Cytotoxic correlation with BACCOR biopharmaceutical mass spectrometry in the biocorrosive process of endodontic files

Fabiano Luiz Heggendorn[1]
Gabriela Cristina Carvalho da Silva[2]
Lúcio Souza Gonçalves[3]
Walter Barreiro Cravo Junior[4]
Marcia Teresa Soares Lutterbach[5]
Sabrina de Castro Brasil[6]
Viviane Oliveira Freitas Lione[7]

Abstract

This study evaluated the biopharmaceutical BACCOR, developed to promote the biocorrosion of fractured endodontic files inside the root canal. The cytotoxicity of cell extracts was evaluated from supernatants and pellets, without the occurrence of biocorrosion of two species *Desulfovibrio desulfuricans*, oral strain and environmental strain, and *Desulfovibrio fairfieldensis* in different culture media. The results were correlated with the most probable number of each bacterial strain and with the analysis of chemical species obtained in energy dispersive spectrometry in RX under a scanning electron microscope. L929 and Vero cell lines were used for MTT cytotoxicity tests. Strains were inoculated into modified Postgate E culture medium (MCP-E mod), Modified Postgate E without Agar-agar (MCP-E s/Ag), Postgate C at 4 g./l. of Agar-agar (MCP-C 4.0 g/l Ag) and Postgate C without Agar-agar (MCP-C s/Ag). After the 42-day cultivation periods cell extracts from Supernatant and Cell Pellet were prepared. All MCP-C supernatants showed lower cell viability when compared to the others using MCP-E. The cell viability of the supernatants was lower than the cell viability of their respective pellets. The test compounds originated from metabolic products and cell lysates of the Sulfate-reducing Bacteria strains showed better results in the groups with MCP-E s/Ag and MCPE-mod, being classified as non-cytotoxic and with moderate cytotoxicity, according to ISO categorization, with viability cell over 50%, which makes this type of inoculum acceptable as a biomaterial.

Keywords: *Desulfovibrio*, oral microbiology; Sulfate-reducing bacteria; Endodontic Files

[1] Post Graduate Program in Dentistry (PPGO) - UNIGRANRIO
[2] Post Graduate Program in Dentistry (PPGO) - UNIGRANRIO
[3] Federal University of Rio de Janeiro School of Pharmacy, Pharmaceutical Bioassays Laboratory
[4] Federal University of Rio de Janeiro School of Pharmacy, Pharmaceutical Bioassays Laboratory
[5] Faculty of Dentistry - Estácio de Sá University
[6] Post Graduate Program in Dentistry (PPGO) - UNIGRANRIO
[7] Federal University of Rio de Janeiro School of Pharmacy, Pharmaceutical Bioassays Laboratory

Resumo

Este estudo avaliou o biofármaco BACCOR, desenvolvido para promover a biocorrosão de limas endodônticas fraturadas no interior do canal radicular. A citotoxicidade dos extratos celulares foi avaliada a partir de sobrenadantes e pellets, sem a ocorrência de biocorrosão de duas *espécies Desulfovibrio desulfuricans*, cepa oral e cepa ambiental, e *Desulfovibrio fairfieldensis* em diferentes meios de cultura. Os resultados foram correlacionados com o número mais provável de cada cepa bacteriana e com a análise das espécies químicas obtidas em espectrometria de energia dispersiva em RX sob microscópio eletrônico de varredura. As linhas celulares L929 e Vero foram usadas para testes de citotoxicidade MTT. As cepas foram inoculadas em meio de cultura Postgate E modificado (MCP-E mod), Postgate E modificado sem ágar-ágar (MCP-E s/Ag), Postgate C a 4 g./l. de Agar-agar (MCP-C 4,0 g/l Ag) e Postgate C sem Agar-agar (MCP-C s/Ag). Após os períodos de cultivo de 42 dias, os extratos celulares do Sobrenadante e do Cell Pellet foram preparados. Todos os sobrenadantes de MCP-C apresentaram menor viabilidade celular quando comparados aos demais utilizando MCP-E. A viabilidade celular dos sobrenadantes foi inferior à viabilidade celular dos seus respectivos pellets. Os compostos de testes oriundos de produtos metabólicos e lisados celulares das cepas de Bactérias Redutoras de Sulfato apresentaram melhores resultados nos grupos com MCP-E s/Ag e MCPE-mod, sendo classificados como não citotóxicos e com citotoxicidade moderada, de acordo com a categorização ISO, com viabilidade celular superior a 50%, o que torna este tipo de inóculo aceitável como biomaterial.

Palavras-chave: *Desulfovibrio*; microbiologia oral; bactérias redutoras de sulfato, limas endodônticas.

INTRODUCTION

In vitro cytotoxicity assay is the first step in the assessment of a medical device, allowing the determination of factors influencing cell growth when exposed to the medical device under study, and checking its tissue compatibility. Although the standard from the International Organization for Standardization rules the use of *in vitro* cytotoxicity assays, defining the parameters (ISO 1099-1, 2003; ISO 10993-5, 2009; NARAYAN, 2009), the literature still mentions the use of different methodologies, exposure times and cell lines for the same medical device and/or biomaterial. There is no standardization of the type of cell line to

be cultured and the type of biomaterial, medical device and *in vitro* clinical situation (HEGGENDORN *et al.,* 2020a).

Most reports found employ L929 mouse fibroblast cell lines (ISO 10993-5, 2009; NARAYAN, 2009; HAUMAN AND LOVE, 2003), for the different materials being studied without taking into account the cell types of the place where the biomaterial will act *in vivo*. Yet, since it is possible that the biomaterial comes into contact with the vascular system, enabling total or partial excretion of metabolites produced by such biomaterial, its cytotoxic evaluation in cell lines of renal origin becomes critical.

The parameters evaluated in cytotoxicity assays can be quantitative and qualitative. Being able to classify the biomaterial, from cell viability (VC), as non-cytotoxic (VC ≥ 90%), slightly cytotoxic (80% - 89%VC), moderately cytotoxic (50% - 79%VC), severely cytotoxic (1% - 49 %VC) and toxic (VC = 0) (ISO 10993-1, 2003; JORGE *et al.,* 2004; ISO 10993-5, 2009; NARAYAN, 2009; HEGGENDORN *et al*., 2020a).

In the dental field, the ISO 7405 (2008) standard guides the assessment of biocompatibility of pharmacological agents, biological components and bioactive endodontic materials or those that claim to stimulate the formation of apical hard tissues. Indeed, initial cytotoxicity tests show limitations when correlated with clinical situations, although they are important tools to evaluate the behavior of the biomaterial and /or medical device. Thus, the search for cytotoxicity test standardization may represent the convergence of the results of different studies enabling determining and refining the biological behavior of materials and /or their components when the reports of different research groups are compared (HEGGENDORN *et al.,* 2020a).

On the other hand, Sulfate-Reducing Bacteria (SRB) are fastidious microorganisms that can be found in different terrestrial and aquatic environments. In the human body, most reports indicate its participation in the intestinal microbiota (HEGGENDORN *et al.,* 2009). In 118 saliva samples, 35 were positive for the presence of SRB, being related to systemic conditions of gastritis (14.4%) and diabetes (3.4%) and to oral conditions of periodontitis (11%) and traumatic fibroma (4.2%) (HEGGENDORN *et al*., 2013). The importance of

analyzing the cytotoxicity of SRB products is reflected in the fact that they are bacteria isolated from the oral cavity with an incidence of 66.67%, of the cases analyzed, including dental remains, teeth with vertical bone loss and mobility and healthy teeth, with indication of extraction for orthodontic reasons (HEGGENDORN *et al.,* 2014).

