Hindawi
Journal of Nanomaterials
Volume 2019, Article ID 8148219, 8 pages
https://doi.org/10.1155/2019/8148219

Research Article

Comparative Study of Antitumor Activity between Lipophilic Bismuth Nanoparticles (BisBAL NPs) and Chlorhexidine on Human Squamous Cell Carcinoma

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Received 2 August 2019; Revised 10 October 2019; Accepted 26 October 2019; Published 5 December 2019

Academic Editor: Ruibing Wang

The objective of this study was to compare the antitumor activity of lipophilic bismuth nanoparticles (BisBAL NPs) and chlorhexidine (CHX) on human squamous cell carcinoma. BisBAL NPs were synthesized by colloidal method and characterized by energy dispersive X-ray spectroscopy in conjunction with scanning electron microscopy (EDS-SEM). The effect of BisBAL NPs and CHX on oral cancer cell line (CAL-27) and nontumor control cell human gingival fibroblasts (HGFs) was determined by MTT cell viability assay. The obtained results showed selective inhibition of CAL-27 cell growth by BisBAL nanoclusters. A 24 h exposition to 25 μM BisBAL NP decreased 91% of CAL-27 cell growth, while nontumor HGFs cells were unaffected by BisBAL NPs showing 90% of cell viability. In contrast, CHX kills both CAL-27 and HGFs with the same efficacy. 25 μM of CHX decreased 97% and 80% of tumor and nontumoral cell growth. BisBAL NP and CHX alter cell permeability suggesting that action mechanism may include loss of cell membrane integrity. Also, CHX and not BisBAL NP presented genotoxicity on genomic DNA of tumor cells. As conclusion, BisBAL NPs have a selective antitumor activity on human squamous cell carcinoma, unlike CHX which was cytotoxic for both tumoral and nontumoral control cells.

1. Introduction

Head and neck squamous cell carcinomas (HNSCC) are important public health challenges worldwide, being the 11th most common cancer detected with approximately 50-300, 000 new cases each year [1, 2]. It is expected that 10, 030 people die in 2018 due to these cancers only in the USA, but the number could be higher in less developed regions [2]. Different risk factors have been described like alcohol, smoking, human papillomavirus (HPV), dietary deficiencies, and fungal infections [3]. HNSCC are progressive chronic diseases in which healthy epithelium is modified into a carcinogenic tissue through one or more risk factors in each specific individual. HNSCC are more frequent in men than in women and their prognosis is poor, especially in late diagnosis [4].

Although chemotherapy is an important treatment option for most cancers after surgery, it has several limitations, amongst which are a lack of target specificity, the development

of resistance, and severe side effects [5]. For example, the side effects of the commonly used anticancer agent cisplatin (CIS) include nephrotoxicity, neurotoxicity, bone marrow suppression, and vomiting [6, 7]. The use of the anticancer drug, docetaxel (DCT), is limited due to liver injury [8, 9], while doxorubicin (DOX) induces cardiotoxicity and resistance [10]. Unfortunately, these kinds of drugs encourage patients to leave chemotherapy due to their nondesired secondary effects. It is urgent to develop selective drugs for cancer treatment.

CHX is a broad-spectrum antimicrobial agent that is found as an active ingredient in mouthwash and is frequently used in dentistry to reduce bacterial load. Previous reports have described the *in vitro* antitumoral effect of CHX on breast and oral cancer cell lines [11, 12]. CHX competes effectively with positive drugs like Adriamycin; however, it is cytotoxic for nontumoral cells [13]. Earlier, it was described that CHX inhibits protein-protein interactions mediated by the antiapoptotic protein Bcl-xL at physiological concentrations inducing apoptosis in several tumor cell lines derived from the tongue and pharynx [14].

