Biocompatibility Analysis of Chemomechanical Caries Removal Material Papacárie on Cultured Fibroblasts and Subcutaneous Tissue

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ABSTRACT

Chemomechanical caries removal allies an atraumatic technique with antimicrobiotic characteristics, minimizing painful stimuli and maximally preserving healthy dental structures. The purpose of this study was to compare the cytotoxic effects of papain-based gel (Papacárie) and another caries-removing substance, Carisolv, to a nontreatment control on cultured fibroblasts in vitro and the biocompatibility in subcutaneous tissue in vivo. The cytotoxicity analysis was performed on fibroblast cultures (NIH-3T3) after 0-, 4-, 8-, and 12-hour exposure (cell viability assay) and after 1-, 3-, 5-, and 7-day exposure (survival assay). In the in vivo study, the 2 compounds were introduced into polyethylene tubes that were implanted into subcutaneous tissues of rats . After 1, 7, 14, 30, and 60 days, tissue samples were examined histologically. Cell viability did not differ between the 2 experimental groups. The control group, however, showed significantly higher percentage viability. There were no differences in cell survival between the control and experimental groups. The histological analysis revealed a moderate inflammatory response at 2 and 7 days and a mild response at 15 days, becoming almost imperceptible by 30 and 60 days in both experimental groups. The 2 tested substances exhibited acceptable biocompatibilities and demonstrated similar responses in the in vitro cytotoxicity and in vivo implantation assay.

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The clinical management of dental caries has evolved considerably over recent decades. This is primarily due to findings elucidating the pathogenesis of dental caries that have resulted in less invasive methods for treating infected dental tissue and to the development of adhesive resin bonding systems.[1-4]

Procedures for caries removal include the conventional mechanical method; air abrasion with aluminum oxide, chemomechanical methods, atraumatic restorative therapy (ART), and most recently, laser treatment. Independent of the technical procedure employed, however, the removal of infected dentin is of great importance during restorative

caries treatment. The main objective of infected dentin removal is the elimination of the softened, infected, and necrotic tissue to control evaluation of the lesion and to support the restorative procedure.[5,6]

The traditional means of cavity preparation employs both high-speed handpieces and slowly rotating instruments. Such means of cavity preparation usually induce pain, annoying sounds, and vibration. Drilling often removes healthy tooth parts as well as the decayed areas. This weakens the tooth, which becomes less durable in the long-term. Thus, the chemomechanical removal of caries has been developed as an alternative to conventional methods. This removal process involves the application of a chemical solution to the carious tissue followed by gentle removal using hand-held instruments. Furthermore, in contrast to conventional, high-speed rotary cutting instruments, the effects of heat and pressure on the pulp are also eliminated by this procedure.[3,4,7]

The chemomechanical technique for caries removal was first introduced in 1972, and other materials have been developed since. The first substance employed to remove organic dentin material was sodium hypochlorite,[8] which is, however, too instable and overly aggressive for healthy tissues. Subsequently, the Sorensen solution was developed, containing a mixture of sodium hydroxide, sodium chloride, and glycine denominated GK 101.[9] In 1984, the Food and Drug Administration (FDA) authorized the use of Caridex (National Patent Medical Products Inc, NJ), developed from efforts to improve GK101 performance, which was very slow to remove caries. Caridex proved to be very efficient in removing infected dentin together with caries, leaving a healthy, mineralized dentin surface adequate for restored. Caridex, however, is less efficient in cases of arrested dentinal caries.[7]

The chemomechanical caries removal system known as Caridex was developed in 1985.[4] Its efficacy in caries removal, however, required improvement, and its hand-held instruments were not effective. Use of this system required a large reservoir with a pump and large quantities of solution. Furthermore, the product was overly expensive and had a short shelf life.[3,4] To overcome these major drawbacks, a new chemomechanical caries removal agent, Carisolv, was developed. This product employed a minimally invasive method of removing carious dentin while preserving sound dentin. The material consists of a 2-component gel used together with a number of special hand-held instruments. The gel consists of a transparent, highly viscous fluid that contains 3 amino acids (glutamic acid, leucine, and lysine), sodium chloride, erythrocin, and sodium hydroxide, and a transparent fluid consisting of a low concentration of sodium hypochlorite.[1,2,10] Carisolv instruments possess a sharp edge and a blunt cutting angle, which are designed to scrape rather than cut, and are apparently leaving the healthy tooth tissue intact.[3] This system is claimed to minimize the need for anesthesia and to reduce the use of rotary instruments while avoiding excessive tissue removal.[3,11]

