UNIVERSIDADE ESTADUAL PAULISTA
"Júlio de Mesquita Filho"

João Rafael Amadeu

DISSERTAÇÃO DE MESTRADO

Avaliação da citotoxicidade e resposta tecidual de soluções irrigadoras experimentais a partir de biovidro e biovidro modificado com cobalto

ARAÇATUBA - SP

2020

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Dissertação de Mestrado apresentada à Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista "Júlio de Mesquita Filho" - UNESP para obtenção do título de Mestre em Ciência Odontológica, área de concentração em Biomateriais.

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Coorientador: Prof. Alberto Carlos Botazzo Delbem
Coorientador: Prof. Luciano Tavares Angelo Cintra

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Dedicatória

Dedico este trabalho a duas grandes mulheres sem as quais não teria logrado êxito algum em fazê-lo.
A minha mãe, Cleusa da Silva Amadeu, que sempre me ajudou e fez de tudo para que eu pudesse chegar até onde estou hoje e a minha orientadora Francine Benetti pelo excelente trabalho e por sempre acreditar em mim e me incentivar a ir mais longe.

Avaliação da citotoxicidade e resposta tecidual de soluções irrigadoras experimentais a partir de biovidro e biovidro modificado com cobalto

Resumo

Introdução: Novas formulações de materiais vítreos apresentaram atividade antimicrobiana e osteoindutora. **Objetivos:** O objetivo deste estudo foi avaliar a citotoxicidade e a biocompatibilidade de soluções produzidas a partir de novas formulações de biovidros, o F18 (biovidro experimental) e o F18 com Cobalto (F18-Co; biovidro experimental dopado com cobalto), comparadas à água de cal, obtida a partir de solução com hidróxido de cálcio ($Ca(OH)_2$). **Material e métodos:** O F18 foi preparado e moído, e parte deste foi dopada com cobalto. As soluções foram preparadas com cada material (1:10 de pó para água), formando os grupos F18, F18-Co e $Ca(OH)_2$. Células L929 foram cultivadas, e a viabilidade celular avaliada a partir das soluções e de suas diluições (½, ¼, 1/8 e 1/16 diluição) pelo teste MTT, após 24 e 48 horas. Para avaliar a biocompatibilidade, tubos de polietileno foram preenchidos com esponjas de fibrina embebidas nas soluções não diluídas, e tubos embebidos em soro fisiológico serviram de controle. Os tubos foram implantados no dorso de 16 ratos. Após 7 e 30 dias (n = 8), os ratos foram eutanasiados, e os tubos com o tecido circundante foram processados para coloração de hematoxilina-eosina (H.E.) e análise da inflamação através de escores. Os dados paramétricos (citotoxicidade) foram submetidos aos testes de normalidade para definir o teste estatístico a ser empregado, e os dados não-paramétricos (H.E.), foram avaliados pelos testes de Kruskal Wallis e Dunn ($p < 0,05$). **Resultados:** As soluções não diluídas dos materiais, e as diluições de ½ e ¼, reduziram a viabilidade celular em 24 h ($p < 0,05$). As diluições de 1/8 e 1/16 do F18 e F18-Co apresentaram viabilidade celular semelhante ao controle ($p > 0,05$), o que não ocorreu com $Ca(OH)_2$ ($p < 0,05$), que foi citotóxico. Em 48 h, apenas as soluções não diluídas e diluições de ½ e ¼ do F18 foram similares ao controle ($p > 0,05$), e as demais foram citotóxicas. Mas as diluições de 1/8 e 1/16 do F18-Co teve um aumento na viabilidade celular comparadas às soluções do $Ca(OH)_2$ ($p < 0,05$), e foram semelhantes ao controle ($p > 0,05$). Aos 7 dias, controle, F18 e F18-Co apresentaram inflamação moderada, e $Ca(OH)_2$, severa ($p > 0,05$); a cápsula fibrosa foi espessa. Aos 30 dias, controle e F18-Co apresentaram

inflamação leve comparados ao F18, com inflamação moderada ($p < 0,05$); $Ca(OH)_2$ teve inflamação leve ($p > 0,05$); a cápsula fibrosa foi fina na maior parte dos espécimes. **Conclusões**: Soluções experimentais de F18 e F18 dopado com cobalto são citocompatíveis, diferentemente da solução de $Ca(OH)_2$; todas as soluções apresentaram biocompatibilidade.

Palavras-chave: biocompatibilidade, biovidro, citotoxicidade, hidróxido de cálcio.

Evaluation of cytotoxicity and tissue response of experimental irrigating solutions from bioglass and bioglass modified with cobalt