Nanomedicine is a recent discipline that uses nanotechnology to develop "*smarter drugs*" [15] that promise to be more efficient than traditional anticancer drugs [16]. Nanomedicine constitutes an interesting field to develop new antitumor drugs. In the last years, several reports have described the employment of different kinds of nanoparticles to treat cancer, underlining selenium, silver, and zinc nanoparticles and nanoformulations for drug delivery [17–20]. Earlier, our group described the selective antitumor activity of lipophilic bismuth nanoparticles (BisBAL NPs) on breast human cancer cells showing a specific and dose-dependent phenomenon [21]. However, it is unknown if BisBAL NPs are effective against oral cancer cells.

The aim of this study is to compare the antitumor effect of BisBAL NPs and CHX on human squamous cell carcinoma. Our hypothesis is that BisBAL NPs will be better as an antitumor agent than CHX based on their lipophilic and cationic properties.

2. Material and Methods

2.1. Synthesis and Characterization of BisBAL NPs. The BisBAL NPs were prepared by colloidal method like previously described [22]. Briefly, 0.485 g of $Bi(NO_3)_3{\cdot}5H_2O$ was dissolved in 20 mL propylene glycol, heated to 80°C, and agitated for 2 h to obtain a 50 mM Bi^{3+} solution. A 2 : 1 molar ratio of Bi^{3+} (Bis) to 2,3-dimercapto-1-propanol (BAL) was prepared by adding 25 μL 10 M BAL to 10 mL 50 mM Bi^{3+} solution. The distribution of BisBAL NP's size and shape were analyzed with scanning electron microscopy (SEM; FEI Tecnai G2 Twin, Hillsboro, OR, USA; 160 kV accelerating voltage). The specific presence of bismuth was corroborated by energy-dispersive X-ray spectroscopy (EDS) SEM (Oxford INCA X-Sight, Tubney Woods, UK).

2.2. Cell Culture. The human squamous cell carcinoma (CAL-27) was obtained from the American Type Culture Collection (ATCC CRL-2095; Rockville, MD, USA). The primary culture of human gingival fibroblasts (HGFs) was used as a nontumor control cells. The cell were cultivated in Dulbecco's modified Eagle's Medium/Ham's F12 (DMEM/F12) supplemented with 10% fetal bovine serum (FBS; Gibco Invitrogen, Carlsbad, CA, USA), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Sigma-Aldrich Corporation, St. Louis, MO, USA) in cell culture flasks (Corning Inc., Corning, NY, USA) at 37°C in a humidified atmosphere with 5% CO_2. Cells of a confluent monolayer were harvested by scraping, washed three times with 10 mM phosphate-buffered saline, pH 7.4 (PBS), and counted with a hemocytometer.

2.3. MTT Cell Viability Assay. The effect of BisBAL NPs on the viability of human squamous cell carcinoma (CAL-27) and nontumor control cells (HGFs) was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay (Biotium, Hayward, CA, USA) [21, 23]. Briefly, 1×10^5 cells were incubated (24 h, 37°C, 5% CO_2) with 0, 1, 10, 25, 50, and 100 μM of BisBAL NP or CHX (Ultradent Products, South Jordan, UT) in solution and 10 μM docetaxel (Zurich Pharma, Mexico city, Mexico) as a positive control. The treatment was terminated by washing the cells with PBS. Next, cells were incubated with MTT (10 μL/well, 2 h, 37°C, 5% CO_2) in the dark according to the provider's instructions. Next, the medium was removed and 100 μL dimethylsulfoxide (DMSO) was added to dissolve the reduced MTT formazan product. To quantify the reduced MTT, the absorbance at 570 nm (A_{570}) was measured with a microplate absorbance reader (BioTek, Winooski, VT); DMSO served as a blank. The assay was performed in triplicate to assess the veracity of results.

