LUTING AGENTS DIFFERENTIALLY MODULATE INFLAMMATION AND MATRIX METALLOPROTEINASES IN CONNECTIVE TISSUE

Alexandra Mussolino de **Queiroz** [1], Thais Helena Andreolli do **Amaral**[1], Francisco Wanderley Garcia **Paula-Silva**[1], Paulo **Nelson-Filho**[1], Raquel Assed Bezerra da **Silva**[1], Patrícia Maria **Gaton-Hernández**[2], Léa Assed Bezerra da **Silva**[1*]

[1] Department of Pediatric Clinics, School of Dentistry of Ribeirão Preto, University of Sao Paulo, Ribeirão Preto, Sao Paulo, Brazil.
[2] University of Barcelona, Barcelona, Catalonia, Spain.

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RESUMO

Objetivo: Avaliar a resposta tecidual e a expressão de metaloproteinases da matriz (MMP) -2 e -9 frente a um cimento resinoso e um cimento ionomérico, após implantação no tecido conjuntivo subcutâneo de camundongos. **Métodos**: O cimento resinoso RelyX™ Unicem (CR; n=30), o cimento ionomérico Ketac™ Cem Easymix (CI; n=30) e tubo de polietileno vazio (controle; n=30) foram implantados no tecido subcutâneo dorsal de camundongos isogênicos BALB/c e os tecidos removidos para análise histológica após 7, 21 e 63 dias. Foram analisadas a resposta celular local, por meio da contagem de células inflamatórias e a espessura da cápsula fibrosa. A expressão de MMP-2 e -9 foi investigada por meio de imunohistoquímica. Os dados foram submetidos à análise estatística (α=5%). **Resultados**: Foi observado que CR induziu uma inflamação leve aos 7 e 21 dias com aumento do número de células inflamatórias aos 63 dias ($p<0,05$). CI induziu uma resposta inflamatória mononuclear mais intensa aos 7 e 21 dias ($p<0,05$), com redução do infiltrado aos 63 dias, semelhante ao observado no controle ($p>0,05$). Em todos os grupos a espessura da cápsula foi considerada fina ($p>0,05$). MMP-2 foi detectada em períodos precoces para CR e CI, com diminuição com o passar do tempo. MMP-9 apresentou um padrão semelhante ao controle para o CI, enquanto para o CR houve aumento com o passar do tempo. **Conclusão**: O cimento resinoso RelyX™ Unicem induziu uma resposta inflamatória e a expressão de MMP-9 mais tardia no tecido conjuntivo subcutâneo que foi diferente da resposta induzida pelo cimento ionomérico Ketac™ Cem Easymix.

Keywords: Luting Agents. Resin Cements. Glass Ionomer Cement. Connective Tissue Response. Matrix Metalloproteinases.

ABSTRACT

Objective: The objective of this study was to evaluate the tissue response and expression of matrix metalloproteinases (MMP)-2 and -9 to resinous and glass ionomer cements in direct contact with the subcutaneous connective tissue. **Methods**: RelyX™ Unicem resinous cement (RC; n=30), Ketac™ Cem Easymix glass ionomer cement (GI; n=30), and polyethylene empty tubes (control; n=30) were implanted in the dorsal subcutaneous tissue of isogenic BALB/c mice, and the tissues were biopsied after 7, 21, and 63 days for histological analysis. The inflammatory cells and fibroblasts were counted, and the fibrous capsule thickness was measured. MMP-2 and MMP-9 expression levels were investigated by immunohistochemistry. Data were analyzed statistically (significance level=5%). **Results**: We found that RC induced a low inflammation at day 7 and 21, which was increased at day 63 ($p<0.05$). GI induced a more intense mononuclear inflammatory response at day 7 and 21 ($p<0.05$), which was reduced at day 63 to levels similar to the control ($p>0.05$). The fibrous capsule thickness was thin for RC, GI, and control ($p>0.05$). MMP-2 was detected early for GI and RC and decreased afterwards. MMP-9 presented a similar pattern for GI, whereas the MMP-9 expression was late for RC. **Conclusion**: Resinous cement RelyX™ Unicem induced an inflammatory response and late MMP-9 expression in the subcutaneous connective tissue that was different from that induced by Ketac™ Cem Easymix glass ionomer cement.