Abstract

Introduction: New formulations of vitreous materials showed antimicrobial and osteoinductive activity. **Objectives:** The aim of this study was to evaluate the cytotoxicity and biocompatibility of solutions produced from new formulations of bioglass, F18 (experimental bioglass) and F18 with Cobalt (F18-Co; experimental bioglass doped with cobalt), compared to lime water, obtained from a solution with calcium hydroxide ($Ca(OH)_2$). **Material and methods**: The F18 was prepared and ground, and part of it was doped with cobalt. The solutions were prepared with each material (1:10 powder to water), forming groups F18, F18-Co and $Ca(OH)_2$. L929 cells were cultured, and cell viability assessed from solutions and from its dilutions (½, ¼, 1/8 and 1/16 dilution) by the MTT test, after 24 and 48 hours. For biocompatibility analysis, polyethylene tubes were filled with fibrin sponges embedded in the non-diluted solutions, and tubes embedded in saline solution served as controls. The tubes were implanted on the dorsum of 16 rats. After 7 and 30 days (n = 8), the rats were euthanized, and the tubes with the surrounding tissue were processed for staining of hematoxylin-eosin (H.E.) and analysis of inflammation through scores. The parametric data (cytotoxicity) were subjected to normality tests to define the statistical test to be used, and the non-parametric data (H.E.), were evaluated by the Kruskal Wallis and Dunn tests ($p < 0.05$). **Results:** Undiluted solutions of the materials, and dilutions of ½ and ¼, reduced cell viability in 24 h ($p < 0.05$). The 1/8 and 1/16 dilutions of F18 and F18-Co showed cell viability similar to the control ($p > 0.05$), which did not occur with $Ca(OH)_2$ ($p <0.05$), which was cytotoxic. At 48 h, only undiluted solutions and dilutions of ½ and ¼ of F18 were similar to the control ($p > 0.05$), and the others were cytotoxic. However, the 1/8 and 1/16 dilutions of F18-Co had an increase in cell viability compared to $Ca(OH)_2$ solutions ($p <0.05$), and were similar to the control ($p > 0.05$). At 7 days, control, F18 and F18-Co showed moderate inflammation, and $Ca(OH)_2$, severe ($p > 0.05$); the fibrous capsule was thick. At 30 days, control and F18-Co showed mild inflammation compared to F18, with moderate inflammation ($p < 0.05$); $Ca(OH)_2$ had mild inflammation ($p > 0.05$); the fibrous capsule was thin in most specimens. **Conclusions**:

Experimental solutions of F18 and F18 doped with cobalt are cytocompatible, unlike the $Ca(OH)_2$ solution; all solutions showed biocompatibility.

Keywords: biocompatibility, bioglass, cytotoxicity, calcium hydroxide.

SUMÁRIO

Páginas

1. Introdução e justificativa

A polpa dentária é um elemento integrante da estrutura do dente, responsável por apoiar as estruturas dentárias mineralizadas, nutrindo estes tecidos. Preservar a vitalidade pulpar é essencial para manter a vascularização do dente; a proteção contra invasores, através dos prolongamentos odontoblásticos, fluido dentinário e produção de dentina terciária, que reduzirão a mortalidade pulpar; e a completa formação radicular (Alqaderi et al. 2014).

O tratamento radical do canal radicular é considerado o tratamento padrão de atendimento para dentes cariados com pulpite irreversível, com resultados muito favoráveis (Friedman & Mor 2004). Entretanto, a pulpotomia é um procedimento que consiste em amputar a polpa coronária inflamada e infectada, geralmente realizado quando o tecido pulpar coronário é exposto por cárie ou trauma (Fuks 2002). Este procedimento é particularmente importante nos dentes decíduos e dentes permanentes jovens, por permitir a permanência do tecido pulpar dos canais radiculares, para que ocorra o término da formação radicular (Lin et al. 2014). O objetivo é remover os tecidos inflamados coronais, que geralmente contêm microrganismos, e permitir a cicatrização na entrada do canal radicular, com tecido pulpar essencialmente saudável (Lin et al. 2014).

Após a amputação da polpa coronária, o remanescente do tecido pulpar pode ser tratado com vários agentes (Chen et al. 2019, Paula et al. 2019). Extrema atenção é dada à medicação e ao material reparador colocados em contato com o tecido pulpar permanentemente, mas pouco se estudou sobre os agentes irrigantes utilizados durante este procedimento.

O hidróxido de cálcio é o agente mais popular para ser colocado em contato direto com o tecido pulpar, e manter a vitalidade deste, devido à sua capacidade de liberar íons hidroxila e íons cálcio, após sua dissociação ao entrar em contato com o tecido (Paula et al. 2019). Este biomaterial é o intensamente estudado e documentado em vários estudos laboratoriais, com animais e clínicos, e apresenta resultados satisfatórios com taxas de sucesso de até 80%, sendo considerado o padrão-ouro (Paula et al. 2019). Além disso, a reação dos íons cálcio do material com o dióxido de carbono do tecido resulta em partículas de carbonato de cálcio, que servem como matriz para posterior mineralização tecidual (Holland et al. 1977). A partir do hidróxido de cálcio, é obtida a água de

cal, também utilizada em tratamentos endodônticos conservadores (Gandolfi et al. 2015).

A propriedade de induzir a formação de tecido mineralizado confere ao material sua bioatividade (Renno et al. 2013), e o torna favorável para o uso na endodontia, onde almeja-se o reparo do tecido pulpar, e formação de dentina terciária (Hench 1998). Outros materiais também conhecidos pela bioatividade são os biovidros, que possuem elevada atividade osteoindutora e osteocondutora (Hench 1991, Osorio et al. 2012, Wang et al. 2016).

Esses materiais são intensamente utilizados para fins biomédicos, apresentando diferentes características morfológicas (Boccaccini & Gough 2007). Certas composições de biovidros podem ser reabsorvidas, promovendo a liberação de íons cálcio, sódio, silício e fosfatos, que são metabolizados pelo organismo, tendo efeitos como a angiogênese e ação antimicrobiana (Vargas et al. 2013, Benetti et al. 2016).