2.4. Cell Membrane Permeability by Fluorescence Microscopy. To explore the effect of BisBAL NPs and CHX on the cell membrane of tumor cells, intracellular calcein-AM assay [24] and fluorescence microscopy were employed. This assay was used to analyze the cell membrane permeability of CAL-27 cells after a 24 h exposure to 0, 10, and 50 μM of BisBAL NP and 50 μM CHX; cells exposed to the pure culture medium, served as growth control. After incubation, cells were washed three times with PBS and stained with 2 μM calcein-AM (Biotium, Hayward, CA, USA) and 4′,6-diamidino-2-phenylindole (DAPI) (Abcam Inc., Cambridge, UK) for 30 min. at 37°C. Next, cells were washed again with PBS and air-dried in the dark. Cell morphology was observed with FITC and DAPI filters at 485 nm and 358 nm, respectively (Thornwood, NY).

2.5. DNA Ladder Assays. To evaluate the possible genotoxic effect of BisBAL NP on tumor cells, DNA ladder assays were carried out following the protocol previously described [25]. CAL-27 cells were exposed for 24 h to 50 μM BisBAL NPs and 50 μM of CHX, and as negative control, growing cells with only culture media were used. After treatment, tumor cells were collected and genomic DNA was extracted using Isolate II Genomic DNA Kit (Bioline, London, UK) following manufacturer's instructions. The obtained DNA samples were analyzed by electrophoresis in 1% agarose gel

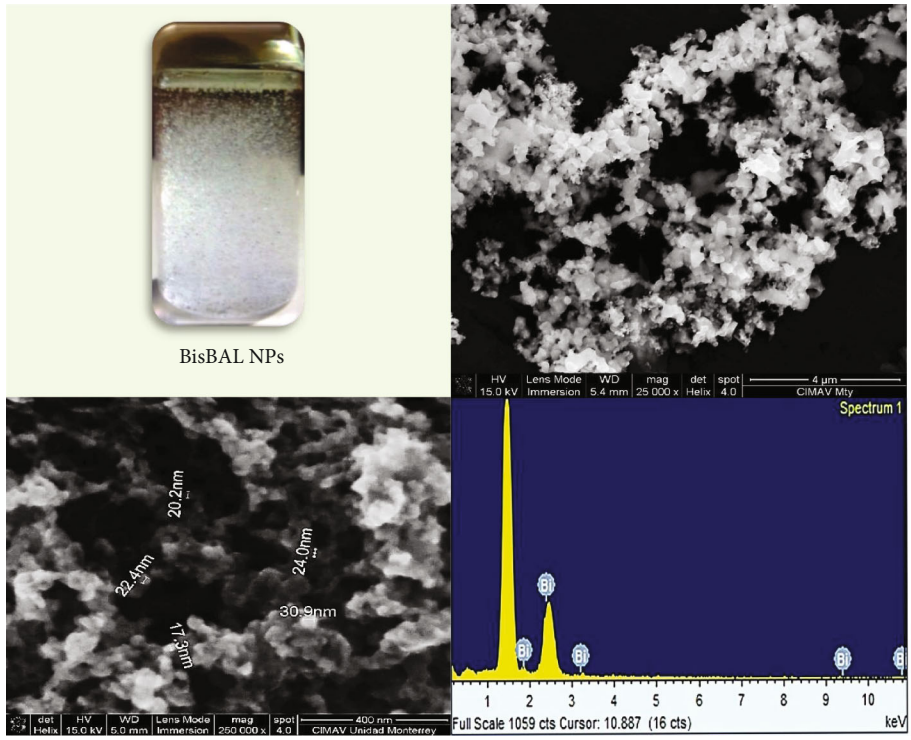

Figure 1: Characterization of BisBAL NPs. Shape, size, and distribution of BisBAL NPs were obtained by scanning electron microscopy (SEM). The specific presence of bismuth was confirmed by energy-dispersive X-ray spectroscopy (SEM-EDS).

and ethidium bromide staining. The presence of several DNA fragments (ladder shape) or a tail will indicate a damaged DNA.

2.6. Statistical Analysis. A multiple comparison 2-way ANOVA with Tukey's correction was used to compare among groups. For all statistical tests, a significance level of $\alpha = 0.05$ was considered.

