Original Research Article

Inhibitory Effect of Gallium Nitrate Against Staphylococcus aureus and Its Action on The Formation of Biofilms on Titanium Surfaces

ABSTRACT

Aims: Under the era of seeking new potential agents able to weaken the development and spread of multi-resistant bacteria towards traditional drugs, the use of the semimetal gallium (Ga^{3+}) has stood out as an important strategy due to its physicochemical characteristics. This study aimed to investigate the effect of gallium nitrate [$Ga(NO_3)_3$], an inorganic antimicrobial agent, against the growth and biofilm formation of *Staphylococcus aureus*.

Study design: This study is a laboratory investigation involving the determination of the inhibitory concentration (IC) of $Ga(NO_3)_3$ against the planktonic strain of *S. aureus* and biofilms formation on titanium coupons.

Place and Duration of Study: Sample: Center of Science and Technology for Sustainability (CCTS), Federal University of São Carlos, SP, Brazil, between March 2020 and March 2022.

Methodology: The inhibitory concentration of gallium nitrate was determined in a 96-well microtiter plate in Muller Hinton broth. The potential of the antimicrobial agent to inhibit biofilm formation by *S. aureus* on titanium surfaces was evaluated by Scanning Electron Microscopy (SEM). The cytotoxicity potential of $Ga(NO_3)_3$ was determined on V79 cells.

Results: The results showed that the susceptibility of gallium nitrate against S. aureus was 1.40 µM, while SEM images revealed that concentrations of 90 µM inhibited biofilm formation by S. aureus.

Conclusion: This research has shown promising results regarding gallium nitrate's potential of inhibiting the growth of both planktonic and sessile S. aureus cells. In addition, coating titanium surfaces with $Ga(NO_3)_3$ would be an extra alternative to prevent implant-associated infections due to its non-toxicity to cells.

Keywords: antimicrobial; biofilms; gallium nitrate; Staphylococcus aureus; titanium.

1. INTRODUCTION

Despite being considered a Gram-positive opportunistic pathogen, *Staphylococcus aureus* is found in both the environment and as part of the human microbiota, colonizing the skin and nasal mucosa of healthy individuals (Tong et al., 2015). The strains cross the border of pathogenicity when entering the bloodstream or internal tissues, causing a wide variety of infections. As its reproduction can occur aerobically and anaerobically at temperatures ranging from 18 to 40 °C, several clinical manifestations are favored by these conditions (Le and Otto, 2015; Rasigade and Vandenesch, 2014).

The areas most affected by *S. aureus* include skin and soft tissues, originating infections such as folliculitis, bacteremia, and pneumonia. In addition, medical devices are also impacted by these bacteria, due to their versatility when found in different hosts and environments or for being capable of withstanding a wide range of temperatures (Tong et al,. 2015; Unakal, 2022;).

For decades, titanium devices have been reported as a product of successful prosthetic rehabilitation and dental implants based on their biocompatibility and stability. However, despite the prosperous rise of these instruments, they were also found to exhibit biofilm-associated infections (Thukkaram et al., 2020). In the past, the risk of failure in these titanium-based instruments was still considered significant, as a result of incompatibility between tissue and biomaterial, surgical trauma, and material imperfections. However, while these barriers were overcome, another issue arose: the emergence of bacterial infections - a contemporary problem with yet no clear solution (Godoy-Gallardo et al., 2014).

In order to prevent infections associated with bacterial biofilms on titanium devices, surface modification and chemical element deposition have been reported, avoiding bacterial colonization. The association of silver, zinc, copper, or gallium ions with titanium surfaces produced inhibitory effects against biofilms (Dong et al*.,* 2017; Giti et al., 2021; Juan et al., 2010; Petrini et al., 2006; Zhu et al., 2015).

