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Bactericidal effect of magnesium ions over planktonic and sessile *Staphylococcus epidermidis* and *Escherichia coli*.

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Abstract

Magnesium and its alloys are in focus to produce implants due to their excellent mechanical properties, facility to decompose in the body, enhancement of new bone formation and antibacterial properties. However, there is still a lack of consensus about the origin of such antibacterial effect. Up to date, most antibacterial studies were performed over newly synthesized MgO particles, nanotubes or nanowires in the form of slurries, integrated within polymeric matrixes or as coatings of other bulk materials whereas the effect of pure Mg^{2+} ions over bacteria viability, in the absence of secondary processes, has not been assessed yet. Hence, in this study, we have characterized the bactericidal effect of Mg^{2+} ions over two different bacteria strains, *Staphylococcus epidermidis* (Gram-positive) and *Escherichia coli* (Gram-negative) controlling both pH and ion concentration. Also, we have considered the ion effect on dissimilar planktonic and sessile cells on surfaces with different hydrophobicity.

Keywords: Biomaterials, Magnesium ions, antibacterial ability, Hydrophobicity, pH.

Subject classification numbers (PACS): 87.85.J- Biomaterials, 87.85.jj Biocompatibility

1. Introduction

Magnesium (Mg) is becoming the focus for the development of new materials for biomedical purposes. The interest on magnesium is promoted by its mechanical properties, the possibility of

being used for biodegradable devices and its biocompatibility [1–7]. However, the high degradation rate of Mg would prevent its usage because due to the fast, massive and detrimental release of hydrogen and hydroxide anions once it is in contact with physiological fluids or any aqueous media [8]. While the former can lead to the formation of gas cavities, the latter can induce a pH rise, usually up to 10 [9]. Nevertheless, alternatives to slow down the degradation rate are in progress. Magnesium alloys, surface treatments and coverages, or the use of particles of magnesium or magnesium alloys embedded in a polymer matrix are promising options [10–19].

An additional property of magnesium and magnesium-based materials is their bactericidal and bacteriostatic potential. It actually represents a valuable property because infections are one of the most adverse complications related to the implantation of any device. The antibacterial effect of magnesium-based materials has been evaluated in previous studies [20–31] but there is still a lack of consensus about the origin of such effect. On the one hand, it is argued that it is a consequence of the high reactivity showed by Mg with H_2O. This results in the production of superoxide ions (O^{2-}) which are allowed to attack bacteria cells by its peptide linkages [20]. Furthermore, other authors went beyond this point and proposed that contact between MgO particles and bacteria cells is a crucial factor for the occurrence of its antibacterial activity [30]. On the other hand, it is supported that the induced pH increase after Mg corrosion is liable for bacteria viability-loss, regardless the ion concentration [22,26,27]. Besides, some other authors ascribed this behaviour not only to a single factor but a synergic effect [31–33].

Nevertheless, to the best of our knowledge, these effects were studied in experimental setups where bacteria were in a direct contact with the surface of the magnesium or magnesium-based materials. In those conditions, it is hard to discern the individual contribution of each of those proposed mechanisms to induce the bacterial damage or killing.

In this study, we are aimed to characterize both the bactericidal and the bacteriostatic effect of the sole presence of Mg^{2+} ions in the media over sessile and planktonic bacteria since the metabolic state of sessile cells changes in respect to its planktonic state [34–36]. In this line, we will also consider if the attachment is done to a hydrophilic or a hydrophobic surface. We will test a Gram-negative and a

Gram-positive strain to account the effect of Mg^{2+} on different cell wall properties. The magnesium concentration range is planned to cover that found at human blood plasma in normal conditions and beyond, aiming to simulate the effect of a moderate Mg^{2+} ion supplementation, regardless its origin, taking to account its non-toxicity in concentrations up to 6 times the level in blood plasma [37].