Although Carisolv is an extremely efficient product, it is inconveniently expensive, which prevents its widespread use. Aiming to expand the chemomechanical removal of decayed tissue and promote this technique particularly in the public health area, a new low-cost formula was deve-loped in Brazil in 2003, consisting of a papain gel known as Papacárie. Papacárie is a gel product, used in the chemomechanical removal of decayed tissue, that consists of papain, toluidene blue, and chloramine. The combination of these 3 substances in the formula provides the safe removal of decayed tissue, together with a local anti-inflammatory effect and bactericidal and bacteriostatic actions.[1,2,10-14]

Papain acts by cleaving collagen molecules, partially destroyed by the action of caries, and is able to digest dead cells, eliminating the fibrin coat formed by the caries process. Only the infected tissue is affected by papain, owing to the absence of α-trypsin, a plasma antiprotease that prevents proteolitic activity in normal tissues.[15] Chloramine is a compound produced from chlorine and ammonia possessing bactericidal and disinfectant properties. In the chemomechanical caries removal procedure, chloramines are used to chemically soften the decayed dentin.

The biocompatibility of materials used in dentistry is of considerable importance since it influences outcome and clinical application. One of the requirements for an ideal dental material is that it does not irritate living connective tissue. High biocompatibility renders a material useful in applications that require contact with vital tissues.[16] To demonstrate the safe use of this new chemomechanical caries removal system, the biocompatibility of Papacárie was evaluated in cultured fibroblasts in vitro and in rat subcutaneous tissue in vivo compared to Carisolv.

METHODS

IN VITRO ASSAY (CELL RESPONSE)

The toxicities of Carisolv and Papacárie were assayed in vitro. The response of rat fibroblasts coming into contact (cell viability) with these materials and their long-term survival (cell survival, which evaluates cell self-renewal capacity) were investigated.

CELL CULTURE

An NIH-3T3 cell line (ATCC CRL 1658) obtained from embryonic mice was cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma Chemical Company, St Louis Mo) supplemented with 10% fetal bovine serum (Cultilab Ltda, Campinas, Sao Paulo, Brazil) and 1% antibiotic/antimycotic solution (Sigma, St. Louis, MO, USA). Embryonic fibroblasts were incubated under standard cell culture conditions (37°C, 100% relative humidity, and a 95% air/5% CO_2 atmosphere). The cells were kept in an incubator at 37°C in a humidified 5% CO_2 atmosphere. Cultures were supplied with fresh medium every other day. Cells between the fifth and tenth passages were used in all experimental procedures.

EXPERIMENTAL GROUPS

Three groups were evaluated: group 1: control, group 2: Carisolv; and group 3: Papacárie. At culture experiments a plain coverslips without (control) or with materials (Carisolv or Papcarie) were used. At the *in vivo* experiments a tube without (control) or with materials were used.

SHORT-TERM ASSAY (CELL VIABILITY)

Cells ($2x10^4$) were plated onto 35-mm diameter Petri dishes. Three days later, the cultures received coverslips. After 0, 4, 8, and 12 hours, the cells in 3 dishes per group were counted and viability curves were plotted. Since cultured cells can be prepared in a uniform suspension in most cases, the use of a large number of replicates is often unnecessary. Often 3 replicates are sufficient, and for many simple observations (like cell counts), duplicates may be sufficient.

CELL SURVIVAL ASSAYS (LONG-TERM)

After cell culture, the cells were plated onto 60-mm diameter culture plaques. For the cell growth assay (long-term), $1x10^4$ cells per plaque were plated as previously described.[17] After 4 hours, the cultures received the coverslips. The control cultures received the coverslip alone without the experimental materials. After 1, 3, 5, and 7 days in contact with the substances, cell counts were performed by counting viable cells in a hemocytometer, using the Trypan blue dye exclusion assay.