3. Results

3.1. Characterization of BisBAL NPs. The obtained BisBAL NPs were round in shape with an average diameter of 24 nm (Figure 1). Bismuth presence was specifically confirmed by EDS-SEM (Figure 1). The BisBAL NPs formed electrodense clusters, a typical characteristic of this kind of nanostructures (Figure 1). A fresh batch of BisBAL NPs was used to study the antitumor activity on human squamous cell carcinoma.

3.2. Antitumor Activity. BisBAL NPs inhibited 86% of the growth of the CAL-27 cell line since 10 μM BisBAL NP after 24 h exposition (Figure 2). The highest reduction of tumor cell growth was obtained at 50-100 μM BisBAL NP, with 90% of inhibition in comparison with growth control (Figure 2). On the other hand, control nontumor cell HGFs showed 84% of cell viability when they were exposed to 50 μM BisBAL NP (Figure 2). The positive control docetaxel (a commercial antitumor drug) at a final concentration of 10 μM inhibits 98% of tumor cell growth, but at the same time reduces 50% of nontumor cell HGFs growth. In the case

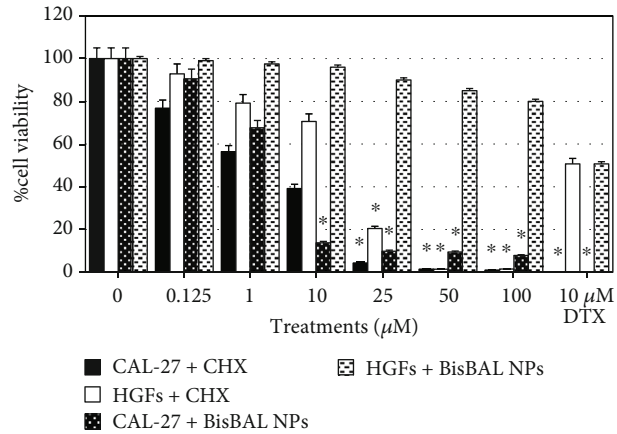

Figure 2: Antitumor effect of BisBAL NPs and CHX on CAL-27 and HGF cell lines. Cell viability was evaluated with the MTT cell viability assay after a 24 h exposure to 0, 0.125, 1, 10, 25, 50, and 100 μM BisBAL NP or CHX and 10 μM DTX (positive control of cytotoxicity). Readings were performed in triplicate. Results are representative of three independent experiments. After a multiple comparison 2-way ANOVA with Tukey's correction, asterisk indicates statistical differences ($p < 0.0001$) ($\alpha = 0.05$). Error bars indicate mean ± SD ($n = 7$).

of CHX, the highest reduction of CAL-27 cell growth was achieved at 25 μM with a 97% decrease, but also inhibited 80% that of nontumor cells (Figure 2). Altogether, these results suggest that only BisBAL NPs have a selective antitumor activity on CAL-27 cell line and compete in efficacy with commercial antitumor drugs.

