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Cytotoxicological Correlation with Baccor Biopharmaceutical Mass Spectrometry-Analysis of 7 Day Bacterial Extracts

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Abstract

Objective: This paper presents the cytotoxic evaluation of the BACCOR biopharmaceutical, developed to promote the biocorrosion of fractured endodontic files inside the root canal.

Methods: The cytotoxicity of bacterial extracts from supernatants was evaluated in sulfate reducing bacteria (SRB) cultures of Desulfovibrio desulfuricans, oral and environmental strain, and Desulfovibrio fairfieldensis, correlating with the analysis of chemical species obtained in dispersive energy spectrometry (EDS) on RX in the scanning electron microscope (SEM). L929 and Vero cell lines were used for MTT cytotoxicity tests. Test compounds were prepared from three bacterial strains in Modified Postgate E culture media without Agar-agar (MCP-E without Ag) and Postgate C medium without Agar (MCP-C without Agar). They were analyzed for chemical species in SEM EDS mode and correlated with cytotoxicity results.

Results: The test compounds with MCP-E s/Ag showed the best results regarding cell viability when compared to the other compounds. As for chemical analysis, the highest concentrations of Sulfur (S) were in the test compounds with the lowest cell viability.

Conclusion: The test compounds derived from bacterial metabolic products of SRB strains showed better results when employed with MCP-E s/Ag, with moderate cytotoxicity which makes this type of inoculum acceptable as a biomaterial.

Keywords: Biopharmaceutical, biologic drug, cytotoxicity tests, desulfovibrio, endodontics

INTRODUCTION

The *in vitro* cytotoxicity assay is the first step in the evaluation of a medical device, allowing the determination of factors that influence cell growth when exposed to the medical device under study, and verifying tissue compatibility.[1-3] Although the International Organization for Standardization (ISO) regulates the use of *in vitro* cytotoxicity assays, setting parameters,[1-3] the literature still mentions the use of different methodologies, exposure times and cell lines for the same medical device or biomaterial. There is no standardization in the type of biomaterial, medical device and clinical situation *in vitro*.

Most of the articles found employ L929 mouse fibroblast cell lines for the different materials studied without taking into account the cell types of the site where the biomaterial will act *in vivo*.[2-4] However, as it is possible for the biomaterial to come into contact with the vascular system, allowing the total or partial excretion of metabolites produced by this biomaterial, its cytotoxic evaluation in renal cell lines becomes critical.

ISO 7405 guides the biocompatibility assessment of medical devices used in dentistry, including pharmacological agents or biological components as an integral part of the device, such as bioactive endodontic materials or applications for stim-

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ulation of apical hard tissue formation.[5] In fact, initial cytotoxicity tests have limitations when correlated with clinical situations, although they are important tools for assessing biomaterial and / or medical device behavior.

The objective of the cytotoxicity tests was to determine the cytotoxicity index ($CI_{50\%}$) of the different test compounds to evaluate the biocompatibility of BACCOR without biocorrosive action. With the absorbance value obtained from each sample was calculated to obtain the $CI_{50\%}$ of each test compound comparing it to the positive control (cells not exposed). The parameters evaluated in cytotoxicity assays may be quantitative and qualitative. The first measures the number of cells after cell proliferation or inhibition, the number of colonies formed or quantifies cells by counting their components as proteins and mitochondria, while the second analyzes cells microscopically, observing morphological changes such as cytoplasmic vacuolization and lysis of its membranes. The material can then be classified as non-cytotoxic, slightly cytotoxic, moderately cytotoxic, severely cytotoxic or toxic.[1-3]

This paper presents the cytotoxicology evaluation step of the BACCOR biopharmaceutical, developed to promote the biocorrosion of fractured endodontic files inside the root canal.[6]

The aim of this paper was to evaluate the cytotoxicity of bacterial extracts, supernatants, without the occurrence of biocorrosion, from aerobic cultures of sulfate-reducing bacteria (SRB) of 7 days, of the especies *Desulfovibrio desulfuricans*, oral strain and environmental strain, and *Desulfovibrio fairfieldensis* in different culture media, correlating with chemical species analyzes obtained by RX dispersive energy spectrometry in the scanning electron microscope.