From this set of information, through the biocorrosive action of SRB, a biopharmaceutical called BACCOR was developed and patented (PI 1105441-7). The formation of SRB biofilm on the metallic surface of endodontic files makes it possible to promote biocorrosion, which can help in the removal of these files when fractured inside the root canals (HEGGENDORN *et al.,* 2018a).

The biocorrosive capacity of BACCOR was demonstrated in endodontic files fractured in root canals *in vitro* for 477 days (HEGGENDORN *et al.,* 2019), while an active SRB biofilm after 84 and 119 days was characterized, showing a low rate of corrosion by loss of dough in these files. When visualizing the metallic surface of these files, several areas of pits of corrosion were identified, which can help in the removal of fractured endodontic files (HEGGENDORN *et al*., 2020).

Therefore, the cytotoxicological evaluation of the biopharmaceutical BACCOR, developed to promote the biocorrosion of fractured endodontic files inside the root canal (HEGGENDORN *et al.,* 2015), becomes essential, in addition to helping to determine the true role of SRBs in the oral cavity.

The aim of this article was to evaluate the cytotoxicity of cell extracts with 42 days of anaerobic cultures, supernatants and cell pellets, without the occurrence of biocorrosion, of SRB from two species *Desulfovibrio desulfuricans*, oral strain and environmental strain, and *Desulfovibrio fairfieldensis* in different culture media. The cytotoxicological frameworks were correlated with the Most Probable Number technique and with the analysis of chemical species obtained in energy dispersive spectrometry in RX under a scanning electron microscope.

MATERIALS AND METHODS

Cell Lines

The cells used were established in culture, in cell monolayer, at the Laboratory of Pharmaceutical Bioassays (LaBioFar) of the Department of Drugs and Medicines of the Faculty of Pharmacy of the Federal University of Rio de Janeiro (UFRJ). Two cell lines were used: L929 (mouse fibroblasts) (Rio de Janeiro Cell Bank, BCRJ: 0188; lot: 000599) and VERO (African Green Monkey Kidney Epithelial Cell) (Rio de Janeiro Cell Bank, BCRJ: 0245; lot: 000590).

Inoculation Vehicles

The Modified Postgate E (MCP-E mod) and Modified Postgate E without Agar-Agar (MCP-E s/Ag) Culture Media had the following composition in g./L. (Postgate 1984): 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.

The Postgate C Media with 4 g./l. of Agar-agar (MCP-C 4.0 g/l Ag) and Postgate C without Agar-agar (MCP-C s/Ag) had the following composition in g./l. (Postgate 1984): 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). For MCP-C 4.0 g/l Ag. 4.0 g/l was added of agar-agar.

The Media were prepared on a hot plate (IKA, C-MAG HS7) at 220°C, under constant stirring and nitrogen purging, and the pH adjusted to 7.6 with NaOH. At the end of the procedure, all 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, Culture in DMEM 5% FBS (Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum), White control, isopropanol, and cell extracts from supernatant and cell Pellet from 42 days of anaerobic culture, described below.

Cell Extracts: Supernatant and Cell Pellet from 42 days of anaerobic culture

The culture media were inoculated under anaerobic, purging conditions and incubated for 42 days at 30 ºC. After the incubation period, cell growth was verified through the formation of iron sulfide in the culture media (Tab. 1). In all samples there was cell growth, except in the two samples of environmental D. *desulfuricans* cultivated in MCP-C s/Ag, which were discarded.

Table 1- Distribution of cultures to obtain test compounds from supernatant and 42-day cell pellet.

Culture medium	Cell inoculation / Indicative of cell growth
Postgate C culture medium with 4 g/l Agar-agar	*D. desulfuricans* environmental(n=2) / Positive growth *D. desulfuricans* oral (n=2) / Positive growth *D. fairfieldensis* (n=2) / Positive growth
Postgate C culture medium without Agar-agar	*D. desulfuricans* environmental (n=2) / Negative growth *D. desulfuricans* oral (n=2) / Positive growth *D. fairfieldensis* (n=2) / Positive growth
Postgate E modified culture medium	*D. desulfuricans* environmental (n=2) / Positive growth *D. desulfuricans* oral (n=2) / Positive growth *D. fairfieldensis* (n=2) / Positive growth
Postgate E culture medium without Agar-agar	*D. desulfuricans* environmental (n=2) / Positive growth *D. desulfuricans* oral (n=2) / Positive growth *D. fairfieldensis* (n=2) / Positive growth

n: number of samples prepared

After the incubation period, cultures were centrifuged at 10,000 g/5 min. for separation of bacterial cells, resulting in two distinct samples, supernatant and cell pellet, pellet. The cell pellet of each sample was resuspended in phosphate buffered saline (PBS) (DULBECCO AND VOGT, 1954), at a volume of 35 ml in each sample, under vortex agitation (Phonex Ap56). This solution was previously

prepared, from the following methodology and composition (g./l. of distilled water): NaCl (8.01); KCl (0.20); Na_2HPO_4 (1.44); KH_2PO_4 (0.24). The pH of the solution was adjusted to 7.4 with NaOH, autoclaved for 15 minutes at 121°C and stored at 4°C until use.

Subsequently, an aliquot of 1.0 ml was removed from each sample to determine the cell concentration by the Most Probable Number (MPN) technique and the excess of each sample was stored at -15°C until the time of use.

Most Probable Number (MPN)

For the MPN technique, modified Postgate E culture medium and reducing solution for anaerobic bacteria were prepared in 15 ml penicillin-type flasks, distributed in a volume of 10 and 9 ml, respectively. For each sample, with an aliquot of 1.0 ml obtained in the previous step, eight serial decimal dilutions in Reducing Solution for anaerobic bacteria, from 10^{-1} to 10^{-8}, were followed. From each of these dilutions, 1.0 ml aliquots were transferred to three flasks containing modified Postgate E culture medium. As a control, a flask of modified Postgate E culture medium without inoculation was used. All flasks were incubated for 28 days at 30 °C and then positive cultures were identified. By the number of positive tubes, black precipitate in the culture medium, in each of the employed dilutions, the MPN per ml was determined, based on the statistical table of MCCRADY (1915).

CYTOTOXICITY TEST CONDITIONS

For the cytotoxicity assays, the exposure conditions diluted in 1:2 DMEM 5% FBS were evaluated, without the presence of fungizone (antifungal), L-glutamine (amino acid) and penicillin (antibiotic).

The cytotoxicological evaluation was carried out in two independent assays for each cell condition: with 1 x 10^4 cells seeded per well of the L929 lineage, in the 3° and 11° passage, and of the Vero renal lineage, in the 195° and 210°

passage. The Positive Control, H_2O_2 0.5%, and the Negative Control, DMEM 5% FBS were also included.

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

Cytotoxicity Test

Cells of the L929 and Vero lineages were cultivated in 96 well culture plates with flat bottom in cell monolayer, with culture medium DMEM 5% FBS, fungizone (antifungal), L-glutamine (amino acid) and penicillin (antibiotic) per an incubation period of 24 hours in an oven (ThermoLab) at 37°C and 5% carbon dioxide (CO_2) to resume exponential growth.