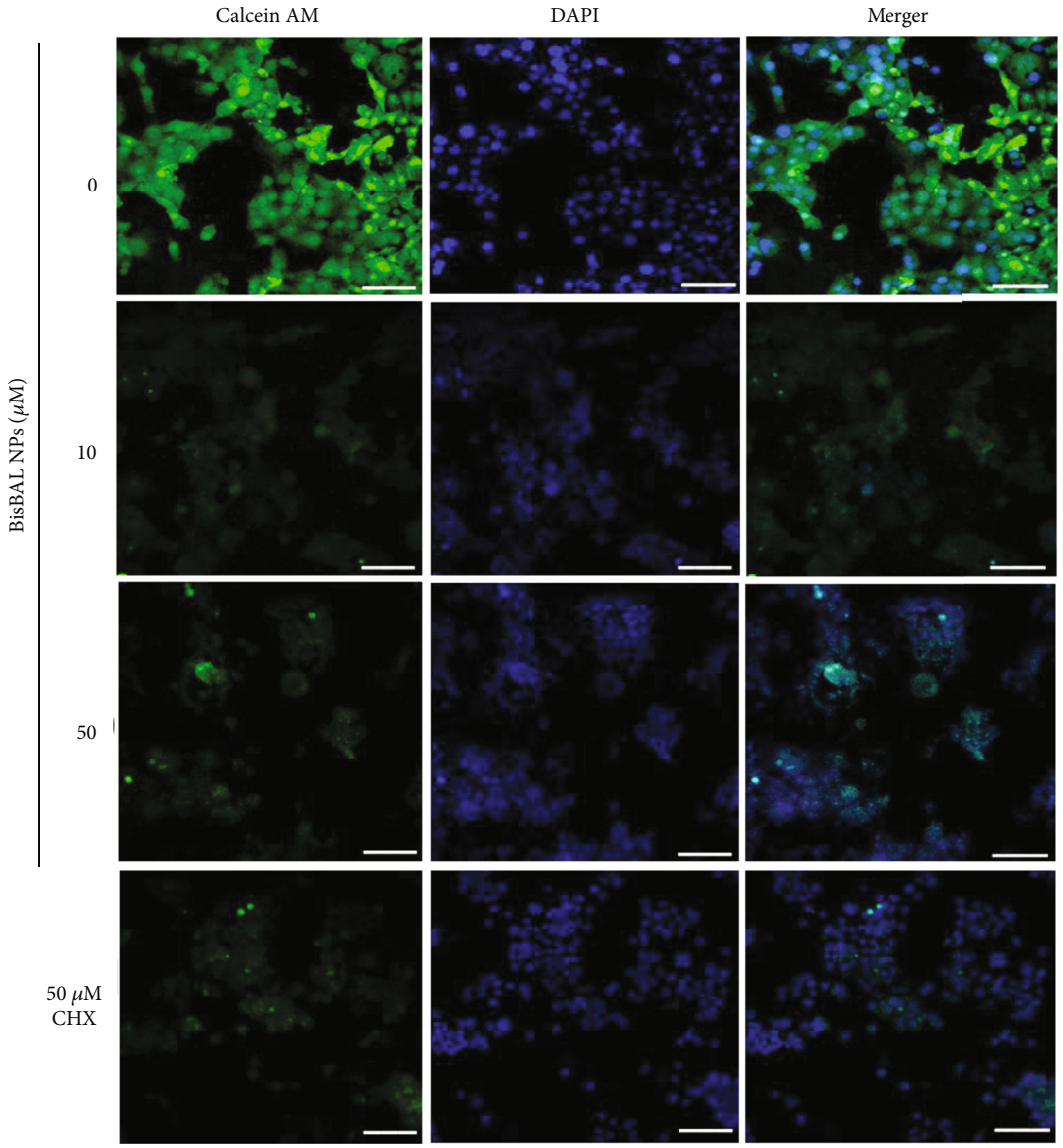

Figure 3: Cell membrane permeability of tumoral cells was determined after exposition to BisBAL NP or chlorhexidine by calcein-AM assay and fluorescence microscopy. CAL-27 cells were treated with 0, 10, or 50 μM of BisBAL NP or 50 μM of CHX for 24 h. After exposition, cells were stained with calcein-AM and DAPI. Bar indicates 5 μm.

3.3. Cell Membrane Permeability Assays. To get an insight about the action mechanism of BisBAL NP and CHX on tumoral cells, cell membrane permeability assays were carried out to explore possible damage at the cell membrane of CAL-27 and HGF cells. After 24 h exposition to 0, 10, and 50 μM of BisBAL NP and 50 μM of CHX, cell morphology was observed by fluorescence microscopy. Tumoral cells showed clear damage after being exposed to 10 μM of BisBAL NPs, and it was more evident when 50 μM of BisBAL NPs were used (Figure 3). Similar results were obtained when tumoral cells were treated with 50 μM of CHX (Figure 3). On the other side, HGFs treated with 0-50 μM of BisBAL NP did not show evidence of cytotoxicity as can be seen in Figure 4. Fibroblasts look their typical long shape and also the nucleus lack signals of toxicity after exposition to 10-50 μM of BisBAL NP. In contrast, 50 μM of CHX showed a strong cytotoxic effect on HGFs (Figure 5), promoting calcein-AM release and amorphous nucleus. These data support our previous results about a selective antitumor activity of BisBAL NPs on human squamous cell carcinoma unlike CHX.

