Research Article

In Vitro Toxic Effect of Biomaterials Coated with Silver Tungstate or Silver Molybdate Microcrystals

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Purpose. This study evaluated the cytotoxicity of antimicrobial silver tungstate (Ag2WO4) or silver molybdate (Ag2MoO4) microcrystals coating biomaterials. Materials and Methods. The coating procedure was performed onto titanium, zirconia, and acrylic resin specimens. Eluates of the coated specimens were obtained, which were used for cytotoxicity analyses, including Alamar Blue, MTT, and CytoTox-ONE tests. Data were analyzed using two-way ANOVA, followed by the Tukey test (α = 0.05). The results of each experimental group were also compared to those of the control of living cells, taken as 100% cell viability. Results. In general, it was observed that the percentage of living cells from all biomaterials coated with both microcrystals was statistically different compared to the ones from the uncoated sample groups, except for the results from MTT of specimens of Ti coated with α-Ag2MoO4. All uncoated biomaterials were classified as noncytotoxic by the three assays used in the present study. It was observed that the microcrystals in solution were strongly cytotoxic, with death of almost 100% of cells, from the analysis of the results of the Alamar Blue assay. Conclusion. The most biomaterials coated with both microcrystals showed some degree of cytotoxicity in the different assays. The results described herein should be seen as an alert to the use of microcrystals, which can expose patients to health risks.

1. Introduction

Several therapies have been proposed to prevent and treat microbial infections, including those caused by biofilms. In general, the microbial biofilm formation is a multistep growth process involving pretreatment of the substrate by the formation of a layer called conditioning film, cell attachment, cell colonization, and extracellular matrix formation. Moreover, the biofilm formation can result in tolerance of microorganisms to high concentrations of various antimicrobials. This fact has an important clinical relevance because, even in the presence of treatment modalities, resistant biofilms can cause chronic infections [1]. Therefore, instead of just treating, effective therapies to prevent biofilm formation on surfaces of implanted and restorative materials are considered an essential measure against biofilm-dependent diseases.

Surfaces with antimicrobial properties are highly desirable in applications requiring a protective barrier against infection. In this context, coating surfaces with nanoparticles or microcrystals have been adopted [2–6]. In medicine and dentistry, different biomaterials, such as polymethylmethacrylate, ceramics, and titanium, could be coated with nanoparticles to improve their antimicrobial properties, especially in hindering adhesion and proliferation of microorganisms [7–12]. Silver nanoparticles (AgNPs) have been
shown to have significant antimicrobial activity against planktonic cells and biofilms of Candida glabrata, Candida tropicalis, Staphylococcus aureus, and methicillin-resistant Staphylococcus aureus (MRSA) [13–17]. Recent studies have shown the antimicrobial effect of Ag as a microcrystal [18–21]. The ability of Ag₂WO₄ in fighting Candida albicans is related to the imperfect and crystalline patterns of atom arrangements in its orthorhombic structure and good photocatalytic capacity under visible light [21]. Also, Ag particles can increase the interaction and penetration into the cell membrane and, consequently, their antimicrobial activity [13, 16, 17].

The same mechanisms involved in killing microorganisms via Ag particles may cause human cell death, limiting its clinical application. Studies have revealed that the consequences of using Ag nanoparticles include potential changes in the cognitive, sensory, and motor functions, which result in brain and liver damage [22, 23]. Thus, it is paramount to perform biocompatibility studies to elucidate this issue. In spite of the expanding application of medical treatments, the residual monomer [26–29] can cause brain and liver damage. Thus, it is paramount to perform biocompatibility studies to elucidate this issue.

2. Materials and Methods

The synthesis and characterization by X-ray diffraction (XRD) patterns of Ag₂WO₄ and Ag₂MoO₄ microcrystals were performed in the Functional Materials Development Center, directed by Professor Elson Longo, and published in the previous study [25]. In the same way, shapes and sizes of the Ag₂WO₄ and Ag₂MoO₄ microcrystals were observed by field emission scanning electron microscopy (FE-SEM), using the same methodology described by Santana et al. [25].