2. Materials and methods

2.1. Mg^{2+} ions solutions

Mg^{2+} solutions were prepared from pure magnesium particles of about 100 µm, kindly supplied by the National Center for Metallurgic Research, CENIM-CSIC, Madrid, Spain. Previously to their use, the particles were cleaned. They were sonicated in pure acetone and ethanol for periods of 10 min each and stored in a desiccator for 24 h. After that, they were soaked in 0.15 M phosphate buffered saline (PBS, pH 6.8), followed by 15 min of sonication and allowed immersed for 24 h. Then, the solution was filtered through 1.2 µm pore size filters (Millipore, USA). Subsequently, the particles were soaked in freshly 0.15 M PBS, sonicated for 15 min and allowed immersed for 48 h. This time was previously checked as enough to reach a saturated solution of Mg^{2+} with a pH value of 7.9 ± 0.1. Similar pH trend due to Mg^{2+} dissolution has been reported previously for HEPES buffer reaching values within 7.7-8.1 [38]. Finally, the supernatant solution was filtered with a 0.22 µm membrane (Nylon filter, Millipore, USA). The Mg^{2+} concentration was 1374 ± 35 mg L^{-1} as determined by mass spectrometry (ICP-MS, NEXion 300D, Perkin Elmer, USA).

From this solution (referenced as 100% Mg), two other solutions with 50% and 25% content in Mg ions were prepared by dilution in PBS. 25% Mg solution matched the concentration of Mg^{2+} in human blood plasma [37]. Their pH values were 7.3 ± 0.1 and 6.9 ± 0.1, respectively.

2.2. Bacteria culture

S. epidermidis ATCC 35983, a Gram-positive bacteria, and *E. coli* ATCC 25922, a Gram-negative bacteria, were obtained from the Spanish Type Culture Collection (CECT) and were stored at 80 °C in porous beads (Microbank, Pro-Lab Diagnostics, USA). From the frozen stock, Columbia blood agar plates (Oxoid, ltd., Madrid, Spain) were inoculated and incubated at 37 °C to obtain cultures.

From these cultures, tubes of 4 mL of Trypticase Soy Broth (TSB) (BBL, Becton Dickinson, USA) were inoculated for 10 h at 37 °C, and then 25 µL of this pre-culture was used again to inoculate 50 mL flasks of TSB at 37 °C for 14 h. For E. coli, no pre-cultures were needed, so direct cultures from the agar plates were inoculated for 19 h on 50 mL flasks of TSB at 37 °C. These times were previously checked as sufficient to guarantee that both strains were at the end of the exponential phase of growth. All inoculations were carried out with an agitation of 100 rpm. Bacteria were harvested by centrifugation for 5 min at 1000 g (Sorvall TC6, Dulont, USA) and washed three times with 0.15 M phosphate buffered saline (PBS, pH 6.9) pre-conditioned at 37 °C. For adhesion experiments, the bacteria were re-suspended in PBS at a concentration of 3×10^8 bacteria mL^{-1}.

2.3. Tests on planktonic bacteria

Bacterial suspensions at 3×10^8 bacteria mL^{-1} were prepared in PBS solutions with 100% Mg, 50% Mg, 25% Mg and 0% Mg (control), respectively. Previously, the pH of all the solutions was raised until 8.0 ± 0.1 adding some drops from a NaOH solution. Suspensions were sonicated for 5 min in an ultrasonic bath at 110W (Ultrasons, J.P. Selecta, Spain) to avoid the formation of bacterial aggregates. Then, wells of Petri dishes were filled with 10 ml of each suspension and were maintained for 60, 150 and 240 min at 37 °C under orbital shaking at 20 rpm (Heidolph Rotamax 120, Heidolph Electro GmbH and Co, Germany).

After these periods of time, the pH value was measured and the bacteria viability tested with two methods. A part of the bacterial suspension was tested with the Live/Dead Backlight L-7012 (Invitrogen SA, Spain), following the manufacturer protocol. Another part of the suspension was tested by the serial dilution method. For this latter test, 25 µL of bacterial suspensions were deposited on TSB agar plates, incubated aerobically at 37 °C for 24 h and the colony forming units (CFU) were then visually counted. Then, the antibacterial rate (AR) was determined from (1) [39]:

$$AR\ (\%) = 100 \times (N_1 - N_2)/N_1 \quad (1)$$

where N_1 and N_2 are the CFU values from the control solution and the Mg^{2+} ion solution considered, respectively.