After a period of 24 hours and the presence of a uniform cell monolayer close to confluence was evaluated, the DMEM culture media were removed and the cell plate in uniform monolayer was washed twice with 200µl of 0.1M PBS, added with 200µl of DMEM 5% FBS medium and the cells were incubated in a 5% CO_2 incubator at 37 °C for 30 minutes. Then, the DMEM 5% FBS medium was discarded and the cells were exposed to 100µl of test compounds and incubated for 24 hours in a 5% CO_2 oven at 37 °C. During the entire experiment, the cells were observed under an inverted microscope (ZeisAxiovert) in relation to the confluence of the cell monolayer.

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

After this period, the MTT was removed and the forman crystals diluted with 100µl Isopropanol (Tedia). The cell plate was homogenized for 30 seconds at 500 rotations per minute (RPM) and reading was performed in an ELISA reader (Thermo Plate, TP-READER) on a 570nm filter. Two independent experiments were carried out in triplicate.

STATISTICAL ANALYSIS

The absorbance data of the samples were analyzed by the method of analysis of variance (ANOVA) with post-test tukey, comparison in pairs between groups, with the criterion of statistical significance adopted at $p < 0.05$ using the graphPad Prism 5 software.

CHEMICAL ANALYSIS OF TEST COMPOUNDS

Scanning electron microscopy

Subsequently, the test compounds were analyzed in relation to chemical species. For each sample, a microfiltration membrane (Sartotius Stedim biotech) with 0.2µm porosity was used where a volume of 0.5 ml of each test compound was deposited, thus preparing the compounds for chemical analysis in the scanning electron microscope (MEV-FEI-Inspect-S50) in EDS (X-Ray Energy Dispersive Spectrometry) mode, which allows the immediate identification of chemical elements, demonstrating the relative count of chemical elements obtained in the surface analysis, thus determining qualitatively and quantitatively the elements present in the sample (GEESEY *et al.,* 2002; OUNSI *et al.,* 2008).

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

RESULTS

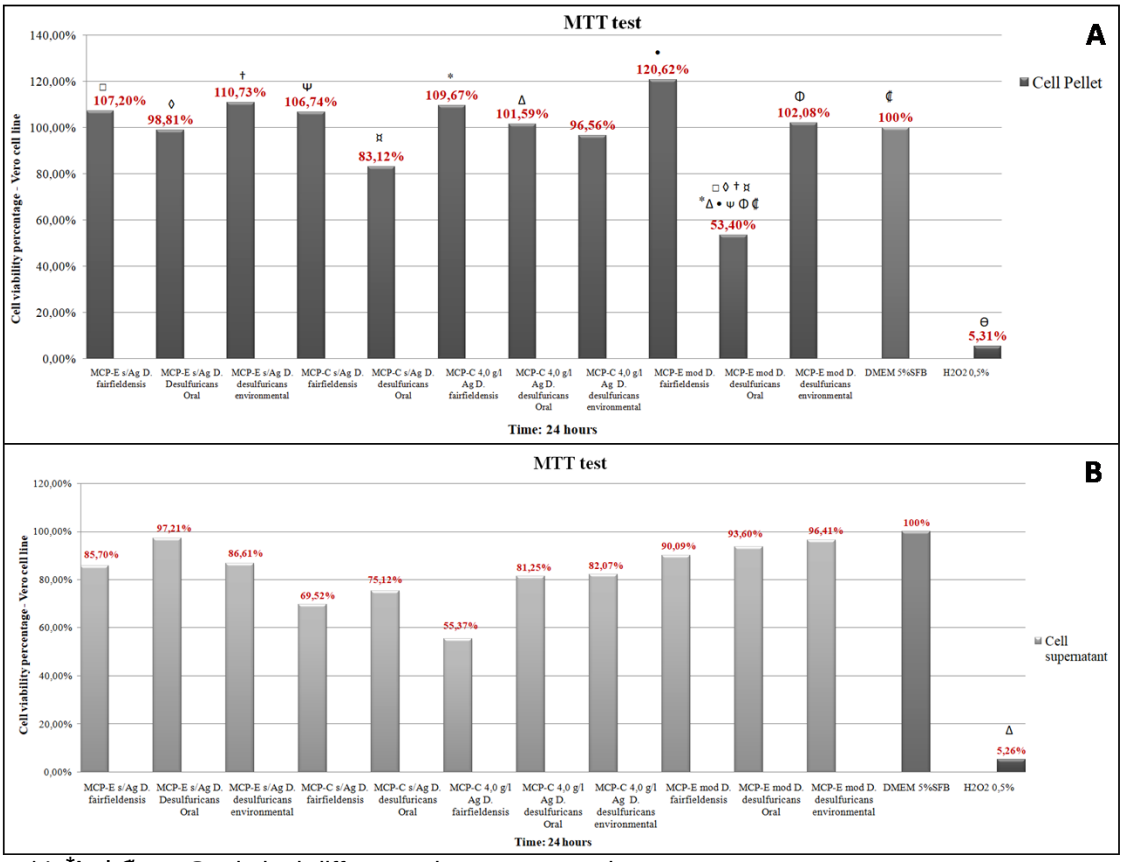
All peaks in MCP-E mod obtained from the test compounds evaluated were negative for SRB cell growth after the 28 day incubation period, with no change in the culture media, thus ensuring that cytotoxicity analyzes occurred only in cell extracts bacteria without bacterial activity.

CYTOTOXICITY OF CELL PELLET AND SUPERNATANT

The MTT test using the Renal Vero cell line, at a concentration of 1 x 10^4 cells per well, revealed a non-cytotoxic framework (VC ≥ 90%) in most of the diluted cell pellets, even extrapolating the cell viability of the control group, with better results in MCP-E mod with *D. fairfieldensis* (120.62% VC) and MCP-E s/Ag with *D. desulfuricans* environmental (110.73% VC). The MCP-C s/Ag with *D. desulfuricans* Oral (83.12% VC) was classified as slightly cytotoxic (80%-89% VC) while MCP-E mod with *D. desulfuricans* environmental (53.4% VC), as moderately cytotoxic (50%-79% VC). Regarding cell supernatants, only 4 compounds were non cytotoxic MCP-E s/Ag with *D. desulfuricans* Oral (97.21% VC), MCP-E mod with *D. fairfieldensis* (90.9% VC) and *D. desulfuricans* oral (93.6% VC) and environmental (96.41% VC). There was an increase in the number of compounds classified as mildly cytotoxic, MCP-E s/Ag *D. fairfieldensis* (85.70% VC) and *D. desulfuricans* Environmental (86.61% VC) and MCP-C 4.0gl/Ag *D. desulfuricans* oral (81.25% VC) and environmental (82.07% VC), as well as in the framework of moderately cytotoxic compounds, MCP-C s/Ag *D. fairfieldensis* (69.52% VC) and *D. desulfuricans* Oral (75.12% VC) and MCP-C 4.0g/l Ag *D. fairfieldensis* (55.37% VC).

All MCP-C had the lowest cellularity when compared to the other supernatants. It was also observed that all cell supernatants showed lower biocompatibility when compared to their respective cell pellets, except in MCP-E Mod with *D. desulfuricans* oral (**Gráf.1**).

Graphic 1 - Mean of the 1st and 2nd independent assays of the Vero renal cell lineage exposed to cell extracts from 42 days of culture, with exposure of the cell pellet (A) and cell supernatant (B).