As a result of its toxicity to bacteria, antimicrobial metal compounds containing gallium (Ga^{3+}) – a transition metal element from group 13 of the periodic table with relevant chemical properties that are similar to Fe^{3+} – make it a pioneering antimicrobial agent, by interfering with iron regulation in microorganisms. Primordial bacterial activities such as metabolism, cellular respiration, and DNA synthesis are Fe^{3+} dependent, so the Ga^{3+} strategy relies on its mimicry to "trick" the Fe^{3+} receptors and enter the cell (Antunes et al., 2012, Chitambar, 2017). In the cell, Ga^{3+} cannot be functional, resulting in disruptions in the microorganisms' metabolism.

In this work, we aimed to explore the antimicrobial effect in order to determine the inhibitory concentration of gallium nitrate in solution against the common bacterial strain reported in titanium device infections: *S. aureus.* Meanwhile, cytotoxicity tests in cells were performed and scanning electron microscopy (SEM) was chosen to evaluate the biofilm inhibition by $Ga(NO_3)_3$.

2. MATERIAL AND METHODS

2.1 Chemicals, microbial strain, and culture conditions

Staphylococcus aureus ATCC 6538 was cultivated under anaerobic conditions at 37°C for 18 h in Tryptic Soy Agar (TSA). In the susceptibility test, *S. aureus* was grown aerobically at 37°C in Mueller-Hinton Broth (MHB; Difco™, USA) in a 96-well microplate.

The analysed gallium compound, $Ga(NO_3)_3 \cdot xH_2O$ (crystalline, 99.9% trace metals basis; Sigma-Aldrich®, USA), was dissolved in 1.0 M sodium citrate [$(CH_2COONa)_2 \cdot 2H_2O$] buffer.

Initially, when gallium nitrate was diluted in water, the pH of the solution decreased to the range of 2.3 – 2.5 and there was a change in the turbidity caused by the formation of a precipitate that interfered with the spectrometric measurements. Thus, to reduce these variations, especially at higher concentrations, $Ga(NO_3)_3$ was diluted in 1.0 M sodium citrate buffer, and pH adjusted between 7.0 and 8.0 in order to reduce turbidity.

2.2 Susceptibility testing

In order to determine the inhibitory concentration (IC) of $Ga(NO_3)_3$, 200 µL of MHB supplemented with gallium nitrate at concentrations ranging from 0.17 to 90 µM were transferred to 96-well microplates and inoculated with approximately 10^7 cfu/mL of *S. aureus* ATCC 6538 previously grown in the same broth. According to Xu et al*.,* 2017, the $Ga(NO_3)_3$ solution has a few flocs at higher concentrations, so 10 µL of 0.2 % EDTA solution was added to each well in order to eliminate any interference with the absorbance reading.

Afterward, the microplate was incubated for 24 h at 35 ºC and read at 540 nm with a microplate reader (Synergy HTX Multimode Reader, Brazil).

2.3 Effect of $Ga(NO_3)_3$ on biofilm formation by *S. aureus* on titanium surfaces

Titanium coupons (10 × 10 mm, 2 mm in thickness, grade II) were polished with #100, #300, #500, and #800 grit SiC abrasive papers. Subsequently, the coupons were washed with liquid-neutral detergent and water, followed by rinsing with distilled water and then immersing in 70 % ethyl alcohol for 1 h to remove fat. The titanium coupons were then rinsed again with distilled water and air-dried under UV light. The cleaned coupons were immersed for 18 h at 37°C in MHB supplemented with 90 µM of $Ga(NO_3)_3$ without bacteria - denominated negative control; in MHB with approximately 1×10^7 CFU/mL of exponentially growing *S. aureus* cells (optical density at 600nm [OD_{600}] of 0.2) – named positive control; and in MHB supplemented with 90 µM $Ga(NO_3)_3$ containing growing cells – denominated treatment.

To evaluate the inhibition effect of gallium nitrate on the biofilm formation, the titanium coupons were rinsed in sterile distilled water after incubation, dried under laminar airflow, and observed via SEM imaging at 300x and 1500x magnification (JEOL – JSM-IT200A, Brazil). Cleaned and sanitized coupons also were observed by SEM.