2.4. Surfaces for adhesion

Ti6Al4V allows the modification of its hydrophobicity using UV-C radiation. This property permits to have surfaces of the same material with different hydrophobicity without any chemical change. Disks of Ti6Al4V (25 mm in diameter) were obtained and cleaned as detailed in reference [40] before their use. In short, disks were mechanically polished with diamond paste and finished with colloidal silica. Then, they were carefully cleaned with DSF disinfectant (DERQUIM DSF 11; Panreac Quimica S.A., Spain) and rinsed with both acetone and ethanol solutions before drying. Also, a second set of samples were prepared similarly and then exposed to a UV-C source for a 2 h period, which was sufficient to guarantee a complete hydrophilization of the surface [41].

2.5. Tests on sessile bacteria

The adhesion of bacteria to the Ti6Al4V surface was carried out with the help of silicone chambers (FlexiPERM, Greiner Bio-One, Germany) fixed to the Ti6Al4V surface. 2 mL of bacterial suspension was added to the silicone chambers and the contact of bacteria for adhesion on the Ti6Al4V surface was allowed for 60 min. Afterwards, the supernatant bacterial suspension was removed and replaced by 2 mL of Mg^{2+} solutions (without pH modification) or PBS (with pH adjusted to 8.0 ± 0.1) and maintained for 60, 150 and 240 min in contact with the Ti6Al4V surface with the adhered bacteria. Both processes were done at 37 °C under orbital shaking at 20 rpm. After these periods, pH was controlled and the magnesium solutions were removed. Then, the Ti6Al4V samples were carefully immersed twice in a volume of 50 mL of freshly prepared PBS to remove weakly bound bacteria. The viability of adhered bacteria was checked with the kit Live/Dead Backlight L-7012, and cells on surfaces were counted with the software NIS-Elements BR 4.10 (Nikon Instruments INC, Melville, USA) on 5-10 different positions of the surface to obtain an average data for each surface.

Statistical analysis of the adhesion rates was made with paired samples, i.e., between irradiated and non-irradiated surfaces to study the influence of surface hydrophobicity. For that purpose, neither the Mg^{2+} ion concentration nor the exposition times were taken into account. Indeed, both factors are referred to stages after bacterial adhesion.

2.6. Mg^{2+} uptake by cells

Bacteria magnesium uptake was studied by mass spectrometry (ICP-MS, NEXion 300D, Perkin Elmer, USA). The experimental setup was the same as described for the tests on planktonic bacteria (Sec. 2.3). Specifically, 100% Mg solutions and 240 min of interaction time were the selected conditions for this assay with suspensions of 2 x 10^{10} bacteria mL^{-1}. Once the contact time was elapsed, bacteria were harvested by centrifugation for 5 min at 1000 g (Sorvall TC6, Dulont, USA), washed twice with 0.15 M PBS pre-conditioned at 37 °C, and desiccated in an oven at 75 °C for 24 h. Subsequently, they were subjected to digestion with a mixture of HNO_3 and H_2O_2 (Fluka, Sigma-Aldrich, USA) before the ICP-MS analysis. Control experiments were done with the control, 0% Mg, solution.

All the experiments have been done in triplicate, at least, with independent bacteria culture.

3. Results and discussion

3.1. Bactericidal and bacteriostatic effect of Mg^{2+} ions onto planktonic bacteria

The viability Live/Dead test on bacteria suspended in Mg-rich media gives qualitative but relevant information on the effects of Mg^{2+} on cells. Figure 1 contains the images obtained for *S. epidermidis* after being suspended in Mg^{2+} ion solutions (100%, 50% and 25%) and control samples (PBS) for 60, 150 and 240 min. Similar images are obtained for *E. coli* (not shown). Bacteria in control samples are 100% viable (green fluorescent), while a progressive loss in viability is displayed when increasing both contact time and Mg^{2+} concentration in suspension. It is observed a slightly higher influence by Mg^{2+} concentration rather than by the contact time. Bacteria that were in 25% Mg solutions stains from almost green up to yellow-like as the contact time increases. Bacteria that were in 50% Mg solutions change progressively from yellow-like up to orange-like, and bacteria that were in 100% Mg solutions appear orange-red after 60 min but look utterly red after 150 min of contact. These images indicate that bacteria were severely compromised or injured. Nevertheless, Huang *et al*. [42] have pointed out that in some cases cell wall damages can be repaired during the subculture of cells onto nutrient-rich media. For that reason, we have assessed the bacteria viability accounting the CFUs

by the serial dilution method. Figure 2 shows the agar plates incubated for the smaller order of dilution at which some colonies were found for every analyzed condition for *S. epidermidis* and Figure 3 summarizes the antibacterial rates (AR) obtained.