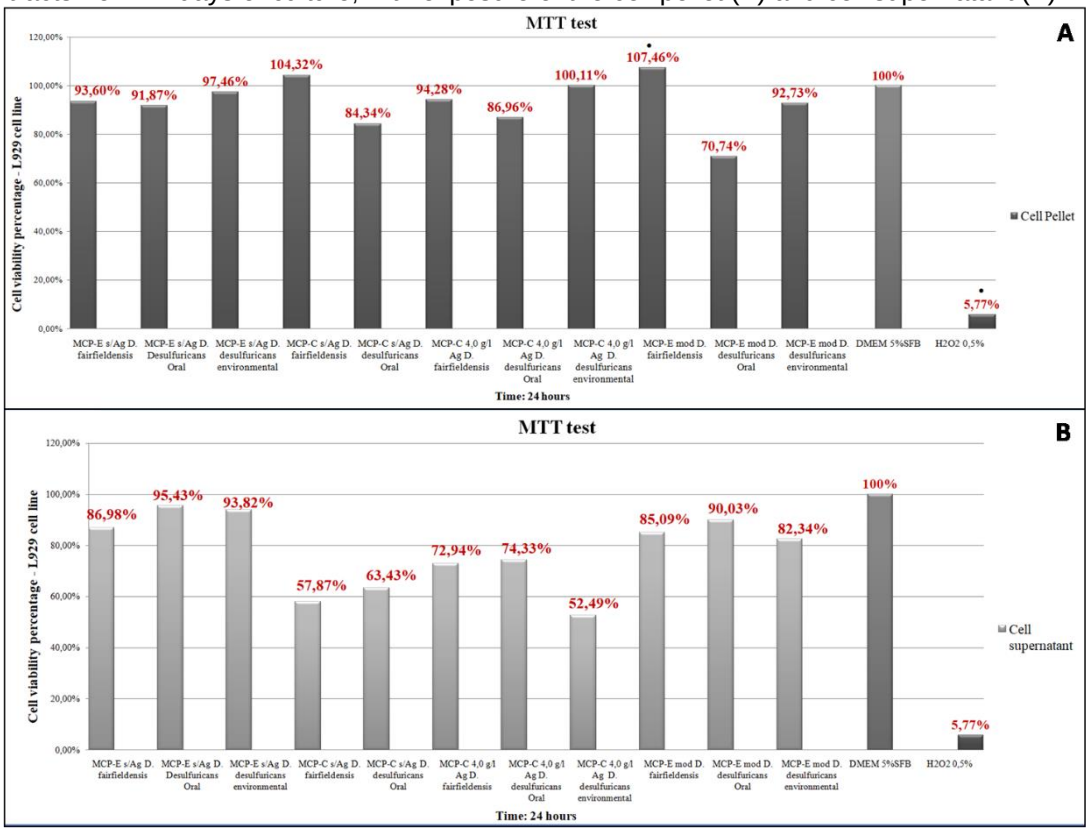
ɵ□◊†¤Δ•ΦЄ Ψ - Statistical difference between samples

The MTT test using the L929 cell line at a concentration of 1 x 10^4 cells per well revealed a noncytotoxic framing in most Cell Pellet test compounds, exceeding the control group cell concentration in three test compounds. Mildly cytotoxic framing was identified in the test compound *D. desulfuricans*oral in MCP-C s/Ag (84.34% VC), MCP-C 4.0 g/l Ag (86.96% VC) and MCP-E mod (70.74% VC). In this condition, the MCP-E Mod with *D. desulfuricans* oral, repeated the same level of cell viability revealed in the Vero renal lineage, presenting the lowest VC, when compared to the other test-compounds.

The analysis revealed that all cell supernatants had lower cell viability when compared to cell pellets, except in MCP-E Mod with *D. desulfuricans* oral, as well as in the Vero cell line. In this analysis, all compounds that used MCP-E mod and MCP-E s/Ag were between non cytotoxic and slightly cytotoxic. The compounds

that used MCP-C s/Ag and MCP-C s/Ag were classified as moderately cytotoxic (**Gráf. 2**).

Graphic 2 - Mean of the 1st and 2nd independent assays of the L929 cell line exposed to cell extracts from 42 days of culture, with exposure of the cell pellet (A) and cell supernatant (B).



•Statistical difference between samples

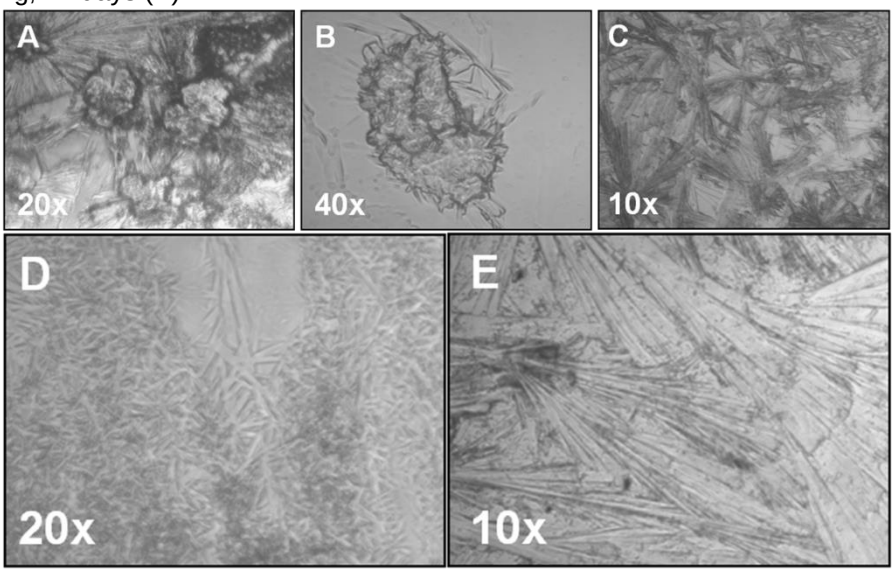
The results of the MTT assay for the two cell lines indicated that all MCP-C supernatants had lower cell viability when compared to MCP-E supernatants. In most conditions, supernatants showed lower cellularity when compared to pellets in the same condition. This finding suggests that the cell supernatant concentrates metabolic substrates derived from bacterial culture, the metabolic products produced by SRB during the culture time, while cell pellets concentrate the lysed cells.

The use of test compounds with higher concentrations of Agar-agar, MCP-E mod and MCP-C 4g/l Agar, did not represent significant changes in cell viability when compared to the same compounds without the use of Agar-agar, MCP-E s/Agar and MCP-C s/Agar, with statistical difference being found only in the

cellular Pellet of MCP-E mod *D. desulfuricans* oral (53.40% VC) in the Vero renal lineage.

The observation of the cell monolayer after a period of time of 24 hours under an inverted microscope (Zeis Axiovert) revealed, in some wells, unidentified artifacts with images suggestive of crystalline formations, in some images it was also noted irradiated crystal structures as well as structures similar to prisms (**Fig. 1**). Such findings can be correlated with the vehicle of inoculation of the test compounds, however it was not possible to find a correlation between these findings and cell viability.

Figure 1 – Photomicrograph of crystal structures observed in L929 cell culture after 24 hours of exposure to the compounds: cell pellet of *D. fairfieldensis*in MCP-E s/Ag, 42 days (A and B); Cell pellet of *D. fairfieldensis* in MCP-C s/Ag, 42 days (C and D); cell pelletof *D. desulfuricans* Oral in MCP-C s/Ag, 42 days (E).



Correlation between MPN and cell viability

When correlating the MPN cell concentration with the IC_{50} cell pellet result, there was a tendency for the lowest MPN cell concentrations to be in the highest pellet IC_{50} results, with some exceptions. Therefore, this data suggests that there is an inverse relationship between the cellular concentration of SRB and the cell viability of cell lines. This situation can be answered by the relation of a greater bacterial number promoting a greater concentration of metabolic substrates

derived from bacterial culture, which could represent a greater cytotoxicity in the presented tests (**Tab.2**).