METHODS

The cells used were established in monolayer culture at the Pharmaceutical Bioassay Laboratory (LaBioFar) of the Department of Drugs and Medicines form the Faculty of Pharmacy of the Federal University of Rio de Janeiro (UFRJ). Two cell lines were used: L929 (mouse fibroblasts) (Rio de Janeiro's cell bank, BCRJ: 0188; lot: 000599) and VERO (African green monkey kidney epithelial cell) (Rio de Janeiro cell bank, BCRJ: 0245; lot: 000590).

The inoculation carriers were the culture media described below.

Main Points

- Addresses the initial development of a biopharmaceutical
- New concept of biopharmaceutical from the action of the metabolic product
- The biopharmaceutical did not indicate cytotoxicity, being promising
- New biomaterial concept for the removal of fractured endodontic files

Postgate E Modified Culture Media without Agar-Agar (MCP-E s/Ag) had the following composition in g/L.: KH_2PO_4 (0.5); NH_4Cl (1.0); Na_2SO_4 (1.0); $CaCl_2.2H_2O$ (0.67); $MgCl_2.6H_2O$ (1.68); Sodium lactate (7.0); Yeast extract (1.0); Ascorbic Acid (0.1); NaCl (5.0); Resazurin (4.0 mL) and $FeSO_4.7H_2O$ (0.5). For the MCP-E mod, 1.9 g/L. was added of Agar-agar.[7]

Postgate C Media without Agar-agar (MCP-C s/Ag) had the following composition in g/L.: KH_2PO_4 (0.5); NH_4Cl (1.0); Na_2SO_4 (4.5); $CaCl_2.2H_2O$ (0.04); $MgSO_4.7H_2O$ (0.06); Sodium lactate (6 mL); Yeast extract (1.0); NaCl (0.5); Resazurin (4.0 ml); $FeSO_4.7H_2O$ (0.004) and sodium citrate dihydrate (0.3).[7]

The media were prepared on a heating plate (IKA, C-MAG HS7) at 220°C, under constant stirring and purging of nitrogen and the pH adjusted to 7.6 with NaOH. At the end of the procedure, all media were autoclaved and stored at 4 °C until the time of the experiment.

Test Compounds

The following test-compound groups were prepared for cytotoxicity assays: Positive control, 0.5% hydrogen peroxide solution (0.5% H_2O_2); Negative Control, DMEM (Dulbecco's modified Eagle's medium) Cultivation at 5% SFB; White control, isopropanol (Isop.) and cell extract from seven day aerobic culture cell supernatant, as described below.

In the cellular extract of Cellular Supernatant from seven days of aerobic culture, the test compounds were obtained from aerobic SRB cultures without purging to guarantee the anaerobic condition of the culture, from cellular supernatants from SRB cultures in MCP-E s/Ag and MCP-C s/Ag, with an incubation time of seven days at 30° C, as shown in Table 1.

After the incubation period (7 days/10 mL of culture), the SRB cultures were centrifuged at 10,000 g/5 min. for separation of bacterial cells resulting in two distinct samples, supernatant and cell pellet. They are then stored at -15°C along with the other samples until occasion of use.

Cytotoxicity Test Conditions

For the cytotoxicity assessment of the test compounds, the diluted exposure conditions in 1:2 DMEM 5% SFB and the pure assessment condition were evaluated. The compounds

Table 1. Description of cell supernatant samples from cultures with incubation time of seven days: Culture medium used and cell species cultured to obtain test compounds

Culture Medium	Cell Inoculation
Postgate C without Agar-agar	Environmental *Desulfovibrio desulfuricans* (n = 1)
	Oral *Desulfovibrio desulfuricans* (n = 1)
	Desulfovibrio fairfieldensis (n = 1)
Postgate E without Agar-agar	Environmental *Desulfovibrio desulfuricans* (n = 1)
	Oral *Desulfovibrio desulfuricans* oral (n = 1)
	Desulfovibrio fairfieldensis (n = 1)

n, number of samples

diluted in 1:2 DMEM 5% SFB occurred without the presence of fugizone (antifungal), L-glutamine (amino acid) and penicillin (antibiotic).

Cytotoxicological evaluation took place in two independent assays for each cell condition: 1 x 10^4 cells seeded per well of the L929 strain on the 3rd and 11th Passage and the Vero renal strain on the 195th and 210th Passage. Also included were Positive Control - H_2O_2 0.5%; Negative Control - DEM 5% SFB.