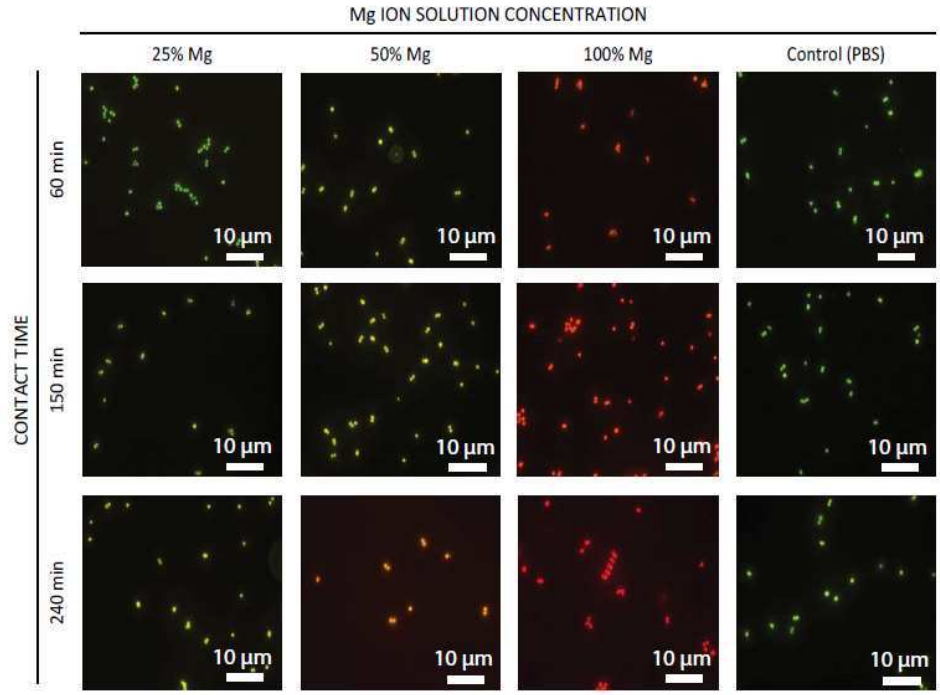

Figure 1. *Qualitative images of S. epidermidis cells' viability as a function of* Mg^{2+} *ions concentration and contact time. Control images are referred to cell viability on PBS pH 8.0 solutions.*

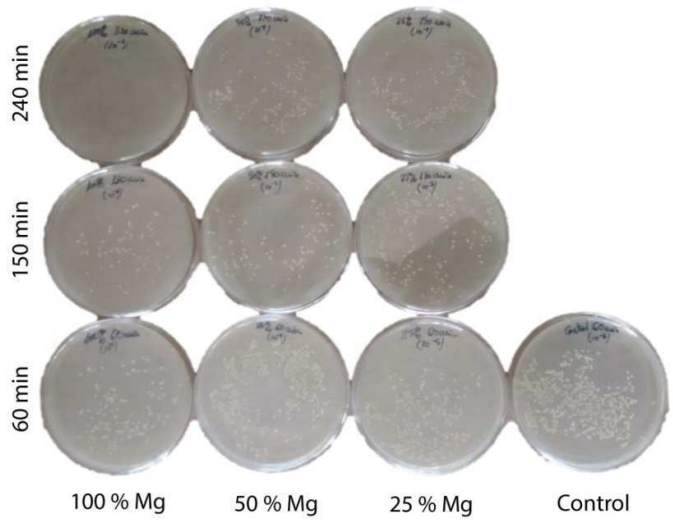

Figure 2. *S. epidermidis agar plates incubated for the smallest order of dilution at which some colonies were found, for every analyzed condition. The right bottom includes the control experiment agar plate from cells suspended on PBS pH 8.*