The number of viable cells harvested from each dish was obtained from the following equation: $UCxDx10^4/nSQ$, where UC is the unstained cell count (viable cells); D, the dilution of the cell suspension; and nSQ, the number of squares counted in the hemocytometer.

The percentage viability of the cell population in each dish was obtained from the following equation: UC/TCx100, where UC is the unstained cell count (viable cells); and TC, representing the total cell count (stained plus unstained cells).

STATISTICAL ANALYSIS

Each data point corresponds to the standard deviations of either the cell count or the percent cell viability from 3 plaques for each experimental period. The data were compared using an analysis of variance procedure followed by Tukey's test. Significance was set at 5% ($P \leq 0.05$).

IN VIVO ASSAY (TISSUE RESPONSE)

This study was conducted under the approval of the Ethics Committee of Nove de Julho University, São Paulo, Brazil. Twenty-five male Wistar rats (*Rattus norvegicus albinus*) of average weight (250-300 g) were fed before and during the experimental period with solid rations and water ad libitum. The animals were maintained in an animal room with a shifted light/dark cycle (typically dark from 10:30 p.m. to 9 a.m. and light from 9 a.m. to 10:30 p.m. and kept 5 animals per cage). Each cage was identified with the appropriate animal numbers and ear tag numbers. The investigation followed the guideline principles on ethics and animal experimentation provided by the Brazilian School of Animal Experimentation (COBEA), an entity affiliated with the International Council of Laboratory Animal Science (ICLAS).

Polyethylene catheters (Sondaplast, São Paulo), 10 mm in length and 1.5 mm in diameter, were washed in ethanol and distilled water and autoclaved before being filled with the test substances. The materials were introduced into the catheters using a lentulo bur. The rats were anesthetized prior to surgery employing a mixture of xylazin and ketamine (0.15 ml per 100 g of body weight). The back of each animal was shaved and disinfected with 5% iodine in ethanol. Incisions (5 mm) were made in the dorsum, and 3 subcutaneous pockets were prepared by blunt dissection and the catheters were implanted subcutaneously 15 mm from the skin incision. After implantation, the incisions were closed with surgical nylon sutures.

Each animal received an implant of 2 tubes, containing the materials to be tested, placed separately in each tube. An empty polyethylene catheter was used as a control.

At the end of each period (1, 7, 14, 30, and 60 days later), the rats were sacrified with an anesthetic overdose. The catheters were removed along with the surrounding tissue and immersed in 10% buffered formalin. After fixation for 48 hours, the tissues were processed for paraffin embedding and serial sections were taken. The sections were stained with hematoxylin and eosin. Tissue responses were graded as mild, moderate, or severe, according to the criteria suggested by Olsson et al[18] and Orstavik and Mjör.[19] The criteria for scoring the inflammatory tissue response were:

1. Grade 1 (no/slight inflammation): the thickness of the reaction zone is similar to or only slightly wider than the thickness of the catheter, and no or few inflammatory cells are observed.
2. Grade 2 (moderate inflammation): an increased reaction zone in which macrophages, plasma cells, or both are present.
3. Grade 3 (severe inflammation): an increased reaction zone in which macrophages and plasma cells and occasional foci of neutrophil granulocytes, lymphocytes, or both are present.

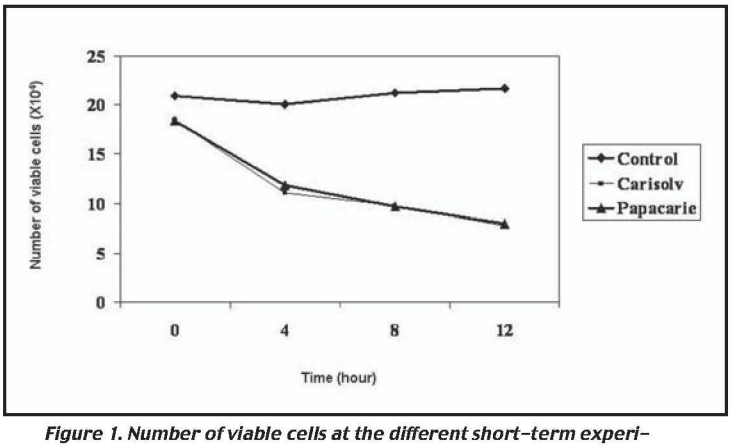

Figure 1. Number of viable cells at the different short-term experimental periods.