Table 2 - Descending order of cell concentration of cultures by the MPN technique with IC_{50} of cell lines exposed to different cell pellets.

Cultivation / Strain	Cell concentration of SRB cultures	IC_{50} ranking	
		Vero cell line	L929 cell line
		Cell Pellet	Cell Pellet
MCP-C 4g/l Ag *D. desulfuricans* Oral	2,40 x 10^7 mnp/ml	7 (101,59%)	9 (86,96%)
MCP-E s/Ag *D. desulfuricans* Oral	2,1 x 10^6 mnp/ml	9 (95,81%)	8 (91,87%)
MCP-E mod *D. desulfuricans* Oral	1,2 x 10^6 mnp/ml	11 (53,40%)	11 (70,74%)
MCP-C s/Ag *D. desulfuricans* Oral	5,3 x 10^5 mnp/ml	10 (83,12%)	10 (84,34%)
MCP-C 4g/l Ag *D. desulfuricans* environmental	4,4 x 10^5 mnp/ml	8 (96,56%)	4 (100,11%)
MCP-E s/Ag *D. desulfuricans* environmental	4,4 x 10^5 mnp/ml	2 (110,73%)	5 (97,46%)
MCP-E mod *D. desulfuricans* environmental	1,1 x 10^4 mnp/ml	6 (102,08%)	5 (92,73%)
MCP-C s/Ag *D. fairfieldensis*	4 x 10^2 nmp/ml	5 (106,74%)	2 (104,34%)
MCP-C 4g/l Ag *D. fairfieldensis*	4 mnp/ml	3 (109,66%)	6 (94,28%)
MCP-E s/Ag *D. fairfieldensis*	No growth	4 (107,20%)	7 (93,60%)
MCP-E mod *D. fairfieldensis*	No growth	1 (120,62%)	1 (107,46%)

MPN/ml: Most Probable number per ml

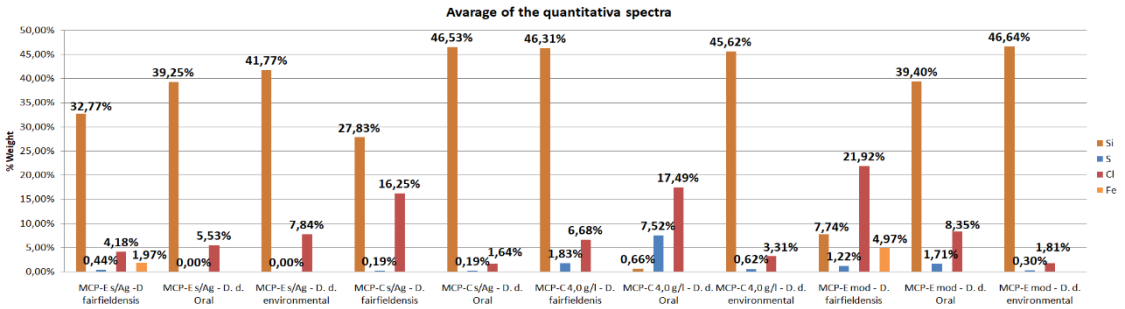
CHEMICAL ANALYSIS OF COMPOUNDS BY QUANTITATIVE SPECTROMETRY

The analyzes obtained from the energy spectra of the test Compounds of the 42 day cell pellets were correlated with the cell viability data of the two cell lines studied, L929 and Renal Vero (**Tab. 3** and **Gráf. 3**).

Table 3- Mass percentage of test compounds from the Cellular Pellet group with 42 days of biocorrosion.

Test compounds Cell Pellet	C %	O %	Na %	Mg %	AL %	Si %	S %	Cl %	K %	Ca %	P %	Fe %
MCP-E s/Ag -*D fairfieldensis*	12,72	21,94	12,20	1,38	2,76	32,77	0,44	4,18	1,36	6,88	1,39	1,97
MCP-E s/Ag - *D. d.* Oral	10,23	16,70	10.67	1,60	3,30	39,25	0,00	5,53	1,78	8,97	2,19	
MCP-E s/Ag - *D. d.* environmental	8,19	11,68	8,82	1,41	3,30	41,77	0,00	7,84	2,15	10,54	4,30	
MCP-C s/Ag - *D. fairfieldensis*	12,13	19,59	14,13	1,00	2,44	27,83	0,19	16,25	1,11	5,17	0,16	
MCP-C s/Ag - *D. d.* Oral	9,11	16,26	9,25	1,73	3,94	46,53	0,19	1,64	2,15	9,21		
MCP-C 4,0 g/l - *D.fairfieldenis*	6,99	10,92	7,80	1,57	3,80	46,31	1,83	6,68	2,73	9,29	2,08	
MCP-C 4,0 g/l - *D. d.* Oral	32,20	15,10	16,96	0,10	0,00	0,66	7,52	17,49	2,95	2,18	4,85	
MCP-C 4,0 g/l - *D. d.* environmental	8,09	16,60	8,94	1,63	3,71	45,62	0,62	3,31	2,31%	9,17		
MCP-E mod - *D. fairfieldensis*	28,92	9,32	11,28	0,87	0,41	7,74	1,22	21,92	2,18%	5,02	6,16	4,97
MCP-E mod - *D. d.* Oral	9,76	11,03	10,37	1,36	3,17	39,40	1,71	8,35	2,43%	9,33	3,08	
MCP-E mod - *D. d.*environmental	8,16	17,59	8,60	1,76	4,10	46,64	0,30	1,81	2,08%	8,82	0,15	

Graphic 3 – Mass percentage of test compounds from the Cell Pellet group correlated with cell viability of Vero and L929 strains.



Test Compound MCP-E mod *D. fairfieldensis*, cell pellet, had the highest cell viability in both cell lines, L929 and renal Vero. This compound had one of the lowest levels of Si while the oral MCP-E Mod *D.desulfuricans* pellet, which had the lowest cell viability among the test compounds, showed a high level of Si. Regarding Cl, the analysis is maintained, the highest level of Cl was identified in the test compound MCP-E Mod *D. fairfieldensis*, pellet, which showed the highest cell viability in both cell lines (**Tab. 3** and **Gráf. 3**).

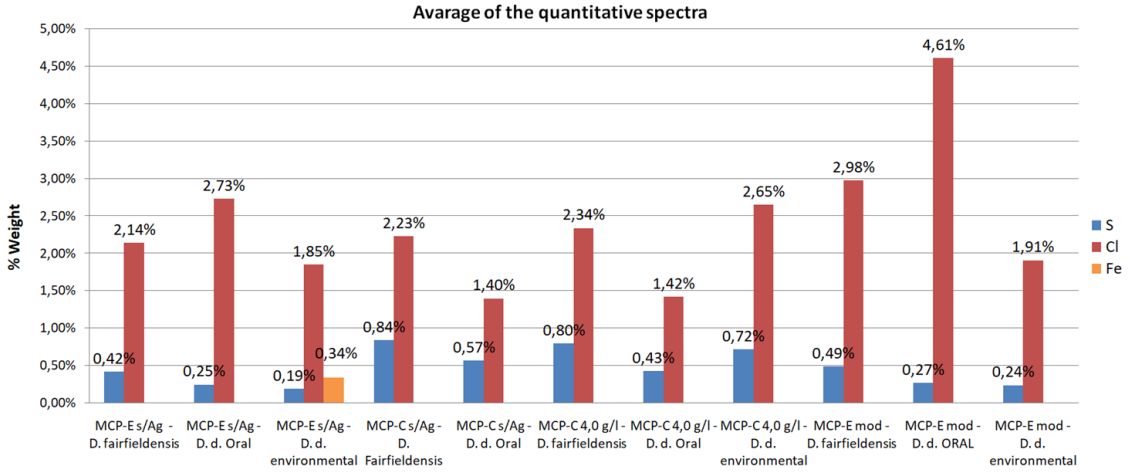
The presence of Fe was detected in the cell pellet of MCP-E mod *D. fairfieldensis* and MCP-E s/Ag *D. fairfieldensis*, not negatively influencing cell viability, since these compounds had the highest levels of cell viability. On the other hand, the presence of S, the main metabolite released by the SRB, showed absence or the lowest levels in the test compounds that did not use Agar, MCP-E s/Ag and MCP-C s/Ag. The results also suggested that Pellets with the highest concentrations of S had the lowest cell viability, as in oral MCP-C 4.0 g/l *D. desulfuricans* (86.96% VC, L929) and MCP-E mod *D. desulfuricans* oral (53.40% VC, Vero and 70.74% VC, L929) (**Tab. 3** and **Gráf. 3**)