After exposure of the cell lines to the test compounds, the samples were subcultured in a 1.0 mL volume in MCP-E mod to verify the occurrence of cell activity, remaining incubated for 28 days at 30°C for later reading samples.

Cytotoxicity Assay
L929 and Vero cells were grown in 96-well flat-bottomed cell monolayer culture plates with DMEM culture medium supplemented with 5% fetal bovine serum (FBS), fugizone (antifungal), L-glutamine (amino acid) and penicillin (antibiotic) for a 24-hour incubation period (ThermoLab) at 37°C and 5% carbon dioxide (CO_2) to resume exponential growth.

After 24 hours and the presence of a uniform and near confluent cell monolayer was evaluated, the DMEM culture media was removed and the uniform monolayer cell plate was washed twice with 200 µL of 0.1M PBS buffer, 200 µL of DMEM-5% SFB medium was added and the cells were incubated in an oven at 37°C and 5% CO_2 for 20 to 30 minutes. Then DMEM-5% SFB medium was discarded and cells were exposed to 100 µL of test compounds and incubated for 24 hours in a 5% CO_2 oven at 37°C. Throughout the experiment the cells were observed under inverted microscope (Zeis Axiovert) for cell monolayer confluence.

Subsequently, the test compounds were removed and the cells were washed with 0.1 M PBS (200 µL), followed by inoculation of 50 µL Thiazolyl Blue Tetrazolium Bromide (MTT; Sigma) solution (1 mg/mL) and incubation in greenhouse for 1h at 37°C and 5% CO_2.

After this time, MTT was removed and formazan crystals diluted with 100 µL Isopropanol (Tedia). The cell plate was homogenized for 30 seconds at 500 RPM and read on an ELISA reader (Thermo Plate, TP-READER) on the 570 nm filter. At least two independent experiments were performed in triplicate.

Statistical Analysis
The absorbance data of the samples were also analyzed by the variance analysis method (ANOVA) with post-test tukey, pairwise comparison between groups, with a statistical significance criterion adopted of $P < .05$ using graphPad Prisma 5 software.

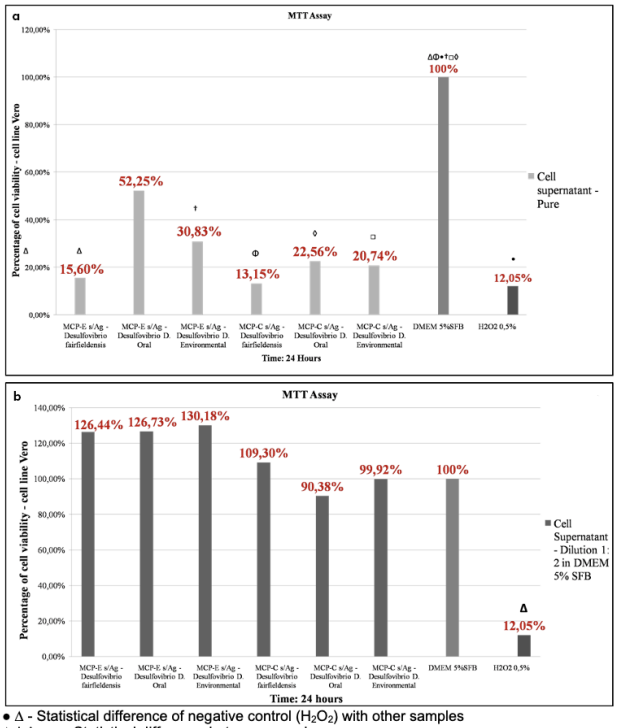
Chemical Analysis of Test Compounds
Test compounds used in Groups 1 were also analyzed for chemical species. A 0.2 µm porosity microfiltration membrane (Sartotius Stedein biotech) was used for each sample, where a volume of 0.5 mL of each test compound on each microfiltration membrane was deposited on its surface with a micropipette, thus preparing the compounds for chemical analysis in the scanning electron microscope (SEM-FEI-Inspect-S50) in EDS (X-ray Dispersive Energy Spectrometry) mode, which allows the immediate identification of the chemical elements with spatial distribution mapping of these elements generating x-ray compositional maps (x-ray mapping) or point analyzes and an energy spectrum demonstrating the relative count of the chemical elements obtained in the surface analysis, with penetration power of 1 µm of the electron beam, determining thus qualitatively and quantitatively the elements present in the sample.[8,9]

Subsequently the results obtained in the quantitative spectra were correlated with the cell viability verified in the cytotoxicity assays.