The results were analyzed statistically employing the Kruskal-Wallis test. Interpretation of the results establishes whether the material can be accepted (biocompatible) or rejected (not biocompatible) based on Federation Dentaire International[16] criteria as following:

1. Absent to slight reaction at both 2 and 12 weeks is acceptable. Absent to slight reaction at 2 weeks increasing to a moderate or severe reaction at 12 weeks is not acceptable.
2. Moderate reaction at 2 and 12 weeks is not acceptable. A moderate reaction at 2 weeks that diminishes at 12 weeks is acceptable.
3. A severe reaction at any moment is unacceptable.

RESULTS

IN VITRO ASSAY: IMMEDIATE CELL RESPONSE–SHORT-TERM EXPERIMENT

Figure 1 shows the number of viable cells at the different short-term experimental periods for groups 1, 2, and 3.

Immediate contact of the NIH–3T3 cell cultures with the compounds led to a decrease in the number of viable cells compared to group 1. Group 3 showed a greater number of viable cells than group 2 over the different exposure periods.

The percentage analysis of viable cells provided in Figure 2 shows no statistically significant differences between groups 2 and 3. Compared to group 1, cell viability was maintained at between 80% and 100% during the initial 12 hours.

CELL SURVIVAL–LONG-TERM EXPERIMENT

The long-term experiment showed that the number of viable cells in groups 2 and 3 was significantly less than in group 1. Cell viability in group 3, however, was greater than in group 2 during the different experimental periods, as shown in Figure 3.

The percentage cell viability showed no statistically significant differences among the 3 groups. Percentage of cell viability was established at 80% to 100%, decreasing after 7 days, as shown in Figure 4.

IN VIVO ASSAY

The histopathological scores for the in vivo assay are summarized in Table 1. There were no statistically significant differences among the histological scores for the test material and control groups.

Representative photomicrographs of the histological findings are shown in Figure 5. On day 1 of the experiment, all groups showed a moderate inflammatory process, the presence of neutrophil granulocytes, scant collagen fiber proliferation, and angiogenesis at the catheters extremities (Figure 5a). After 7 days, groups 2 and 3 showed a slight or moderate inflammatory process, with a predominance of lymphocytes associated with moderate fibrosis and angiogenesis (Figure 5b). Group 1 exhibited a slight inflammatory process, lymphocytes, and minimal vessel and fiber proliferation. A marked effect of Papacárie was seen in 3 rats and in 1 Carisolv-treated rat, where an increase in the chronic inflammatory process was noted.

After 14 days of contact, groups 2 and 3 exhibited a slight inflammatory process, the presence of lymphocytes, fibroblasts, and angiogenesis (Figure 5c). The areas around the implanted material exhibited a foreign body reaction. A response to Carisolv in group 2 and Papacárie in group 3 was seen in 3 rats where there was a moderate

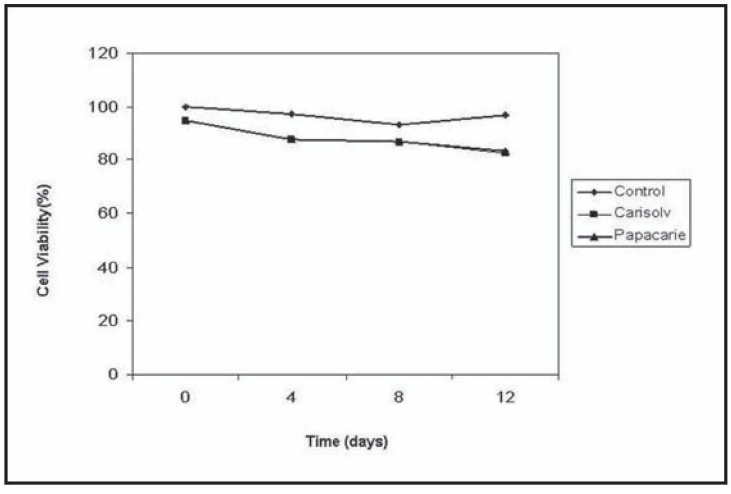

Figure 2. Percentage analysis of viable cells at the different short-term experimental periods.

