

Biocompatibility of a New Dental Glass Ionomer Cement with Cellulose Microfibers and Cellulose Nanocrystals

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Developing new restorative materials should avoid damage to tissue structures. This study evaluated the biocompatibility of a commercial dental glass ionomer cement (GIC) mechanically reinforced with cellulose microfibers (GIC+CM) or cellulose nanocrystals (GIC+CN) by implantation of three test specimens in subcutaneous tissue in the dorsal region of 15 *Rattus norvegicus albinus* rats. Each rat received one specimen of each cement, resulting in the following groups (n=15): Group GIC (Control), Group GIC+CM and Group GIC+NC. After time intervals of 7, 30 and 60 days, the animals were sacrificed and the following aspects were histologically evaluated: type of inflammatory cells, fibroblasts, blood vessels, macrophages, giant cells, type of inflammatory reaction and capsule thickness (μm). These events were scored as (-) absent, (+) light, (++) moderate and (+++) intense. The results were statistically analyzed by Kruskal-Wallis test and Mann-Whitney post test. At 7 days, Group GIC+NC showed more favorable tissue repair because quantitatively there were more fibroblasts ($p=0.022$), fewer macrophages ($p=0.008$) and mononuclear cells ($p=0.033$). Polymorphonuclear neutrophils and giant cells were absent in all experimental periods. At 60 days, test specimens in Group GIC+NC were surrounded by a fibrous tissue capsule with reduced thickness ($26.72\pm 2.87 \mu\text{m}$) in comparison with Group GIC+CM ($41.21\pm 3.98 \mu\text{m}$) ($p=0.025$). In general, all biomaterials showed satisfactory biocompatibility, but glass ionomer cement modified with cellulose nanocrystals showed a more advanced tissue repair.

Introduction

Glass ionomer cement (GIC) is a most important milestone in dentistry, due to its fluoride-releasing properties, adhesion to dental structure, thermal expansion coefficient similar to that of dental structures and biocompatibility (1-3). Nevertheless, these cements have some limitations like a deficient mechanical integrity and capacity to bear fracture loads (4). Therefore, when a material like chlorhexidine is added to their formulations, it may increase cell death significantly (5). In the last few years, increasing worldwide interest in sustainable technologies led to the creation of products with lower impact on the environment (6). Thus, a vast range of researches and work were applied in the area of polymeric materials and composites.

Among them cellulose attained an outstanding place, since it may be used in its microcrystalline form or in the form of nanocrystals (7,8). There are various nanostructured materials, like carbon nanotubes and inorganic nanocrystals (9), nevertheless, cellulose nanocrystals present several advantages over them, such as the low cost, easy formation process and mainly better mechanical properties in comparison with those materials (10).

Silva et al. (11) added cellulosic fibers to glass ionomer cement in an endeavor to enhance the mechanical strength of this material. Studies have reported that the cellulosic fiber-modified glass ionomer cement showed increased compressive strength and abrasion resistance and higher bond strength to dental structures. In a recent study (12), glass ionomer cement was reported as completely interlaced with cellulose nanocrystals randomly distributed throughout the cement matrix, suggesting union between them. Therefore, following the logic of the experiments, the aim of this study was to evaluate the biocompatibility of a commercial glass ionomer cement modified with cellulose microfibers and cellulose nanocrystals.

Material and Methods

Composite Development

The cellulose microfibers (CM) were obtained by processing 6 g of eucalyptus cellulose fibers hydrolyzed with hydrochloric acid. The suspension was filtered and washed with distilled water until the pH was equal to the distilled water. The product was mixed with distilled water and sonicated for five cycles of 2 min each, at a

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controlled temperature. The material obtained was frozen in liquid nitrogen and freeze-dried. To obtain the cellulose nanocrystals (CN) the eucalyptus containing α -cellulose (96-98%) was hydrolyzed with sulphuric acid (13) at 50 °C for about 40 min. The dispersion was diluted twice and washed three times with deionized water by centrifugation, dialyzed against deionized water until it attained pH~6, immediately ultrasonicated for 5 min. and filtered. The final concentration of cellulose nanocrystals in the dispersion was approximately 1% by mass (12).