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*** Correspondence to:**
Profa. Dra. Léa Assed Bezerra da Silva
Faculdade de Odontologia de Ribeirão Preto da Universidade de São Paulo. Avenida do Café, s/n. 14040-904. Ribeirão Preto. SP. Brazil. Phone: 55 16 3315-3984. Fax: 55 16 3315-4102. e-mail: lea@forp.usp.br

INTRODUCTION

Glass ionomer and resin cements are widely used for cementation of indirect restorations. Studies have shown that glass ionomer cement causes low irritation when applied near the pulp,[1-3] allowing it to be used as a restorative material without requiring the use of a cavity base.[4] *In vitro* studies revealed that Ketac™ Cem presents low cytotoxicity and it is not genotoxic,[5] although moderate inflammatory responses in the pulp have been reported *in vivo*.[6] Resin-modified glass ionomer, on the other hand, induces severe inflammation and large areas of tissue necrosis when directly applied as a pulp capping agent.[7]

The resinous cement RelyX™ Unicem induces a slight reduction in cell viability *in vitro.*[8] *In vivo,* when applied to the dentin after indirect pulp capping, RelyX™ Unicem caused minimal inflammation,[9,10] resulting in a small amount of tissue disorganization. In contrast, there was a severe inflammatory response when it was accidentally exposed to the pulp,[9] demonstrating that the material is unsatisfactory for direct contact with connective tissue. Usually, teeth prepared to receive total crowns present extensive decay and luting agents are applied nearly to the dental pulp. Because dentin and pulp are closely related tissues,[11] materials applied to the dentin might diffuse through pulpal tissue and induce inflammatory response. For that reason, the mechanisms involved in the tissue disorganization and inflammatory response induced by glass ionomer and resin cements should be further investigated.

Proteases responsible for extracellular matrix degradation, such as matrix metalloproteinases (MMPs), are involved in the connective tissue breakdown.[12] Previous studies have demonstrated that MMP-9 can be detected in teeth with pulp inflammation[13,14] and that the collagenolytic activity was increased in the dentin-pulp complex after an application of self-curing dental adhesives.[15,16] Additionally, dental pulp cells that were stimulated with tumor necrosis factor-α (TNF-α) expressed MMP-1, -2, and -13, demonstrating their importance during pro-inflammatory events.[17] However, the effect of direct contact between glass ionomer- or resin-based cements and the connective tissue on the *in vivo* MMP expression has not been investigated.

Therefore, the aim of this research was to study the histopathological tissue response and the matrix metalloproteinase-2 and -9 expression patterns resulting from two cements, one glass ionomer-based and the other resin-based, applied to the subcutaneous connective tissue in mice.

MATERIALS AND METHODS

All animal procedures were performed according to the protocols reviewed and approved by the Animal Care Committee of the University of Sao Paulo in compliance with the applicable ethical guidelines and regulations of the international guiding principles for biomedical research involving animals (#11.1.540.53.9).

Subcutaneous Connective Tissue Implantation in Isogenic Mice

Ninety isogenic male BALB/c mice, which were 6-8 weeks old and weighed 18-20 g, were obtained from the central vivarium of the University of São Paulo. Polyethylene tubes, measuring 1 cm in length and 2 mm in diameter, were sterilized using ethylene oxide and then filled with the materials to be tested. One end of the tube was closed with heated clinical tweezers. Dental materials were prepared according to the manufacturers' instructions under aseptic conditions and immediately prior to their implantation in the dorsal region. For the RelyX™ Unicem cement group, the material was added to the polyethylene tube and then photopolymerized for 20 seconds at 450 mW/cm^2, as measured with a curing radiometer (Ultralux; Dabi Atlante, Ribeirão Preto, Brazil).

The animals were anesthetized by an intramuscular injection of 10% ketamine chlorhydrate (100 mg/kg; Agener União Química Farmacêutica Nacional S/A, Embu-Guaçu, Brazil) and xylazine (10 mg/kg; Dopaser, Laboratórios Calier S/A, Barcelona, Spain) into their hind flank. Next, trichotomy was performed on the animal's dorsal region with a razor, and 1% chlorhexidine digluconate was used for antisepsis. The surgical procedure consisted of a 1-cm incision on the dorsal region, which was followed by divulsion using Kelly forceps.

Each animal was implanted with a polyethylene tube in its dorsal region containing the following compounds: Group 1 (experimental; 30 animals) - the glass ionomer cement Ketac™ Cem Easymix (3M ESPE, Seefeld, Germany); Group 2 (experimental; 30 animals) - the resin cement RelyX™ Unicem (3M ESPE, Seefeld, Germany); and Group 3 (control; 30 animals) - an empty polyethylene tube. The animals were randomly allocated to the groups, the operator was blind to experimental procedure and sample size was determined in a pilot study as recommended by ISO 10993-6: 2007.[18]

After positioning the tube in the connective tissue, the skin was sutured with silk thread (Ethicon, Johnson & Johnson, USA). The surgery was performed under aseptic conditions while aiming to minimize the trauma to the implant area. The animals were maintained and fed *ad libitum* in the vivarium during the experimental periods and periodically observed for local, systemic, and behavioral abnormalities.

