Barium titanate nanoparticles exhibit cytocompatibility in cultured bovine fibroblasts: a model for dermal exposure

ABSTRACT

The emergence of barium titanate nanoparticles ($BaTiO_3$ NPs) represents an advancement in various fields such as technology, health, and agribusiness. However, increased production heightens the risk of their dispersion into the environment, thereby raising concerns about potential exposure to animals and humans, including the risk of dermal exposure. This study explores the chemical-physical properties of $BaTiO_3$ NPs and their cytocompatibility using a bovine fibroblast cell model. The size and Zeta potential of the NPs were analyzed using scanning electron microscopy and dynamic light scattering technique. Raman spectroscopy was used to characterize the composition of the $BaTiO_3$ NPs. Bovine fibroblasts were exposed in vitro to NPs (0.1 to 100 µg mL^{-1}) for 24 hours to evaluate the cytocompatibility using the Thiazolyl Blue Tetrazolium Bromide assay. The data were evaluated by analysis of variance and the means compared by the Tukey test. Scanning electron microscopy revealed that $BaTiO_3$ NPs measured approximately 100 nm. Dynamic light scattering analysis indicated a hydrodynamic size of 149.27 nm with a polydispersion index of 0.37, and the Zeta potential was -13mV. Raman spectroscopy analysis highlighted the cubic phase of $BaTiO_3$ NPs. Cytotoxicity tests demonstrated that $BaTiO_3$ NPs did not affect cell viability, with 10 µg mL^{-1} resulting in enhanced cell proliferation. Overall, these findings underscore the non-toxic characteristics of $BaTiO_3$ NPs in fibroblast cells, positioning them as promising and cytocompatible nanomaterials.

Keywords: Ceramic nanomaterial; In vitro models; Piezoelectricity; Safe nanomaterials.

1. INTRODUCTION

Nanotechnology involves the manipulation of matter at atomic, molecular, and supramolecular levels to create novel materials, devices, and systems with unique properties and functionalities. With promising solutions across diverse domains, case studies abound, highlighting how nanoscale materials hold the potential to address contemporary challenges within the biomedical and agribusiness sectors [1,2]. The distinct ability to manipulate materials at the nanoscale makes nanotechnology unparalleled. On this scale, the forces governing matter differ significantly from those at the micro or macro scale [3]. Nanomaterials possess a defining characteristic: at least one dimension (height, width, or depth) measures less than 100 nanometers (nm), or 10^{-9} meters [4]. Consequently, nanomaterials exhibit unique physical and chemical properties, including significantly increased surface area and heightened reactivity compared to larger-scale materials [5].

Barium titanate nanoparticles ($BaTiO_3$ NPs) are perovskite bioceramic materials and have been considered emerging nanomaterials due to their ferroelectricity and piezoelectric properties [3,6]. Notably, recent reports suggest that $BaTiO_3$ NPs could be used in a wide

range of applications including imaging diagnostics [7,8,] neural stimulation networks [9], drug nanocarriers [10], tissue engineering [11,12], and agribusiness [13].

The increasing concern over the toxicity of $BaTiO_3$ NPs arises from their vast range of potential applications, given that the rapid spread of these nanomaterials could potentially impact both animal and human health. Previous studies have demonstrated that toxicological risks are related to the fact of substantial differences in the chemical and physical properties of nanoscale materials compared to the same materials in bulk form [14,15]. Current research on the *in vitro* cytotoxicity of $BaTiO_3$ NPs in biological models is restricted. Especially, these studies incorporated $BaTiO_3$ NPs alongside other materials, potentially mitigating toxicity factors through functionalization [16-18].