Three groups of cylindrical-shaped test specimens measuring 5x3 mm were fabricated for the biocompatibility evaluation using a conventional glass ionomer brand (Vidrion R, SS White, Rio de Janeiro, RJ, Brazil): Group GIH (control, n=15), Group GIC+CM, n=15) and Group GIC+NC (n=15). Group GIC samples were obtained by agglutinating the powder (sodium fluorosilicate, calcium, aluminum, barium sulphate, polyacrylic acid, pigments) with the glass ionomer cement liquid (tartaric acid, distilled water). Group GIC+CM samples were obtained by previous preparation of 0.010 g by weight concentration of cellulose microfibrils and adding them to glass ionomer cement during the manipulation. In Group GIC+NC the nanocrystals were prepared and added to the glass ionomer cement liquid in concentrations of 0.4% of their total mass. The composites were prepared respecting the 1:1 powder:liquid ratio and mixed in accordance with the manufacturer's recommendations. The test specimens were stored in distilled water at 37 °C (± 1 °C) until use.

Biocompatibility Test - Subcutaneous Implant

The research protocol was approved by the Research Ethics Committee on the Use of Animals of the UFVJM, Brazil, in compliance with the ethical guidelines for animal experimentation.

Fifteen young male rats (*Rattus norvegicus albinus*, Holtzman), five months old, and mean weight of 141.15 g were used. The animals were obtained from the Animal Care Facility laboratory of the Basic Science Department, Federal University of Minas Gerais, Brazil. During the study, the animals were maintained in cages identified according to the group and study period. The animals were fed solid ration, (except for the 12 h preoperative period) and water *ad libitum*. For the surgeries, the animals were anesthetized with 1 mL (mg/kg of the animal's live weight) of Ketamine (Chloramine - ketamine hydrochloride, injectable IM/IV-50 mg/mL - Biochimico, Rio de Janeiro, RJ, Brazil), administered intraperitoneally. Subsequently, the dorsal region was shaved and cleaned with 0.12% chlorhexidine solution (Farmoderm, São Paulo, SP, Brazil). The entire surgical operation was performed by a trained, experienced researcher (JLM) and under aseptic conditions.

The evaluator (RMS) was blinded for the microscopic analysis. A 10 mm incision was made in the median dorsal region using a #15 scalpel blade and tissue division was performed with blunt-end scissors. Thus three surgical sites, approximately 18 mm deep, were formed in the connective tissue, to receive one test specimens of each material. In the Group GIC, test specimens were placed in the left superior region; in Group GIC+CM, in the right superior region; and those of Group GIC+NC in the central inferior region. The distance between samples was 1 cm. Careful asepsis was implemented throughout the operation. No case developed purulent exudation. The skin edges were closed with nylon 5-0 (Ethicon; Johnson & Johnson, São José dos Campos, SP, Brazil). The animals were daily observed to identify local, systemic and behavioral abnormalities such as edema, exudation, suture dehiscence, lack of appetite and prostration. After the time intervals of 7, 30 and 60 days, the rats were sacrificed by anesthetic overdose and the test specimens including the surrounding tissues (skin and subcutaneous connective tissue) were removed for histopathological analysis. Thus, there were 5 animals for each study period, totaling 15 samples at the end of the evaluated periods. The tissue samples were fixed in 10% buffered formalin for 48 h and then dehydrated and embedded in paraffin. Serial sections 5 μ m-thick were obtained at every 50 μ m, up to 12 sections per specimen. The sections were stained with hematoxylin-eosin (HE) and analyzed by a trained pathologist (JLM), using a light binocular microscope (Zeiss - PrimoStar, Oberkochen, Germany) coupled to an image capture system (Axio com ERc5s, Jena, Germany) and AxioVision LE 4.8.2.0 (Oberkochen, Germany) software for Windows. The total area of the field analysis was 55.896 μ m². Each tissue sample was subjected to qualitative, descriptive, morphological and morphometric analyses of the connective tissue around the implant in two superior and two inferior fields of the fibrous capsule, at 10x, 40x and 400x magnifications, considering the following histopathological events: I: type of inflammatory infiltrate: prevalence of polymorpho nuclear neutrophils or mononuclear cells; II: presence of fibroblasts and blood vessels; III: macrophage activity: presence of macrophages and inflammatory multinucleated giant cells; IV: type of inflammatory reaction: acute or chronic; V: fibrous capsule thickness.