O biovidro foi capaz de regular a proliferação e diferenciação osteoblástica (Polak et al. 2001), e se espera o mesmo com os odontoblastos, visto as semelhanças morfológicas e funcionais destas células (Benetti et al. 2019a). Para formar um biovidro, a concentração de dióxido de silício (SiO_2) é reduzida, aumentando sua solubilidade e dissociação iônica. Assim, ocorre uma alta concentração de sódio, mantendo pH neutro ou levemente alcalino, com formação de camadas bioativas, e permitindo a ação antimicrobiana (Granito et al. 2009). Ainda, há evidências crescentes dos efeitos positivos do vidro bioativo na vascularização dos tecidos (Polak et al. 2001, Gorustovich et al. 2010).

Pesquisadores do Laboratório de Materiais Vítreos (LaMaV, UFSCar, São Carlos, Brasil) desenvolveram uma composição de biovidro altamente reativo que não cristaliza durante o processamento, o F18 (Gabbai-Armelin et al. 2015, Souza et al. 2017a; 2017b). Este material mostrou biocompatibilidade em forma de fibras ou scaffold (Gabbai-Armelin et al. 2015) e foi desenvolvido com o objetivo de regenerar tecidos moles, ampliando suas indicações (Gabbai-Armelin et al. 2015; 2017b). Estimulam a proliferação de fibroblastos, e são potencialmente biodegradáveis (Gabbai-Armelin et al. 2017a; 2017b). Assim, destaca-se o potencial deste biomaterial para atuar no reparo do tecido pulpar, como se almeja em procedimentos de pulpotomia ou capeamento pulpar direto.

O pó de biovidros misturado com água destilada mostrou resultados positivos na remineralização de estruturas dentárias (Tirapelli et al. 2010; 2011, Pintado-Palomino & Tirapeli 2015), e foram eficazes na regeneração do esmalte e da dentina (Tirapelli et al. 2010; 2011, Pintado-Palomino & Tirapeli 2015). Efeito antimicrobiano também tem sido demonstrado, com ação mesmo em bactérias que apresentam resistência ao tratamento endodôntico, como *Enterococcus faecalis* (Martins et al. 2011). Em um estudo anterior, observamos que pastas a partir de biovidros mostraram biocompatibilidade, indução à osteogênese, e atividade antimicrobiana comparável à pasta de hidróxido de cálcio (Amadeu *et al*. 2019, Araújo-Lopes et al. 2020).

Assim, o pó de biovidros possui propriedades capazes de sugerir seu uso em endodontia, podendo compor medicação intracanal ou soluções irrigadoras. No entanto, a citotoxicidade e biocompatibilidade de soluções a partir destes materiais no tecido conjuntivo não mineralizado ainda não foi avaliada, e é importante determiná-la antes que os materiais entrem em contato com a polpa. Ainda, sabe-se que a adição de íons cobalto às biopartículas vítreas induz a hipóxia tecidual, estimulando a angiogênese (Littmann et al. 2018). Então, propomos fazer a dopagem do biovidro F18 com íons cobalto.

Desta forma, o objetivo deste estudo foi avaliar a citotoxicidade e a biocompatibilidade de soluções experimentais preparadas a partir do biovidro F18 e do biovidro F18 dopado com cobalto. A água de cal, obtida a partir do hidróxido de cálcio, foi utilizada para comparação.

II. Artigo

Dental Materials

Comparison of solutions from bioglass, bioglass modified with cobalt, and calcium hydroxide for conservative treatment of pulp tissue

Abstract

Objectives: To evaluate the cytotoxicity and biocompatibility of solutions from new formulations of bioglass, F18, and F18 with cobalt (F18-Co), compared to lime water (from $Ca(OH)_2$). **Methods**: The F18 was prepared, and part of it was doped with cobalt. The solutions were prepared with materials (1:10 powder to water), forming F18, F18-Co and $Ca(OH)_2$ groups. The cell viability was assessed by MTT assay (undiluted, ½, ¼, 1/8 and 1/16 dilution; 24/48 h). Polyethylene tubes were filled with fibrin sponges embedded in the solutions or in saline solution (control) and implanted in the 16 rats. After 7 and 30 days (n = 8), the rats were euthanized, and the specimens were processed for hematoxylin-eosin. The inflammation was scored. Statistical tests were performed ($p<0.05$). **Results**: Undiluted materials solutions, and ½ and ¼ dilutions, reduced cell viability in 24 h ($p<0.05$). The 1/8 and 1/16 dilutions of F18 and F18-Co showed cell viability similar to the control ($p>0.05$), unlike $Ca(OH)_2$ ($p<0.05$), that was cytotoxic. At 48 h, regarding undiluted solutions and ½ and ¼ dilutions, only F18 was similar to control ($p>0.05$). However, the 1/8 and 1/16 dilutions of F18-Co had increased cell viability compared to $Ca(OH)_2$ solutions ($p<0.05$), and were similar to the control ($p>0.05$). At 7 days, the groups showed moderate to severe inflammation ($p>0.05$), and thick fibrous capsule. At 30 days, control and F18-Co showed mild inflammation compared to F18, with moderate inflammation ($p<0.05$); $Ca(OH)_2$ had mild inflammation ($p>0.05$); overall, the fibrous capsule was thin. **Significance**: The solutions of F18 and F18 doped with cobalt are cytocompatible and biocompatible.