Exposure of the skin to nanomaterials poses potential risks that require thorough understanding and mitigation [19].Nanomaterials have the capability to penetrate the skin barrier and interact with underlying cells, potentially causing adverse effects such as cellular damage [20]. In this context, there is a gap regarding the possible toxic effects of skin exposure to $BaTiO_3$ NPs. Therefore, it is important to investigate the effects of nanomaterial exposure on skin cells using appropriate models. *In vitro* models utilizing cultured fibroblasts offer a valuable tool for studying cutaneous exposure to nanomaterials. Fibroblasts are a type of skin cell found in the dermis and play a crucial role in maintaining skin structure and function [21]. This *in vitro* cellular model allows for the study of cytotoxic mechanisms, encompassing changes in cell morphology, alterations in metabolism, and cell death, in response to exposure to materials [22,23]. Therefore, the present work aimed to study the chemical-physical characteristics of $BaTiO_3$ NPs and their cytocompatibility with bovine fibroblast cells model cultured *in vitro*.

2. MATERIAL AND METHODS

2.1 Materials

$BaTiO_3$ NP (Product Number 467634, CAS Number 12047-27-7, purity> 99%, density of ~6,08 g/cm^3), Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin antibiotics, HCl and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) thiazolyl blue tetrazolium bromide (MTT) assay kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypan Blue was purchased from Invitrogen (Carlsbad, CA, USA).

2.2 Dynamic light scattering analysis

$BaTiO_3$ NP suspensions (100 µg mL^{-1}) were dispersed in DMEM medium by sonication using an ultrasonic instrument for 1 minute (cycle 0.5, amplitude 70) (UP200S, Hielscher, Teltow, Germany). The average size and Zeta potential were determined using the dynamic light scattering (DLS) technique with the Malvern 3000 ZetasizerNanoZS equipment (Malvern Instruments, UK).

2.3 Scanning electron microscopy (SEM)

The size distribution and morphology of $BaTiO_3$ NPs were characterized through scanning electron microscopy (SEM) analysis. This analysis was conducted using a high-resolution Zeiss-DSM 950 microscope (manufactured by Zeiss, Germany) operating at an accelerating voltage of 6 kilovolts (kV).

2.4 Raman spectroscopy

Raman Spectroscopy was performed with a Bruker RFS 100 (Bruker, USA) spectrometer excited with a Nd + 3/YAG laser operating at 1064 nm, equipped with an InGaAs detector cooled with liquid nitrogen. The spectra were acquired at 4 cm^{-1} resolution. An average of 1024 scans were collected with a laser power rating of 110 mW.

2.5 Cell culture

Primary fibroblast cell cultures derived from bovine skin biopsies were stored in the cell bank of the Brazilian Agricultural Research Corporation. All relevant biosafety regulations were strictly followed, following the approved procedures of the Internal Biosafety Commission under protocol number 03/2012. To establish the cell bank, fibroblasts from the skin biopsies were cultured in DMEM supplemented with 10% FBS and 100 units/ml of penicillin-streptomycin. The cells were incubated at 37°C, 5% CO_2, and 95% humidity (Model 4130, Thermo Fisher Scientific, Waltham, MA, USA), and cryopreserved after the second passage. Before their use in this study, fibroblasts were thawed and subsequently cultured up to the third passage.

2.6 *In vitro* nanoparticle exposure

In vitro cytotoxicity assays were based on ISO 10993-5 [24]. Samples of $BaTiO_3$ NPs at a concentration of 5 mg mL^{-1} were dispersed in DMEM cell culture medium, supplemented with 10% FBS. Then, fibroblast cells (60% confluence) were seeded into 96-well plates at a $6x10^3$ cells/well. Cells were incubated with $BaTiO_3$ NPs at different concentrations: 0 (vehicle control), 0.1, 1, 10, 50, and 100 µg mL^{-1} for 24 hours.

2.7 Cell morphology analysis

Monitoring the growth and cell morphology of fibroblasts was performed using light microscopy (Nikon TS100F, Nikon Instruments Inc., Melville, NY, USA) to evaluate the possible impact of cell exposure to $BaTiO_3$ NPs on cell morphology.