A qualitative descriptive analysis of the events I to IV was performed using the following scores: (-) absent, (+) light, (++) moderate and (+++) intense (Silva et al. 2009). In the quantitative analysis, fibrous capsule thickness (μ m) was measured in four distinct regions, using the AxioVision (Oberkochen, Germany) software.

The results of the histological events were analyzed by SPSS (Statistical Package for Social Sciences, IBM Inc.,

New York, NY, USA), version 17.0. The Shapiro-Wilk and Levene tests verified the normality and homogeneity of variance of the collected data. The Kruskal-Wallis test and Mann-Whitney post-test were used to analyze the data of histopathological events and the capsule thickness. Correlation between the capsule thickness and histopathological events was also analyzed. A p-value less than 0.05 was considered statistically significant.

Results

Table 1 shows a summary of the qualitative microscopic analysis results. Polymorphonuclear neutrophils and giant cells were not associated with biomaterials in any of the experimental periods. At day 7, there was moderate presence of mononuclear cells, blood vessels and macrophages only for Group GIC+CM animals that had moderate chronic inflammatory reaction underneath the capsule. Group GIC and Group GIC+NC animals developed a slight chronic inflammation. GIC+NC showed moderate occurrence of fibroblasts, whereas for Group GIC and Group GIC+CM, there was a slight presence at 7 days. In the time intervals of 30 and 60 days, there was the same pattern of histological events for the three evaluated groups. Figure 1 shows the histopathological events according to biomaterials and experimental times.

The Kruskal-Wallis test revealed a statistically

significant difference between the groups when fibroblasts (p=0.022) and macrophages (p=0.019) were evaluated. In the post-test analyses, this difference occurred between the Group GIC and Group GIC+CM for macrophages (p=0.022), Group GIC and Group GIC+NC for macrophages (p=0.008) and mononuclear cells (p=0.033), and between Group GIC+CM and Group GIC+NC there was difference for fibroblasts (p=0.010). Therefore, the best histological aspects were for Group GIC+NC at 7 days. In the analyses of the time intervals at 30 and 60 days, the Kruskal-Wallis test revealed no significant difference between the groups.

Table 2 presents the descriptive quantitative measurements of the fibrous capsule thickness at 7, 30 and 60 days. Only at 60 days there was a statistically significant difference between Group GIC+CM and Group GIC+NC (p=0.028). Figure 2 presents the histological features of the fibrous capsule and surrounding tissues at all experimental times.

Spearman's correlation showed that at day 7, the Group GIC and Group GIC+CM showed positive correlation between mononuclear cells and macrophages (p=0.001), and Group GIC+NC showed positive correlation between fibroblasts and macrophages (p=0.001). After 30 days, Group GIC and Group GIC+NC showed positive correlation between mononuclear cells and macrophages, while for Group GIC+CM this correlation occurred between

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Table 1. Descriptive qualitative analysis of the histopathological events at 7, 30 and 60 days according to established scores

Histopathological events	7 days			30 days			60 days		
	Group GIC (Control)	Group GIC+CM	Group GIC+NC	Group GIC (Control)	Group GIC+CM	Group GIC+NC	Group GIC (Control)	Group GIC+CM	Group GIC+NC
Polymorphonuclear neutrophils	-	-	-	-	-	-	-	-	-
Mononuclear	+	++	+	+	+	+	+	+	+
Fibroblasts	+	+	++	++	++	++	++	++	++
Blood vessels	+	++	+	+	+	+	+	+	+
Macrophages	+	++	+	+	+	+	+	+	+
Inflammatory Giant Cells	-	-	-	-	-	-	-	-	-

Table 2. Descriptive quantitative measure of the fibrous capsule thickness at 7, 30 and 60 days

Group	Capsule Thickness (µm)						
	Median (Range) 7 days	p value*	Median (Range) 30 days	p value*	Median (Range) 60 days	p value*	Post-hoc**
GIC (Control)	60.24 (93.45)		53.23 (101.68)		30.15 (49.38)		AB
GIC+CM	49.14 (71.36)	0.223	43.52 (167.18)	0.459	41.58 (82.75)	0.022	A
GIC+NC	60.48 (77.94)		46.24 (68.88)		27.83 (36.45)		B

*Kruskal-Wallis Test (p<0.05). **Mann-Whitney Test. Different letters within Post-Hoc column indicate statistically significant difference (p<0.05).

mononuclear cells, macrophages and capsule thickness ($p=0.001$). After 60 days, there was a higher correlation among the histopathological events for the three groups, and between them and capsule thickness.