1. Introduction

The dental pulp is responsible for supporting mineralized dental structures, nourishing these tissues. Preserving pulp vitality is essential to maintain protection against invaders, in addition to being necessary for a complete root formation (Alqaderi et al. 2014).

The radical treatment of the root canal is considered the standard of care for decayed teeth with irreversible pulpitis, with very favorable results (Friedman & Mor 2004). However, pulpotomy - a procedure that consists of amputating the inflamed and infected coronary pulp - is particularly important in young permanent teeth, as it allows the pulp tissue of the root canals to remain, so that root formation ends (Fuks 2002, Uesrichai et al. 2019). The European Society of Endodontology recently issued a statement recommending that teeth with deep caries and asymptomatic exposures to dental pulp could benefit from conservative treatment of vital pulp (Duncan et al. 2019).

After amputation of the coronary pulp, the remainder of the pulp tissue can be treated with various agents (Chen et al. 2019, Paula et al. 2019). Extreme attention is paid to medication and repair material placed in contact with the pulp tissue, however, few studies has been done about the irrigating agents used during this procedure. Calcium hydroxide is the most popular agent to be placed in direct contact with the pulp tissue, and to maintain its vitality, due to its ability to release hydroxyl ions and calcium ions, when in contact with the tissue (Chen et al. 2019, Paula et al. 2019). The reaction of the material's calcium ions with the tissue's carbon dioxide results in calcium carbonate particles, which serve as a matrix for later tissue mineralization (Holland et al. 1977). Lime water is obtained

from calcium hydroxide, also used in conservative endodontic treatments (Gandolfi et al. 2015).

The property of inducing the formation of mineralized tissue gives the material its bioactivity (Renno et al. 2013), favorable where pulp tissue repair is desired, and the formation of tertiary dentin (Hench 1998). Other materials also known for bioactivity are bioglass, which have high osteoinductive and osteoconductive activity (Hench 1991, Osorio et al. 2012, Wang et al. 2016). Certain compositions of bioglass can be reabsorbed, promoting the release of calcium, sodium, silicon and phosphates, which are metabolized by the body, having effects such as angiogenesis and antimicrobial action (Vargas et al. 2013).

Bioglass was able to regulate osteoblastic proliferation and differentiation (Polak et al. 2001) and the same is expected with odontoblasts, given the morphological and functional similarities of these cells (Benetti et al. 2019a). These materials have a high concentration of sodium, which keeps the pH neutral or slightly alkaline, with the formation of bioactive layers (Granito et al. 2009). There is growing evidence of the positive effects of bioactive glass on vascularization of tissues (Polak et al. 2001, Gorustovich et al. 2010).

A new highly reactive bioglass formulation that does not crystallize during processing has been developed and named F18 (Gabbai-Armelin et al. 2015, Souza et al. 2017a; 2017b). This material was developed with the objective of regenerating soft tissues (Gabbai-Armelin et al. 2015; 2017b), stimulates the proliferation of fibroblasts and is potentially biodegradable (Gabbai-Armelin et al. 2017a; 2017b). Thus, its potential to act in the repair of pulp tissue stands out.

Bioglass powder mixed with distilled water was effective in regenerating enamel and dentin (Tirapelli et al. 2010; 2011, Pintado-Palomino et al. 2015).

Antimicrobial effects have also been demonstrated, with action against *Enterococcus faecalis* (Martins et al. 2011). A recent study showed that pastes from bioglass are biocompatible, induce osteogenesis, and have antimicrobial activity comparable to calcium hydroxide paste (Araújo-Lopes *et al*. 2020).

Thus, the bioglass powder has properties capable of suggesting its use in endodontics, being able to compose intracanal medication or irrigating solutions. However, the cytotoxicity and biocompatibility of solutions from these materials in non-mineralized connective tissue has not yet been evaluated, and it is important to determine it before the materials come into contact with the pulp. It is known that the addition of cobalt ions to the vitreous bioparticles induces tissue hypoxia and stimulates angiogenesis (Littmann et al. 2018). Therefore, we propose to doping the F18 bioglass with cobalt ions.

Thus, this study evaluated the cytotoxicity and biocompatibility of experimental solutions prepared from bioglass F18 and bioglass F18 doped with cobalt. Lime water, obtained from calcium hydroxide, was used for comparison. Null hypotheses were adopted, that there would be no difference between (a) cell viability and (b) biocompatibility, from these different solutions

2. Material and Methods

2.1. Synthesis of glass particles

The fusion of the bioglass (F18; Souza et al. 2015) was made in an electric oven at 1200 ºC / 4 h, using a platinum crucible. Among the reagents used for the synthesis of the material, there will be sodium carbonate (Na_2CO_3), potassium (K_2CO_3), and calcium ($CaCO_3$), phosphorus pentoxide (P_2O_5), magnesium oxide

(MgO) and zinc (ZnO), and silicon dioxide (SiO_2), as previously described (Souza et al. 2015). The reagents were purchased from Sigma-Aldrich (Inc, St Louis, MO) and Santa Rosa LTDA (GO, Brasil). Before the fusion, the reagents were homogenized in a jar spinner (12 h). The glass was poured into water to obtain frit, which was dried in an oven (100 ºC, 24 h) and subjected to grinding in a planetary mill (500 rpm) for variable times (5-30 min), for the initial grinding. Afterwards, the F18 went through milling and sieving steps to obtain particles smaller than 25 µm.