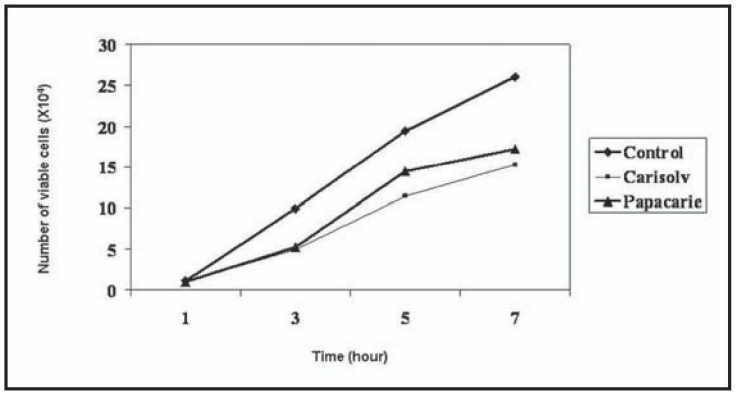

Figure 3. Number of viable cells at the different long-term experimental periods.

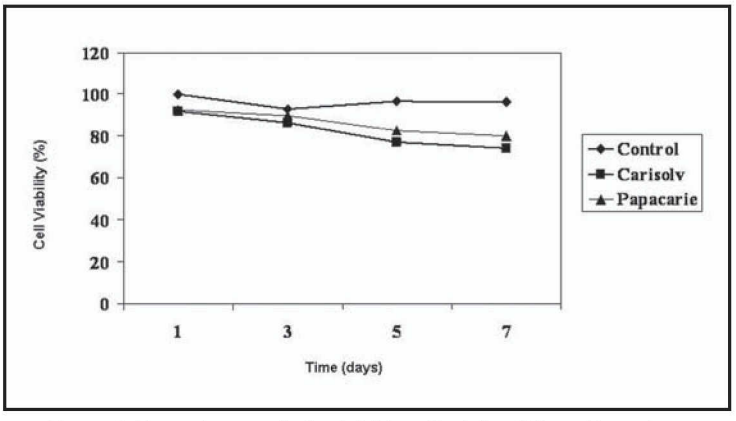

Figure 4. Percentage analysis of viable cells at the different long-term experimental periods.

Table 1. Histological Tissue Response (mean±SD)

Group	1 day	7 days	14 days	30 days	60 days	Interpretation
Carisolv	2.20±0.44	1.80±0.44	1.20±0.44	1.00±0.00	1.00±0.00	Acceptable
Papacárie	2.40±0.54	1.60±0.54	1.20±0.44	1.20±0.44	1.20±0.44	Acceptable
Control	2 .00±0.00	1.60±0.54	1.20±0.44	1.00±0.00	1.00±0.00	Acceptable

** Grade 1=no/slight inflammation; grade 2=moderate inflammation; grade 3=severe inflammation*

chronic inflammatory process with the formation of a thin fibroblast capsule. Necrosis was not found. Rare, multinucleate giant cells were occasionally noticed around the material. The group 1 showed no inflammatory process, with well-organized fibrosis and angiogenesis.

The findings for the 3 groups were similar by 30 days post-implantation. There was an absence of inflammatory reaction, with intense reparative processes characterized by angiogenesis and fibrosis (Figure 5d). The areas around the implanted material exhibited a foreign body reaction (Figure 5f).

The findings for all groups were similar by 60 days, revealing only fibrosis at the catheter extremities (Figure 5e).

DISCUSSION

The cytotoxic effects of materials used in chemomechanical carious removal were tested in vitro using a fibroblast cell line and in vivo in rat subcutaneous tissue. This study furnishes the investigator with the information on the effect of the materials when in contact with vital tissues, aiding in choosing the best material for clinical applications.