Discussion

The present study sought to evaluate the biocompatibility of glass ionomer cements modified by the addition of cellulose microfibrils and nanocrystals. Cellulose is the most abundant organic compound on the planet and became a classical example of a natural resource to produce organic elements for reinforcements, including cellulose microfibrils and nanocrystals. These particles present the following advantages: a renewable resource, low cost, low density, highly specific mechanical properties, non-abrasive and

easy processing (14,15).

According to Silva et al. (12), the different concentrations of CM or CN used to prepare the groups were due to the relative size and properties of each particle. When the concentrations of CN were over 1%, the nanoparticles aggregated, causing the composites' mechanical properties to fail. On the other hand, for CM concentrations smaller than 3%, the amount of filler was not sufficient to enhance the mechanical properties of the GIC. This behavior is characteristic of larger fillers, when relatively large concentrations are required to achieve reinforcement, whereas the advantage of nanofillers as the CN, is that very small concentrations are required for reinforcement, due to the large specific area of the nano-materials.

SEM/EDS and FTIR analyses of the modified biomaterials

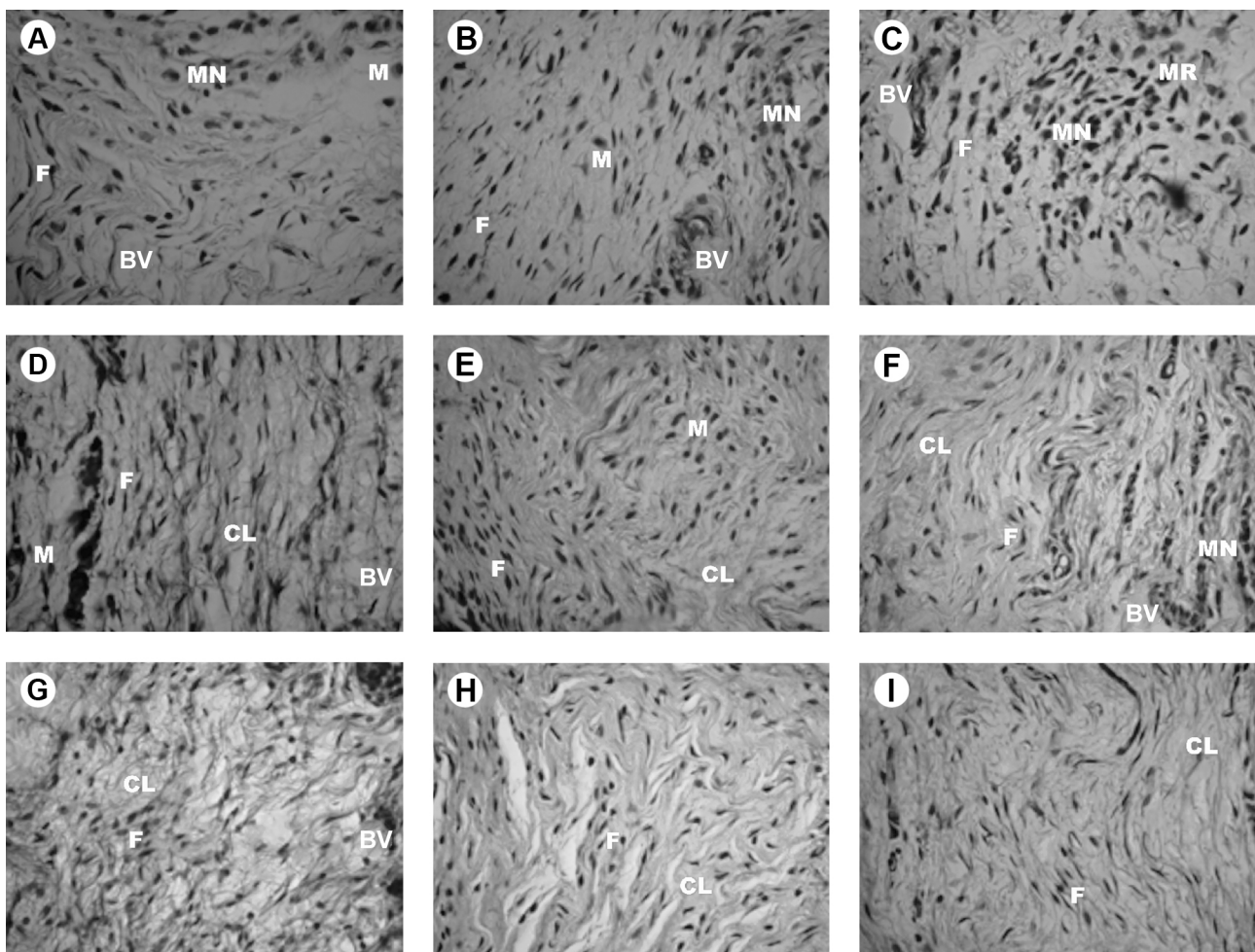


Figure 1. Histological features according to groups and experimental times. 7 days. A: Group GIC exhibiting macrophages (M) (+), other mononuclear cells (MN) (+) and fibroblasts (F) (+). B: Group GIC+CM exhibiting macrophages (++) , other mononuclear cells (++) , blood vessels (BV) (++) and fibroblasts (+). C: Group GIC+NC exhibiting macrophages (+), other mononuclear cells (+), blood vessels (+) and fibroblasts (++) . 30 days. D: Group GIC exhibiting macrophages (+), blood vessels (+), fibroblasts (++) and moderate collagenization (CL). E: Group GIC+CM exhibiting macrophages (+), fibroblasts (++) and moderate collagenization. F: Group GIC+NC exhibiting macrophages (+), blood vessels (+), fibroblasts (++) and moderate collagenization. 30 days. G: Group GIC exhibiting innumerable fibroblasts (++) , scarce blood vessels (+) and abundant collagenization. H: Group GIC+CM exhibiting numerous fibroblasts (++) and abundant collagenization. I: Group GIC+NC exhibiting numerous fibroblasts (++) and abundant collagenization. HE 400x.