2.4 Cytotoxicity evaluation

To evaluate the cytotoxic potential of gallium nitrate, normal Hamster lung cells (V79 lineage) were used. Cells were plated at a concentration of 1×10^5 cells/well in 96-well plates and incubated at 37°C under 5 % CO_2. After 24 h, the cells fully adhered to the polystyrene surface were exposed to $Ga(NO_3)_3$. The exposure was carried out under increasing concentrations of gallium nitrate, ranging from 39.10 to 273.71 µM, for a period of 24 h at 37°C under 5 % CO_2. After incubation, the $Ga(NO_3)_3$ solution was removed from the culture and the cells were washed with phosphate-buffered saline (PBS). Then, 100 µL of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide solution (MTT) at a concentration of 0.5 mg/mL (v/v) were added to each well and the plate was incubated again for 3 h at 37°C under 5 % CO_2. Finally, the MTT solution was removed, and for cell fixation, 100 µL of dimethyl sulfoxide (DMSO) was added to each well. Cell viability analysis was performed using the microplate reading equipment (RCHISTO Tecan Infinite®, Brazil) at a wavelength of 570 nm.

2.5 Statistics

Statistical analysis employed a one-way analysis of variance (ANOVA) followed by Tukey's test (significance level of $p<0.05$), performed using OriginPro 2021b 9.85v.

3. RESULTS AND DISCUSSION

3.1 Effect of $Ga(NO_3)_3$ on the growth of *S. aureus*

The inhibitory concentration of gallium nitrate against *S. aureus* ATCC 6538 was investigated in MHB. In higher concentrations, the $Ga(NO_3)_3$ precipitation during cultivation interfered with absorbance measurements avoiding the correct determination of the inhibitory concentration (Figure 1). However, the study still revealed that the inhibitory concentration was able to inhibit 50 % of the strain when compared to the absorbance results of the pathogen's positive control ($p<0.05$). The negative control, MHB supplemented with only $Ga(NO_3)_3$, did not show any interference with the absorbance. The results showed an inhibition effect of gallium nitrate at 1.40 µM against *S. aureus* (Figure 1).