Part of the F18 was doped with Cobalt (1% CoO; Santos et al. 2017, Souza et al. 2017a), to obtain the F18-Co particles. The frit was made again, and the milling steps were repeated to obtain the final product.

2.2. Preparation of solutions

Experimental solutions based on F18, F18-Co and calcium hydroxide ($Ca(OH)_2$) were produced by mixing the powder of these materials with distilled water, both weighed and prepared in a 1:10 ratio of powder for water.

2.3. Cytotoxicity analysis

L929 cell line fibroblasts were cultured under standard cell culture conditions in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin, at 37 ºC, 100 % humidity, 95 % air, and 5 % CO_2. Cells were subsequently seeded in 96 well plates (10^4 cells/well) and incubated for 24 h under standard cell culture conditions to allow the cells to adhere before adding the solutions. Afterwards, several dilutions of the solutions were applied to the cells, being: undiluted, ½

dilution, ¼ dilution, 1/8 dilution, and 1/16 dilution. Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, after 24 and 48 hours. For that, the culture medium and dilution of the solutions from each well were removed and 100 µL of MTT solution (0.5 mg/mL) in DMEM without SFB (1:10) was added to each well. The MTT solution was removed after 4 hours of incubation, the formazan crystals were dissolved in 100 µL of isopropyl alcohol. The plate was left at room temperature in a darkroom for 30 minutes on a rotary shaker. The absorbance of the plates was assessed at 570 nm using an Elisa reader (Eon Microplate Spectrophotometer, BIOTEK, USA).

2.4. Biocompatibility analysis

2.4.1. Animals

Sixteen 2-month-old male rats (*Rattus albinus*, Wistar) were used, weighing between 250g and 280g. The animals were kept in an environment with temperature between 22 and 24ºC, controlled light cycle (12 hours light and 12 hours dark) and in collective cages (four rats per cage), fed throughout the experimental period with solid diet and water "*ad libitum*". The institutional ethics committee approved the experimental protocol. The entire study was conducted according to ARRIVE guidelines.

2.4.2. Preparation of the tubes

For *in vivo* analysis, we used the implantation of tubes filled with the evaluated materials, as well as empty tubes for control, in the subcutaneous tissue of rats. This is a method recommended by ISO 10993 (International Organization for Standardization 2016).

A total of 64 polyethylene tubes (Abbot Lab. Do Brasil Ltda., São Paulo) with 1.0 mm internal diameter, 1.6 mm external diameter and 10.0 mm long were used (ISO 10993-6: 2007). The tubes were filled with fibrin sponge (Cintra et al. 2014), and soaked in solutions (sixteen tubes for each solution: F18, F18-Co and $Ca(OH)_2$) previously prepared from each material, as described in item 2.2 of this section. Other sixteen tubes with fibrin sponge were soaked in 0.9% saline water to serve as control group.

2.4.3. Surgical procedure

The surgical procedure was performed following previous studies (Cintra et al. 2017, Benetti et al. 2019b). The rats were anesthetized, their dorsa were shaved, and a 2.0-cm incision was made in a head-to-tail orientation with a #15 Bard-Parker blade (BD, Franklin Lakes, NJ, USA). The skin was reflected to create two pockets on the right side and two pockets on the left side of the incision. Thus, each animal received four tubes, three from the experimental groups (F18, F18-Co and $Ca(OH)_2$), and an tube of control group. The tissue was sutured with 4.0 non-resorbable silk thread (Ethicon, Johnson & Johnson, São Paulo, SP, Brazil), and the final antisepsis was performed. The animals were monitored until they recovered from anesthesia. Thus, after the experimental procedures, groups were formed according to each material used, and can be seen in Table 1.

Table 1. Distribution of experimental groups for 7 and 30 days

Groups	Material inside the tube	n
Control	0.9% Saline solution	8
$Ca(OH)_2$	Lime water (1:10 powder for distilled water)	8
F18	F18 solution (1:10 powder for distilled water)	8
F18-Co	F18-Co solution (1:10 powder for distilled water)	8

2.4.4. Histological analysis

At 7 and 30 days after implantation, the rats were euthanized with an overdose of anesthetic (150 mg/kg, sodium thiopental, Thipentax, Cristália - Produtos Químicos Farmacêuticos LTDA, Itapira, Brazil). After, the tubes were removed together with the surrounding tissues and were fixed in a solution of 4% buffered formaldehyde for 24 h, at pH 7.0. The specimens were processed and embedded in paraffin, and serially sectioned in sections of 5 µm, and stained with hematoxylin and eosin (H.E.).

The histological sections were analyzed under bright field illumination under an optical microscope (Optiphot-2, Nikon, Japan) by a single, calibrated investigator in a blinded manner. The tissue inflammation in contact with the materials at the open end of the tubes was evaluated under scores, according to previous studies (Cintra et al. 2017, Benetti et al. 2019b): 1, inflammatory cells absent and no inflammatory reaction; 2, less than 25 cells per field and mild reaction; 3, between 25 and 125 cells and moderate reaction; and 4, more than 125 cells and severe reaction. The most central sections of the tube were used,

one section per specimen, and the scores will be applied in an area of 1.2×0.6 mm (400 × magnification), in the center of the tube opening (Benetti et al. 2019b). The fibrous capsule was considered thin when less than 150 µm and thick when greater than or equal to 150 µm.