suggested that the composites maintained the main characteristics of their precursors (12). Normally, in level I biocompatibility tests the biomaterials are inserted in polyethylene or silicone tubes (16-20). However, in the present study, the test specimens were implanted directly in subcutaneous tissue. This methodology may be used safely, as it is based on the results of Silva et al. (11), who evaluated the solubility and disintegration of this modified cement and found that did not exceed 0.78%. This value agrees with the international specifications (21). Corroborating previous studies, no multinucleated giant cells or acute inflammation were identified around the implanted materials, in every experimental time interval (16,17,19). The complete absence of these cells indicated that the biomaterials had low potential to induce foreign body giant cell reactions. This type of granulomatous tissue

reaction has been related for resin-modified glass ionomer cement (17) and some ionomer cements with an acid-base reaction (22,23).

After *in vivo* implantation of the biomaterial, it was expected that there would be a mechanism for normal wound healing, a complex process that involves the dynamic interaction of different types of cells. These responses were tested in the experiment presented here, because all the cell populations involved in the reaction of healing around the test specimens were quantified and correlated. Fibroblasts are cells that play a pivotal role in tissue regeneration, due to their capacity to produce extracellular matrix components, like collagen. These cells are also able to release growth factors that produce tissue homeostasis (24). Angiogenesis is the process of new blood vessel formation from pre-existing vessels, to assure the transport of oxygen, nutrients