Currently, many recommended tests are available for the biological evaluation and acceptance of dental materials. One of the *in vitro* tests, that provides a general toxicity profile for such materials is cytotoxicity using cultured cells.[20,21] A secondary test that evaluates the biocompatibility of dental materials uses implantation into the connective tissue of small animals.[22,23] Following the FDA protocol in this study, the substances were used in the same formulation employed for clinical purposes. Since the chemomechanical caries removal system comes into contact with vital tissues, particularly the pulp, is important to demonstrate biocompatibility and the safety of such materials.

Our findings show that both materials tested, Carisolv and Papacárie, exhibited low cytotoxicity in vitro and acceptable biocompatibility in vivo.

We performed 2 tests of in vitro cytotoxicity that, in the clinical context, may provide information regarding effects on cells close to the material both in the short term and in the long term. In the short term, both Papacárie and Carisolv caused a decrease in the number of viable cells compared to the group control. The percentage analysis of viable cells revealed approximately 80% viability, however, reflecting low initial cytotoxicity possibly associated with constituent components such as chloramine in Papacárie and sodium hypochlorite in Carisolv.

In this in vitro study, we analyzed the reaction of the cells in contact with the materials constructing growth and cell viability curves, characterizing a long-term experiment. Cell viability in the presence of the tested materials was approximately 80% at to the end of the experiment, at 7 days. This finding show that, despite the initial cytotoxicity, the cells remained viable.

Our findings are similar to those of Sepet et al,[24] who tested Carisolv in another cell line (FM3A). They observed a significant decrease in cell growth 20 minutes after Carisolv application compared to the control and 1-minute treatment groups. These authors concluded that the prolonged application of Carisolv does not affect cell viability, but reduced cell growth in the FM3A cell line.

Our histological analysis revealed a moderate inflammatory response after 1 and 7 days and a mild response at 14 days, becoming almost imperceptible by 30 and 60 days in both experimental groups. There were no statistically significant differences among the test and control groups.

The moderate inflammation seen in the early tissue responses (1 and 7 days) to the test materials may relate to initial toxicity before contact of the substances with the connective tissue. The presence of inflammatory cells in the tissue adjacent to the 2 materials, compared to the empty catheter, provides evidence of tissue irritability by the materials. Leukocytes are short-lived, approximately 3 to 4 days and upon dying release enzymes that activate factors chemotactic for mononuclear cells. Their presence at 14 days may be considered evidence of a cumulative toxicity effect of the material, surgical trauma, and the continued seepage of particles of the materials tested into the surrounding tissue. In this study, however, the inflammatory reactions to both chemomechanical caries removal materials decreased with time. By 15 days, the tissue responses were significantly less than those at 1 day. After 30 and 60 days, a fibrous capsule with fibroblasts appeared with a few inflammatory cells. This appears to be a normal tissue response, since such a capsule was also present along the walls of the relatively nontoxic polytetrafluoroethylene catheters.