Euthanasia, biopsy, and histological evaluation

At the end of each experimental period (7, 21, and 63 days), 10 animals from each group were randomly selected and anesthetized for careful removal of the implant together with the surrounding tissues (skin and connective tissue). Next, the animals were euthanized by means of an anesthesia overdose. The excised tissue was fixed in a 10% formaldehyde solution for 36 hours and submitted to routine histological processing. After embedding the specimens in paraffin, serial 5-micrometer thick sections were made parallel to the tube axis and were stained with hematoxylin and eosin or with Brown and Brenn for bacterial detection.

A blinded histological evaluation was performed using a

Zeiss Axio Imager M1 binocular light microscope (Upright microscope; Carl Zeiss AG, Göttingen, Germany) with 5, 10, 20, 40, 63, and 100x magnification. The intra-examiner calibration showed a kappa index of 0.83 in the pilot study. The number of inflammatory cells [both mononuclear and polymorphonuclear (PMN) cells] and fibroblasts were counted using the 63x magnification for 3 fields near the open end of the tube as follows: the center of the tube, 250 µm to the right of the tube center, and 250 µm to the left of the tube center. The global inflammatory cell counts were accomplished by video microscopy using the ImageJ 1.42q cell counter tool (National Institutes of Health, Bethesda, MD, USA) and an AxioCam MRc5 video camera (Carl Zeiss AG). The thickness of the fibrous capsule was measured (in micrometers) in images of 3 regions of each section at 5x magnification. Three sections were used per specimen.

Dichotomic data were analyzed by means of a Fisher's exact test. Continuous data were analyzed with a 2-way analysis of variance, which was followed by a Bonferroni multiple comparison post-hoc test (significance level= 5%).

Immunohistochemistry

The Goat ImmunoCruz™ assay (Santa Cruz Biotechnology Inc., La Jolla, CA, USA) was used for the immunohistochemical analyses. Briefly, tissue sections were quenched in peroxidase buffer for 5 min, and antigen retrieval performed by boiling sections in 10 mM sodium citrate (pH 6.0) at 93ºC for 10 min. Nonspecific binding was blocked by treating the sections with donkey serum blocker for 30 min. Next, the sections were incubated for 1 hr with primary antibodies for MMP-2 (5.0 µg/ml; Santa Cruz Biotechnology Inc., sc-8835) or MMP-9 (5.0 µg/ml; Santa Cruz Biotechnology Inc., sc-6840). Then, the sections were incubated with an anti-goat secondary antibody for 30 min, which was followed by streptavidin-conjugated horseradish peroxidase for 20 min with 3,3'-Diaminobenzidine as the enzyme substrate for 5 min. The tissues were counterstained with Harris's hematoxylin and mounted using standard protocols. Negative controls consisted of replacing the primary antibody with goat IgG. Microscopic analysis was performed to evaluate the presence or absence of positively labeled cells and the location of the staining, using the Zeiss Axio Imager M1 binocular light microscope with the 20, 40, 63, and 100x magnification.

RESULTS

Histological analysis

Two specimens from the 21-day samples and 2 from the 63-day samples of the resinous cement were lost during the histological processing. Two specimens were lost from the empty tube (control) group from each experimental time point. No specimens from the glass ionomer cement group were lost.

A comparison of the glass ionomer cement group with the control (empty tube) group showed that the cellular response induced by glass ionomer cement was more intense than by the control tubes at days 7 and 21, as a higher percentage of mononuclear and PMN cells were observed at day 7 ($p<0.05$), and more mononuclear cells were observed at day 21 ($p<0.05$). A higher percentage of fibroblasts was observed in the control group at day 21 ($p<0.05$). At the end of the experiment (day 63), no difference among fibroblasts, mononuclear and PMN was observed when glass ionomer cement was compared to empty tube ($p>0.05$) (Figures 1 and 2).

A comparison of the resinous cement group and the control group showed that fibroblasts were more common in the resinous cement group than in the control group ($p<0.05$) at days 7 and 21, and mononuclear cells were more common in the control group ($p<0.05$). At the end of the experiment (day 63), a higher percentage of mononuclear and PMN cells and a lower number of fibroblasts were observed in the resinous cement group, compared to empty tube ($p<0.05$) (Figures 1 and 2).