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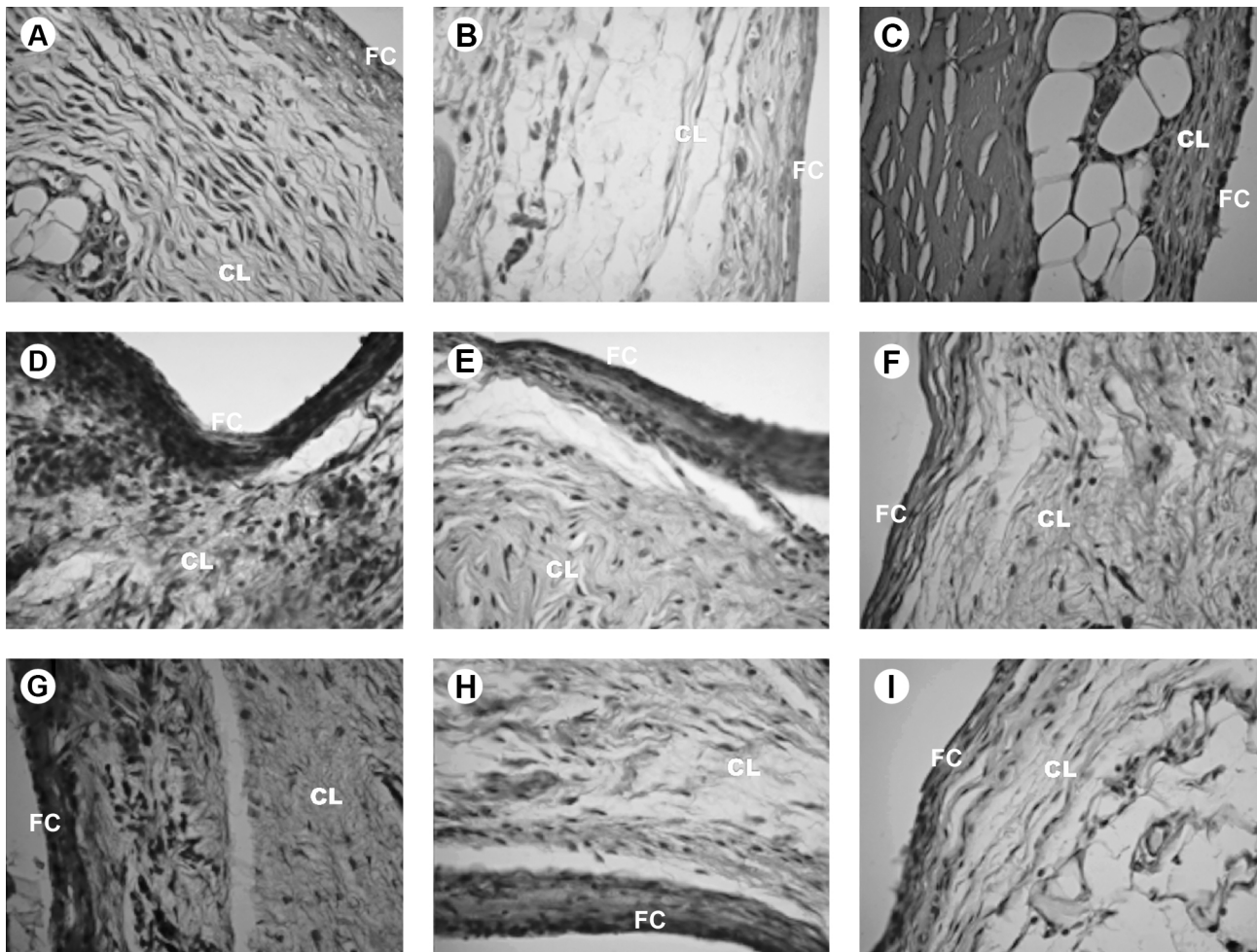


Figure 2. Histological features of the fibrous capsule (FC) and surrounding tissues in each experimental times: At 7 days. A: Group GIC exhibiting thick fibrous capsule (62.86 ± 30.65) little collagenization (CL). B: Group GIC+CM exhibiting medium thick fibrous capsule (50.49 ± 17.65) and little collagenization. C: Group GIC+NC exhibiting thick fibrous capsule (61.83 ± 23.91) and little collagenization. At 30 days. D: Group GIC exhibiting medium thick fibrous capsule (56.08 ± 25.02) and moderate collagenization. E: Group GIC+CM exhibiting thick fibrous capsule (65.76 ± 59.88) and moderate collagenization. F: Group GIC+NC exhibiting medium thick fibrous capsule (50.2 ± 17.63) and moderate collagenization. At 60 days. G: Group GIC exhibiting thin fibrous capsule (30.15 ± 4.28) and abundant collagenization. H: Group GIC+CM exhibiting thin fibrous capsule (41.21 ± 3.98) and abundant collagenization. H: Group GIC+NC exhibiting thin fibrous capsule (26.72 ± 2.87) and abundant collagenization. HE 400x.

and growth factors to promote vascularization and tissue remodeling at the implant site (25). Moreover, macrophages and mononuclear cells participate in the protection against tissue injuries, and also stimulate fibroblast differentiation and proliferation in the healing process (16,24).

