9.0 μg/mL induced increased ROS production and had antiproliferative and apoptotic effects in the lymphoma cell line L5178Y.

The results obtained in the present study demonstrate that tumor cell proliferation, viability, mitochondrial membrane potential (Supplementary material) and ROS production are more affected by AgCl-NP and Ag/AgCl-NP treatments than the nontumor cell line RPE-1. Compared with MDA-MB-436 cells, BT-474 cells were the least sensitive to treatment with nanoparticles, with deleterious effects observed only after treatment with the highest concentrations. A similar result was obtained by Swanner *et al*. [42], who compared the effect of AgNPs on MDA-MB-231 (triple-negative breast cancer), BT-549 (triple-negative breast cancer), SUM-159 (triple-negative breast cancer), MCF-7 (breast cancer), MCF-10A (nontumor mammary gland cells), HMEC (nontumor epithelial cells) and 184B5 (nontumor mammary gland cells) cell lines. The results showed that nontumor cells are less affected by nanoparticles than tumor cell lines, and triple-negative lines are the most sensitive, thus corroborating the results obtained in the present study, which showed that MDA-MB-436, a triple-negative cell line, was more sensitive to AgCl-NP and Ag/AgCl-NP treatments than the BT-474 luminal B breast cancer cell line. Swanner *et al*. [42] attributed this phenomenon to the fact that triple-negative tumor cells have higher amounts of oxidized proteins and phosphorylated γH2AX histones, which are used as indicators of oxidative stress and DNA damage, respectively. The more pronounced effects observed in tumor cells treated with silver-based nanoparticles may also be related to the microenvironment. The tumor microenvironment is known to be acidic [43], and silver nanoparticles can release more Ag+ ions when in an acidic environment [44]; thus, greater ion release could lead to greater cytotoxicity.

Our results also demonstrated that Ag/AgCl-NPs were more cytotoxic than AgCl-NPs, this difference may be due to the composition or size of nanoparticles. AgCl-NPs have diameters between 1.6 and 34.4 nm and an average diameter of 9.8 ± 5.7 nm [32], while Ag/AgClNPs have diameters between 2 and 22 nm with an average diameter of 6.9 ± 4.5 nm [26]. Smaller nanoparticles have greater interaction with surrounding microenvironment due to their greater surface area by volume [45].

In conclusion, the results obtained in the present study showed that silver-based nanoparticles reduced tumor cell proliferation and viability, possibly via necrosis and apoptosis, which may be triggered by damage to mitochondrial function, leading to increased ROS production. Although additional studies are needed, our data suggest that AgCl-NPs and Ag/AgCl-NPs are promising antitumor agents because they affect the viability and proliferation of tumor cells with minimal effects on nontumor cells. In the future, silver-based nanoparticles may be a viable treatment option for cancer types that currently have no effective treatments, which would offer a better prognosis for many patients.

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**Figure Caption**

**Figure 1:** Effect of AgCl-NPs or Ag/AgCl-NPs on proliferation of RPE-1 and breast cancer cells: (a, b) RPE-1, BT-474 (c, d) and MDA-MB-436 (e, f) cells were cultured for 72h with 0 - 40 µg/ml (a, c) AgCl-NPs or (b, d) 0 - 12.5 µg/ml Ag/AgCl-NPs. \* P <0,05, \*\* P <0,01, \*\*\* P <0,001 (Anova Two-way).

**Figure 2**: Effect of AgCl-NPs or Ag/AgCl-NPs on viability of RPE-1 and breast cancer cells: (a, b) RPE-1, BT-474 (c, d) and MDA-MB-436 (e, f) cells were cultured for 72h with 0 - 40 µg/ml (a, c) AgCl-NPs or (b, d) 0 - 12.5 µg/ml Ag/AgCl-NPs. \* P <0,05, \*\* P <0,01, \*\*\* P <0,001 (Anova Two-way).

**Figure 3**: Effect of AgCl-NPs or Ag/AgCl-NPs on apoptosis percentage of RPE-1 and breast cancer cells: (a, b) RPE-1, BT-474 (c, d) and MDA-MB-436 (e, f) cells were cultured for 72h with 0 - 40 µg/ml (a, c) AgCl-NPs or (b, d) 0 - 12.5 µg/ml Ag/AgCl-NPs. \* P <0,05, \*\* P <0,01, \*\*\* P <0,001 (Anova Two-way).

**Figure 4**: Effect of AgCl-NPs or Ag/AgCl-NPs on ROS production of RPE-1 and breast cancer cells: (a, b) RPE-1, BT-474 (c, d) and MDA-MB-436 (e, f) cells were cultured for 72h with 0 - 40 µg/ml (a, c) AgCl-NPs or (b, d) 0 - 12.5 µg/ml Ag/AgCl-NPs. \* P <0,05, \*\* P <0,01, \*\*\* P <0,001 (Anova Two-way).

**Figure S1**: Effect of AgCl-NPs or Ag/AgCl-NPs on mitochondrial membrane potential of RPE-1 and breast cancer cells: (a, b) RPE-1, BT-474 (c, d) and MDA-MB-436 (e, f) cells were cultured for 48h with 0 - 40 µg/ml (a, c) AgCl-NPs or (b, d) 0 - 12.5 µg/ml Ag/AgCl-NPs.\* P <0,05, \*\* P <0,01, \*\*\* P <0,001 (Anova One-way).

**Figure S2**: Effect of AgCl-NPs or Ag/AgCl-NPs on lysosomal acidification of RPE-1 and breast cancer cells: (a, b) RPE-1, BT-474 (c, d) and MDA-MB-436 (e, f) cells were cultured for 48h with 0 - 40 µg/ml (a, c) AgCl-NPs or (b, d) 0 - 12.5 µg/ml Ag/AgCl-NPs.\* P <0,05, \*\* P <0,01, \*\*\* P <0,001 (Anova One-way).

**Figure S3**: Effect of AgCl-NPs or Ag/AgCl-NPs on microfilament polymerization of RPE-1 and breast cancer cells: (a, b) RPE-1, BT-474 (c, d) and MDA-MB-436 (e, f) cells were cultured for 48h with 0 - 40 µg/ml (a, c) AgCl-NPs or (b, d) 0 - 12.5 µg/ml Ag/AgCl-NPs.\* P <0,05, \*\* P <0,01, \*\*\* P <0,001 (Anova One-way).

**Figure S4**: Effect of AgCl-NPs or Ag/AgCl-NPs on cell area of RPE-1 and breast cancer cells: (a, b) RPE-1, BT-474 (c, d) and MDA-MB-436 (e, f) cells were cultured for 48h with 0 - 40 µg/ml (a, c) AgCl-NPs or (b, d) 0 - 12.5 µg/ml Ag/AgCl-NPs.\* P <0,05, \*\* P <0,01, \*\*\* P <0,001 (Anova One-way).