A comparison of the resinous cement and glass ionomer cement-induced inflammatory responses in mice at day 7 showed that the two materials had similar percentages of fibroblasts, mononuclear cells, and PMN cells ($p>0.05$). At day 21, the percentage of fibroblasts was higher in the resinous cement ($p<0.05$) group, and mononuclear cells were most prevalent in the glass ionomer cement ($p<0.05$) groups. There was no difference in the number of PMN cells ($p>0.05$). At day 63, the percentage of mononuclear and PMN cells was higher in the resinous cement ($p<0.05$) group, and the fibroblasts were higher in the glass ionomer cement group ($p<0.05$) (Figures 1 and 2).

At day 7, the resinous cement group showed a larger fibrous capsule thickness than the glass ionomer cement group and the empty tube group ($p<0.05$). At day 21, the thickness of the fibrous capsule in the resinous cement group was similar to the thickness of the empty tube ($p>0.05$). The glass ionomer cement group showed a thicker fibrous capsule than the empty tube ($p<0.05$) at this time point. At day 63, the thickness of the fibrous capsule in the resinous cement and glass ionomer cement groups was similar to that in the empty tube group ($p>0.05$) (Figures 1 and 2).

Overall, glass ionomer cement showed a tissue response similar to the control group, while resinous cement induced a late inflammatory response. Bacteria were not found in any group at any time point.

Matrix metalloproteinase-2 expression

Glass ionomer cement (Figure 3)

Positive staining was detected in the subcutaneous

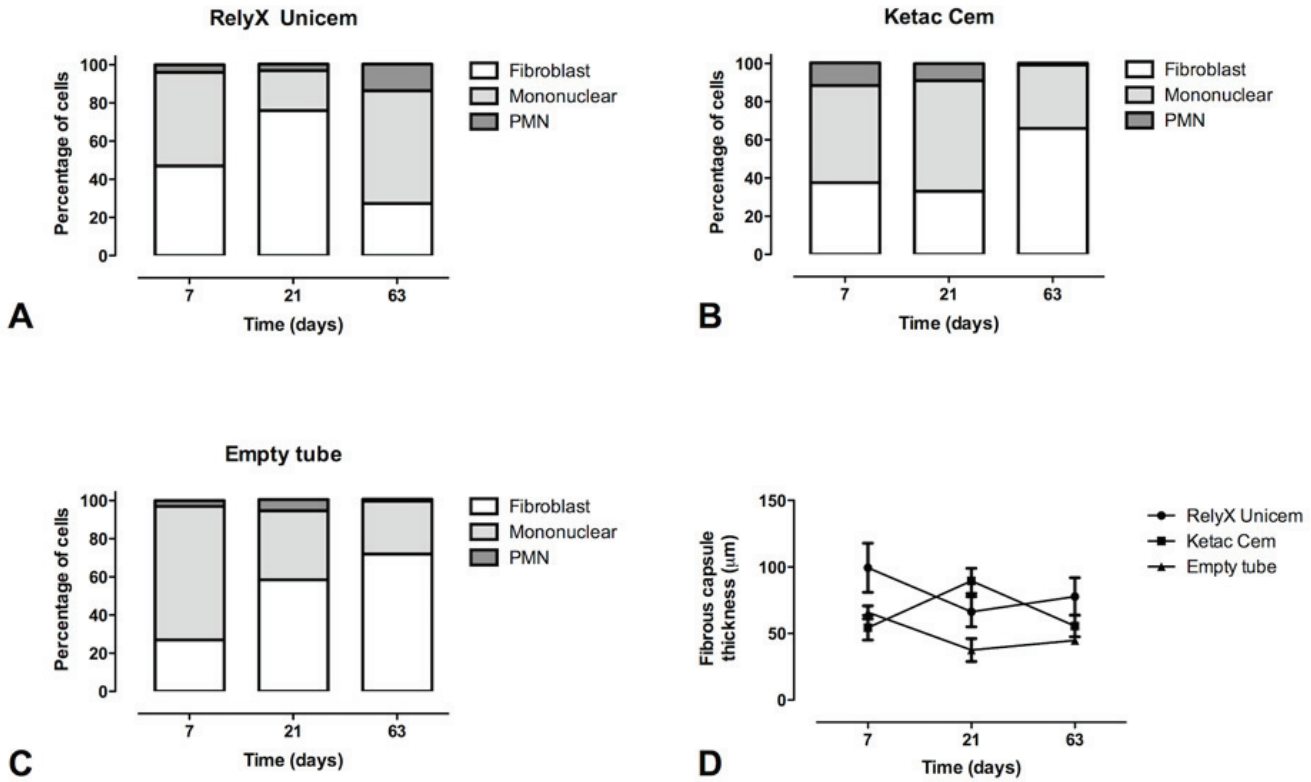

***Figure 1**: Graphs showing the percentages of cells [fibroblasts, mononuclear, and polymorphonuclear (PMN) cells] within the fields of vision at 63x for each of the groups (A- Ketac™ Cem, B- RelyX™ Unicem, and C- empty tube) at different time points (7, 21, and 63 days). D- The thickness of the fibrous capsule in µm for each group (Ketac™ Cem, RelyX™ Unicem, and empty tube) at day 7, 21, and 63.*