Thirty-six discs of each titanium (Ti), zircon (Zi), and acrylic resin (AR) were prepared (8 mm in diameter and 2 mm in thickness) and distributed in the experimental groups as described in Figure 1. Pure titanium alloy discs were donated by Conexão Sistemas de Prótese (Arujá, SP, Brazil). Zirconia Lava™ (3M Espe, Saint Paul, MN, USA) discs were prepared using the Ceramill Motion software system. Acrylic resin specimens were prepared in a metal matrix for discs with the resin denture base Vipi Wave (VIPI Indústria e Comércio Exportação e Importação de Produtos Odontológicos Ltda, Pirassununga, SP, Brazil), according to the manufacturer’s instructions. All AR samples were left in distilled water at 37°C for 48 hours to release the residual monomer [26–29].

The coating procedure of microcrystals on discs was performed using a precipitation technique. Suspensions of Ag₂WO₄ or Ag₂MoO₄ microcrystals at a concentration of 1 mg/mL each in isopropyl alcohol were subjected to ultrasound for 20 minutes. Then, 5 μL of each suspension was dripped onto the upper surface of the discs. After 20-minute drying, the dripping was repeated 5 times. After deposition of all layers, the samples were heat treated at 250°C (Ti and Zi) or 100°C (AR) for 2 hours. Scanning electron microscopy (SEM) analysis was carried out to demonstrate the deposition of microcrystals onto disc surfaces.

To analyze the cytotoxic effect, the coated biomaterials were subjected to procedures to obtain eluates of soluble substances. Specimens were ultrasonically cleansed in distilled water for 20 minutes and kept for 20 minutes under ultraviolet light to prevent microbial contamination [26–29]. Then, three specimens of each biomaterial (coated or not with the different microcrystals) were placed in polypropylene tubes with 2.5 mL of DMEM culture medium and incubated (37°C/24 hours) [26–29]. Another tube containing only 2.5 mL of culture medium was stored under the same conditions, thus serving as a negative control group.
bottles of 75 cm² with DMEM culture medium containing 10% of fetal bovine serum (FBS), with 2.0 mmol/L L-glutamine, 10,000 μg mL⁻¹ penicillin G, 10,000 μg mL⁻¹ streptomycin, and 25 μg mL⁻¹ amphotericin (Sigma-Aldrich, Saint Louis, Missouri, USA), at 5% CO₂/37°C. For maintenance culture, the cells were cultured until they reach confluence (90%), washed with phosphate buffer 1X PBS (140 mmol L⁻¹ NaCl, 3.0 mmol L⁻¹ KCl, 4.30 mmol L⁻¹ Na₂HPO₄, and 1.4 mmol L⁻¹ KH₂PO₄), removed with trypsin solution (0.05%, containing 0.53 mmol L⁻¹ EDTA), and then subjected to centrifugation (400 × g/5 min). Next, the cells were resuspended in DMEM culture medium and counted in a Neubauer chamber. The cells were plated at 1.5 × 10⁴ cells/well in sterile 96-well plates and incubated (5% CO₂/37°C). After 24 hours, the cells were exposed to eluates and incubated for another 24 hours. The exposed cells were subjected to three cytotoxicity assays. In addition, the cell morphology was observed with an inverted optical microscope (Model 403, Optiphase, Van Nuys, CA, USA).