3.4. DNA Ladder Assays. When the genotoxic effect of BisBAL NP and CHX was studied on tumoral cells, DNA ladder assays were developed. Our findings showed a typical ladder shape in the sample treated with 50 μM of CHX (Figure 4, line 4). In contrast, DNA genomic exposed to 50 μM of BisBAL NP did not show signals of degradation (Figure 4, line 3), presenting a band similar to DNA genomic control (Figure 4, line 2). These results suggest that only CHX and not BisBAL NP present a genotoxic effect on tumoral cells.

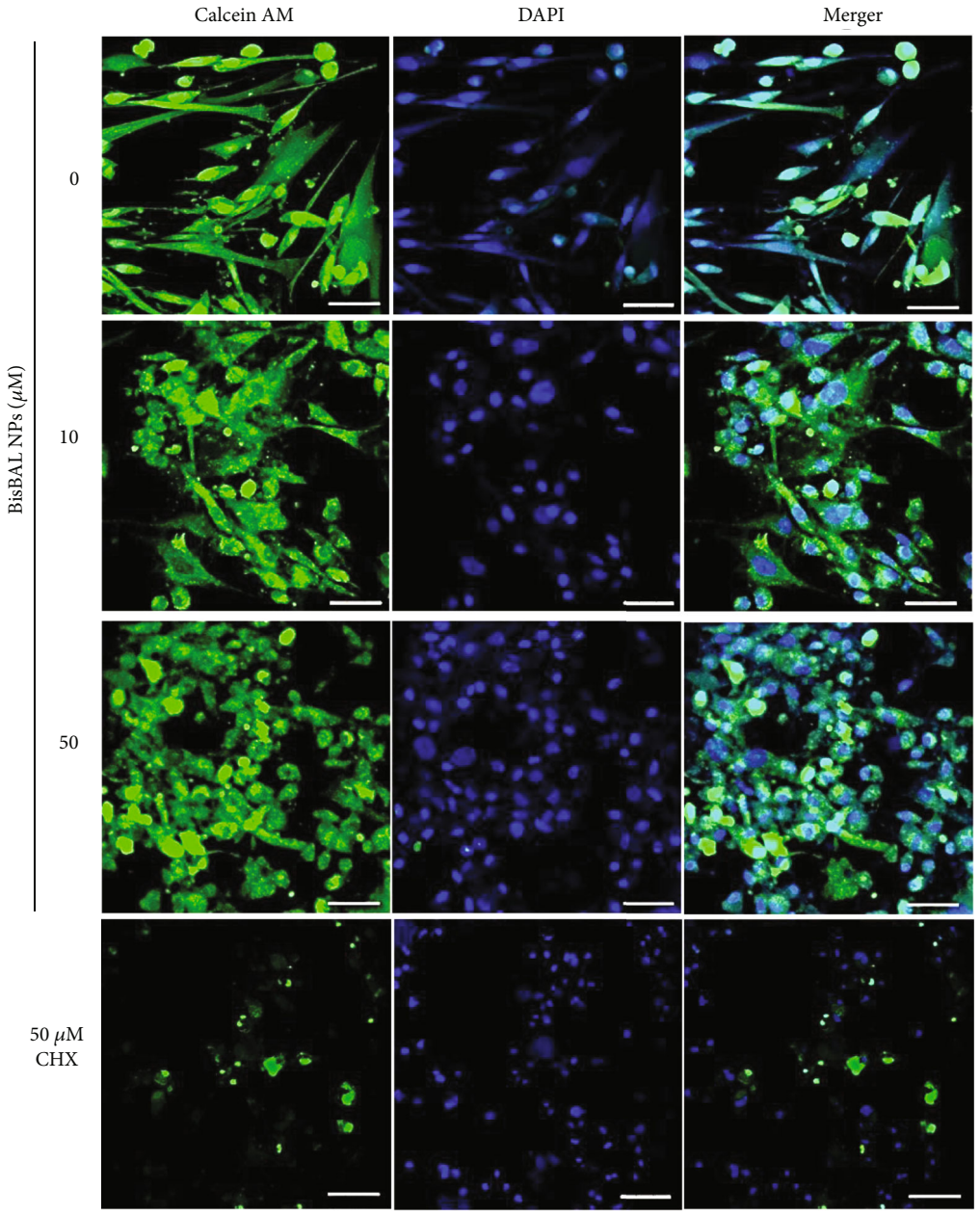

Figure 4: Cell membrane permeability of nontumoral control cells (HGFs) was determined after exposition to BisBAL NP or chlorhexidine by calcein-AM assay and fluorescence microscopy. HGF cells were treated with 0, 10, or 50 μM of BisBAL NP or 50 μM of CHX for 24 h. After exposition, cells were stained with calcein-AM and DAPI. Bar indicates 5 μm.

4. Discussion

The first option of treatment for patients with oral cancer is surgery if it is possible [26]. However, the employ of chemotherapy and immunotherapy in oral cancer has increased recently. With the objective of reducing the incidence of metastatic recurrence, chemotherapy is advisable after surgery in most types of cancer [26]. In spite of good intentions, alone or in combination with radiation, chemotherapy can cause strong cytotoxic effects on nontumor cells of healthy tissue leading to cancer patients leaving treatment. Antitumor drugs like doxorubicin, docetaxel, and cisplatin