connective tissue in contact with the material at the open end of the polyethylene tube and along its sidewalls at day 7. Staining was weak in the extracellular matrix and in the fibroblast and mononuclear cell cytoplasms, but the PMN cells were strongly stained.

At day 21, weak staining at the material border and surrounding the polyethylene tube was observed. There was low staining in the extracellular matrix and in the cytoplasm of fibroblasts and mononuclear cells. When present, the PMN cells were inconsistently stained.

Staining at the material border or around the polyethylene tube was not observed at day 63. Weak staining was observed extracellularly and intracellularly in the mononuclear cells, fibroblasts, and PMN cells.

Resinous cement (Figure 4)

Positive staining was observed in the subcutaneous connective tissue in contact with the material at the open end of the polyethylene tube and along its sides at day 7. Staining was moderate in the extracellular matrix and more evident in the fibroblast and mononuclear cell cytoplasms, while the PMN cells were clearly marked.

Positive staining in the subcutaneous connective tissue in contact with the open end of the polyethylene tube and along its sides was evident at day 21. Staining was moderate in the extracellular matrix and weak in the fibroblast, mononuclear cell, and PMN cell cytoplasms.

Weak staining was observed at the material border and surrounding the polyethylene tube at day 63. There was weak to no staining in the extracellular matrix and in the fibroblast, mononuclear cell, and PMN cell cytoplasms.

Empty tube (control)

Positive staining was observed in the subcutaneous connective tissue at the edge of the material at the open end of the polyethylene tube and along its sidewalls at day 7. The staining was weak in the extracellular matrix and in the fibroblast, mononuclear cell, and PMN cell cytoplasms and was restricted to the tube/tissue interface. The staining pattern in the control group was different from the staining pattern in the experimental groups.

Weak staining was observed at the material border and near the polyethylene tube at day 21. When present, the staining in the extracellular matrix and in the fibroblast, mononuclear cell, and PMN cell cytoplasms was weak. The line marking the division between the underlying connective tissue was less evident.

No staining was observed at the material border or around the polyethylene tube at day 63. Slight extracellular or intracellular staining was observed in the mononuclear cells, fibroblasts, and PMN cells. The line between the tube and the connective tissue, which was observed at day 7 and 21, was absent by the end of the experiment.

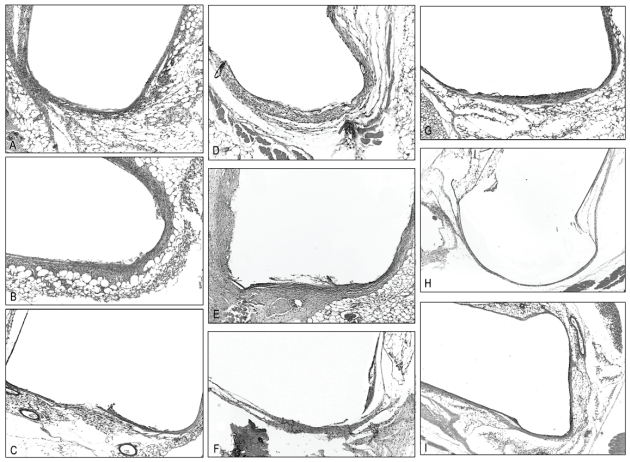

***Figure** 2: Representative photomicrographs of the tissue response in the mouse subcutaneous connective tissue at 7, 21, and 63 days after the implantation of a polyethylene tube filled with Ketac™ Cem (A, B, C), RelyX™ Unicem (D, E, F) or an empty tube (G, H, I). Original magnification: 5× (A, B, D, H, I), 10× (C, E, F, G). The thickness of the fibrous capsule decreased over time for resinous cement and the empty tube. However, glass ionomer cement caused an increase in the capsule thickness until day 21, which subsequently decreased until it was similar to the empty tube at day 63.*

Matrix metalloproteinase-9 expression

Glass ionomer cement (Figure 5)

Positive staining was detected in the subcutaneous connective tissue in contact with the material at the open end of the polyethylene tube and along its sidewalls at day 7. The staining was moderate in the extracellular matrix and in the fibroblast, mononuclear cell, and PMN cell cytoplasms. Some specimens had a bright line at the polyethylene tube wall, and PMN cells, fibroblasts, and mononuclear cells were stained in this region.