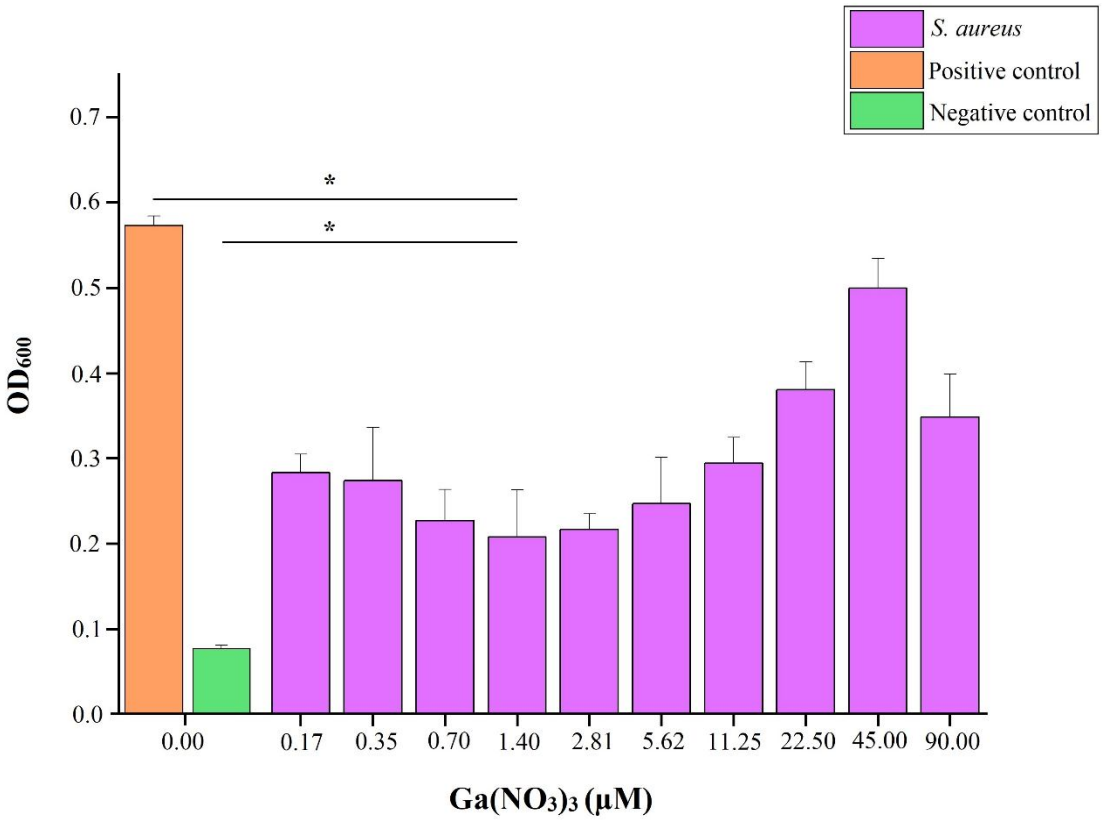

Figure 1. Susceptibility of *S. aureus* treated with $Ga(NO_3)_3$. Statistical significance between the absorbances data of positive and negative control against the pathogen data is indicated by asterisk (* *P* < 0.05).

3.2 Biofilm inhibition

Scanning electron microscopy was used to evaluate the ability of *S. aureus* to form biofilms on titanium surfaces in the absence and presence of gallium nitrate. The uniformity of the cleaned and sanitized titanium coupon surfaces is presented in Figure 2A and Fig. 2B, at 300x and 1500x magnification, respectively. Figure 2C and 2D show the titanium surfaces under the same magnifications after its immersion in MHB supplemented with $Ga(NO_3)_3$ for 18 h The positive control is shown in Figure. 2E and 2F, which represent *S. aureus*' typical biofilm structure.