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was carried out to assess the cellular metabolism. The HaCaT cells exposed to eluates from coated materials were washed with 150 μL of 1X PBS. Next, 150 μL of MTT solution (5.0 mg mL⁻¹; Sigma-Aldrich, Saint Louis, Missouri, USA) was added to each well of a 96-well microplate, followed by incubation (37°C/5% CO₂/4 hours in the dark). Then, the formazan crystals were solubilized in 75 μL of acidified 2-propanol with 0.04 N HCl. After stirring and checking the homogeneity of the solutions, absorbance reading was performed at 570 nm in Microplate Reader EZ 400 (Biochrom, Cambourne, Cambridge, UK). Cell proliferation of the keratinocytes was also assessed using the Alamar Blue® assay. The HaCaT cells exposed to eluates were washed with 150 μL of 1X PBS. Then, an aliquot of 150 μL of diluted Alamar Blue ( Molecular Probes, Invitrogen Corporation, Waltham, Massachusetts, USA) solution (10% of Alamar Blue solution plus 90% of DMEM with 10% FBS) was added to each well of a 96-well microplate, followed by incubation (37°C/5% CO₂/4 hours). After this period, the contents of the wells were transferred to a sterile black 96-well plate with a flat bottom. The fluorescence was read immediately using Fluoroskan Ascent FL, with a filter of 544 nm of emission and 590 nm of transmission. The assays were performed in three separated experiments.

Besides the assessment of cytotoxicity of biomaterials after coating with both microcrystals, cytotoxicity of Ag₂WO₄ and Ag₂MoO₄ microcrystals in solution was also analyzed. For the preparation of solutions, each sintered powder, after sterilization, was dispersed in sterile distilled water in Falcon tubes, with the final concentration of 1 mg/mL. To perform the cytotoxicity assay, serial dilutions, in DMEM culture medium with 10% FBS, were made to reach concentrations of 0.25 mg/mL and 0.125 mg/mL for both microcrystals. These concentrations were selected according to the minimum inhibitory concentrations (MIC) determined after previous microbiological tests [18, 19, 30]. The cells (HaCaT cell line 0341) were plated at 1.5 × 10⁴ cells/well in sterile 96-well plates and incubated (5% CO₂/37°C). After 24 hours, the cells were exposed to microcrystals in solution and incubated for another 24 hours. Cells that were not exposed to the solution of microcrystals served as a negative control. All cells were subjected to the Alamar Blue assay, as previously described.

In the quantitative analysis, the results of the viable cells obtained on different tests (MTT, Alamar Blue, and CytoTox-ONE) were tabulated and subjected to the tests of normality (Shapiro-Wilk) and variance homogeneity (Levene) to verify the distribution of the variables. To assess the cytotoxicity of the three biomaterials coated with Ag₂WO₄ or Ag₂MoO₄ microcrystals, a two-factor analysis of variance (two-way ANOVA) was applied, followed by the Tukey multiple comparison test, with 5% significance level for decision-making. In these analyses, the percentage of living cells of all the experimental groups was compared to that of the control. For the qualitative analysis, the results of each experimental group were compared with those of the control group (taken as 100% viability). The biomaterials and microcrystals used were ranked according to the cytotoxic effect [31]: nontoxic (inhibition less than 25% compared to the control group), slightly cytotoxic (inhibition between 25% and 50% compared to the control group), moderately cytotoxic (inhibition between 50% and 75% compared to the control group), and strongly cytotoxic (inhibition greater than 75% compared to the control group).

3. Results

Results about the synthesis and characterization by X-ray diffraction (XRD) patterns of Ag₂WO₄ and Ag₂MoO₄ microcrystals can be observed in the study reported by Santana et al. [25]. Figure 2 shows Ag₂WO₄ and Ag₂MoO₄ microcrystals on the surfaces of biomaterials: titanium (Ti), zircon (Zi), and acrylic resin (AR).

Table 1 shows the results of living cell percentages relative to control (considered 100% living cells), for all experimental conditions. It is noteworthy that for the CytoTox-ONE test, the data was subtracted from 100% to determine the number of living cells since the test determines the number of dead cells by quantification of released LDH. In general, it was observed that the percentage of living cells from all biomaterials coated with both microcrystals was statistically different compared
to the ones from the uncoated sample groups, except for the results from MTT of specimens of Ti coated with Ag₂MoO₄. In the Alamar Blue test, for all the three materials tested, coating with both microcrystals significantly reduced \((p < 0.05)\) the percentage of living cells in comparison to the uncoated groups. When the coated materials were compared, there were no significant differences among them \((p > 0.05)\), regardless of the type of microcrystal used. Within the groups of uncoated biomaterials, only the Ti was considered statistically similar \((p > 0.05)\) to the control of living cells (considered 100%) and different from the other groups \((p < 0.05)\), which showed no significant differences between them \((p > 0.05)\). The MTT assay showed that, except for the Ti specimens coated with Ag₂MoO₄, the percentage of

### Table 1: Mean (standard deviation) of living cell percentages compared to control, obtained in the different tests, according to the biomaterial and the employed coating.

