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Experimental furcal perforation treated with mineral trioxide aggregate plus selenium: immune response

Marcela Carvalho ESPALADORI[a]
Kamilla Faria MACIEL[a]
Luciana Carla Neves de BRITO[b]
Toshihisa KAWAI[c]
Leda Quércia VIEIRA[d]
Antônio Paulino RIBEIRO SOBRINHO[a]

[a]Universidade Federal de Minas Gerais – UFMG, School of Dentistry, Departament of Operative Dentistry, Belo Horizonte, MG, Brazil.

[b]Universidade de Itaúna, School of Dentistry, Itaúna, MG, Brazil.

[c]The Forsyth Institute, Department of Immunology, Boston, MA, USA.

[d]Universidade Federal de Minas Gerais – UFMG, Institute of Biological Sciences, Departament of Biochemistry and Immunology, Belo Horizonte, MG, Brazil.

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Corresponding Author:
Antônio Paulino Ribeiro Sobrinho
E-mail: sobrinho.bhz@gmail.com

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Abstract: The aim of this study is to evaluate the expression of cytokines in response to mineral trioxide aggregate (MTA) plus selenium in germ-free mice with experimental furcal perforation. The first left maxillary molar was opened, and the furcal area was perforated and treated with post-MTA-Se (experimental group). The same surgical intervention was performed for the maxillary right first molar, which was treated with MTA (control group). Fifteen mice were sacrificed 7, 14, and 21 days after furcal perforation, and periapical tissue samples were collected. The mRNA expression levels of the cytokines TGF-β, TNF-α, IFN-γ, HPRT, IL-10, IL-4, RANK, RANKL, IL-1, and IL-17 were assessed by using real-time polymerase chain reaction. In the experimental group, at 21-days post-MTA-Se sealing, the mRNA levels of TNF-α and IL-10 were upregulated compared with those in the control group ($p < 0.05$). Futher assessment revealed basal mRNA expression levels of IL-1α, IFN-γ, RANK, RANKL, IL-17A, IL-4, and TGF-β, over long experimental times, in both the experimental and control groups ($p > 0.05$). In conclusion, MTA+Se sealing favoured increased expression of IL-10 and TNF-α at later time points (day 21).

Keywords: Selenium; Cytokines; Immunophenotyping; Models, Animal.

Introduction

Furcal perforation is mechanically driven or pathologically induced communication between root canals and supportive dental tissues.[1] Perforations usually result from resorption or, caries or may be produced iatrogenically.[1,2] Such perforations are the second most common cause of failed endodontic treatments, with serious clinical consequences.[1] Treatment consists of using a biocompatible material to seal the cavity and aims to prevent bacterial invasion, inflammation dissemination, and periradicular bone destruction.[1]

Periradicular bone destruction around the perforation depends on the balance between proinflammatory (type 1) and anti-inflammatory (type 2) cytokines.[3,4,5] Proinflammatory cytokines, such as IFN-γ, TNF-α, and RANKL, play a key role in lesion progression. IFN-γ and TNF-α act on bone resorption by inducing the release of RANKL, which helps to differentiates and activates osteoclasts.[4,6] Conversely,

the production of cytokines such as IL-4, IL-10, and IL-13 by lymphocytes inhibits bone resorption and initiates tissue repair.[7]

Mineral trioxide aggregate (MTA) is the main material used to seal root canal perforations given its biocompatibility, insolubility, and sealing ability, among other properties.[2,8] It has been demonstrated that MTA affects neither phagocytosis nor the ability of macrophages (M1 and M2) to eliminate microorganisms.[9] Additionally, an evaluation of its effect on the adaptive immune response revealed that it did not interfere with the expression of IL-10, TNF-α, or RANKL by memory T cells.[10]

Selenium (Se) is being used in several medical applications, including cancer treatment, as a bacterial growth inhibitor on instruments or biological prostheses, in bone metabolism, and as an immune system booster.[11,12] Se may slow bone growth by changing bone metabolism.[13] The blood concentration of Se is inversely related to the rate of bone renewal and positively associated with low bone mineral density in humans.[14] Low intake of Se correlates with a high risk of bone diseases.[15,16]

Se is an essential mineral present in selenocysteine, an amino acid involved in various biological activities, such as antioxidant defence, cell division and differentiation, and mainly participates in maintaining the proper function of the immune system.[17] The lack of Se leads to a reduction in mature T-cell reserves and the activation of faulty T-cells.[18,19,20,21,22]

Several studies have demonstrated that MTA presents excellent properties to treat root canal perforations. With that in mind, our experiment involved supplementing MTA with an essential mineral such as Se, which is capable of inhibiting bacterial growth and stimulating the immune system and bone renewal, as a way to enhance the properties of MTA. In the present study, experimental furcal perforations were generated in mandibular molars of germ-free mice. Perforations were sealed with MTA or MTA plus Se in the form of sodium selenite. Animals were longitudinally sacrificed, and the expression of cytokines, namely TGF-β, TNF-α, IFN-γ, IL-10, IL-4, RANK, RANKL, IL-1, IL-17, and HPRT, was evaluated by using real-time polymerase chain reaction (RT-PCR).