The analysis of cell supernatants showed the presence of Fe only in the test-compound MCP-E s/Ag *D. desulfuricans* environmental, suggesting that there was no change in cell viability in the cytotoxicology assays involving the Vero and L929 strains (**Tab. 4** and **Gráf. 4**).

Table 4 – Mass percentage of test compounds from Cell supernatant group with 42 days of biocorrosion**.**

Test compounds Cell Pellet	C %	O %	Na %	Mg %	AL %	Si %	S %	Cl %	K %	Ca %	P %	Fe %
MCP-E s/Ag - *D. fairfieldensis*	8,86	17,40	8,78	1,80	3,96	45,60	0,42	2,14	2,04	8,87	0,13	
MCP-E s/Ag - *D. d.* Oral	8,83	15,97	8,82	1,77	3,96	46,02	0,25	2,73	2,07	9,43	0,14	
MCP-E s/Ag - *D. d.*environmental	9,37	23,79	10,00	1,85	3,68	40,06	0,19	1,85	1,69	7,18		0,34
MCP-C s/Ag - *D. Fairfieldensis*	7,52	16,19	9,44	1,70	3,81	45,71	0,84	2,23	2,41	10,16		
MCP-C s/Ag - *D. d.* Oral	7,76	17,89	8,94	1,90	4,02	46,42	0,57	1,40	2,08	9,02		
MCP-C 4,0 g/l - *D. fairfieldensis*	9,28	16,76	9,59	1,74	3,80	44,59	0,80	2,34	2,08	8,88	0,13	
MCP-C 4,0 g/l - *D. d.* Oral	10,40	22,80	9,50	1,68	3,86	40,72	0,43	1,42	1,83	7,26	0,11	
MCP-C 4,0 g/l - *D. d.* environmental	7,80	16,44	9,05	1,79	4,04	46,33	0,72	2,65	1,99	9,03	0,11	
MCP-E mod - *D. fairfieldensis*	9,52	16,98	9,53	1,75	3,94	43,99	0,49	2,98	2,10	8,49	0,24	
MCP-E mod - *D. d.* Oral	6,51	12,20	8,27	2,03	4,14	48,01	0,27	4,61	2,75	10,30	0,91	
MCP-E mod - *D. d.* environmental	8,00	15,35	8,73	1,73	4,19	48,48	0,24	1,91	2,22	9,09	0,05	

Graph4 –Mass percentage of test compounds from Cell supernatant group at 42 days with elements correlated with cell viability of Vero and L929 strains.



The Cl ementum maintained the same characteristic, with high concentration in samples with high cell viability classified as non-cytotoxic and

slightly cytotoxic, as in oral MCP-E mod *D. desulfuricans* (93.60% VC Vero and 90.03% VC L929) and MCP-E mod *D. fairfieldensis* (90,09% VC Vero and 85,09% VC L929).

However, a correlation flow Cl concentration was identified in test compounds with lower cell viability, as in oral MCP-C s/Ag *D. desulfuricans* (75.12% VC Vero and 63.43% VC L929) and MCP-C 4, 0 g/l Ag *D. desulfuricans* Environmental (82.07% VC Vero and 52.49% VC L929). S had higher concentrations in the test compounds MCP-C s/Ag and MCP-C 4.0 g/l. The high concentration of S in the supernatants that had lower cell viability and the lower concentration of S in the supernatants with the highest cell viability, in the two strains studied, suggests that there is an inverse relationship between the concentration of S in the supernatants and the cell viability of the strains used in the cytotoxicity assays (**Tab. 4** and **Graph.** 4).

DISCUSSION

The process of biocorrosion by SRB is a phenomenon of rupture of the passive film of steel by the corrosive metabolites released by SRB in the medium. The presence of chloride ions, associated with the biogenic sulfides, released by the SRB, potentiate this process, leading to an increase in the speed of attack on the metal (VIDELA, 2003; LARRY AND HAMILTON, 2007). REMOUNDAKI *et al.* (2008) described a cloudy haze corresponding to the deposition of metallic sulphides on the surface of the SRB cell wall and/or area adjacent to the bacterial cell, such findings are in agreement with the findings. In the spectrometry analysis revealing S and Fe concentrations in the supernatant and cell pellet analysis. Biofilm formation and biocorrosion capacity of SRB in endodontic files were evaluated for 445 days demonstrating high concentrations of Ca, P, C, S and Fe (HEGGENDORN *et al*., 2018a). The presence of S, Cl and O was correlated with the biocorrosion of endodontic files, further releasing metal alloy forming products such as Fe, Ni and Cr in the medium (HEGGENDORN *et al*., 2019). Such analyzes demonstrated the importance of analyzing the supernatant and pellet of SRB cultures both in the cytotoxic and chemical aspects by means of mass

spectrometry due to the chemical diversity present in these compounds, correlating them with possible biological and cellular responses. Responding in part to the findings of the images of crystalline structures visualized after the cytotoxicity assay, REMOUNDAKI *et al*., (2008) stated that solid structures in the form of rods could be bacterial cells encapsulated by zinc and iron sulfides, which would help to respond to the existence of such structures. The differences found in IC_{50} between cell lines may be related to the existence of different cytotoxic responses between different cell lines exposed to the same compound, with an influence on the number of passages of the lines when the cytotoxic response is evaluated (WATAHA *et al*., 1994).

Even under controlled and similar conditions of cultivation, the test compounds showed variation in chemical composition, in bacterial population number, directly reflecting the cytotoxic response of the test compounds. The growth kinetics of two microbial species of the genus *Desulfovibrio*, *Desulfovibrio desulfuricans* (oral and environmental strain) and *Desulfovibrio fairfieldensis*, under aerobic and anaerobic conditions were analyzed in MCPE-S/Ag and MCPC-S/Ag, demonstrating that such bacteria hinder in exponential phase between 10 and 40 hours of cultivation, showing divergence in the reading of the growth curve with the formation of iron sulfide in the modified Postgate E culture medium without agar (HEGGENDORN *et al*., 2018), corresponding to the variation of iron sulfide concentration in the test compounds analyzed here, so cultures at the same time, microbial species and conditions may have different concentrations of chemical elements and bacterial products.