2.8 Mitochondrial metabolism assay

Cell proliferation was evaluated after exposure of cells to different experimental groups by the Thiazolyl Blue Tetrazolium Bromide assay (MTT), which assesses mitochondrial activity. After NP exposure, MTT aliquots at the concentration of 5 mg mL^{-1} were prepared, and then incubated with the cell cultures using this solution in the culture medium, for 4 hours at 37ºC, in a humidified atmosphere containing 5% $CO_2$and 95% atmospheric air. After the culture medium was removed and isopropanol-acid 0.04 M HCl was added to complete the solubilization of the precipitate. Then, the absorbance was determined using the wavelength of 570 nm in the Eon Microplate Reader equipment (BioTek, Winooski, USA). Cell viability results in the groups exposed to $BaTiO_3$ NP were expressed as a percentage compared with the control group.

2.9 Trypan blue assay

The trypan blue exclusion test assessed the effect of $BaTiO_3$ NP on cell viability [23]. After NP exposure, the cells were trypsinized and stained with 0.4% Trypan Blue solution. Then, the cells were counted using a hemocytometer.

2.10 Statistical analysis

Data from the cell viability assay were evaluated by variance analysis (ANOVA) and the means were compared by the Tukey test. P values lower than 0.05 were considered significant. The results were presented as means ± standard error (EP) of the mean.

3. RESULTS AND DISCUSSION

Initially, we carried out the physicochemical characterization of the $BaTiO_3$ NPs to gain deeper insights into their inherent properties. The hydrodynamic size of the $BaTiO_3$ NPs was measured at 149.27±0.81 nm, with a polydispersion index of 0.37. Furthermore, the suspension of $BaTiO_3$ NPs displayed a Zeta potential of -15.3±0.78 mV. The dispersion status of nanomaterials can significantly influence their impact on biological systems [25]. A polydispersion index of 0.37 characterizes a moderately polydisperse sample [26], suggesting a potential instability of the $BaTiO_3$ NP suspension. Zeta potential, also known as electrokinetic potential, serves as an indicator of dispersion stability and is a crucial factor influencing the interaction of nanomaterials with cellular components. Generally, values exceeding 30 mV (in modulus) for particulate matter are deemed stable due to electrostatic repulsion [27]. Instabilities in the suspension may result in the formation of clusters consisting of weakly linked nanomaterials, or aggregates comprising strongly bonded nanomaterials that are challenging to separate. These occurrences are common when the Zeta potential falls below 20 mV [28].

The morphological assessment of the $BaTiO_3$ NP suspensions obtained via SEM revealed the presence of aggregates (Figure 1), with the nanoparticles appearing to be approximately 100 nm in size.

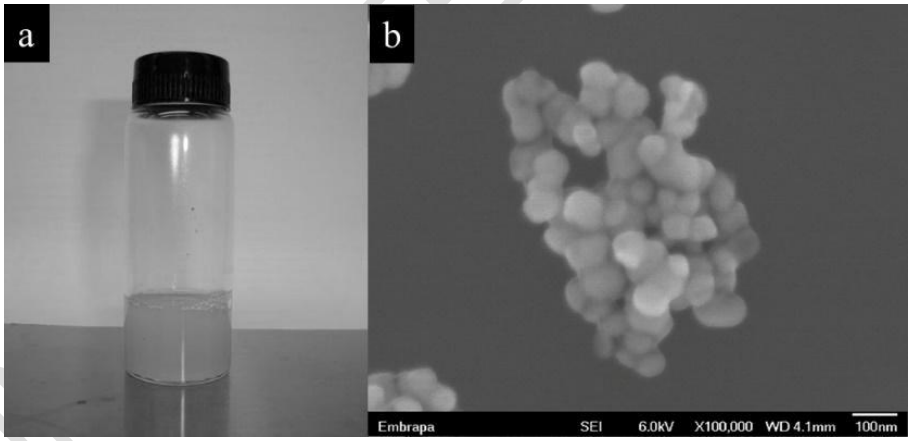

Fig. 1. (a) Suspensions of barium titanate nanoparticles (BaTiO3 NPs) in Dulbecco's Modified Eagle Medium (100 µg mL^{-1}). (b) Scanning electron microscopy (SEM) images of BaTiO3 NPs. Scale bar =100 nm.