Methodology

Animals

Fifteen germ-free mice of both sexes aged between 4 and 8 weeks, were used in this experiment (Swiss/NIH, ICB, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil). All animals were maintained in Trexler isolators (Madison, USA) and transferred to micro-isolators (UNO, Roestvastaal BV, Zevenaar, New Zealand) to perform the procedures. All procedures were performed under sterile conditions in laminar flow cabinets (Veco, Campinas, Brazil). The animals were fed *ad libitum*. The present study was approved by the Ethics Committee for the Use of Animals in Research (67/2014 CEUA/UFMG).

Experimental furcal perforation

The procedures were performed as described previously.[23,24,25] In brief, before any procedure, animals were anaesthetized intraperitoneally with 100mg/kg ketamine hydrochloride (Dopalen, Division Vetbrands Animal Health, Jacareí, Brazil) and 10mg/kg xylazine (Anasedan, Agribands do Brasil LTDA, Paulínia, Brazil). Pulp chambers of the first upper molars were accessed with a ½ sterile carbide drill (KG Sorensen, Barueri, Brazil) attached to a controlled speed motor (Driller, São Paulo, Brazil), under endodontic operative microscopy (Alliance, São Paulo, Brazil). Furcal perforation was performed using ¼ sterile carbide drills, perpendicular to the furcal zone and parallel to the long axis of the tooth (Figure 1 shows a schematic representation). Perforation cavities of the right molars (control) were sealed with MTA Bios (Ângelus, Londrina, Paraná, Brazil), while those of the left molars (experimental) were sealed with MTA plus Se. All clinical crowns were sealed with Coltosoll® (Coltène/Whaledent AG Altstätten, Suisse).

MTA manipulation

MTA was prepared following the manufacturers' instructions under sterile conditions. Subsequently, 1-μM sodium selenite solution was incorporated into MTA. To define an adequate Se concentration, macrophages ($1x10^5$ cells mL^{-1}) isolated from Swiss/NIH

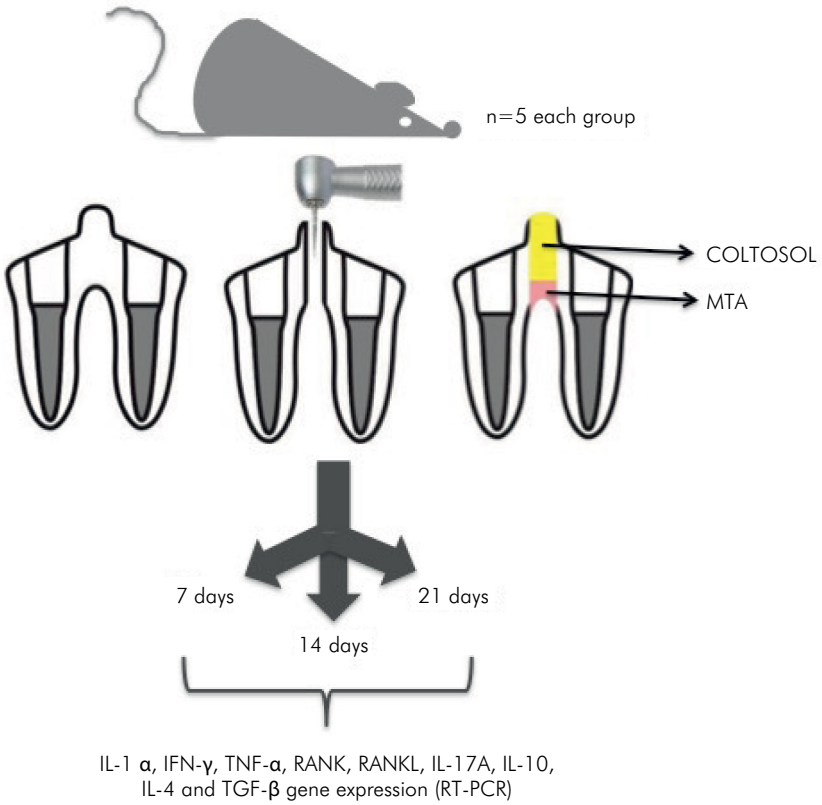

Figure 1. Germ free mouse model of experimental furcation perforation: schematic representation of the treatment protocol.

mice were cultured in 1 mL of medium in 24-well culture plates (Nunclon; Nalge Nunc International, Naperville, USA) in the presence of several dilutions of Se. At Se concentrations ≤10 μM, the viability, adherence and phagocytosis ability of macrophages were not modified (data not shown).