have severe adverse side effects such as nephrotoxicity, bone marrow suppression, and cardiotoxicity. Early updates of meta-analysis evaluating the effectiveness of chemotherapy in the treatment of patients with oral cancer did not show benefit [27]. Therefore, it is urgent to develop selective and biocompatible anticancer drugs. Nanotechnology is a new area that explores biomolecular structures, developing "*smart drugs*" with a lot of applications in material science and medicine. Nanostructures are more reactive than common elements or molecules with a higher ratio of volume/area on their surface. Metal nanoparticles with different biological properties have been described like antibacterial, antimycotic,

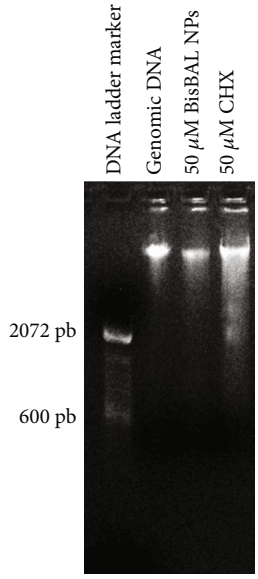

Figure 5: Effect of BisBAL NP and CHX on genomic DNA of CAL-27 cell line. Genomic DNA was extracted from tumoral cells exposed to 50 μM of BisBAL NP or 50 μM CHX for 24 h. DNA was analyzed by electrophoresis in agarose gel and ethidium bromide staining.

antibiofilm, antitubercular, and antiviral activities [28, 29]. With antitumor activity, several metal nanoparticles have been reported including silver, iron, zinc, selenium, copper, iron, and vanadium [18, 19, 30]. In this study, we synthesized BisBAL NP by colloidal method and they were characterized by SEM like several early reports of our group [21, 22, 31, 32]. BisBAL NP showed the same circular shape, with an average size of 24 nm, and agglomerates were evident in SEM images.

