An Endodontic Sealer Induces a Pathological Condition when Associated with Persistent Tissue Toxicity and Presence of Myofibroblasts

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The aims of this study were to evaluate the ratio between inflammatory reactions induced by four endodontic sealers and the occurrence of fibrosis and the number of myofibroblasts with positivity to α-smooth-actin muscle (α-SMA). Polyethylene tubes were filled with a root canal sealer (Endofill, AH Plus, Acroseal and Epiphany) and inserted into 4 site at the dorsal region of 24 Wistar rats; 2 empty tubes (control) were grafted in 6 rats. After 7, 21, and 45 days, 8 animals were euthanized, providing 6 specimens per test group and 2 specimens from the control group. The fragments were subjected to histological processing and immunohistochemical analysis for anti-α-SMA protein. All specimens, except those from the control group, presented severe inflammatory reaction on the 7th postoperative day, which also coincided with a large number of myofibroblasts. On the 21st and 45th days post-surgery, the inflammatory reaction induced by Endofill, AH Plus and Acroseal decreased significantly, which coincided with reduced presence of myofibroblasts and usual collagen deposition. In contrast, in the group filled with Epiphany, significant inflammatory cell infiltrate was present in all analyzed periods. The persistence of an inflammatory reaction induced by endodontic sealer may also induce the development of fibrosis in combination with presence of myofibroblasts.

Key Words: endodontic sealers, fibrosis, biocompatibility, myofibroblasts.

INTRODUCTION

In the endodontic practice, several types of sealer have been used to fill and seal the root canals (1,2). Although these materials are manufactured to be used only within the root canal, they commonly extrude through the apical constriction (3). The contact of sealers with living tissue produces interactions with the complex biologic systems around where it occurs, resulting in a biologic response represented by the immune reaction.

Since there are no inert materials in dentistry (4) to minimize the toxic reactions to tissues, endodontic sealers are based on various formulations according to their main component (e.g., sealers based on zinc-oxide and eugenol, epoxy resin, methacrylate and calcium hydroxide), and these different formulations may produce different results in terms of toxicity, affecting the inflammatory response and tissue repair (5,6).

During a usual repair and/or reconstitution of connective tissue, fibroblasts migrate into the wound, where they produce and remodel extracellular matrix, resulting in wound closure. These events commonly are mediated by a specialized form of fibroblasts, denominated myofibroblasts (7).

The myofibroblasts are terminally differentiated cells that express α-smooth-actin muscle (α-SMA) filaments and share a hybrid phenotype between...
fibroblasts and muscle cells (8). After differentiation, these cells participate in wound healing by promoting the contraction necessary to restore homeostasis in damaged tissue, closing the repair process (9). The persistence of or changes in the life cycle of myofibroblasts is a hallmark of fibrotic lesions (10) similar to pathological fibro-proliferative conditions, such as scleroderma, kidney and lung fibrosis or myelofibrosis in bone.

Since inflammatory cytokines, especially interleukins and growth factors, are the main inducers of α-SMA phenotype development and collagen synthesis and deposition (7,11), it was hypothesized that the immune reactions induced by endodontic sealers could also contribute to pathological fibrosis. Thus, the aim of this study was to analyze the correlation between tissue toxicity developed by four endodontic sealers (Endofill, AH Plus, Acroseal and Epiphany) and the presence of fibrosis and myofibroblasts.

MATERIAL AND METHODS

Twenty-four 60-70-day old Wistar rats (Rattus norvegicus) weighing 250-280 g and with no history of disease were used following a protocol approved by the Animal Care and Use Committee of Positivo University, Brazil (Protocol #144/2008). The animals were kept in a room with controlled temperature (approximately 22°C) and maintained under a 12 h light-dark cycle.

Before surgery, the animals were anesthetized with 8×10^{-3} mL/g of ketamine hydrochloride and 4×10^{-3} mL/g of 2% xylazine hydrochloride (Virbac do Brasil Indústria e Comércio Ltda., São Paulo, SP, Brazil). Dorsal shaving was performed and the area was disinfected using an iodine-polyvinylpyrrolidone (PVPI) solution.