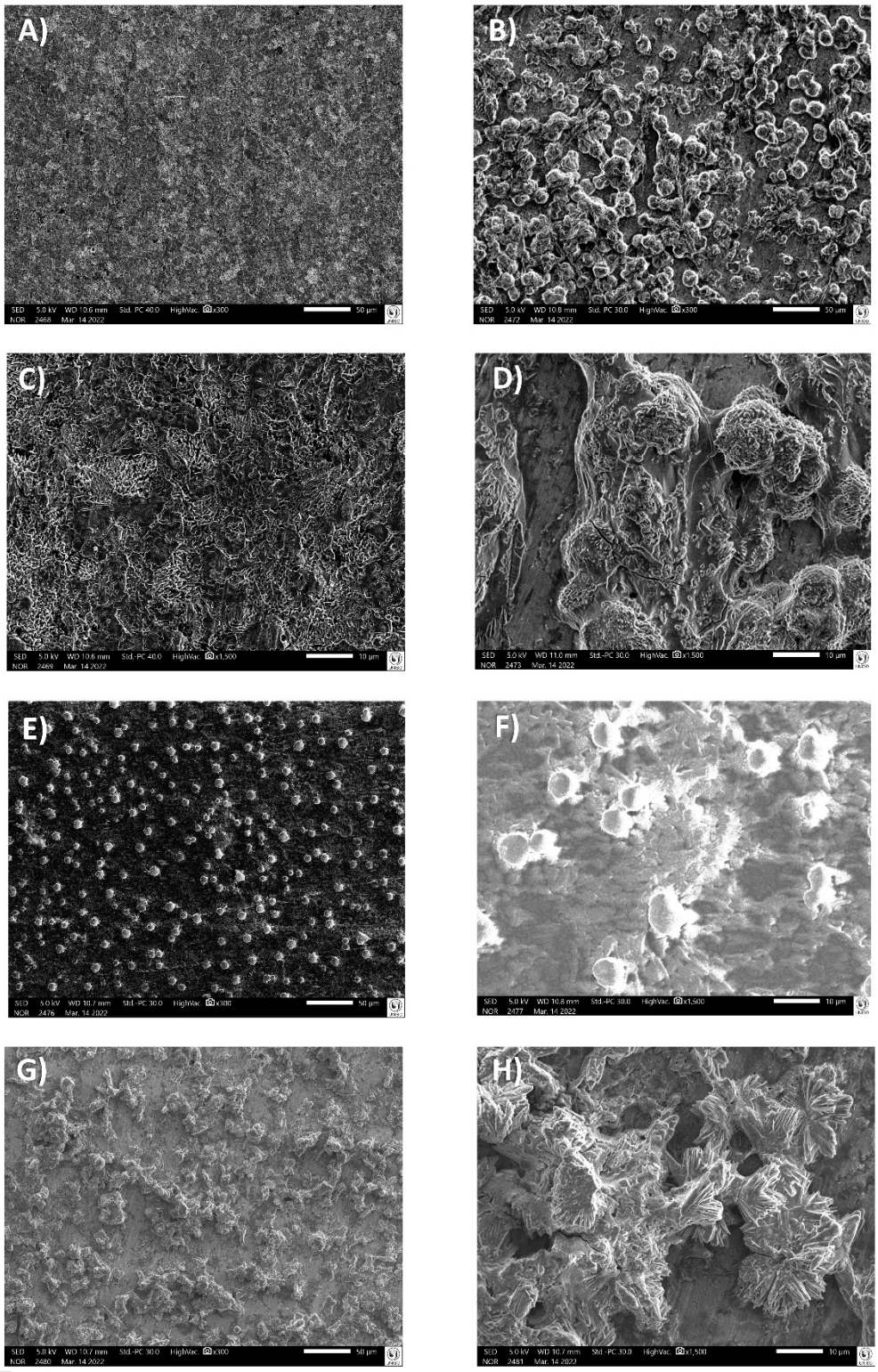

Fig. 2. Scanning electron microscopy of *S. aureus* biofilm on titanium surfaces in the presence and absence of $Ga(NO_3)_3$. Images were captured for a clean and sanitized titanium coupon (A and B), a titanium coupon treated with MHB supplemented $Ga(NO_3)_3$ (C and D), a titanium coupon immersed in MHB with the addition of *S. aureus* (E and F), and a titanium coupon immersed in MHB supplemented with $Ga(NO_3)_3$ with the addition of *S. aureus* (G and H). Two resolutions were used to obtain the images: 300x (A, C, E, and G) and 1500x (B, D, F, and H).

When titanium coupons were immersed in MHB supplemented with 90 µM of gallium nitrate and inoculated with *S. aureus*, no typical biofilm structure was observed (Fig. 2G and 2H). The topography noted in the images is characteristic of the microstructure of gallium adsorbed on the metal surface.

3.3 Cytotoxic effect of $Ga(NO_3)_3$ in cell lines

The cytotoxicity of gallium nitrate was evaluated on V79 cells. The MTT assay revealed low cytotoxicity for all tested concentrations of $Ga(NO_3)_3$ (Figure 3), indicating that the antimicrobial agent does not inhibit the growth of healthy cells, acting only against pathogenic ones.