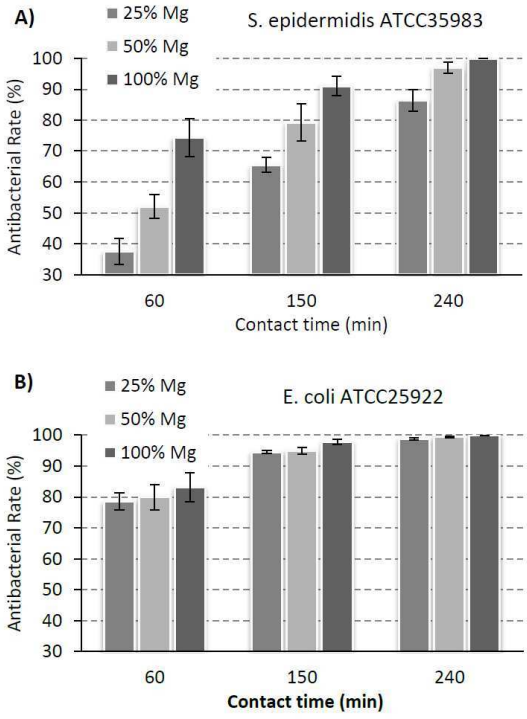

Figure 3. *Antibacterial rates (ARs) calculated from culture plate counts for a) S. epidermidis cells and b) E. coli cells, for every analysed condition. Error bars represent standard deviations.*

Figure 3.A shows the results from *S. epidermidis* experiments. For this strain, AR increases progressively with both contact time and Mg^{2+} concentration, reaching a 100% damaged bacteria after

240 min of contact with the 100% Mg solution. Although *S. epidermidis* viability seems to be more influenced by Mg^{2+} concentration at the shortest exposure times, differences vanish when the period is extended.

In the case of the Gram-negative *E. coli* (Figure 3.B), its evolution with contact time and Mg^{2+} concentration have a similar trend than for *S. epidermidis*. These results support the qualitative information previously obtained by the staining method since reddish damaged bacteria are not viable anymore. However, for each condition, the AR values for *E. coli* are higher than those for *S. epidermidis*. It appears that *E.coli* viability, in contrast with *S. epidermidis*, is more influenced by the exposure time rather than by Mg^{2+} concentration for the whole range studied. This behavior can be ascribed to their different cell wall structure. The thick cell wall in Gram-positive bacteria is composed of many layers of peptidoglycan and teichoic acids whereas the Gram-negative bacterial cell wall is relatively thinner, with an outer membrane containing lipopolysaccharides and lipoproteins bilayers [43,44]. Indeed, it can be expected that such damage on bacteria walls allows the uptake of large amounts of magnesium from the suspensions.

Table 1 includes the ng of Mg^{2+} per mg of bacteria pellets measured by ICP-MS. Results from the control pellets show that both bacterial strains contained a noticeable amount of Mg^{2+} on their structures. However, these values rose after being in contact with the concentrated Mg^{2+} solutions, especially for *E.coli*. Differences between both strains show that they are not equally permeable to Mg^{2+}, in agreement with their properties as Gram-positive and Gram-negative cells, respectively, and confirm that cell wall characteristics play an essential role for bacterial viability when they are endangered by external agents like Mg^{2+} ions. Nevertheless, it has to be taken into account that according to Lusk *et al*. [45] not only the entry of Mg^{2+} into *E. coli* cells can be hampered by the permeability of the wall, but also its release can be inhibited by a reduction of the available metabolic energy.

Table 1. *Mg^{2+} ion concentrations measured on S. epidermidis and E. coli cells after being in contact either with PBS (control) or 100% Mg ion solution, for 240 min. Δ refers to the relative increment of Mg^{2+} concentration.*

Sample	Mg^{2+} ion concentration (ng/mg)		
	PBS (Control)	100% Mg ion solution	Δ (%)
S. epidermidis	848.2 ± 46.3	1030.2 ± 78.3	21.4 ± 7.1
E. coli	1485.3 ± 104.7	1948.9 ± 172.5	31.2 ± 8.7

It is important to highlight that the pH of all solutions for this set of experiments was adjusted to 8.0 ± 0.1 before bacteria inoculation (including PBS 0%-Mg control solutions) and, within the experimental uncertainty; no modifications of the pH were detected afterwards. Consequently, it can be concluded that the observed bacterial viability behavior should be a direct consequence of the presence of Mg^{2+} ions within solutions regardless of the solutions pH. More specifically, we suggest that at constant pH, a larger concentration of Mg^{2+} ions yields to a high osmotic stress over the cell's wall, which in the end cause their viability-loss. This interpretation is, indeed, in agreement with other studies carried out on bulk Mg and Mg alloys [32,33] but here we isolate the effect of Mg^{2+} ions by keeping constant the solution pH.