At day 21, the extracellular staining was weak and homogenous with few stained cells (PMN cells, fibroblasts, and mononuclear cells). A clearly defined line marked the edge of the polyethylene tube.

Diffuse staining was observed in the extracellular matrix at day 63. The staining was more visible near the polyethylene tube. PMN cells, fibroblasts, and mononuclear cells were sporadically stained.

Resinous cement (Figure 6)

Strong staining was observed around the tube, both at the opening and along the sidewalls, at day 7. The extracellular matrix was weakly stained with positive staining in both the mononuclear cells and fibroblasts.

The extracellular matrix staining around the tube was weak at day 21. Fibroblasts and mononuclear cells were lightly stained.

Intense, well-defined staining was observed around the tube at day 63 with positive staining in the extracellular matrix and the cytoplasms of fibroblasts and mononuclear cells. The PMN cells were not stained.

Empty tube (control)

Weak positive staining of the extracellular matrix and a stronger level of staining at the polyethylene tube interface were observed at day 7. Few cells (mononuclear cells and fibroblasts) were stained.

Weak positive staining of the extracellular matrix and at the polyethylene tube interface was observed at day 21 and 63. Few cells (mononuclear and fibroblast cells) were stained.

DISCUSSION

The group of mice implanted with polyethylene tubes filled with glass ionomer cement in the subcutaneous connective tissue showed a high percentage of fibroblasts, a low percentage of mononuclear and PMN cells, and a thin fibrous capsule, which is a state compatible with tissue repair. In contrast, the group of mice implanted with polyethylene tubes filled with resinous cement in the connective tissue showed an increased inflammatory response at the end of the experiment that was characterized by increased percentages of mononuclear and PMN cells with decreased numbers of fibroblasts, indicating a late inflammatory response.

The PMN and mononuclear cell numbers peaked at day

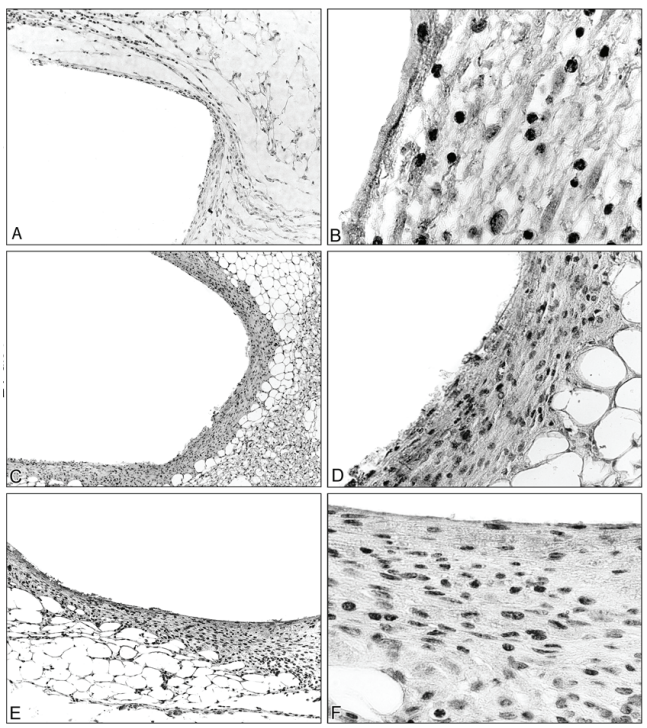

***Figure 3**: Immunohistochemical staining of MMP-2 in the subcutaneous tissue of mice at 7, 21, and 63 days after the implantation of a polyethylene tube filled with Ketac™ Cem ionomer cement. Original magnification: 10× (A, C), 20× (E), 63× (D), and 100× (B, F). Positive staining in the extracellular matrix and in fibroblast, mononuclear cell and PMN cytoplasm were detected at day 7, decreased at day 21, and had disappeared by day 63.*

