Cellular and Molecular Tissue Response to Triple Antibiotic Intracanal Dressing

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Abstract

Introduction: The aim of this study was to characterize the response of mouse subcutaneous tissue to triple antibiotic paste (TAP) using conventional light microscopy and real-time PCR (qRT-PCR). **Methods:** Polyethylene tubes containing TAP or calcium hydroxide (CH) (ie, the control group) were implanted in mouse subcutaneous tissue. Animals that received empty tubes or no tubes were used as additional controls. After periods of 7, 21, and 63 days postimplantation, the specimens were removed and subjected to histologic processing. The number of inflammatory cells and vessels, vessel areas, vascular density, and relative percentage of collagen were evaluated. Gene expression of proinflammatory (interleukin-1 beta, tumor necrosis factor alpha, and interleukin 17) and anti-inflammatory (transforming growth factor beta) cytokines and angiogenic factors (vascular endothelial growth factor and hypoxia-inducible factor-1 alpha) was quantified by 7 and 21 days postimplantation. Results were analyzed using the Student *t* test, analysis of variance, and the Tukey test ($\alpha = 0.05$). **Results:** TAP induced an exuberant inflammatory and angiogenic response, with higher numbers of inflammatory cells, higher vascular area and density, and lower relative percentage of collagen compared with CH. In general, the expression of genes involved in inflammation and angiogenesis was higher in the TAP group compared with animals that received CH or empty tubes. **Conclusions:** The response of mouse subcutaneous tissue to TAP was characterized by exuberant and persistent inflammatory and angiogenic responses with no repair and high gene expression of biomarkers associated with inflammation and angiogenesis. *(J Endod 2014;40:499–504)*

Key Words

Biocompatibility, calcium hydroxide, intracanal dressing, triple antibiotic paste

Pulp necrosis in teeth with incomplete root formation promotes interruption in root development (1). Traditionally, in this situation, calcium hydroxide (CH) is the intracanal dressing of choice for inducing the development of a calcified apical barrier (2). Another treatment is the placement of an apical barrier of mineral trioxide aggregate (MTA) (3). However, after these procedures, the roots remain short with thin and fragile walls being more susceptible to fracture (4, 5).

Recently, the use of a triple antibiotic paste (TAP) composed of ciprofloxacin, metronidazole, and minocycline (6, 7) has been suggested as an intracanal dressing in immature necrotic teeth. The root canal is irrigated with sodium hypochlorite filled with TAP (8–16), which remains in the canal from days to months (4, 8, 17–21). After the removal of TAP, bleeding is induced to promote the formation of a blood clot, and a layer of MTA is placed in the root canal (8, 10–12, 17, 22). The goal of this treatment is to promote revascularization and allow continued root formation, resulting in roots with normal morphology (5, 16, 18, 22).

TAP is recommended for its antibacterial efficacy (6–8, 18, 23), but its use has been supported mainly by reports of clinical success in the revascularization of immature necrotic teeth (4, 17–22). Nevertheless, no consensus has been reached regarding the biological action of TAP. Gomes-Filho et al (24) showed that TAP has similar biocompatibility to CH paste. On the other hand, it has been speculated that TAP may be toxic (13, 25) and capable of inducing lesions and delayed repair (12) in dog teeth.

Therefore, because of the lack of consensus on the biological action of TAP and the lack of molecular studies evaluating the tissue reactions to it, the objective of the present work was to assess the subcutaneous tissue response to TAP by conventional light microscopy and real-time PCR (qRT-PCR) in order to provide more precise scientific basis for its clinical use.

Materials and Methods

All animal procedures conformed to the applicable ethical guidelines and regulations of the University's Animal Research Ethics Committee, which approved the project. Seventy male BALB/c mice weighing 20–25 grams were used. Each animal received 2 dorsal implants composed of polyethylene tubes (Embramed, São Paulo, SP, Brazil) filled with the same paste: TAP or CH paste (control) (Calen; SS White Artigos Dentários Ltda, Rio de Janeiro, RJ, Brazil). Some animals received empty tubes or no tubes and were used as additional controls for qRT-PCR. TAP was

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prepared before insertion by mixing equal parts of pure ciprofloxacin, metronidazole, and minocycline (Bioquant, Ribeirão Preto, SP, Brazil) in sterile distilled water, obtaining a paste with creamy consistency (10, 11, 12, 25). The tubes had 1 end closed, were sterilized, and were filled with the pastes before implantation. Thus, only the 1 end of the tube that remained open allowed the tested material to come into contact with the subcutaneous connective tissue. Animals were anesthetized with 25 mg/kg ketamine and 10 mg/kg xylazine; the dorsal region was shaved and disinfected with 2% chlorhexidine. Two 0.5-cm incisions were performed on the dorsal scapular regions of each animal. Each site received 1 implant and was sutured.

Histopathological Evaluation

Five mice from the CH group and 5 from the TAP group were randomly selected and euthanized with an overdose of anesthetic solution at 7, 21, and 63 days postimplantation (DPIs). The implants and surrounding tissues were removed (n = 10 implants per group for each experimental period), fixated in 10% neutral-buffered formalin, and subjected to histologic processing. Each tissue sample was sectioned in 6-μm- and 7-μm-thick semiserial sections and stained with hematoxylin-eosin or picrosirius red.