In the experimental group, four 17 mm-long incisions were performed on the dorsal region of each animal. The incisions were arranged in quadrants, equidistant from the center of the dorsum. Two incisions were made on the scapular region (one on the right side and the other on the left side - positions I and II, respectively) and the other 2 incisions were created on the pelvic region (one on the right side and the other on the left side - positions III and IV, respectively).

Polyethylene tubes (10 mm long and 0.8 mm internal diameter) were autoclaved, had one of the ends closed and were filled with the following sealers, prepared according to the manufacturers’ instructions: Group I - Endofill (Dentsply Ind. e Com. Ltda., Petrópolis, RJ, Brazil); Group II - AH Plus (Dentsply DeTrey, Konstanz, Germany); Group III - Acroseal (Septodont Brasil Ltda., Tamboré, SP, Brazil); and Group IV - Epiphany (Sybron Endo, Orange, CA, USA). The tubes containing the sealers were inserted into subcutaneous spaces created by blunt dissection. The tubes were grafted into the surgical cavities, parallel to the incisions, with the open end pointing to the animal’s tail. Two animals per period were used as control and received an empty tube in the subcutaneous tissue of the dorsal region. Tissues were repositioned and the incisions were sutured with 5-0 nylon silk.

All animals were euthanized at 7, 21 and 45 days postoperatively (6 animals per period in the test groups) by brief exposure to a CO2 chamber until they ceased moving.

Tissue Processing

Tissue fragments from the areas that contained the tubes were carefully necropsied with a 2-cm safety margin, and all fragments were fixed in 10% buffered formalin for 24 h. The specimens were washed in tap water, dehydrated, cleared and embedded in paraffin.

Serial 3-µm-thick sections perpendicular to the long axis of the tubes were cut with a microtome (RM2155; Leica Microsystems GmbH, Nussloch, Germany) and stained with hematoxylin-eosin and Masson’s Trichrome to detect deposition of collagen fibers.

Immunohistochemistry Processing

Three-micrometer-thick serial sections of these specimens were deparaffinized and subjected to antigen retrieval in 10 mM citrate solution (pH 6.0) for 45 min in a double boiler at 95°C. The slides containing the histological specimens were incubated overnight with the primary antibody anti-α-SMA (clone 1A4; Dako, Glostrup, Denmark) with dilution factor 1:150. The labeled streptavidin biotin (LSAB plus; Dako) antibody-binding detection system was used to detect the primary antibodies, and the specimens were then submitted to a solution composed of 3,3’ diaminobenzidine tetrachloride (Sigma, St. Louis, MO, USA) for 3 min to reveal the reaction, producing a brown precipitate at the antigen site. All specimens were counterstained with Harris hematoxylin. For each specimen, 3 slides were used for incubation with antibody.
**Image and Statistical Analysis**

Images of histological and immunohistochemistry sections were captured with a digital camera coupled to a light microscope with ×200 original magnification. Each digital image was collected and saved with 600 dpi resolution, producing a virtual picture of 117 × 80 cm. Five histological fields were analyzed in each specimen.

The analysis of the presence of fibrous tissue and the number of inflammatory and \( \alpha \)-SMA\(^+ \) cells was performed using the software Image Tool 2.00 (University of Texas Health Science Center; San Antonio, TX, USA). The perimeters of histological fibrous tissue and total area were carefully traced, and the following areas were computed while the quantity of the inflammatory and \( \alpha \)-SMA\(^+ \) cells per total cells were counted and tagged. The data were counted manually, transformed in percentage and expressed in scores graded as negative if they were <5%. Positive staining for cell markers was graded as + (5-20%), ++ (21-40%), +++ (41-60%) and ++++ (>60%).

Each parameter was evaluated separately within the established periods. Data were analyzed using Moda, to verify possible similarities between the groups.

**RESULTS**

**Quantitative and Qualitative Analysis of the Inflammatory Infiltrate**

A brief description of the histological features among groups was performed, while the quantitative parameters of the inflammatory response were summarized and demonstrated in Table 1 and in Figure 1, respectively.