<table>
<thead>
<tr>
<th>Cytotoxic assay</th>
<th>Material</th>
<th>Coating Ag₂WO₄</th>
<th>Coating Ag₂MoO₄</th>
<th>Without coating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alamar</td>
<td>Ti</td>
<td>77.3 (2.6)²A</td>
<td>73.3 (1.8)²A</td>
<td>96.9 (2.1)²B†</td>
</tr>
<tr>
<td></td>
<td>Zi</td>
<td>73.4 (1.2)²A</td>
<td>69.2 (1.2)²A</td>
<td>88.6 (1.3)²B</td>
</tr>
<tr>
<td></td>
<td>AR</td>
<td>76.7 (1.6)²A</td>
<td>74.3 (1.0)²A</td>
<td>88.6 (1.8)²B</td>
</tr>
<tr>
<td>MTT</td>
<td>Ti</td>
<td>73.7 (2.8)³B²A</td>
<td>76.3 (3.4)³B²A</td>
<td>84.2 (0.3)³B</td>
</tr>
<tr>
<td></td>
<td>Zi</td>
<td>63.7 (2.6)³A²A</td>
<td>61.2 (2.8)³B²A</td>
<td>85.2 (5.4)³B</td>
</tr>
<tr>
<td></td>
<td>AR</td>
<td>72.5 (3.9)³B²A</td>
<td>71.0 (1.2)³B²A</td>
<td>82.6 (3.1)³B</td>
</tr>
<tr>
<td>CytoTox-ONE</td>
<td>Ti</td>
<td>66.8 (0.8)³A²A</td>
<td>63.5 (1.8)³B²A</td>
<td>86.4 (2.2)³B</td>
</tr>
<tr>
<td></td>
<td>Zi</td>
<td>63.7 (4.0)³B²A</td>
<td>53.3 (2.1)³A²A</td>
<td>75.2 (2.2)³C</td>
</tr>
<tr>
<td></td>
<td>AR</td>
<td>65.1 (1.6)³B²A</td>
<td>59.2 (1.4)³B²A</td>
<td>81.6 (1.1)³C</td>
</tr>
</tbody>
</table>

Means followed by the same letter (uppercase in line or lowercase in the column) were not significantly different (Tukey test: \(p > 0.05\)). †Not significantly different from the living cell control.

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**Figure 2:** SEM representative images of the surface characteristics of each biomaterial before and after the deposition of the microcrystals.
living cells from the other experimental conditions was significantly lower \((p < 0.05)\) than that from the uncoated groups. When the coated materials were compared, in general, the biocompatibility of Zi was lower than that of Ti, regardless of the type of microcrystal used. There were no significant differences \((p > 0.05)\) among the groups of uncoated biomaterials. All conditions tested were statistically different from the control group of living cells by this test. For the CytoTox-ONE test, the results showed that the samples of Zi and AR coated with Ag\(_2\)MoO\(_4\) had the lowest percentages of alive cells, which were significantly different \((p < 0.05)\) from the percentage obtained for the same materials coated with Ag\(_2\)WO\(_4\). In addition, all uncoated materials demonstrated higher values \((p < 0.05)\) of viable cells than the coated ones. When the biomaterials were compared, there were no significant differences \((p > 0.05)\) among the Ti, Zi, and AR samples coated with Ag\(_2\)WO\(_4\). When the specimens coated with Ag\(_2\)MoO\(_4\) were compared, the Zi samples showed the lowest percentage of viable cells. In addition, within the uncoated material group, there were no significant differences \((p > 0.05)\) between Ti and AR samples, which were statistically different from Zi samples \((p < 0.05)\). All conditions tested were statistically different from the control group of living cells by this test.