3.2. Bactericidal effect of Mg^{2+} ions onto sessile bacteria

Adhesion of bacteria to a surface induces changes in its metabolic state compared to the planktonic case. Since infections related to the presence of external materials begin with the colonization of their surface by bacteria, the study of the influence of Mg^{2+} ions over adhered bacteria is of vital importance. Thus, bacteria adhesion experiments were carried out over Ti6Al4V discs, whose hydrophobicity can be modified with UV-C light irradiation [41], beyond any chemical change on its surface composition.

Figure 4 shows the number of adhered *S. epidermidis* and *E. coli* cells by unit surface area for hydrophobic and hydrophilic Ti6Al4V surfaces, being *E. coli* adhesion lower in both situations. Also, the results indicate that retention on the hydrophilic Ti6Al4V surface is seriously reduced in respect to the hydrophobic state, reaching an about 40% and 75 % reduction for *S. epidermidis* and *E. coli*, respectively. These results are consistent with those obtained in previous works dealing with the

changes observed in Ti6Al4V hydrophobicity after UV irradiation [41] and its effect on bacterial retention [46].

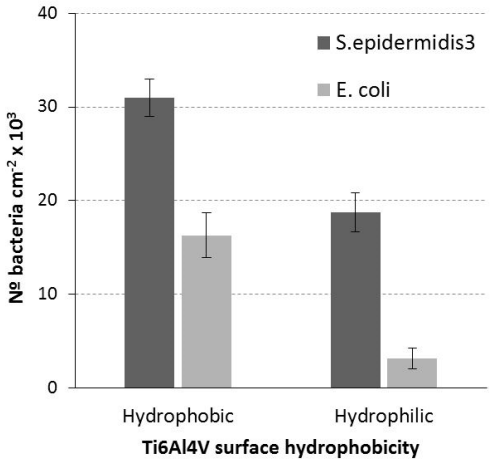

Figure 4*. Number of adhered S. epidermidis and E. coli cells (viable and non-viable) by area as function of Ti6Al4V hydrophobicity. Error bars represent standard deviations.*

The dependence of the adhered *S. epidermidis* viability to hydrophobic and hydrophilic Ti6Al4V samples with both Mg^{2+} concentration and contact time is shown on figure 5. Similar images are obtained for *E. coli* (data not shown). As for planktonic bacteria experiments, control samples are 100% viable, regardless surface hydrophobicity and contact time. Thus, the surface hydrophobicity does not have any influence on adhered bacteria viability. From Figure 5 it is clear that the effect of magnesium on the viability of sessile bacteria appears since the lowest Mg^{2+} concentration and the shortest contact time tested. Cells glitter orange-like near red-like even for the smallest concentration (25% Mg) while cells stain entirely red at all further conditions. It is remarkable that the effect of magnesium on sessile cells is acuter than that on planktonic cells. Sessile bacteria suffer from metabolic changes in respect to their planktonic state that leads, among other effects, to the production of exopolymeric substances (EPS) [47]. There are many negatively charged functional groups in EPS, like carboxyl, phosphoric, sulfhydryl, and hydroxyl groups, that can bind with divalent cations, such as Mg^{2+} [48]. This disruption on the typical cell behavior could be associated with the affectation of the cell viability by a more efficient internalization of magnesium ions [49]. Besides, results suggest that the differences in the adhesion mechanism to hydrophobic and hydrophilic surfaces do not compromise the bactericidal effect of Mg^{2+} ions.