**Group I (Endofill)** - On the 7\(^{th} \) postoperative day, the microscopic analysis revealed moderate inflammatory infiltrate, composed predominately of lymphocytes and plasma cells surrounding the polyethylene tubes and edematous areas. On the 21\(^{st} \) day, discrete mononuclear inflammatory infiltrate remained around the tube, although well organized granulation tissue was present. On the 45\(^{th} \) day post-surgery, discrete inflammatory infiltrate was detected focally surrounding the polyethylene tube. In addition, connective tissue exhibiting usual morphology was seen, while the discrete fibrosis areas were observed surrounding the entire tube.

**Group II (AH Plus)** - On the 7\(^{th} \) postoperative day, mononuclear inflammatory infiltration restricted to the tube aperture was identified. In addition, a loose irregular connective tissue was present on the fragment analyzed. On the 21\(^{st} \) and the 45\(^{th} \) days post-surgery, an organized and vascularized connective tissue was apparent, without significant inflammatory response (<5%).

**Group III (Acroseal)** - On the 7\(^{th} \) and 21\(^{st} \) days post-surgery, the histological analysis revealed an evident granulation tissue. On the 7\(^{th} \) postoperative day, a remarkable amount of diffuse inflammation infiltrate on the vascularized tissue was evident. This inflammatory infiltrate was composed predominantly of lymphocytes, plasma cells and macrophages. Presence of this inflammatory tissue decreased gradually on the...
21st and 45th day of analysis. On the 45th day post-
surgery, few inflammatory cells were present, although
a thin fibrous tissue layer was concentrated around the
polyethylene tube.

Group IV (Epiphany) - The histological frame
induced by Epiphany revealed the presence of a
remarkable and diffuse inflammatory response in all
time periods monitored. On the 7th day post-
surgery, the microscopy analysis of these specimens demonstrated
both acute and mononuclear inflammatory infiltrate in
the entire necropsied tissue. It was also observed a focus
of necrosis, especially where there was intimate contact
between the live tissue and sealer (4/6 specimens). On the
21st and 45th days post-surgery, diffuse and predominately
mononuclear inflammation (predominately plasma cells,
lymphocytes and macrophages) was identified, and also
a discrete number of giant cells were observed.

Group V (Control) - Isolated inflammatory

Figure 1. Histological comparison among the groups analyzed. Micrographs A, B and C demonstrate the microscopic features observed
in group I (Endofill) on the 7th, 21st and 45th postoperative days, respectively. Micrograph A shows intense inflammatory reaction
induced by the endodontic sealer, while images B and C show a usual connective tissue repair. These facts occurred similarly in group
II (AH Plus; micrographs D, E and F) and group III (Acroseal; micrographs G, H and I). In contrast, group IV (Epiphany) shows
the presence of an inflammation reaction in all analyzed periods (J - 7th postoperative day; K - 21st postoperative day and L - 40th
postoperative day). All micrographs were stained with hematoxylin and eosin, at original magnification ×100, except for L (original
magnification ×40). cip = chronic inflammation process; acip = acute and chronic inflammation process; ct = usual connective tissue.
response was observed only on the 7th day post surgery. On the 21st and 45th days postoperative, only fibrous tissue surrounding the polyethylene tubes was observed.

**Masson’s Trichrome Analysis**

All groups tested positive for fibrous tissue development (Fig. 2). The moda of score distribution in each group can be seen in Table 1. The groups that received tubes filled with Endofill, AH plus and Acroseal showed well-formed granulation tissue, composed of thin collagen fibers, as soon as the inflammation was finalized. These results were similar to those of the control group, which also presented usual connective tissue on the 7th postoperative day. In addition, fibrosis was observed only peripherally to the polyethylene tubes, especially on the 21st and 45th days post-surgery in groups I, II, III and V. In contrast, specimens filled with Epiphany (Group IV) showed intensive fibrotic tissue deposited on both the 21st and 45th postoperative days, mimicking a pathological fibro-proliferative condition.