RESULTS

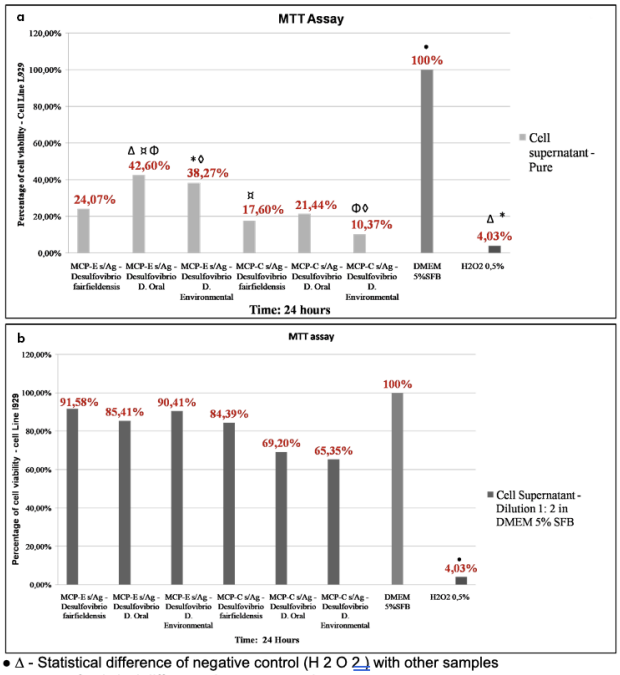
MTT testing using the Vero cell line at a concentration of 1 x 10^4 cells per well revealed biocompatibility of all diluted bacterial supernatants when in contact with cells, even extrapolating the cell concentration. Test compounds using the MCP-E s/Ag vehicle showed better percentages of cell viability when compared to test compounds under pure exposure condition, MCP-E s/Ag with oral *D. desulfuricans* (52.25%). It was the only group that presented biocompatibility, followed by MCP-E s/Ag with environmental *D. desulfuricans* (30.83%) already being classified as severely toxic[2] as well as the other test compounds, which showed a statistically significant difference when compared to the group control. By analyzing the conditions used in the vero cell line it can be verified that the best results were concentrated in the test compounds with MCP-E s/Ag, with better results in MCP-E s/Ag with oral *D. desulfuricans* and environmental *D. desulfuricans* (Figure 1).

As in the Vero cell line, supernatants, when diluted, showed biocompatibility of all supernatant test compounds when exposed to the L929 cell line. The best cell viability was in the MCP-E test compounds with the best MCP-E s/Ag results with *D. desulfuricans fairfieldensis* (91.58%). Pure exposure also followed the same trend in cell viability, with better results in the MCP-E groups. Although no samples in the pure condition presented biocompatibility, there was a statistically significant difference between MCP-E s/Ag test compounds with oral *D. desulfuricans* and MCP-C s/Ag with *D. desulfuricans fairfieldensis*; MCP-E s/Ag with oral *D. desulfuricans* and MCP-C s/Ag with environmental *D. desulfuricans*; MCP-E s/Ag with environmental *D. desulfuricans* and MCP-C s/Ag with environmental *D. desulfuricans*; MCP-E s/Ag with environmental *D. desulfuricans* and Negative Control with H_2O_2 (Figure 2). It was also possible to verify that MCP-C s/Ag environmental *D. desulfuricans* was the group with the lowest cell viability in the pure (10.37%) and diluted (65.35%) condition (Figure 2).



● Δ - Statistical difference of negative control (H_2O_2) with other samples
Δ † ◊ □ ϖ - Statistical difference between samples.

Figure 1. a, b. Mean of 1st and 2nd independent assay of Vero renal cell line exposed to Pure (a) supernatants and diluted in 5% DMEM (b) from 7 days of cultivation



● Δ - Statistical difference of negative control (H 2 O 2) with other samples
Δ † ◊ □ ϖ - Statistical difference between samples.