Glass ionomer cement has shown cellular biocompatibility in human gingival fibroblast cultures (24), in subcutaneous tissue (18,21,22), rat alveoli (25) and in deep cavities prepared in human teeth (3). In the present study, there was a positive correlation between the presence of macrophages, mononuclear cells and fibroblasts and correlation of them with the capsule thickness. These results allowed to infer that these cells probably produced cytokines and growth factors, and acted in an autocrine and paracrine manner, they contributed improve the healing process around the test specimen, denoted by the variable thickness of the fibrous capsule according to the implanted biomaterial. The experimental biocompatibility test of the tested materials also showed progressive reduction in the fibrous capsule with time (21,22,25). As there was a thinner capsule in Group GIC+CM in the present study, in comparison with the Group GIC, the inference was that the microfiber particles interacted well with the cell populations. Group GIC+NC showed a statistically significant thinner fibrous capsule ($26.72 \pm 2.87 \mu\text{m}$) than Group GIC+CM and associated with abundant collagenization. Boaventura et al. (17) found similar biocompatibility of various formulations of conventional glass ionomer cement. On the other hand, ceramic-reinforced glass ionomer demonstrated superior biocompatibility compared with conventional glass ionomer (18). In this study, the nanocrystals interacted structurally with glass ionomer cement and produced an advanced stage of repair. These results suggested further researches to assess the material biocompatibility with the dentin pulp complex as liner material in deep cavities and surrounding the dental pulp.

The results of this study demonstrated that a scaffold based on cellulose microfibers and nanocrystals retained the biocompatibility of the original glass ionomer cement. However, there was a significantly more expressive result obtained by the experimental glass ionomer cement modified with cellulose nanocrystals.

Resumo

Os novos materiais restauradores em desenvolvimento devem evitar danos aos tecidos dentários. Portanto, o objetivo deste estudo foi avaliar a biocompatibilidade de uma marca comercial de cimento de ionômero de vidro convencional (CIV) modificado com microfibras de celulose (CIV+MC) ou nanocristais de celulose (CIV+NC) através da implantação de três amostras em tecido subcutâneo na região dorsal de 15 ratos *Rattus norvegicus albinus*. Cada rato recebeu um exemplar de cada cimento, resultando nos seguintes grupos (n=15): Grupo CIV (controle, n=15), Grupo CIV+MC e Grupo CIV+NC. Nos intervalos de 7, 30 e 60 dias os animais foram sacrificados e os seguintes aspectos foram avaliados

histologicamente: tipo de células inflamatórias, fibroblastos, vasos sanguíneos, macrófagos, células gigantes, tipo de reação inflamatória e espessura da cápsula (μm). Estes eventos foram quantitativamente classificados conforme os escores: (-) ausente, (+) suave, (++) moderado e (+++) intenso. Os resultados foram analisados estatisticamente pelo teste Kruskal-Wallis e pós-teste Mann-Whitney. Aos 7 dias, o Grupo CIV+NC demonstrou um nível mais elevado de reparação tecidual porque havia maior quantidade de fibroblastos ($p=0,022$) e uma menor quantidade de macrófagos ($p=0,008$) e células mononucleares ($p=0,033$). Neutrófilos e células gigantes estavam ausentes em todos os períodos experimentais. Aos 60 dias, o Grupo CIV+NC apresentou cápsula de tecido fibroso com espessura mais reduzida ($26,72 \pm 2,87 \mu\text{m}$) em comparação ao Grupo CIV+MC ($41,21 \pm 3,98 \mu\text{m}$) ($p=0,025$). No geral, todos os materiais apresentaram satisfatória biocompatibilidade, no entanto, o cimento de ionômero de vidro modificado com nanocristais de celulose proveu reparação tecidual mais avançada comparativamente aos demais materiais avaliados.

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