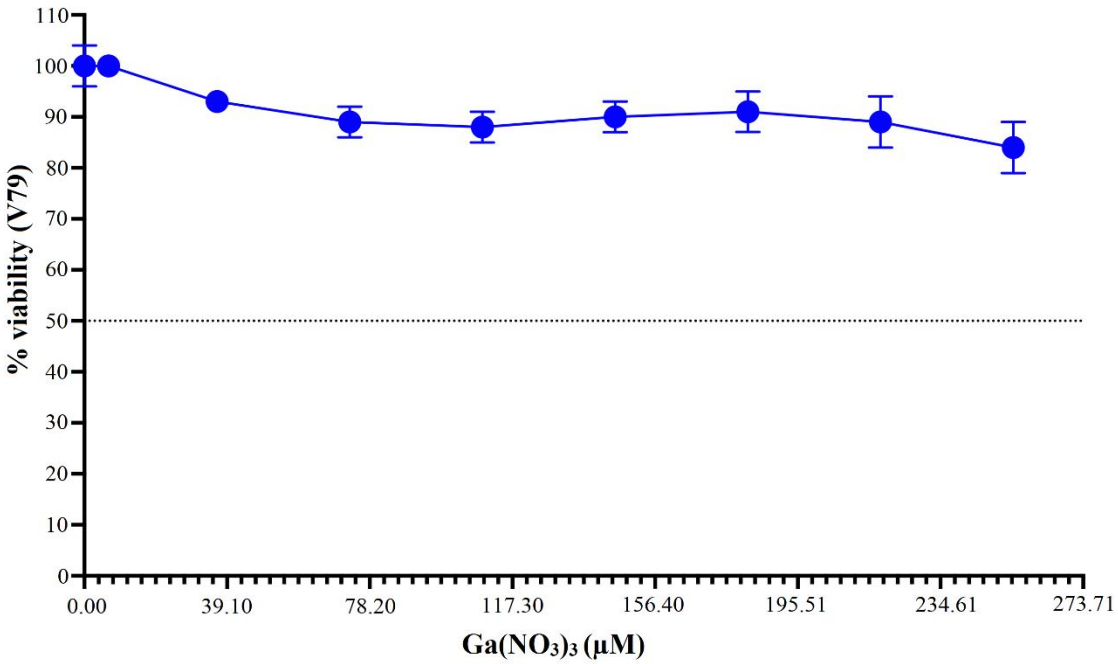

Fig. 3. V79 lineage cell viability in the presence of different concentrations of $Ga(NO_3)_3$.

Among metallic ions, Ga^{3+} stands out for being an antimicrobial agent that acts against several targets, in addition to being able to bind to iron-dependent proteins to enter the cell as a "Trojan Horse" and cause an interruption in the enzymatic metabolism of the microorganism (Li et al., 2022; Michalska et al., 2022). Therefore, due to the increase in arthroplasty and infections associated with these devices by multidrug-resistant microorganisms, such as *S. aureus*, gallium nitrate was used to inhibit planktonic growth and biofilm formation by this important opportunistic pathogen on titanium-based surfaces.

However, after the dilution of gallium nitrate in aqueous medium, a complex began to precipitate, interfering with the turbidity of the solution and causing the acidification of the medium, as previously reported by Tsukatania et al. (2012). According to Zhao et al. (2008), the precipitate in this type of solution corresponds to $Ga(OH)_3$. When $Ga(NO_3)_3$ is added to aqueous solutions, gallium ions react with OH^- ions, which results in precipitate formation, identified as gallium hydroxide [$Ga(OH)_3$] (Ristic et al. 2005). In order to avoid this phenomenon, $Ga(NO_3)_3$ was diluted in sodium citrate solution. There was minimal change in the turbidity even following pH adjustment to neutral values with NaOH. This circumstance corroborates with Rzepishevska et al. (2011), who reported the use of gallium citrate (Ga-Cit) in order to prevent precipitation of $Ga(OH)_3$, allowing the investigation of the minimum inhibitory concentration of gallium nitrate against Gram-negative and Gram-positive planktonic bacteria with minor interference.

Despite the buffer solution supplemented with $Ga(NO_3)_3$ not being fully effective in the control of *S. aureus* ATCC 6538 (Figure 1), for the evaluated concentrations, the results still proved it to be an efficient antimicrobial agent against the pathogen. The required inhibitory concentration to inhibit 50% of *S. aureus* cells was 1.40 µM. This concentration of the antimicrobial, expressed in micromolar, reduced the absorbance by 50% when compared to that found in the positive control.