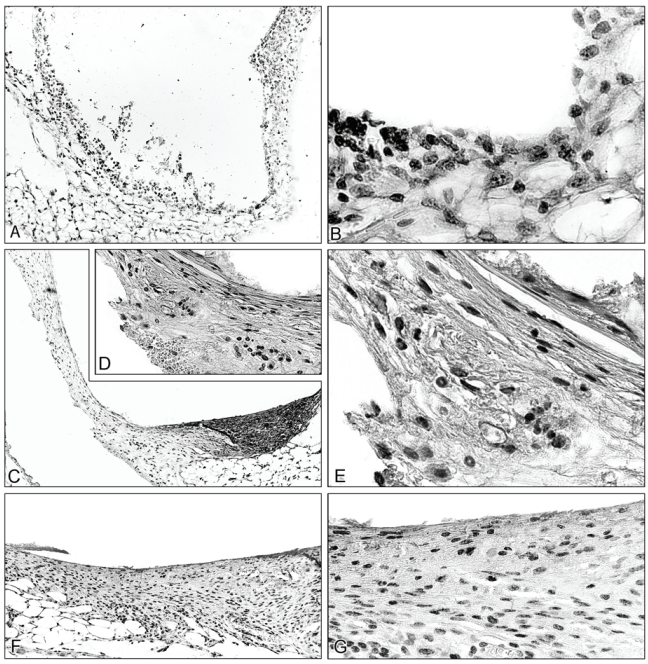

***Figure 4**: Immunohistochemical staining of MMP-2 in mouse subcutaneous tissue 7, 21, and 63 days after implantation of a polyethylene tube filled with RelyX™ Unicem resin cement. Original magnification: 5× (C), 10× (A, F), 20× (D), 40× (G), and 100× (B, E). Positive staining in the extracellular matrix and in the fibroblast, mononuclear cell, and PMN cell cytoplasm was detected at day 7, decreased at day 21, and was weak at day 63.*

21 for glass ionomer cement (mononuclear cells) and at day 63 for resinous cement (mononuclear and PMN cells). One possible explanation for this cell behavior is that glass ionomer cement is more easily dissolved than resinous cement,[19] which results in an early tissue response. The low solubility of RelyX™ Unicem has been attributed to its high concentration of silanized glass powder and treated silica, which prevent its immediate degradation in a humid environment.[20]

The fibrous capsule in this study was thin for both resinous cement and glass ionomer cement across all time points based on previously published parameters, which state that a capsule is thin if it is less than 150 µm thick.[21,22] However, the capsule thickness in the glass ionomer cement group increased between day 7 and 21 and later decreased until it was the same thickness as the control group at the end of the experiment. The increased capsule thickness at day 21 occurred when the mononuclear cell percentages near the tube were also increasing. The thickness of the reactive tissue is a parameter for measuring the tissue's response, as the majority of the reactive tissue is mostly occupied by macrophages in addition to young fibroblasts. Thus, measuring the thickness of the reactive tissue qualitatively measures the prevalence of macrophages in the inflammatory response.[23]

A higher expression of MMP-2 was observed shortly after the polyethylene tube implantation with or without cement, which then decreased over time. At the end of the experiment (day 63), there was low intra- or extracellular MMP-2 expression for both resinous and glass ionomer cements, similar to what was observed in the control group (empty tube). The higher MMP-2 expression observed in both the cement and empty tube groups early in the experiment may be due to tissue remodeling after the polyethylene tube implantation.

The MMP-9 expression was high early in the experiment and decreased over time for the glass ionomer cement and empty tube groups. In contrast, a weak MMP-9 expression was observed early in the resinous cement group that increased at the end of the study (day 63). These results are consistent with the histological analyses, which showed that direct contact of the resinous cement with the connective tissue resulted in a delayed inflammatory response characterized by mononuclear and PMN cells. Previous *in vitro* studies have shown that adhesives can cause MMP-2 expression in fibroblasts isolated from dental pulp, which is possibly due to the acidic nature of the material and the presence of non-polymerized monomers.[24] However, a direct comparison is difficult, as we could not find any *in vivo* studies on the effect of cements on the MMP expression.

According to ISO 10993-6,[18] it is recommended that implants be initially performed in the subcutaneous connective tissue based on a preliminary source of information on the compatibility of the material with the biological activity of the tissues in the area at a macro- and microscopic level.[25-27] Further confirmatory tests are also recommended for applications of the material to the dental