All uncoated biomaterials were classified as noncytotoxic by the three assays used in the present study (Alamar Blue, MTT, and CytoTox-ONE). The same rating was given for Ti and AR specimens coated with Ag\(_2\)WO\(_4\) by the Alamar Blue test and for Ti specimens coated with Ag\(_2\)MoO\(_4\) by the MTT assay. The other biomaterials coated with both microcrystals were classified as slightly cytotoxic because their extracts inhibited cell growth between 25 and 50% compared to the control group.

Figure 3 illustrates the cell morphology after the contact with the eluates from coated or uncoated biomaterials. The black arrows indicate the presence of particles released in the culture medium from the preparation of the extracts.

Figure 4 shows the fluorescence results (Alamar Blue assay) of the solutions of Ag\(_2\)WO\(_4\) and Ag\(_2\)MoO\(_4\) microcrystals. It is possible to observe a substantial reduction of the cell viability when the experimental groups were compared to the control group. Because of this discrepant difference, statistical analysis was not necessary to establish the differences among groups. Figure 5 illustrates the percentage of cell viability for the solutions compared to the control cells (considered 100% viability). It was observed that the microcrystals in solution, in both concentrations, were strongly cytotoxic, with death of almost 100% of cells. Thus, the other tests (MTT and CytoTox-ONE) were not carried out because they were considered unnecessary.
Ag2MoO4 microcrystals in solution) compared to the control cells.

4. Discussion

Surfaces with antimicrobial properties are highly useful in applications requiring a protective barrier against infection, and the use of nanoparticles or microcrystals represents a promising strategy, since they have the ability to inhibit the growth of microorganisms by different mechanisms [13, 32]. Despite the promising and effective antimicrobial activity [18, 19], the use of nanoparticles or microcrystals should be indicated with caution since the same death mechanisms of the microorganisms can also cause the death of human body cells. Genetic changes, systemic sclerosis, rheumatoid arthritis, lupus erythematosus, and chronic kidney disease can be caused by the exposure to nanoparticles [33–35]. Considering this information, this study evaluated the cytotoxicity of silver tungstate (Ag2WO4) and silver molybdate (Ag2MoO4), both in solution and in biomaterial coatings. For this, the cytotoxicity assays, using cultured cells and eluates, was selected because it is considered to be relatively simple, reproducible, effective, and controlled [36, 37].

MTT and Alamar Blue assays showed similar results regarding the effect of coating Ti, Zr, and AR surfaces with Ag2WO4 or Ag2MoO4. When these results were compared to the data from the CytoTox-ONE test, a divergent situation was observed, since the CytoTox-ONE test detected some differences among the experimental conditions that were not observed before. The CytoTox-ONE test seems to be more sensitive than the other ones, due the fact that, in some groups, cells with preserved mitochondrial activity showed changes in the membrane, which were detected by the release of LDH. This indicates that these methods are complementary to each other, each providing a capability lacked by the other, and thus, the results should be interpreted within the context of all data [38, 39].