Regarding the main metabolic product of SRB, hydrogen sulfide, the cytotoxic action of the sulfide occurs through the inactivation of cellular cytochrome oxidase, the splitting of essential disulfide bonds of proteins, and the binding of sulfide to metal ions (PERSSON, 1992). The presence of sulfide in periodontal pockets can interfere with the serum proteins involved in the opsonization of bacteria, inhibiting the elimination of certain bacteria. However, in another bacterial strain, the presence of sulfide did not significantly influence both in aerobic and anaerobic conditions, in addition to not having detected an effect of sulfide on immunoglobulin G antibodies and on opsonin C3, still allowing

the performance of most functions of the polymorphonuclear leukocytes at levels below 2 mM of sulfide (PERSSON, 1992; GRANLUND-EDSTEDT *et al*., 1993).

Subsequently, the invasive capacity of *D.desulfuricans* and *D. fairfieldensis* in oral epithelial cells was demonstrated, inducing the secretion of cytokines, IL-6 and IL-8 (BISSON-BOUTELLIEZ *et al*., 2010). The use of lipopolysaccharides from *Desulfovibrio desulfuricans* in human gingival fibroblasts of the HGF-1 lineage in 24-hour cultures demonstrated low levels of IL-6 and IL-8 secretion by these cells. At concentrations of 1, 3, 10 and 30 µg/mL of lipopolysaccharides, there was no significant inhibition of cell growth, in contrast, when the concentration of 100 µg/mL of lipopolysaccharides was used (DZIERŻEWICZ *et al*., 2010). Likewise, concentrations of 10, 50 and 100 µg/mL of *Desulfovibrio desulfuricans* lipopolysaccharides, isolated from intestinal samples, in tests with Caco-2 cancer cells exposed for one, six, 12 and 24 h demonstrated the ability of lipopolysaccharide to promote alterations in the transcriptional expression genes p65 and IKBα (KAPRAL *et al*., 2010).

In a previous article, the cytotoxicological analyzes presented only in the BACCOR inoculation vehicle without the presence of bacterial strains, suggested that the presence of Agar-agar, in the different Postgate culture media, is the common denominator responsible for the cytotoxic effect on the on cell lines (HEGGENDORN *et al.,* 2016). The best result in pure and diluted exposures with inoculation vehicles was found in MCP-E s/Ag followed by MCP-E mod (HEGGENDORN *et al.,* 2016). Such data follow the same data, in this report, when we evaluated the cytotoxicity of different inoculation vehicles after SRB cultures, under different conditions. Even with a new factor added to the biological system, there was no variation in the pattern of cytotoxicity, the results of the MTT assay for the two cell lines indicated that the cell supernatants of MCP-C showed lower viability when compared to the supernatants using MCP-E. However, the addition of a new factor, cellular material, lysate and cell excretory material, represented alterations that were difficult to correlate with the cytotoxicological results, however, the results of the spectrum analysis suggested that there was a correlation with the concentration of sulfur existing in the compounds, indicating

an inverse relationship between cell viability and the concentration of S in the culture, the main material released by the SRB in their cell cycle.

In the study of the pathogenic activity of hydrogen sulfide in periodontitis, human gingival-derived epithelial cells (Ca9-22) incubated with 50 ng/mL of H_2S showed that after 24 and 48 hours more than a third of the cells were in different stages of apoptosis. and less than 5% in late apoptosis and necrosis, while in the 72-hour period, the levels of late apoptosis and necrosis increased significantly and the levels of apoptosis declined to a quarter of the level present at 48 hours (CALENIC *et al*., 2010). When using the same concentration of 50 ng/mL H_2S in cells isolated from human dentin pulp exposed for 48 hours, KOBAYASHI *et al*., (2011) reported early-stage apoptosis and late-stage apoptosis and necrosis in 16.3% and 7%, respectively. For the authors, these data suggested a relationship between H_2S and the development of pulpitis. In contrast, 100 ng/ml H_2S in human gingival fibroblasts for 72 hours was shown to have a cell necrosis rate of less than 10% (YAEGAKI *et al.,* 2008).

Such findings are in agreement with the analysis of the inverse relationship between sulfur concentration and cell viability in the L929 and Renal Vero cell lines. It is worth mentioning that the in vitro cytotoxicity tests are different from the in vivo tests 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 contact location (ESTRELA, 2001). Therefore, cytotoxicity assays using cell lines have limitations because they use only one cell type, preventing the immune system from acting and/or interacting with other cell types. In addition, it is worth adding that most laboratory cell lines show reduced physiological responses (HANKS *et al*., 1996; HORNEZ *et al*., 2002). In addition to these facts, endodontic materials are intended to be confined within the root canal, coming into contact with the periapical tissues through extrusion through the apex or by leaching (HAUMAN AND LOVE, 2003). This situation corroborates the need to evaluate the different test compounds presented, since in cases of extrusion of this material, via the apex or apical foramen, during endodontic retreatment, these compounds would be extruded

during the permanence of BACCOR in the endodontic canal, for the removal of fractured endodontic files inside the root canal.

CONCLUSION

Test compounds originated from metabolic products and cell lysates of SRB strains, under anaerobic conditions of 42 days of culture, showed better results when used with MCP-E s/Ag and MCPE-mod, being classified between non-cytotoxic and with moderate cytotoxicity, according to ISO categorization, with cell viability above 50%, which makes this type of inoculum acceptable as a biomaterial. Only MCP-E s/Ag was classified as non-cytotoxic in all cytotoxic assays. Spectrum analyzes suggested that there was an inverse relationship between the concentration of sulfur in the compounds and cell viabilit.

REFERENCES

1. BISSON-BOUTELLIEZ, C.; MASSIN, F.; DUMAS, D.; MILLER, N.; LOZNIEWSKI, A. Desulfovibrio spp. Survive withi KB cells and modulate inflammatory responses. **Mol oral Microbiol***.* v. 25, p. 226-235, 2010.
2. CALENIC, B.; YAEGAKI, K.; MURATA, T.; IMAI, T.; AOYAMA, I.; SATO, T.; *et al.* Oral malodorous compound triggers mitochondrial-dependent apoptosis and causes genomic DNA damage in human gingival epithelial cells. **J Periodont Res**. v. 45, n. 1, p. 31-37, 2010.
3. DULBECCO, R.; VOGT, M. Plaque formation and isolation of pure lines with poliomyelitis viruses. **J Exp Med***,* v. 99, n. 2:167-82, 1954.
4. DZIERŹEWICZ, Z.; SZCZERBA, J.; LODOWSKA, J.; WOLNY, D.; GRUCHLIK, A.; ORCHEL, A.; *et al.* The role of *Desulfovibriodesulfuricans* lipopolysaccharides in modulation of periodontal inflammation through stimulation of human gingival fibroblasts. **Arch Oral Biol**. v. 55, n. 7, p. 515-522, 2010.
5. ESTRELA, Carlos. **Metodologia Cientifica** – Ensino e pesquisa em odontologia*.* São Paulo: Artes Médicas, 2001.
6. GEESEY, G. G.; NEAL, A. L.; SUCI, P. A.; PEYTON, B. M. A review of spectroscopic methods for characterizing microbial transformations of minerals. **J Microbiol Methods**. V. 51, n. 2, p. 125-39, 2002.
7. GRANLUND-EDSTEDT, M.; JOHANSSON, E.; CLAESSON, R.; CARLSSON, J. Effect of anaerobiosis and sulfide on killing of bacteria by polymorphonuclear leukocytes. **J Periodontal Res**. v. 28, p. 346-353, 1993.