2.5. Statistical Analysis

For analysis of cytotoxicity, the data of control group was considered as 100%. The data obtained with the analysis of cytotoxicity were subjected to a normality test and then to the appropriate statistical test. When normality was confirmed, one-way ANOVA followed by the Tukey test were performed. When there was no normality, the data were submitted to the Kruskal-Wallis followed by the Dunn test, as well as with data obtained of the histological analysis. For all tests, $P < 0.05$ was considered.

3. Results

3.1. *In Vitro* Study - Cytotoxicity analysis

The data on cell viability are shown in Figure 1. A decrease cell viability was identified by undiluted materials solutions, and ½ and ¼ dilutions at 24 h, when compared to the control ($P<0.05$). The other dilutions (1/8 and 1/16) of F18 and F18-Co solutions had lower cell viability than control, but without significant different ($P>0.05$). Unlike, these dilutions (1/8 and 1/16) of $Ca(OH)_2$ solutions showed significantly reduced cell viability compared to other groups ($P<0.05$), except compared to 1/16 F18 solution ($P>0.05$) in this period.

Regarding of 48 h, undiluted, ½ and ¼ dilutions of $Ca(OH)_2$ and F18-Co solutions had decreased cell viability compared to control ($P<0.05$), unlike the

F18 solutions (undiluted, and ½ and ¼ dilutions), which were similar to the control (*P*>0.05). On the other hand, the other dilutions of F18-Co (1/8 and 1/16) had an increased cell viability than these same dilutions of $Ca(OH)_2$ solution (*P*<0.05), and assimilating to the control (*P*>0.05). Already the 1/8 dilution of F18 was different to control (*P*<0.05), but 1/16 dilution was similar to it (*P*>0.05).

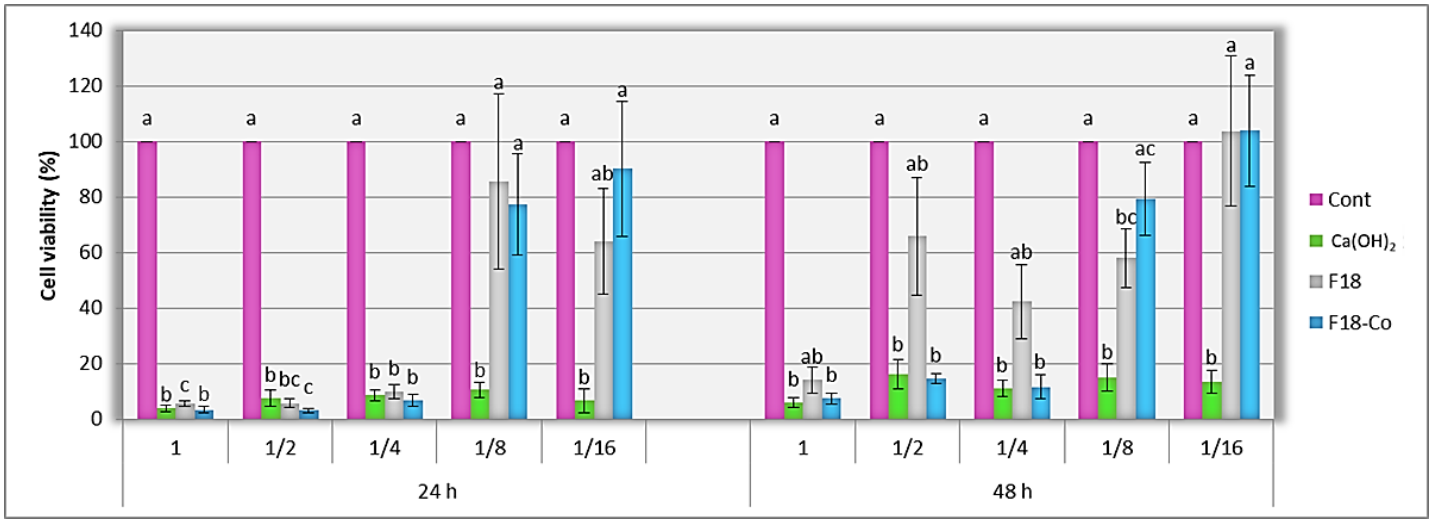

Figure 1 L929 fibroblasts' viability determined by an MTT assay. The same letters indicate no statistical differences among the groups at the same period and in the same dilution.

3.2. *In Vivo* Study - Biocompatibility analysis

Representative images of the tissue responses can be observed in Figure 2, and the histological analysis in Table 2. At 7 days, a moderate infiltration of inflammatory cells (mainly presence of polymorphonuclear cells), was observed in control, F18 and F18-Co groups; the $Ca(OH)_2$ group had severe infiltration of

inflammatory cells in this period. No significant difference was observed among the groups (*P*>0.05), and all specimens exhibited a thick fibrous capsule.

At 30 days, most of the specimens of the control and F18-Co groups had absence to mild inflammation (*P*>0.05), differently from the F18 group, with mild to moderate inflammation (*P*<0.05). The $Ca(OH)_2$ had mild inflammation, similar to other groups (*P*>0.05). The fibrous capsules were thin in most specimens in this period.