The size of the nanomaterial is a critical determinant of its toxicity, which is essential for any biotechnological application [29].Nanomaterial size can affect their penetration into cells, tissues, and organs, as well as their increased interaction with specific membrane receptors, facilitated by their highly reactive nature due to free atoms on their extensive surface area [30]. This diminutive nanomaterial size can induce oxidative stress, DNA damage, and cell death [4, 31,-33]. In this investigation, the moderate polydisperse sample of $BaTiO_3$ NPs

exhibited an average size of 149.27±0.81 nm, indicating a dispersion comprising small particle sizes and a limited number of aggregates, as observed in the SEM image (Figure 1B), likely attributable to the low modulus value of the Zeta potential.

Raman spectroscopy was used to examine the structure of BaTiO3 NPs. Raman peaks of BaTiO3 NPs located at 192 cm^{-1}, 306 cm^{-11}, 511 cm^{-1}, 716 cm^{-1} assigned at [A1(TO), E(LO)]; (B1, E(TO + LO)); (A1(TO), E(TO)) and (A1(LO), E(LO)), respectively (Figure 2). In Raman measurements, the characteristic feature of the tetragonal $BaTiO_3$ NPs phase is observed around 306 cm^{-1}. The broad band at 513 cm^{-1} can be attributed to the intrinsic cubic phase (Figure 2), corroborating data available in the literature that characterizes these nanomaterials with cubic and tetragonal formats [34-37].

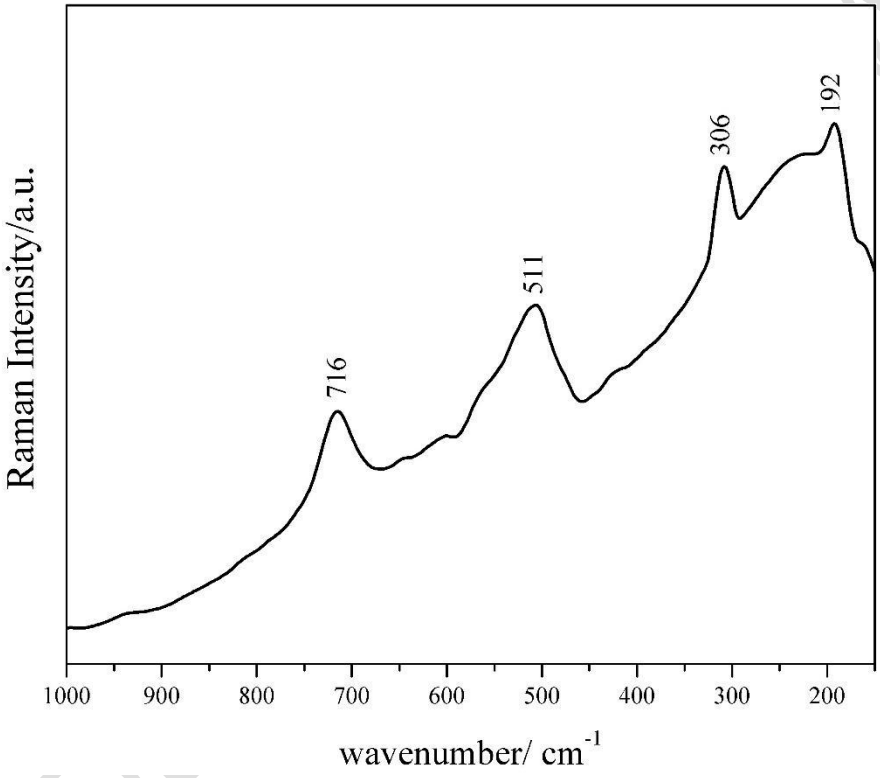

Fig. 2. Raman spectra of barium titanate nanoparticles (BaTiO3 NPs).

Cells were exposed to different concentrations (0.1-100 µg mL^{-1}) of $BaTiO_3$ NPs for 24, 48 h, and cell viability was measured by microscopy analyses, MTT and Trypan Blue assays. The evaluation of cellular morphology is a critical parameter in nanotoxicity studies [38]. In our current study, we observed no significant changes in the cellular morphology of fibroblasts at any concentration of $BaTiO_3$ NPs compared to the control group (Figure 3).