In this work, we tackle the lack of selectivity among antitumoral drugs and describe the antitumor activity of BisBAL NPs and CHX on human squamous cell carcinoma. Early reports have described the antitumor properties of CHX [11, 12]; however, its antitumor activity was not compared with control nontumor cells at the same time to evaluate its selectivity against tumor cells. Our results show that CHX lacks selectivity inhibiting the growth of both tumor and control nontumor cells after 24 h of exposition at a final concentration of 25 μM. Previously, it was reported that 237.7 μM of CHX inhibited 80% of AW13516 cell (poorly to moderately differentiated squamous cell carcinoma of the tongue) growth [12]. In comparison with our results, we obtain higher inhibition of tumor cell growth with 10 times less concentration of CHX. Several previous studies have described the cytotoxic effect of CHX on different kinds of oral cells including fibroblasts and odontoblasts [13, 33–35]. Our results support these reports because 25 μM of CHX interfered with 80% of human gingival fibroblast employed as noncancer cells. BisBAL NPs inhibit 90% of the tumor cell growth when 25 μM was added to the cultured cells. This datum agreed with our early report employing the same nanoclusters on breast cancer cells [21]. Early reports have been described the antitumor activity of silver nanoparticles [36, 37]; however, they cannot evaluate their effect on control nontumor cells at the same time. Unlike CHX, BisBAL NPs showed a selective antitumor activity with more than 80% of cell viability of noncancer cells at 25 μM, suggesting that BisBAL NPs are an innovative alternative to tackle the lack of selectivity among antitumor drugs.

The action mechanism of BisBAL NPs to inhibit the tumoral cell growth seems to be based on their lipophilic properties. Our experiments with calcein-AM showed clear damage in the cell plasmatic membrane of tumoral cells post-treatment with BisBAL NPs leading to cell lysis. Tumoral cells exposed to CHX presented similar damage in their membrane, affecting their permeability. We hypothesize that the lipophilic character of BisBAL NP is associated with their selective effect on tumoral cells. Early reports or our group described an identical phenomenon with breast cancer cells [21]. Employing higher concentrations of BisBAL NPs could increase the damage of the cell membrane, thus promoting faster cell lysis. Interestingly, the genomic DNA of tumoral cells was not damaged after 24 h exposition with 50 μM of BisBAL NPs. In contrast, DNA of tumoral cells treated with 50 μM CHX showed a clear ladder shape, indicating a typical genotoxic effect. Altogether, these results suggest that CHX kill both tumoral and nontumoral control cells in the same way; altering their cell membrane permeability and damaging their genomic DNA lacking selectivity. Several early studies have been described as the cytotoxicity of CHX on different kinds of cells [13, 33, 38]. Previously, it was reported that 1% of CHX shows a cytotoxic effect on human gingival fibroblasts (same control cells used in our study) employing also MTT cell viability assays [39]. Also, it has been described that cytotoxicity and genotoxicity of CHX in a dose-dependent phenomenon on macrophages may be via ROS generation [40].

It is important to underline the low cost of synthesis of BisBAL NPs in comparison with the high cost of common antitumor drugs [41, 42], because, in developing countries, the cost of chemotherapy treatment is an important cause of leaving the treatment [43]. We estimate that bismuth nanoparticle synthesis is 140 times less expensive in comparison with doxorubicin or docetaxel, two of the most common antitumor drugs.

In conclusion, we present evidence of the effectiveness of a BisBAL NP hydrogel as a selective growth inhibitor of human cervix uterine, prostate, and colorectal cancer cell lines. BisBAL NP-loaded hydrogels may be a low-cost, innovative alternative for the topical treatment of cervical, prostate, and colon cancer without adverse effects on nontumor cells.

Data Availability

The data that support the findings of this study are available from the corresponding author, Claudio Cabral-Romero, upon reasonable request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This study was an approved project by the Sectorial Fund for Education Research, CONACyT (CB2017-2018 and A1-S-20148). The authors want to thank to Nayely Pineda-Aguilar from CIMAV-Unidad Monterrey for their technical assistance in SEM analysis of BisBAL NPs.

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