Morphologic and quantitative analyses were performed using a Leica conventional light microscope (Leica DMR; Leica Microsystems GmbH, Wetzlar, Germany) to observe the regions where the pastes remained in contact with subcutaneous tissue. Three sections of each implant were evaluated by a skilled observer blinded to the treatment groups.

Quantitative analysis using the Leica QWin software (Leica Imaging Systems Ltd, Cambridge, England) evaluated the number of inflammatory cells, the number of vessels and their areas, and the vascular density in 4 histologic fields. Vascular density was calculated using a ratio of the vessel area to the number of vessels in each histologic field (26). The relative percentage of collagen per area was analyzed in 4 fields from the sections stained with picrosirius red (27).

qRT-PCR

qRT-PCR was performed in the CH and TAP groups and in the groups that received empty tubes or no tubes at 7 and 21 DPIs. Five animals from each group were used.

Animals were anesthetized, and subcutaneous tissue capsule on the open end of both tubes of each animal was removed and placed in a tube with 500 μL TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) (n = 5 per group for each experimental period). Subcutaneous tissue samples were also obtained from the dorsal region of 5 animals that received no implants.

RNA was extracted using the Illustra RNAspin Mini Isolation Kit (GE Healthcare, Freiburg, Germany). Complementary DNA was made from purified RNA by using the SuperScript III Reverse Transcriptase Enzyme (Invitrogen Life Technologies). Inflammatory (interleukin-1 beta [IL-1β], tumor necrosis factor alpha [TNF-α], and interleukin-17 [IL-17]) and anti-inflammatory (transforming growth factor beta [TGF-β]) cytokines, vascular endothelial growth factor (VEGF), and hypoxia-inducible factor 1 alpha (HIF-1α) were quantified by qRT-PCR using the SYBR Green Mix protocol (Invitrogen Life Technologies). qRT-PCR samples were run in the Step One Plus Sequence Detector (Applied Biosystems, Foster City, CA). Results were normalized against the beta-actin gene and determined based on the cycle threshold values. Relative messenger RNA expression against the group that received no tubes ($2^{-\Delta\Delta Ct}$) was calculated according to the manufacturer's User's Bulletin #2 (P/N 4303859, Applied Biosystems).

Results were analyzed using the Student t test, analysis of variance, and Tukey test (α = 0.05). Data are presented as the means and the standard error of the means.

Results

Persistent Inflammation and Absence Repair in TAP-exposed Tissues

TAP induced the formation of a thick layer of reactive tissue with exuberant inflammatory response, the presence of granulation tissue with a dense network of congested blood vessels, and intense inflammatory infiltration mainly composed of neutrophils and the absence of repair in all periods (Fig. 1*D–F*). The CH group (Fig. 1*A*) showed moderately thick inflammatory reactive tissue with a predominance of neutrophils at 7 DPIs. At 21 and 63 DPIs, few lymphomononuclear cells and fibroblasts interspersed with organized collagen fibers were observed (Fig. 1*B* and *C*).

The TAP group presented a greater number of inflammatory cells (Fig. 1*G*) and greater vascular area and density compared with CH (P < .05) in all periods (Fig. 1*H* and *I*). No difference was observed between the groups (P > .05) regarding the number of blood vessels (data not shown). The collagen percentage in the TAP group was lower at 21 and 63 DPIs than at 7 DPIs (P < .05) and was increased in the CH group at 21 and 63 DPIs (P < .05) (Fig. 2*A–G*).

Differential Gene Expression Is Induced by TAP and CH Dressings

The results are shown in Figure 3*A–F*. The TAP group had a higher expression of genes IL-1β, TNF-α, IL-17, TGF-β, HIF-1α, and VEGF in comparison with the CH and empty tube groups (P < .05) at 7 DPIs. At 21 DPIs, the expression of IL-1β, IL-17, and HIF-1α was still higher in the TAP group compared with the CH and empty tube groups (P < .05), whereas the expression of VEGF, TNF-α, and TGF-β was similar between the CH- and TAP-exposed tissues (P > .05).

An analysis of each group also showed that TAP sustained a high expression of IL-1β and TGF-β genes on 7 and 21 DPIs (P > .05). TNF-α and HIF-1α genes displayed a reduction in expression at 21 DPIs (P < .0001), whereas IL-17 and VEGF had an increased expression in the same period (P < .0001 and P < .05, respectively). In the CH group, IL-1β, TGF-β, and HIF-1α were not differentially expressed in both periods evaluated (P > .05). However, an increased expression of IL-17 and VEGF (P < .05) and a decreased expression of TNF-α (P < .05) were observed at 21 DPIs. No differences in the expression of all genes were observed between the CH and empty tube groups in all the experimental periods (P > .05). Empty tubes induced changes in some gene transcriptions in comparison with connective tissues that had no tubes inserted subcutaneously.

Discussion

The tissue reaction to TAP was characterized by exacerbated and persistent inflammation and the absence of repair, showing an aggressive nature of TAP. In contrast, CH induced inflammation at 7 DPIs and tissue repair at 21 and 63 DPIs. These results indicate that in the initial periods, inflammation induces an angiogenic response with the formation of granulation tissue, which can lead to 2 different outcomes: (1) in tissues exposed to CH, it is believed that the formation of new blood vessels brings oxygen, nutrients, and leukocytes to the wounded area, favoring tissue repair, or (2) because of the continuous irritating action of TAP, leukocyte activation and a sustained release of cytokines were