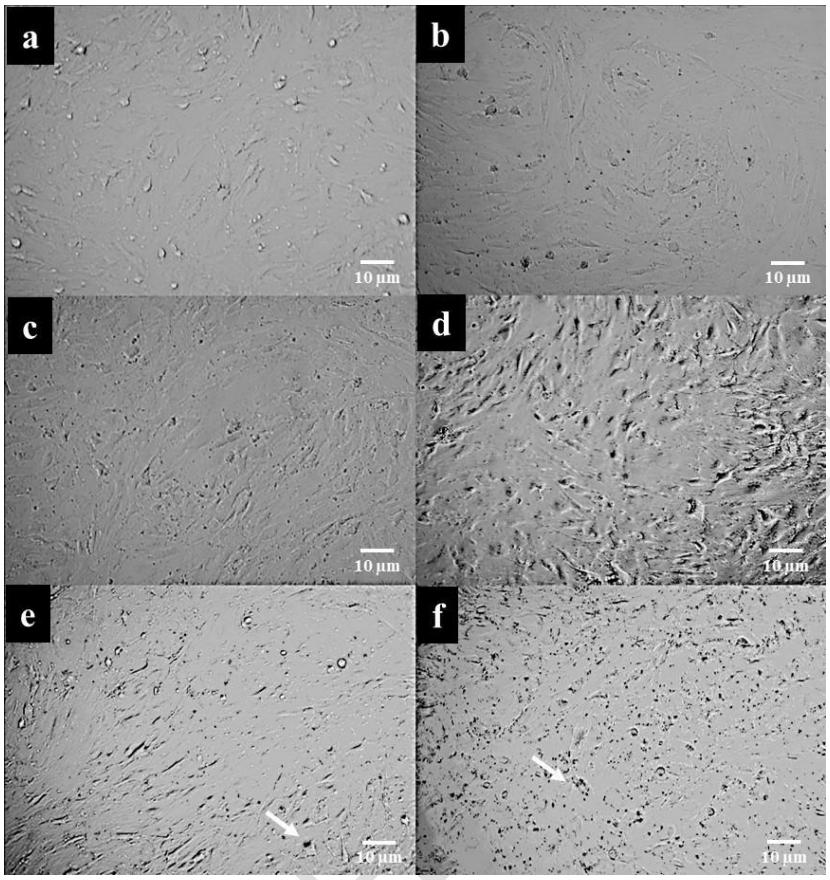

Fig. 3. Cellular morphology evaluation of bovine fibroblasts exposed to barium titanate nanoparticles ($BaTiO_3$ NPs) for 24 hours. (a) 0 (Control), (b) 0.1, (c) 1, (d) 10, (e) 50, and (f) 100 µg mL^{-1}. Magnification 100×. Scale bar = 10 µm.

Similar findings were reported by Amaral et al. [12] who did not detect any changes in the morphology of human mesenchymal stem cells in vitro exposed to $BaTiO_3$ NPs. However, several nanomaterials induce cellular toxicity through the generation of reactive oxygen species, which can impact the dynamics of microtubules, thereby modifying cell morphology [39,40]. Ahamed et al. [41] reported alterations in cell morphology among human lung carcinoma (A549) cells exposed to $BaTiO_3$ NPs, attributing these changes to oxidative stress. However, our investigation did not reveal any significant changes in cell morphology following exposure to BaTiO3 NPs. One potential explanation could be attributed to the specific cellular model utilized, as exposure routes such as air and skin contact may induce distinct toxicological responses.

Importantly, aggregate points of the nanoparticles were evident at concentrations of 50 and 100 µg mL^{-1}, as depicted by the arrows (Figure 3E, F). Despite studies in the literature demonstrating that nanoparticle aggregation induces nanotoxicity [42], our study showed that aggregation also did not induce cytotoxic responses. The presence of $BaTiO_3$ NPs aggregates might have been influenced by the composition of the culture medium containing fetal bovine serum proteins. Consequently, a future study could investigate the impact of varying concentrations of fetal bovine serum on $BaTiO_3$ NP toxicity.