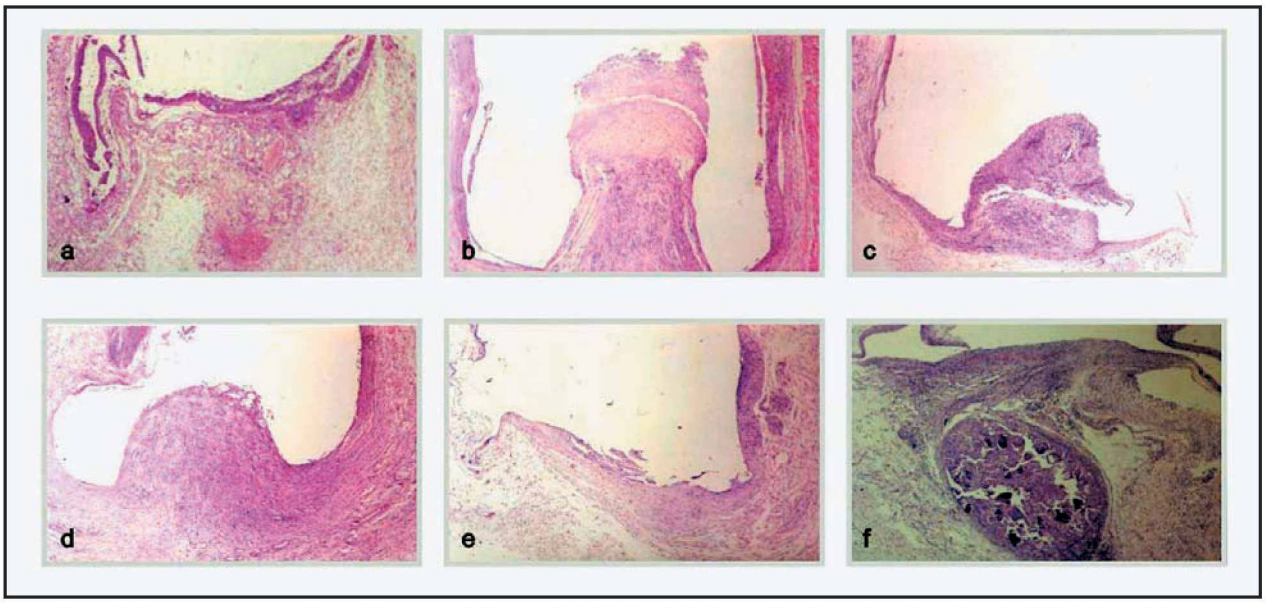

Figure 5. Tissue responses. a. Papacárie group, 1 day, grade 2. Moderate inflammation (hematoxylin and eosin, X40). b. Papacárie group, 7 days, grade 1. Slight inflammation reactions (hematoxylin and eosin, X40). c. Carisolv group, 14 days, grade 1. Slight inflammation reactions (hematoxylin and eosin, X40). d. Papacárie group, 30 days, grade 1. Absence of inflammatory reaction, with intense reparative processes characterized by angiogenesis and fibrosis. e. Carisolv group, 60 days, grade 1. Absence of inflammatory reaction and fibrosis. f. Papacárie group, 30 days. Areas around the implanted material exhibited a foreign body reaction.

The presence of materials within macrophage vacuoles and foreign body giant cells is evidence of phagocytosis and transport of the materials away from the implantation site. One factor contributing to this issue is that movement of the catheter within the tissue can cause displacement of the unset material. This problem has been reported by other investigators under similar experimental conditions.[18]

Our findings corroborate a study by Berto et al[25] that analyzed the reaction of subcutaneous tissue to Carisolv, noting a similar tissue response between the test and control groups and suggesting that Carisolv does not provoke adverse effects compared to a control. The tendency of connective tissue to encapsulate the implants in both groups may due to the presence of materials that are well-tolerated by the organism.

Some studies of Carisolv biocompatibility can be found in the literature.[11,24,25] This is the first investigation, however, to show the behavior of Papacárie, which is used in South America as a Carisolv substitute.

Carisolv has been tested in pulp tissue through histological evaluations of pulp exposed to Carisolv or sterile saline (control) in Class V cavities. The compound is biocompatible with human pulp tissue, although it may produce a hemostatic effect.[26] Carisolv produces superficial necrosis of the pulp tissue that results in inflammation lasting for a few weeks[27] and induces the rapid production of a rigid tissue matrix close to the exposed pulp.[28]

Most effects of Carisolv on the oral mucosa are harmless. Some studies, however, have shown a tendency to increase inflammatory reaction 24 hours after directly application onto the mucous membrane. The reaction was not strong, and 48 hours after application the number of cells was similar to that in the control tissue. Twenty-four and 48 hours after application, no discomfort was discernable clinically.[28]

Under our experimental conditions, the 2 substances tested exhibited acceptable biocompatibilities and showed similar cytotoxicity in vitro and in vivo. Papain has been used in medical practice for decades, and provides excellent results. In plantar ulcer patients, Otuka et al.[29]used papain and observed improvement on the disease and ample acceptance by the patients compared to other methods. Papain acts as a debridant chemical, aiding in the healing process due to its anti-inflammatory and antibacterial activities. Furthermore, it promotes the alignment of collagen fibers and uniform tissue growth.[12-14,30-32]

The need for low-cost materials used to remove caries chemically without adverse reactions has led to the development of Papacárie . Its compound is similar to Carisolv, showing acceptable biocompatibility and lower cytotoxicity.

CONCLUSION

Papacárie and Carisolv exhibit acceptable biocompatibilities and disclose similar response in the in vitro cytotoxicity and in vivo implantation assay so.

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