The results of this study showed that Ag2WO4 and Ag2MoO4 microcrystals, in solution, decreased the cell proliferation by over 75%, when compared to the control group and, thus, were considered extremely cytotoxic. These results agree with other studies [24–40]. The reduction in cell viability could be explained, in part, due to the release of silver ions [41]. Silver nanoparticles have promising antibacterial activity for combating adhesion and biofilm formation, but their small size and high mobility require security concerns due to increased cytotoxic potential [42, 43]. Previous studies have shown that silver nanoparticles have detrimental effects on the cellular membrane [44], causing changes in its structure and, consequently, cell death. In addition, these particles can cause DNA damage and can increase reactive oxygen species, which can irreversibly impair cell function, leading to cell death [45, 46]. Another way that silver nanoparticles with 20 nm in size or smaller can lead to cell death is by penetrating into the cell without endocytosis, being distributed within the cytoplasm [47]. Moreover, it has been accepted that silver nanoparticles can connect onto the cell membrane surface, causing protein denaturation and, consequently, irreversible damage to cells [48]. Nanoparticles can penetrate inside the cell and cause damage by interacting with sulfur and phosphorus compounds, such as proteins and DNA [49]. Additionally, when these nanoparticles are placed in a culture medium, they form complexes of protein and nanoparticles [50]. The formation of these complexes can also have a cytotoxic effect, due to the interaction between the protein complex layer and the cells in culture [51].

The cytotoxic effects of the Ag2WO4 and Ag2MoO4 microcrystals, in solution, could also be explained by molybdate and tungstate singly. Molybdate and tungstate are molybdenum and tungsten oxyanions, respectively, which are metallic chemical elements. Metal oxides are known for their semiconductive properties, allowing electrons to transfer between the nanomaterial and aqueous environments [52, 53]. Shape and size play an important role in determining the reactivity and the cytotoxicity of the nanoparticles [52]. The presence of metals can also participate in one-electron oxidation-reduction reactions and lead to the formation of reactive oxygen species [54], and the highly reactive free radical can interact irreversibly with organic compounds of the cells, causing collapse of the membranes and damaging DNA, RNA, and proteins of the intracellular microorganism system [24].
Currently, many studies have been published on the toxic effects of nanoparticles or microcrystals, while data on their toxicity, when used as coatings on biomaterials, are sparse. For most of the biomaterials coated with Ag₂WO₄ and Ag₂MoO₄ microcrystals, cell inhibition was observed in the order of 25% to 50%, compared to the control group, and both groups were classified as slightly cytotoxic. Although the precipitation technique has been used for coating the samples (Figure 2), the cytotoxic effect was probably due to the release of the nanoparticles from the coated biomaterial during preparation of the extracts, as shown in Figure 3. Therefore, the same effects above described for microcrystals in solution may have caused the cytotoxicity of coated biomaterials because of the release of particles in aqueous medium (eluates). Despite the inhibition of the cellular metabolism, Figure 3 illustrates that the cells maintained their characteristic polygonal epithelial shape, forming a confluent monolayer for all biomaterials coated with Ag₂WO₄ and Ag₂MoO₄ microcrystals.

The results of this in vitro study provide valuable information about the cytotoxicity of biomaterials coated with microcrystals. However, future studies are needed to understand the complex toxicity mechanisms of microcrystals, which cause cell death.

5. Conclusions

According to the results and within the limitations of this study, it can be concluded as follows:

(1) In general, the percentage of living cells from all biomaterials coated with both microcrystals was statistically different from that from the uncoated sample groups

(2) In the Alamar Blue test, for all the three materials tested, coating with both microcrystals significantly reduced the percentage of living cells in comparison to the uncoated groups

(3) In the MTT assay, for the majority of groups, the percentage of living cells from the coated biomaterials was significantly lower than that from the uncoated groups

(4) In the CytoTox-ONE test, the results showed that the samples of Zi and AR coated with Ag₂MoO₄ had the lowest percentages of alive cells, which were significantly different from the percentage obtained for the same materials coated with Ag₂WO₄

(5) In the CytoTox-ONE test, all uncoated materials demonstrated higher values of viable cells than the coated ones

(6) All uncoated biomaterials were classified as noncytotoxic by the three assays used in the present study (Alamar Blue, MTT, and CytoTox-ONE)

(7) The majority of the biomaterials coated with both microcrystals were classified as slightly cytotoxic

(8) The solutions of Ag₂WO₄ and Ag₂MoO₄ microcrystals were ranked as strongly cytotoxic

Data Availability

The statistical data used to support the findings of this study are available from corresponding author upon request.

Conflicts of Interest

The authors declare no conflict of interest.

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