Based on the MTT assay $BaTiO_3$ NPs, except for at 10 µg mL^{-1}, did not significantly impact cell proliferation when compared to the non-exposed control (P>0.05) (Figure 4).

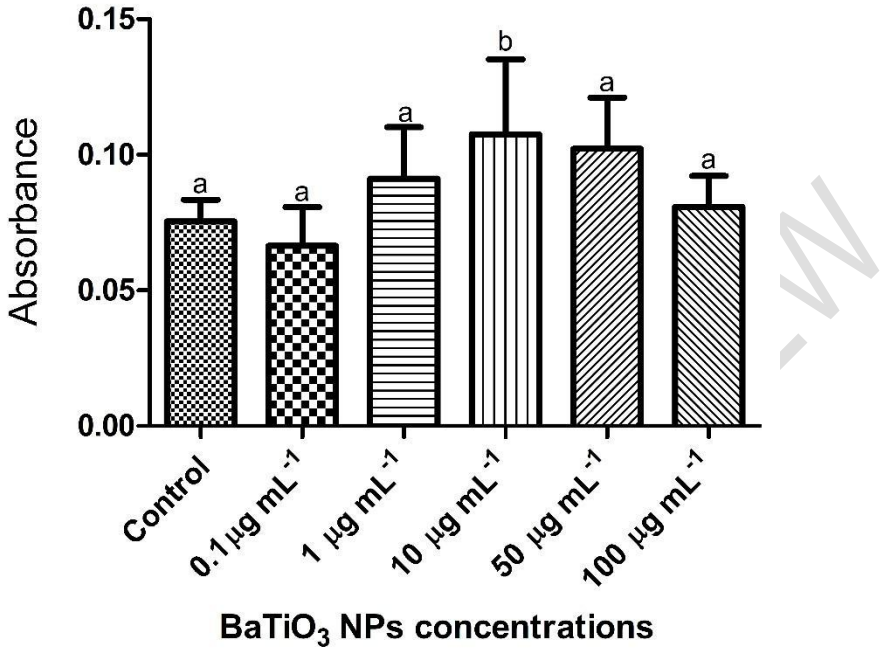

Fig. 4. Analysis of bovine fibroblast cell proliferation exposed to barium titanate nanoparticles ($BaTiO_3$ NPs) using the MTT assay. Mean percentage of viable cells after exposure to concentrations of 0 (control). 0.1, 1, 10, 50 e 100 µg mL^{-1} of $BaTiO_3$ NPs for 24 hours. *P<0.05.

Notably, the concentration of 10 µg $mL^{-1}BaTiO_3$ NPs led to an increase in cell proliferation compared to the control group (P<0.05). The MTT assay, a common colorimetric test used to evaluate cell viability, operates by converting tetrazolium salt into formazan crystals through a mitochondrial membrane, serving as an indicator of cell proliferation [43,44]. Several studies in nanotoxicology utilize this test as an initial parameter to assess the toxicity of nanomaterials [12, 45-47]. In our study, the 10 µg mL^{-1} concentration of $BaTiO_3$ NPs promoted cell proliferation compared to the control group, consistent with previous research demonstrating enhanced adhesion and proliferation of human osteoblastic cells following exposure to $BaTiO_3$ NPs [48]. The increase in cell proliferation at a concentration of 10 µg mL^{-1} might be attributed to the potential stimulation of specific cell signaling pathways or the facilitation of cellular metabolic activities, potentially leading to enhanced cell division. Additionally, this enhanced cell proliferation could be associated with the cytocompatibility of $BaTiO_3$ NPs at this specific concentration, indicating a favorable response of the cells to the nanoparticle stimulus.