Table 2. Inflammatory score and thickness of fibrous capsule according to group, at 7 and 30 days

Time / *P* value	**Groups***	**Scores**				**Median**	**Capsule**		**n**
		1	**2**	**3**	**4**		Thick	Thin	
7 days *P*=0.385	Control[a]	0	0	4	4	3	8	0	8
	$Ca(OH)_2$[a]	0	0	3	5	4	8	0	
	F18[a]	0	0	4	4	3	8	0	
	F18-Co[a]	0	1	5	2	3	8	0	
30 days *P*=0.005	Control[a]	6	2	0	0	1	0	8	8
	$Ca(OH)_2$[ab]	2	5	1	0	2	1	7	
	F18[b]	1	3	4	0	3	1	7	
	F18-Co[a]	6	2	0	0	1	0	8	

*Same letters indicate no statistical difference among the groups in each analysis period (*p*>0.05).

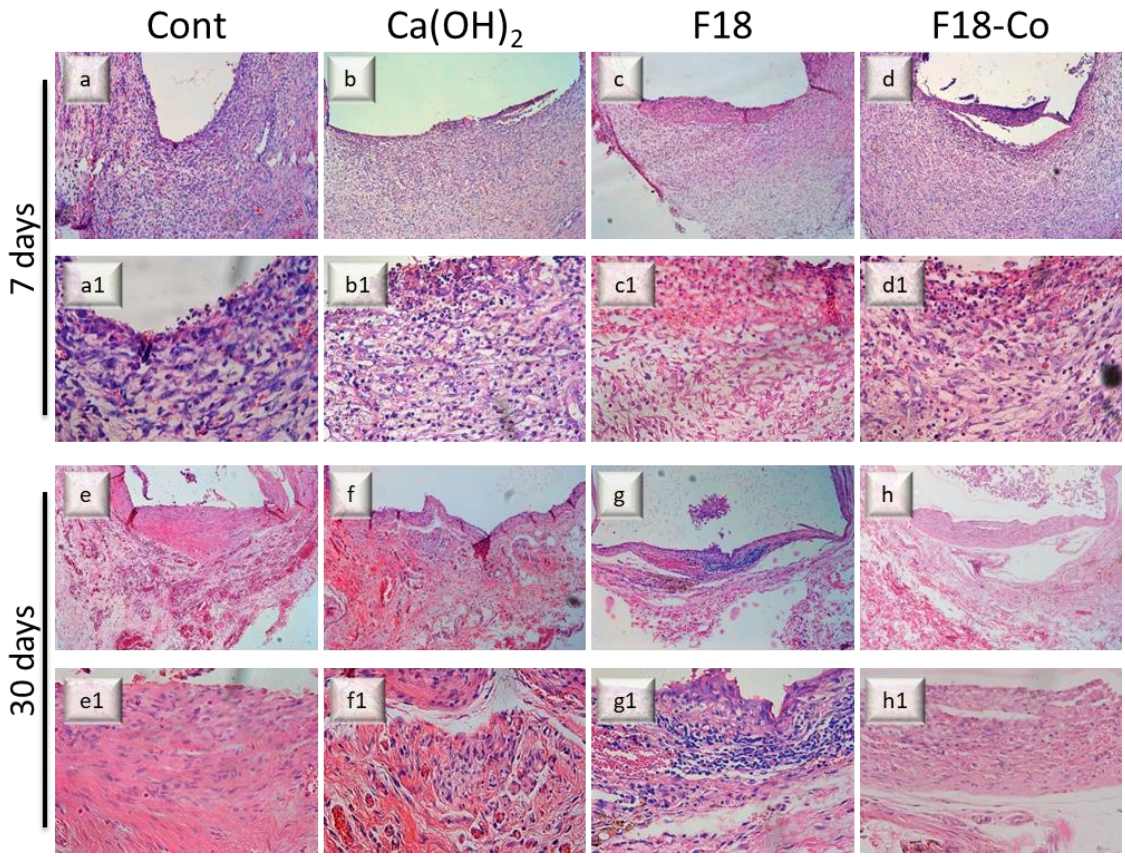

Figure 2 Representative images of the subcutaneous tissue reactions for inflammatory infiltrate. At 7 days: (a,a1) control, (c,c1) F18, and (d,d1) F18-Co groups, with the presence of moderate inflammatory cell infiltration and a thick fibrous capsule, while (b,b1) $Ca(OH)_2$ has severe inflammation. At 30 days: (e,e1) control and (h,h1) F18-Co groups with absence or mild inflammatory infiltration, (f,f1) $Ca(OH)_2$ group with mild inflammation, and (g,g1) F18 groups with mild to moderate inflammation, and thin fibrous capsule. [Hematoxylin-eosin staining; a-h, 100×; a1-h1, 400×]

4. Discussion

This study analysed the cell viability and biocompatibility of solutions from bioglass materials, the F18 and F18 doped with cobalt, compared with water from $Ca(OH)_2$ (or lime water), as possible solutions for use in conservative treatment

of the dental pulp. This study observed that F18 and F18-Co solutions allowed cell viability – mainly F18, unlike water from $Ca(OH)_2$, rejecting the null hypothesis of that there would be no difference in (a) cell viability of solutions; but all solutions were biocompatible *in vivo*, mainly F18-Co, accepting the null hypothesis of that there would be no difference among (b) biocompatibility of solutions.

There is a great effort in studying different materials to be placed in contact with the pulp tissue in conservative treatments, as these will be in permanent contact with the tissue (Cosme-Silva et al. 2019, Silva et al. 2019). Nonetheless, the irrigating solutions to be used during these procedures are poorly studied. The most studied irrigating solutions are sodium hypochlorite and chlorhexidine (Silva et al. 2006, Baldissera et al. 2013, Özgür et al. 2017). However, both show controversial results because some studies indicate that they cause delay in pulp repair (Horsted-Bindslev et al. 2003, Accorinte et al. 2007), besides severe cytotoxic effects when the sodium hypochlorite was evaluated, even in low concentrations (Heling et al. 2001).