**α-SMA⁺ Cells (Myofibroblasts)**

The positivity to α-SMA⁺ cells was observed in all groups analyzed on the 7th postoperative day (Fig. 3). Only in group V (control) was the myofibroblasts identified as surrounding the polyethylene tube and the mature blood vessels (pericytes), while in groups I to IV, the positivity was present on the cells spread on the inflammatory tissue. On the 21st and 45th day post-surgery, the presence of myofibroblasts in groups I, II and III, decreased significantly, while the usual connective tissue and/or discrete fibrosis surrounding the polyethylene tubes were built. During these periods, the presence of the α-SMA was restricted to the blood vessels, characterizing the smooth muscle tissue around the newly formed artery, or surrounding the polyethylene tubes. In contrast, in group IV (Epiphany), the presence of α-SMA⁺ cells remained higher and spread among the

Figure 2. Fibrous deposition on the 40th postoperative day. Micrographs A, B and C reveal thinner and delicate collagen fibers surrounding the polyethylene tube filled by Endofill, AH plus and Acroseal, respectively. Micrograph D exhibits an intense fibrosis peripherally in the tube filled with Epiphany. All micrographs were stained with Masson’s Trichrome, at original magnification ×200.
fibrous tissue in all periods except on the 7th day.

**DISCUSSION**

The toxicity of a material is usually evaluated *in vitro* using methods that allow directing contact between the material and cell cultures. These studies are extremely important because if particular material is considered toxic *in vitro* it is expected to cause adverse tissue response when used *in vivo* (12).

According to the manufacturers, all endodontic sealers tested in this study have a satisfactory tissue response: Figure 3. Immunopositivity to α-SMA antibody (brownish color) in all groups analyzed. Micrographs A, B and C showed the presence of α-SMA+ cells in group I (Endofill) on 7th, 21st and 45th postoperative days, respectively. Micrograph A demonstrated the presence of protein surrounding the blood vessels (pericytes) in the granulation tissue, while B and C also revealed the presence of α-SMA+ cells (myofibroblast) surrounding the polyethylene tube. Micrographs D, E and F showed, respectively, the presence of discrete quantities of α-SMA+ cells in group II (AH plus) restrict to adjacencies of the polyethylene tube on the 7th, 21st and 45th days post-operative. Micrograph G revealed intense α-SMA+ cells among the granulation tissue on 7th day post-surgery, while H and I demonstrate the presence of myofibroblast surrounding the polyethylene tube at 21st and 45th day post-surgery. Figure J showed intense acute inflammatory reaction and scarce α-SMA+ cells in the connective tissue around the tube filled with Epiphany (group IV) on 7th day. Micrographs K and L, respectively for the 21st and 45th postoperative day, revealed higher quantities of α-SMA+ cells (myofibroblasts) among the connective tissue (group IV), similar to pathological fibrosis. Original magnification ×100, except for L (original magnification ×40).
response. However, the results of Epiphany in the present study did not corroborate the manufacturer’s claims. This sealer induced a persistent inflammatory reaction up to the 45th postoperative day together with a greater presence of myofibroblasts and robust deposition of collagen, mimicking a pathological fibrosis.

Fibrosis is the result of the excess synthesis and deposition of collagen, and it is a characteristic of several connective tissue diseases. It constitutes the final stage in a series of pathological events that begins with altered vascular tonus dysfunction, immunological activation, and extravascular inflammation (13).

The literature states that the ratio between inflammatory reaction and fibrosis has been attributed to persistence and elevation of levels of inflammatory cytokines and growth factors, especially the transforming growth factor beta (TGF-β), an ordinary growth factor produced by platelets and white cells in injured sites (14). Thus, when persistent, these inflammatory cytokines have acted as fibrogenic cytokines, constituting fibrosis since it is responsible for a collagen synthesis contributing to an intensive extracellular matrix deposition (9), and in the same way, inhibition of extracellular matrix-degrading enzymes.

This collagen is usually produced by myofibroblasts, which are terminally differentiated cells that express α-SMA filaments and share a hybrid phenotype between fibroblasts and muscle cells (15). The appearance and localization of these cells at sites of wound healing and tissue repair have always been associated with the period of the active fibrous deposition. In fact, they are considered important to the genesis of the extracellular matrix deposition as well as proliferation and differentiation of vascular components among the granulation tissue (16). Furthermore, the literature highlights that the persistence or changes on the life cycle of the myofibroblasts is a hallmark of fibrotic lesions (10). This was also demonstrated by Krieg et al. (13), who analyzed biopsies of fibroproliferative lesions (10). This was also demonstrated by Krieg et al. (13), who analyzed biopsies of fibroproliferative tissue and found a significant increase in the number of myofibroblasts, particularly in the deep derms when compared with usual connective tissue.