It was also possible to notice that the Gram-positive *S. aureus* was sensitive to gallium nitrate, which can be attributed to the presence of only one barrier preventing access to the cytoplasm in Gram-positive strains, resulting in lower required concentrations of the antimicrobial agent. This evidence corroborates those established in the literature. Garcia et al. (2016) studied the action of poly(methyl methacrylate) (PMMA) and calcium sulfate ($CaSO_4$), which are compounds frequently used in the treatment of orthopedic infections involving strains of *Staphylococcus* spp., by delivering $Ga(NO_3)_3$ to infected sites. In addition, the complex's effectiveness as an antimicrobial agent against strains of *S. aureus* and *Staphylococcus epidermidis* was evaluated (Garcia et al., 2016). As a result, data revealed a significant decrease in microbial growth at concentrations above 16 µM and 4 µM, respectively, while concentrations above 64 µM completely inhibited both trialed strains. In our current work, concentrations of 90 µM were found to completely inhibit biofilm formation on titanium surfaces, values similar to those found by Garcia et al. (2016), who observed the inhibition of *S. aureus* biofilms in the presence of concentrations above 128 µM.

In 2017, Dong et al. tested the inhibitory effect of $Ga(NO_3)_3$ on *Pseudomonas aeruginosa* and *S. aureus* biofilms on titanium chips. A thin layer of gallium nitrate was used, which was found to be effective against the *P. aeruginosa* strain, but the results against *S. aureus* were negligible. The need for high concentrations of $Ga(NO_3)_3$ was demonstrated in recent studies. Gugala et al. (2019) evaluated the effectiveness of the antimicrobial on isolated species of *Escherichia coli*, *P. aeruginosa,* and *S. aureus*. They have found the minimal inhibitory concentration of gallium nitrate against these strains, which needed 31,250 µM, 15,630 µM, and 15,620 µM, respectively (Gugala et al., 2019). On the other hand, the effectiveness of much lower concentrations of $Ga(NO_3)_3$ has been demonstrated in the present study, either by inhibiting planktonic growth or by inhibiting the formation of biofilms by *S. aureus* on titanium coupons.

Upon finding the effectiveness of gallium nitrate, cytotoxicity tests were necessary to evaluate the potential to cause damage to healthy mammalian cells. The present study showed that $Ga(NO_3)_3$ is not toxic to hamster lung cells (V79 lineage) under concentrations above 39.10 µM. Similarly, in the study conducted by Best et al. (2020), three polysaccharides (carboxymethyl cellulose, alginate, and pectin) were tested as binding and delivery agents in three different bacteria (*P. aeruginosa, E. coli, and S. aureus*) with potential bioresponsive mode. As a result, gallium loaded with carboxymethyl cellulose showed an effective inhibitory activity against all microbial growth and did not exhibit a cytotoxic effect on human dermal neonatal fibroblasts (HDNF) (Best et al., 2020). Rodríguez-Contreras et al. (2020) highlight the positive antimicrobial results of Ga^{3+} without inducing cytotoxicity when using it on internal and external titanium surfaces obtained by 3D printing. The presence of a layer of calcium titanate containing Ga^{3+} adhered to the substrate was revealed. In addition, Ga^{3+} ions promoted the antibacterial effect against Gram-positive strains, especially *P. aeruginosa*, and did not have cytotoxic action against human osteoblasts (SAOS-2) (Rodríguez-Contreras et al., 2020). Cochis et al. (2019) investigated the addition of Ga^{3+} ions to titanium alloys, producing intermetallides with high stability and an increase of 1 to 23% gallium in weight. These alloys guaranteed the antibacterial effect without any visible cytotoxic effect, evaluated by direct and indirect contact tests with mature osteoblasts and preosteoblasts progenitor cells (Cochis et al., 2019).

4. CONCLUSION

Our study showed the effect of 1.40 µM of gallium nitrate in reducing the planktonic growth of *S. aureus* by 50 %. Thereby, it was shown that $Ga(NO_3)_3$ has an effective role as an antimicrobial agent against biofilms in representative biomaterials used in arthroplasties without visible cytotoxicity under concentrations ranging from 39.10 to 273.71 µM. In addition, besides representing a microbial growth inhibitory tool, gallium nitrate also has the potential to be used in the deposition of biomaterials in order to prevent healthcare-associated infections. In order to advance studies involving medical devices and pathogenic microorganisms, further research is needed, especially regarding the antimicrobial effect of $Ga(NO_3)_3$ on other strains commonly found in hospital environments and its involvement in hospital tools.

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