The $Ca(OH)_2$ induces pulp tissue repair when in contact with this (Fransson et al. 2016, Araújo-Lopes et al. 2020). It was observed that the $Ca(OH)_2$ paste allows the formation of dentin in pulpotomy treatments (Fransson et al. 2016) and shows satisfactory clinical results (Chailertvanitkul et al. 2014), in addition to having excellent antimicrobial action (Araújo-Lopes et al. 2020). Lime water, on the other hand, produced by mixing saline solution with $Ca(OH)_2$ powder, has a low antimicrobial effect, but excellent haemostatic activity by to activate the coagulation factors due calcium ions release (Pérez-Gómez et al. 2007).

Bioactive glass has gained space in dentistry (Tirapelli et al. 2010, Renno et al. 2013). A time-dependent kinetic modification occurs on the surface of vitreous materials when in aqueous solutions (Hench et al. 2006, Hench & Jones 2015), which form a link between the material and the tissues. For example, the formation of a layer of hydroxycarbonate apatite (HCA) on surface of a bioglass - due to the dissolution of the glass in the aqueous medium - is responsible for its attachment to teeth or bone tissues (Jones 2013, Pintado-Palomino et al. 2015). HCA interacts with collagen fibrils and integrates with the tissues (Hench 2006). These leaching reactions from bioactive glasses have been well described previously (Hench 1991).

When inserting the bioglass in an aqueous solution, a release of alkaline elements occurs, and results in a pH increased. Concomitantly, Si-O-Si bonds break through the action of hydroxyl ions (OH), which releases silica into the solution in the form of silicic acid [$Si(OH)_4$]. In the precipitation process, calcium and phosphate ions released by the bioglass in the solution form a layer rich in calcium phosphate (CaP) (Hench & Jones 2015), initially amorphous, and later form the HCA.

Previously, membranes produced from F18 bioglass have increased the metabolic activity of osteoblastic cells (Hidalgo-Pitaluga et al. 2018). We can observed an increased cell viability in cells of fibroblast lineage. Still, the solution of pure F18 in the present study was the one that allowed the increase of the cell viability faster compared to the solutions of the other evaluated materials, in the period of 48 h. When evaluated as a membrane form, a rapid response in cell growth was also observed (Hidalgo-Pitaluga et al. 2018), attributed to its rapid dissolution in particles, releasing ions such as Ca, Na, Si and P in the medium

(Hoppe et al. 2013, Crovace et al. 2016). Moreover, F18 induced the *in vitro* proliferation osteoblast and fibroblast cells (Kido *et al*. 2013, Crovace *et al*. 2016, Gabbai-Armelin *et al*. 2015, 2017b). These data corroborate with results found in the present study.

This rapid ionic dissociation may have been beneficial to fibroblasts in our *in vitro* analysis. Ionic release is beneficial to the tissues to be repaired, as they activate mesenchymal stem cells for the repair process. Similarly, the F18-Co solution also showed satisfactory results when compared to lime water. Studies have revealed that the doping of materials with transition metals, such as cobalt, can improve the anticancer and antioxidant activity of the materials, in addition to the antimicrobial activity (Fang et al. 2008, Nair et al. 2011). Also, doping with cobalt has improved the biological, magnetic and optical characteristics of materials used in biomedicine (Bouaine & Brihi 2007), and when added to bioglass, they stimulated angiogenesis (Littmann et al. 2018). Thus, bioglass solutions can be alternative irrigators to act directly on the pulp tissue, and stimulate its repair.

A bioglass had its biocompatibility tested for the first time in 1981 in the subcutaneous tissue of rats, but in the form of discs (Hench 2006). The material exhibited interaction with connective tissue, and became suitable for clinical use (Hench 2006). Bioglass was also evaluated as scaffold in subcutaneous rat tissue, and chronic inflammatory was reduced over 45 days of implantation, with the formation of a fibrous capsule during this period (Kido et al. 2013), corroborating with the present results.

F18 fibres for bone regeneration demonstrated improvement in the biocompatibility and bioactivity in rat subcutaneous tissue and cell culture

(Gabbai-Armelin *et al*. 2017b). In addition, a previous study showed the *in vivo* degradability of F18 after implantation of its fibers in subcutaneous tissue of rats; there was also observed formation of HCA on the material surface (Gabbai-Armelin et al. 2017b). Thus, besides its bioactivity, the potential of this biomaterial for the regeneration of connective tissues is highlighted, assisting in tissue repair.

Although the beneficial effects of doping with cobalt were mentioned in this study, this was the first study to evaluate an F18 solution with cobalt, and the results found here were satisfactory. The *in vivo* data were extremely positive, where 30 days after implantation it was observed absent to mild inflammation in the F18-Co group, which did not occur with the other materials. However, these are still initial results, and it is necessary to evaluate the effects of these solutions in direct contact with the pulp tissue, in addition to verifying whether cobalt had an effect on angiogenesis.

5. Conclusion

Diluted experimental solutions from F18 bioglass and from F18 doped with cobalt were cytocompatible, and all solutions were biocompatibility.

Conflict of Interest

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

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