In addition, it is noteworthy that the possible ability of myofibroblasts to contribute to fibrosis development may be due to a cause-effect relationship with inflammatory cytokines and growth factors and extracellular matrix deposition (17). In this way, inflammatory cytokines and growth factors, such as TGF-β, are known for their ability to differentiate mesenchymal cells into myofibroblasts, since they regulate the phenotype by inducing α-SMA filament transcription (18). In turn, the contraction of α-SMA cells has been strictly correlated with enhanced force-generating capacities that may secrete TGF-β in an autocrine fashion, which exacerbates the effects of this cytokine, thus contributing to the development of fibrotic conditions (10). This feed-forward loads a mechanism that can help to promote and maintain the myofibroblast differentiation at the same time that the TGF-β conduces myofibroblast survival (14), since this relationship suppresses the apoptotic capacity of myofibroblasts (19).

In the present study, the reparative histological frame showed simultaneously inflammatory infiltrate, fibrosis and a remarkable number of myofibroblasts only in tissues that had close contact with Epiphany. The extrapolation of this result may indicate that the presence of myofibroblasts and fibrosis in the group that received Epiphany can be an important component to limit bone matrix deposition during periapical bone repair. This hypothesis is based on the findings of a recent study (20) that demonstrated that myofibroblasts in a bone microenvironment produced a fibrotic frame rich in collagen III, which is a type of collagen that neither surrounds nor supports the adhesion of bone cells.

In contrast, the results present herein also demonstrated that Endofill, AH Plus and Acroseal had a severe toxic effect on the 7th postoperative day, but it decreased significantly on the 21st and 45th postoperative days, which coincided with decrease in the number of myofibroblast and presence of the usual connective tissue that surrounded the polyethylene tubes.

The findings of the present study are in accordance with those of a previous study that found that the severe toxicity of AH Plus and Acroseal in the earlier period may be attributed to formaldehyde release, which decreases significantly after polymerization of the materials and thus attenuates the inflammatory response (21). On the other hand, the toxic effects of Endofill may be due to the eugenol released during sealer contraction (22). In this regard, Hume (23) has stated that in spite of being recognizably aggressive to the tissues eugenol has antiinflammatory properties.

This way, these factors acting together may help explaining the severe initial inflammatory process and subsequent repair usually observed when Endophill, AH Plus and Acroseal were used.

On the basis of the obtained results, it may be
concluded that the persistence of inflammatory infiltrate induced by an endodontic sealer (Epiphany) may induce the development of fibrosis in combination with the presence of myofibroblasts.

RESUMO

O objetivo deste estudo foi avaliar a relação entre reação inflamatória induzida por quatro cimentos endodônticos e a presença de fibrose e quantidade de miofibroblastos que apresentam positividade para α-SMA. Tubos de policrileno foram preenchidos com o cimento (I: Endofill; II: AH Plus; III: Acroseal; IV: Epiphany) e inseridos em 4 regiões do dorso de 24 ratos Wistar, enquanto 2 tubos vazios (V - controle) foram inseridos em 6 ratos. Após 7, 21 e 45 dias, oito animais foram sacrificados obtendo 6 indivíduos por grupo e 2 para o grupo controle. Os fragmentos foram submetidos ao processamento histológico e à análise imuno-histoquímica para a proteína anti-α-SMA. Todos os grupos, exceto o controle, demonstraram notável reação inflamatória no 7º dia pós-operatório, que também coincidiu com uma grande quantidade de miofibroblastos. No 21º e 45º dia pós-operatório, a reação inflamatória induzida pelo Endofill, AH Plus e Acroseal diminuiu significativamente, o que coincidiu com reduzida presença de miofibroblastos e deposição de colágeno normal. Em contraste, no grupo Epiphany, infiltrado inflamatório significativo esteve presente em todos os periodos analisados. A persistência do infiltrado inflamatório induzido por cimento endodôntico pode também provocar uma fibrose associada com a presença de miofibroblastos.

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