8. HANKS, C. T.; WATAHA, J. C.; SUN, Z. *In vitro* models of biocompatibility: A review. **Dent Mater.** v. 12, n. 3, p. 186-93, 1996.
9. HAUMAN, C. H. J.; LOVE, R. M. Biocompatibility of dental materials used in contemporary endodontic therapy: a review. Part 1. Intracanal drugs and substances. **Int Endod J.** v. 36, n. 2, p. 75-85, 2003.
10. HEGGENDORN, F. L.; GONÇALVES, L. S.; LUTTERBACH, M. T. S.; DIAS, E. P. Physiologic and pathological processes of the sulphate-reducing bacteria of the genus **Desulfovibrio sp. Brasília Méd.** v. 46, p. 247-252, 2009.
11. HEGGENDORN, F. L.; GONÇALVES, L. S.; DIAS, E. P.; SILVA JUNIOR, A.; GALVÃO, M. M.; *et al.* Detection of sulphate-reducing bacteria in human saliva. **Acta Odontol Scand**. v. 71, n. 6, p. 1458-63, 2013.
12. HEGGENDORN, F. L.; GONÇALVES, L. S.; DIAS, E. P.; HEGGENDORN, C.; LUTTERBACH, M. T. S. Detection of Sulphate-Reducing Bacteria and Others Cultivable Facultative Bacteria in Dental Tissues. **Acta Stomatol Croat.** v. 48, n. 2, p.116-122, 2014.
13. HEGGENDORN, F. L.; GONÇALVES, L. S.; DIAS, E. P.; LIONE, V. O. F.; LUTTERBACH, M. T. S. Biocorrosion of endodontic files through the action of two species of sulfate-reducing bacteria: **Desulfovibrio desulfuricans and desulfovibrio fairfieldensis. J Contemp Dent Pract.** v. 16, n. 8, p. 665-73, 2015.
14. HEGGENDORN, F. L.; SILVA, G. C. C.; CARDOSO, E. A.; CASTRO, H. C.; GONÇALVES, L. S.; DIAS, E. P.; *et al.* Initial cytotoxicity assays of media for sulfate-reducing bacteria: An endodontic biopharmaceutical product under development. **Dent Mater** *J*. v. 35, n. 5, p. 762–768, 2016.
15. HEGGENDORN, F. L.; COUTINHO, D. A.; GONÇALVES, L. S.; DIAS, E. P.; LIONE, V. O. F.; CRAVO JUNIOR, W. B.; *et al*. On the growth kinetics of oral and environmental sulfate-reducing bacteria (SRB) in aerobic and anaerobic conditions. **Brazilian Journal of Biosciences**. v. 16, n. 3, p. 81-86, 2018.
16. HEGGENDORN, F. L.; FRAGA, A. G. M.; FERREIRA, D. C.; GONÇALVES, L. S.; LIONE, V. O. F.; *et al.* Sulfate-Reducing Bacteria: Biofilm Formation and Corrosive Activity in Endodontic Files. **Int J Dent.** v. 10, p. 1-12, 2018a.
17. HEGGENDORN, F. L.; GONÇALVES, L. S.; LIONE, V. O. F.; CRAVO JUNIOR, W. B.; LUTTERBACH, M. T. S. Analysis of endodontic files submitted to biocorrosion by sulfate reducing bacteria *in vitro.* **Arq Odontol***.* v. 55, p.1-12, 2019.
18. HEGGENDORN, F. L.; PINTO, L. A. L. T.; GONÇALVES, L. S.; LIONE, V. O. F.; CRAVO JUNIOR, W. B.; LUTTERBACH, M. T. S. Biocorrosive behavior of sulphate-reducing bacteria in kerr endodontic files: Determination of the corrosion. **J Conserv Dent**. v. 23, n. 2, p. 196-200, 2020.
19. HEGGENDORN, F. L.; GONÇALVES, L. S.; CARDOSO, E. A.; LUTTERBACH, M. T. S.; LIONE, V. O. F. Biocompatibility tests: cultivation of animal cells and their applications in toxicity studies in Destistry. **Revista Conexão Ciência**. v. 15, n. 1, p. 67-77, 2020a.

20. HORNEZ, J. C.; LEFÈVRE, A.; JOLY, D.; HILDEBRAND, H. F. Multiple parameter cytotoxicity index on dental alloys and pure metals. **Biomol Eng.** v. 19, p. 103-17, 2002.
21. International organization for standardization. ISO 10993-1: Biological evaluation of medical devices, Part 1: Evaluation and testing, 2003.
22. International organization for standardization. ISO 7405: Dentistry — Evaluation of biocompatibility of medical devices used in dentistry, 2008.
23. International organization for standardization. ISO 10993-5: Biological evaluation of medical devices, Part 5: Tests for *in vitro* cytotoxicity, 2009.
24. KAPRAL, M.; WEGLARZ, B.; PARFINIEWICZ, J.; LODOWSKA, J.; JAWORSKA-KIK, M. Quantitative evaluation of transcriptional activation of NF-KB p65 e p50 subunits and IKBα encoding genes in colon cancer cells by *Desulfovibriodesulfuricans* endotoxin. **Folia Microbiol**. v. 55, n. 6, p. 657-661, 2010.
25. KOBAYASHI, C.; YAEGAKI, K.; CALENIC, B.; ISHKITIEV, N.; IMAI, T.; LI, H.; *et al.* Hydrogen sulfide causes apoptosis in human pulp stem cells. **J Endod.** v. 37, p. 479-484, 2011.
26. LARRY, L. B.; HAMILTON, W. A. **Sulphate-reducing Bacteria Environmental and engineered systems.** New York: Cambridge University Press, 2007.
27. MCCRADY, M. H. The numerical interpretation of fermentation-tube results. ***J Infect Dis*.** v. 17, p. 183-212, 1915.
28. NARAYAN, R. ***Biomedical Materials*.** Boston: Springer, 2009.
29. OUNSI, H. E.; AL-SHALAN, T.; SALAMEH, Z.; GRANDINI, S; FERRARI, M. Quantitative and qualitative elemental analysis of different niquel-titanium rotary instruments by using scanning electron microscopy and energy dispersive spectroscopy. **J Endod.** v. 34, n. 1, p. 53-5, 2008.
30. PERSSON, S. Hydrogen sulfide and methyl mercaptan in periodontal pockets. **Oral Microbiol Immunol.** v. 7, p. 378-379, 1992.
31. POSTGATE, J. R. **The Sulphate-reducing bacteria**, 2rd edn. London: Cambridge, 1984.
32. REMOUNDAKI, E.; KOUSI, P.; JOULIAN, C.; BATTAGLIA-BRUNET, F.; HATZIKIOSEYIAN, A.; TSEZOS, M. Characterization, morphology and composition of biofilm and precipitates from a sulphate-reducing fixed-bed reactor. **J Hazard Mater.** v. 153, p. 514-24, 2008.
33. VIDELA, H. A. **Biocorrosão, Biofouling e Biodeterioração de materiais**. São Paulo: Edgard Biucher, 2003.
34. WATANABE, K.; MIKAMO, H.; TANAKA, K. Clinical Significance of sulfate-reducing bacteria for ulcerative colitis. **Japanese Journal of clinical medicine**. v. 7, n. 65, p. 1337-1346, 2007.
35. YAEGAKI, K.; QIAN, W.; MURATA, T.; IMAI, T.; SATO, T.; TANAKA, T.; KAMODA, T. Oral malodorous compound causes apoptosis and genomic DNA damage in human gingival fibroblasts. **J Periodontal Res**. v.43, p. 391-9, 2008.