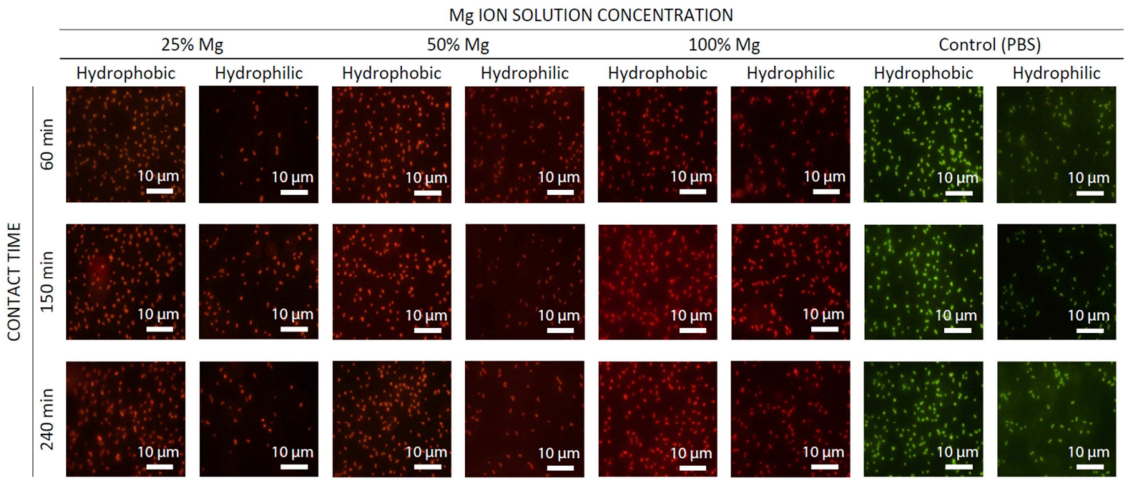

***Figure 5**. Qualitative images of adhered S. epidermidis cells' viability as function of Mg^{2+} concentration and contact time. For every condition, control and irradiated Ti6Al4V surfaces are included. Control images refer to adhered cells in contact with PBS solutions.*

As for planktonic bacteria, the pH of every sample was checked before and after the experiments, being its magnitude constant. Thus, pH is only dependent on Mg^{2+} concentration and it does not experience any modification after being in contact with bacteria cells. It is known that the solution pH can affect the bacteria viability, but there is still an open controversy about its role. For instance, for *E. coli* Mendonca *et al*. [50] claimed that high pH treatments do not damage the cells and Sawai *et al*. [30] reported a very little reduction of its survival ratio at pH 10.5. On the other hand, Imran *et al*. [51] claimed that magnesium antibacterial effect over *Pseudomonas aeruginosa* and *Staphylococcus aureus* strains is exclusively due to the pH raise originated by magnesium corrosion; and Robinson *et al*. [27] claimed that a simple increase in Mg^{2+} ion concentration alone has no antibacterial effect against *E. coli*, *Pseudomonas aeruginosa* and *S. aureus* but the resulted increase of pH.

Taking into account that all our Mg^{2+} ion solutions have a pH comprised within 6.9 ± 0.1 to 8.0 ± 0.1, it can be assessed that, within the conditions here studied, the bacteria viability-loss is a consequence of the high osmotic stresses originated by the presence of Mg^{2+} ions and the solution pH. The effect of this high osmotic stress is clearly more intense over sessile cells than over planktonic ones and in both cases is proportional to the Mg^{2+} ion concentration.

4. Conclusions

Magnesium ions show a remarkable antibacterial behavior over *S. epidermidis* and *E. coli*, even more clearly over sessile bacteria cells. For the first time, the bactericidal effect of magnesium ions has been isolated from the solution pH. At constant pH, a higher concentration of magnesium ions leads to a more intense bactericidal effect due to the larger osmotic stresses originated over the cells. The concentration of dissolved ions appears to have a significantly higher influence on bacteria viability rather than the contact time, but the differences vanish when the exposure time is extended.

Amounts of Mg^{2+} ions in the order of 687 mg L^{-1}, (50% Mg solution) can reduce the bacteria survival ratio for Gram-positive and Gram-negative bacteria over 50% with only 60 min of contact. Therefore, moderate Mg^{2+} ion supplementation coming directly from the prosthesis itself or from oral or intravenous infusion would bring significant benefits after implant surgery, diminishing the chance of ripening an infection focus.

In addition, the results show that hydrophobicity does not influence bacteria viability nor compromises the Mg^{2+} effects. Only a clear impact on bacteria adhesion rates has been found.

Acknowledgments

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