Figure 2. a, b. Mean of the 1st and 2nd independent assay of the L929 cell line exposed to Pure (a) supernatants and diluted in 5% DMEM (b) from 7 days of cultivation

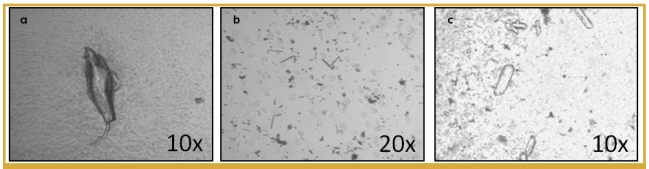


Figure 3. a-c. Photomicrograph of crystalline structures observed L929 cell culture after 24 hours of exposure to compounds: oral D. desulfuricans supernatant in MCP-E s/Ag, diluted 2:1, 7 days (a); D. fairfieldensis supernatant in MCP-C s/Ag, diluted 2:1, 7 days (b) and under environmental D. desulfuricans supernadant in MCP-E s/Ag, Pure, 7 days (c)

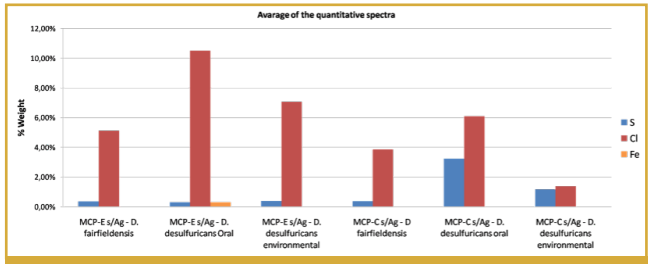


Figure 4. Percentage of mass of test compounds of Cell Pellet group with 7 days of biocorrosion of elements correlated with cell viability of Vero and L929 strains.

Analyzing the results two strains under both conditions, diluted and pure, the test compounds with MCP-E s/Ag showed higher cell viability when compared to those with MCP-C s/Ag, especially MCP-E s/Ag with Environmental *D. desulfuricans* and MCP-E s/Ag with *D. desulfuricans* showed the highest cell viability in 3 of the four tested conditions. In the only condition in which they did not lead the best cell viability, they were after the best condition, which was MCP-E s/Ag with *D. fairfieldensis*.

Observation of the cell monolayer after the 24-hour group 1 time period under inverted microscope (Zeis Axiovert) revealed in some wells artifacts not identified with images suggestive of crystalline formations, in some images irradiated crystalline structures as well as structures were noted similar to prisms (Figure 3). These findings may be correlated with the inoculation vehicle of the test compounds, however it was not possible to find a correlation between such findings and cell viability.

Quantitative Spectrometry

The analyzes obtained on the X-ray Dispersive Energy Spectrometry of the 7-day cell supernatant test compounds were correlated with the cell viability data of the two studied cell lines, L929 and renal Vero (Table 2 and Figure 4). Chloride (Cl) showed higher concentrations in the test compounds with higher cell viability, and it was possible to visualize the same mass percentage ranking of Cl with cell viability ranking of the pure supernatant in the L929 strain. The highest sul-

Table 2. Percentage by mass of test compounds from the Cell Supernatant Group with 7 days of biocorrosion

7 day cultivation supernatant	C	O	Na	Mg	Al	Si	S	Cl	K	Ca	P	Fe
MCP-E s/Ag - *D. fairfieldensis*	12.06%	16.02%	10.86%	1.36%	3.23%	40.05%	0.36%	5.12%	1.91%	8.88%	0.14%	
MCP-E s/Ag - *D. desulfuricans* (Oral)	16.24%	19.22%	15.20%	1.35%	2.49%	26.59%	0.31%	10.51%	1.36%	5.94%	0.49%	0.31%
MCP-E s/Ag - *D. desulfuricans* (environmental)	12.82%	14.72%	11.25%	1.33%	2.86%	37.31%	0.40%	7.07%	1.99%	9.74%	0.50%	
MCP-C s/Ag - *D fairfieldensis*	8.10%	13.18%	7.42%	1.57%	4.10%	49.24%	0.37%	3.87%	2.60%	9.11%	0.44%	
MCP-C s/Ag - *D. desulfuricans* (Oral)	11.81%	12.24%	11.91%	1.17%	2.93%	36.27%	3.24%	6.10%	2.76%	9.75%		
MCP-C s/Ag - *D. desulfuricans* (environmental)	10.21%	23.24%	9.92%	1.72%	3.62%	39.55%	1.17%	1.38%	1.67%	7.52%		

fur (S) concentrations were identified in MCP-C s/Ag when compared to MCP-E s/Ag. In addition, the highest concentrations of S were in the test compounds with the lowest cell viability, MCP-C s/Ag *D. desulfuricans* oral and environmental conditions, in both diluted and pure conditions and in the two strains employed, Vero and L929. Fe was isolated in the sample with higher cell viability, oral MCP-E s/Ag *D. desulfuricans*, in both pure and diluted conditions, suggesting not to interfere with the cell viability of the test compound.