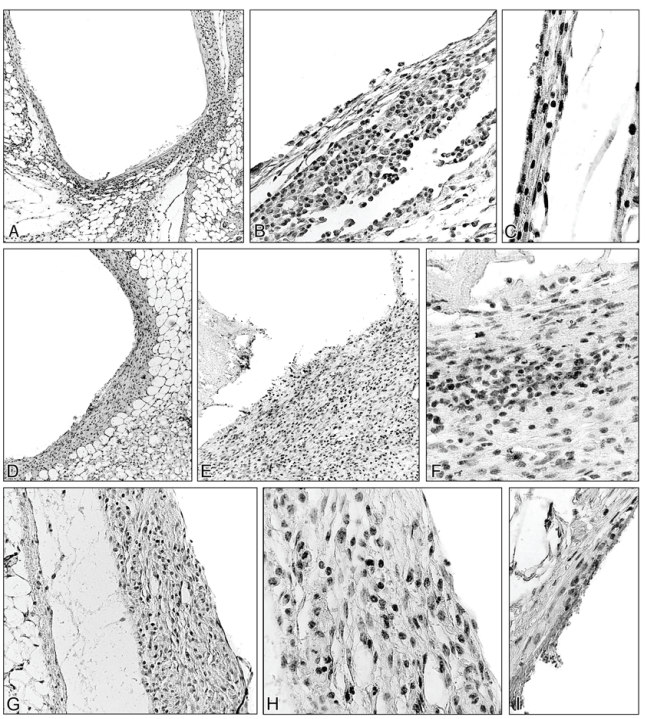

***Figure 5**: Immunohistochemical staining of MMP-9 in the mouse subcutaneous tissue at 7, 21, and 63 days after the implantation of a polyethylene tube filled with Ketac™ Cem ionomer cement. Original magnification: 10× (A, D, E), 20× (G), 40× (B, F), and 63× (C, H, I). Positive staining in the extracellular matrix and in the fibroblast, mononuclear cell, and PMN cell cytoplasm was observed at day 7, which later became weak and homogeneous by day 21. At day 63, diffuse staining of the extracellular matrix was detected, and PMN cells, fibroblasts, and mononuclear cells were sporadically stained.*

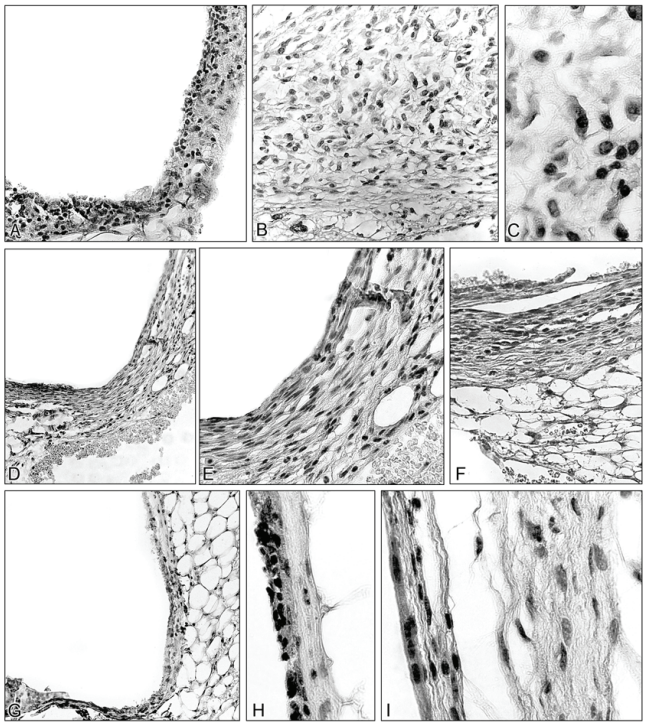

***Figure 6**: Immunohistochemical staining of MMP-9 in the mouse subcutaneous tissue at 7, 21, and 63 days after the implantation of a polyethylene tube filled with RelyX™ Unicem resin cement (A-E, G-I). Negative control = Immunoglobulin G (F). Original magnification: 20× (D, G), 40× (A, F), 63× (B, E, H), and 100× (C, I). The extracellular matrix, fibroblasts, and mononuclear cells were weakly stained at day 7, with staining in the fibroblasts and mononuclear cells decreasing at day 21. At day 63, intense, well-defined staining was observed in the extracellular matrix and in the fibroblast and mononuclear cell cytoplasm.*

tissue to evaluate the response of the pulp, apical, and periapical tissues.[28] Due to the unsatisfactory results obtained for resinous cement, including the inflammatory response and late MMP-9 expression in the mouse subcutaneous connective tissue, studies investigating its clinical application for direct pulp protection are not recommended.

CONCLUSION

The glass ionomer cement Ketac™ Cem showed a good compatibility with mouse connective tissue, unlike the resin cement RelyX™ Unicem. In both the groups, the MMP-2 expression was highest immediately after implantation and decreased over time. However, the RelyX™ Unicem cement caused a late increase of MMP-9 expression, which was not observed in the Ketac™ Cem group, indicating delayed wound healing.

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