On the other hand, the present study did not detect changes in cell proliferation at concentrations of 50 and 100 µg mL^{-1}. This finding is consistent with prior studies, which similarly showed no differences in the MTT test when human mesenchymal stem cells were exposed to $BaTiO_3$ NPs for 24 h, even at elevated concentrations [12]. These results are likely attributed to the observed increase in the formation of nanoparticle aggregates at

concentrations of 50 and 100 µg mL^{-1} $BaTiO_3$ NPs, thereby reducing the availability of free nanoparticles for interaction with cells.

A Trypan Blue test was conducted to investigate the cell viability of fibroblast cells cultured with $BaTiO_3$ NPs (Figure 5). This assay relies on the principle that cells with intact membranes exclude Trypan blue dye [21]. The results demonstrated that none of the $BaTiO_3$ NP treatments significantly altered cell viability when compared to the control group ($P>0.05$).

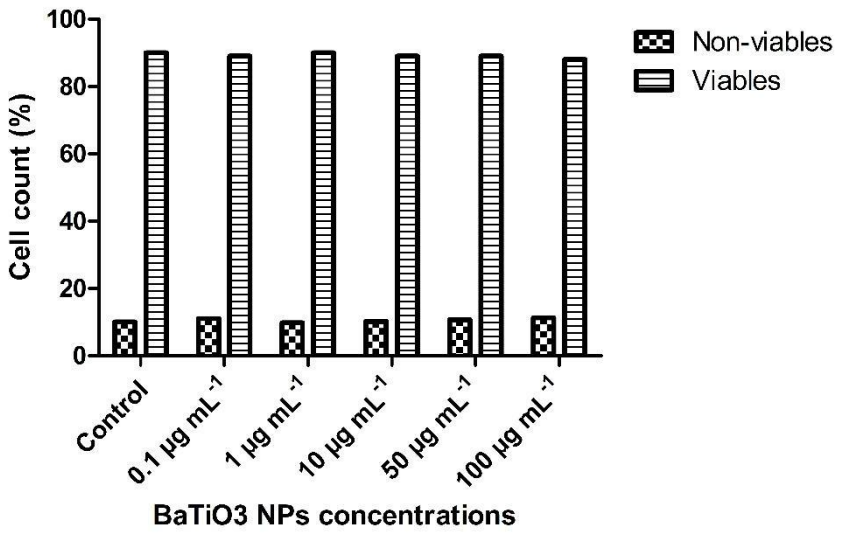

Fig. 5. Analysis of bovine fibroblast cellular viability exposed to barium titanate nanoparticles ($BaTiO_3$ NPs) using the Trypan Blue Assay. Mean percentage of viable cells after exposure to concentrations of 0 (control). 0.1, 1, 10, 50 e 100 µg mL^{-1} of BaTiO3 NPs for 24 hours.

Similarly, a previous study reported no decrease in cell viability in vitro when stem cells were exposed to $BaTiO_3$ NPs at the same concentrations as in our study [12]. However, another study has demonstrated that the cell viability of A549 cells exposed to concentrations of up to 50 µg mL^{-1} $BaTiO_3$ NPs for 24 h was reduced [41]. The differing responses observed in these studies may be attributed to the specific cell lineage under evaluation. It is plausible that the physiological characteristics of the fibroblast cells, including their innate resistance to external stressors and their capacity to adapt to various environmental conditions, played a role in maintaining cell viability despite exposure to $BaTiO_3$ NPs. Moreover, the intricate interplay between the physicochemical properties of the $BaTiO_3$ NPs and the cellular microenvironment could have influenced the cells' response. This includes parameters such as the surface charge, size, and aggregation status of the nanoparticles, as well as the composition of the cell culture medium and the presence of serum components, which might have interacted with the nanoparticles, thereby influencing their overall cytocompatibility.

4. CONCLUSION

Under the experimental conditions of this study, $BaTiO_3$ NPs exhibited cytocompatibility with bovine fibroblasts, with a notable observation that at a concentration of 10 µg mL^{-1}, $BaTiO_3$ NPs led to increased cell proliferation. These findings provide valuable insights into the safety profile of $BaTiO_3$ NPs, particularly in their potential applications within the medical and agricultural industries.

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 where applicable. All experiments have been examined and approved by the appropriate ethics committee.

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