The screening control for certification of lack of cellular activity of bacterial extracts from MCP-E mod peaks of Group 1 test compounds was negative to SRB cell growth after the 28-day incubation period, with no change in culture media. This ensures that cytotoxicity analyzes have occurred only with SRB cell extracts.

DISCUSSION

The process of iron biocorrosion by SRB should be seen as a phenomenon of rupture of the passive steel film by the corrosive metabolites released by the SRB in the medium. This process can be intensified by the presence of chloride ions, associated with biogenic sulfides, released by the SRB, causing a synergy and an increase in the metal attack speed.[10,11] Such analyzes demonstrate the importance of analyzing the supernatant and washed of SRB cultures in both cytotoxic and chemical aspects by mass spectrometry. Remoundaki et al.[12] described through the SEM-EDX a 10µm rod-shaped SRB encapsulation, a cloudy mist corresponding to the deposition of metallic sulfides on the bacterial wall surface and / or adjacent bacterial area,[12] such findings agree with found in spectrometric analyzes revealing the presence of S and iron (Fe) in bacterial supernatant analyzes.

In a previous article, cytotoxicological analyzes presented only in the BACCOR inoculation vehicle without the presence of bacterial strains, suggested that the presence of Agar-Agar in different Postgate culture media be the common denominator responsible for the cytotoxic effect on strains cell phones.[13] The best result in pure and diluted exposures with inoculation vehicles was found in MCP-E s/Ag followed by MCP-E mod.[13] These data follow the same data in this report when we evaluated the cytotoxicity of different inoculation vehicles after SRB cultivation under different conditions. Even with a new factor added to the biological system, there was no variation in the cytotoxicity pattern in Group 1, in both strains, and in both diluted and pure conditions, the MCP-E s/Ag test compounds showed higher cell viability. In addition, MTT assay results for both cell lines indicated that MCP-C cell supernatants were less viable when compared to supernatants using MCP-E. However, the addition of a new factor, the bacterial material, lysate and SRB excretory material, represented alterations that were difficult to correlate with the cytotoxicological results. However, the results of spectrum analyzes suggested a correlation with the sulfur level evaluated in the culture, indicating an inverse relationship between cell viability and S concentration in the culture, the main material released by SRB in its bacterial cycle.

Regarding the images of crystalline structures visualized after the cytotoxicity assay, Remoundaki et al.[12] stated that solid rod-shaped structures may be bacterial cells encapsulated by zinc and iron sulfides, which could help to answer the existence of such structures. Differences found in cell line assays can be better exemplified by Wataha et al.[14], who reported differences in cytotoxic response between different cell lines exposed to identical compounds, such as those used by the authors, as a compound of metal ions. The authors also indicated that the number of passages also affected the cytotoxic response of the studied cell lines.

Regarding the main metabolic product of the SRB, hydrogen sulfide, Persson[15] stressed the need to seek the true mechanism of action of sulfide at the cellular level and its possible toxic effects such as inactivation of cellular cytochrome oxidase, division of disulfide bridges, of proteins and, again, the binding of sulfide to metal ions. However, at 1–2 mM sulfide levels, polymorphonuclear leukocytes are capable of performing most of their functions.[15] Granlund-edstedt et al.[16] simulated the periodontal conditions of sulphide, present in periodontal pockets, on the phagocytic capacity of polymorphonuclear leukocytes in encapsulated group B streptococci and in an unencapsulated variant under aerobic and anaerobic conditions. The results of this study indicated that the presence of sulfide may interfere with the serum

proteins involved in bacterial opsonization, and only in the encapsulated streptococcal strain was a significant inhibition in the elimination of these bacteria.[16] However, the action of polymorphonuclear leukocytes on the other bacterial strain was not significantly influenced by the presence of 2 mM sulfide under both aerobic and anaerobic conditions, and no effect of sulfide on immunoglobulin G antibodies and opsonin C316 was detected. Subsequently, Bisson-Boutelliez et al.[17] demonstrated the invasive capacity of *D. desulfuricans* and *D. fairfieldensis* in oral epithelial cells inducing cytokine secretion, IL-6 and IL-8, by these cells. These findings are reaffirmed by Dzierżewicz et al.[18], who using *D. desulfuricans* lipopolysaccharides in human gingival fibroblasts of the HGF-1 strain in 24-hour cultures demonstrated low levels of IL-6 and IL-8 secretion by these cells. However, at concentrations of 1, 3, 10 and 30 µg/mL lipopolysaccharides there was no significant inhibition of cell growth, in contrast to 100 µg/mL lipopolysaccharides concentration. Kapral et al.[19] reported the use of 10, 50 and 100 µg/mL concentrations of *D. desulfuricans* lipopolysaccharides isolated from intestinal samples in tests with Caco-2 cancer cells. The authors used the times of one, six, 12 and 24 h, where they demonstrated the ability of lipopolysaccharide to promote alterations in p65 and IKBα transcriptional expression genes.

Calenic et al.[20] studied the pathogenic activity of hydrogen sulfide in periodontitis. The authors used human gum-derived epithelial cells (Ca9-22) incubated with 50 ng/mL H_2S at 24, 48 and 72 hours. After 24 and 28 hours, more than one third of the cells were in different stages of early apoptosis and less than 5% in late apoptosis and necrosis. For the 72-hour period, the latter were at significantly increased levels, while apoptosis levels fell to a quarter from the present 48-hour level. In contrast, Yaegaki et al.[21] using human gingival fibroblasts incubated for 72 hours with 100 ng/mL hydrogen sulfide reported a cell necrosis rate of less than 10% of the number of human gingival fibroblasts. Subsequently, Kobayashi et al.[22] reported early stage apoptosis and late apoptosis and necrosis in 16.3% and 7%, respectively, of cells isolated from human dentinal pulp after 48 hours cultivation with 50 ng/mL H_2S. For the authors, these data suggested a relationship between H_2S and pulpitis development.

These findings are in agreement with the inverse relationship analysis of sulfur concentration with cell viability under different conditions. It is noteworthy that *in vitro* cytotoxicity tests differ from *in vivo* assays due to the interaction between the material and the tissue, dependent on a biological interrelation between resident cells, belonging to the tissue in contact with the material, and cells that migrate to place of contact.[23] Therefore, the cell culture system has limitations, such as the fact that it is performed on only one cell type, preventing the immune system acting and/or interacting with other cell types, besides the fact that most laboratory cell lines present reduced physiological responses.[24,25] In addition to these facts, endodontic materials are intended to be confined within the root canal, coming into contact with periapical tissues by extrusion through the apex or by leaching.[4]

The results of this study suggest the applicability of biopharmaceuticals to assist in the biocorrosion of manual endodontic files after fracture inside root canals and may be an auxiliary instrument for the clinical resolution of this endodontic complication.[8,10] The demonstration of moderate cytotoxicity of two compounds MCP-E s/Ag and MCPE-mod opens the way for the continuity of this study to develop a biopharmaceutical that helps the removal of fractured files during the clinical routine.

Test compounds originated from metabolic products and cell lysates of SRB strains, under aerobic conditions of 7 days of bacterial culture, presented better results when employed with MCP-E s/Ag and MCPE-mod, with a moderate cytotoxicity, according to ISO categorization, with cell viability above 50%, which makes this type of inoculum acceptable as a biomaterial.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of the Faculty of Medicine and Antônio Pedro University Hospital, Fluminense Federal University (Approved on October 16, 2013. CEP: CAAE 17247013.3.0000.5243, Nº 426.311).

Informed Consent: Written informed consent was obtained from the patient who participated in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – F.L.H.; Design – G.C.C.S., L.S.G.; Supervision – W.B.C.J., M.T.S.L., V.O.F.L.; Fundings – W.B.C.J., M.T.S.L., V.O.F.L.; Materials – F.L.H., G.C.C.S., L.S.G., W.B.C.J., M.T.S.L., V.O.F.L.; Data Collection and/or Processing – F.L.H.; Analysis and/or Interpretation – F.L.H., W.B.C.J., M.T.S.L., V.O.F.L.; Literature Search – F.L.H.; Writing Manuscript – F.L.H., G.C.C.S., L.S.G.; Critical Review – G.C.C.S., L.S.G.

Declaration of Interests: The authors have no conflicts of interest to declare.

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