Preparation of the tissue samples

Animals were sacrificed at 7, 14, and 21 days after perforation (n = 5). Periradicular tissues adjacent to furcal perforations were aseptically removed and frozen at −70°C. mRNA was isolated by TriZol (Gibco BRL Laboratories, Grand Island, USA), followed by the addition of chloroform and centrifugation at 12,000 ´*g* and 4°C for 10 min. The aqueous phase was collected, and isopropanol was added to precipitate RNA, followed by centrifugation at 12,000 ´*g* and 4°C for 10 min. mRNA was washed with 75% ethanol, dried, dissolved in RNAase-free water, and incubated at 55°C for 10 min. [24,25]

Real-time PCR

Complementary DNA was synthesized by reverse transcription using 2 μg of RNA.[24] The standard PCR conditions were as follows: a holding stage of 95°C for 10 min; a cycling stage with 40 cycles of 95°C for 15 s and 60°C for 1 min; and a melting curve stage of 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. The primer sequences used for the analysis of INF-γ, TNF-α, IL-1β, IL-17, IL-10, TGF-β, IL-4, RANK, and RANKL mRNA expression by quantitative real-time PCR are shown in Table. The primer sequences were designed by using Primer Express software (Applied Biosystems, Foster City, CA, USA) based on the nucleotide sequences available in GenBank. Real-time PCR was performed using the Step One Real-time PCR System (Applied Biosystem). In addition, the Sybr Green detection system (Applied Biosystems) was used to assay primer amplification. The housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT) was used to normalise mRNA expression levels. All

Table. Primer sequences used in PCR reactions.

Gene	Sense and antisense	Length (bp)*	NCBI/GeneBank reference
HPRT	FW: GTTGGATACAGGCCAGACTTTGTT RV: GATTCAACTTGCGCTCATCTTAGG	163	JO0423.4
TNF-α	FW: ATCTTCTCAAAATTCGAGTGACCA RV: TGGAGTAGACAAGGTACAACCC	173	AB062426.1
TGF-β	FW: TGACGTCACTGGAGTTGTACG RV: GGTTCATGTCATGGATGGTGC	170	AY340221.1
RANKL	FW: CATCCCATCGGGTTCCCATAA RV: CCTTAGTTTTCCGTTGCTTAACGAC	100	AF019048.1
RANK	FW: TGCTGGCATGGTGATGGA RV: GAATGATGCCAGGTGGTAGGA	78	AF019046.1
IL-4	FW: ACAGGAGAAGGGACGCCAT RV: GAAGCCCTACAGACGAGCTCA	95	NM_021283.2
IL-17A	FW: TGAGCTTCCCAGATCACAGA RV: TGCAGAACGCCCTCAGACTA	101	NM_010552.3
IL-10	FW: GGTTGCCAAGCCTTATCGGA RV: ACCTGCTCCACTGCCTTGCT	191	NM_01058.2
IFN-γ	FW: CAAGTGGCATAGATGTGGAAGAA RV: TGGCTCTGCAGGATTTTCATG	91	NM_008337.4
IL-1β	FW: CAACCAACAAGTGATATTCTCCATG RV: GATCCACACTCCCAGCTGCA	152	NM_008361.4
IL-6	FW: TTCCATCCAAGTTGCCTTCTTG RV: TTGGGAGTGGTATCCTCTGTGA	102	M24221.1

FW: forward primer; RV: reverse primer. *Amplicon length in base pairs.

samples were run in duplicate in a 20 μL reaction volume with 1 μg of cDNA. Sequence Detection Software version 2.0 (Applied Biosystems) was used to analyse data after amplification. The results were obtained as threshold cycle (Ct) values. Expression levels were calculated using the comparative C(T) method.[26] The Ct values are expressed as the mean of two independent measurements, and the mRNA expression levels for all samples are expressed as the ratio between the expression of the gene of interest and the expression of HPRT.[24,26,27]

Statistical analysis

Statistical analysis was performed by the normality test (Shapiro-Wilk test) and homogeneity test (Levene test). Subsequently, analysis of variance (for data with a normal distribution) and the Kruskal-Wallis test (for data with a non-normal distribution). Followed by a post hoc test (with Bonferroni correction), were used for analysing more than two independent variables. For analysing two independent variables, Student's t-test (for a normal distribution) and the Kolmogorov-Smirnov *Z* test (for non-normal distribution) were used. The level of significance was 95%, and a p-value < 0.05 was considered significant.

Results

The expression of cytokinesIL-1α, IFN-γ, TNF-α, RANK, RANKL, IL-17A, IL-10, IL-4, and TGF-β was assessed by using real-time PCR and normalised to the HPRT gene. At all time points evaluated and in both groups, basal expression of IL-1α, IFN-γ, RANK, RANKL, IL-17A, IL-4, and TGF-β was observed ($p > 0.05$; data not shown). At 21 days after sealing the perforation with MTA/Se, TNF-α expression was significantly

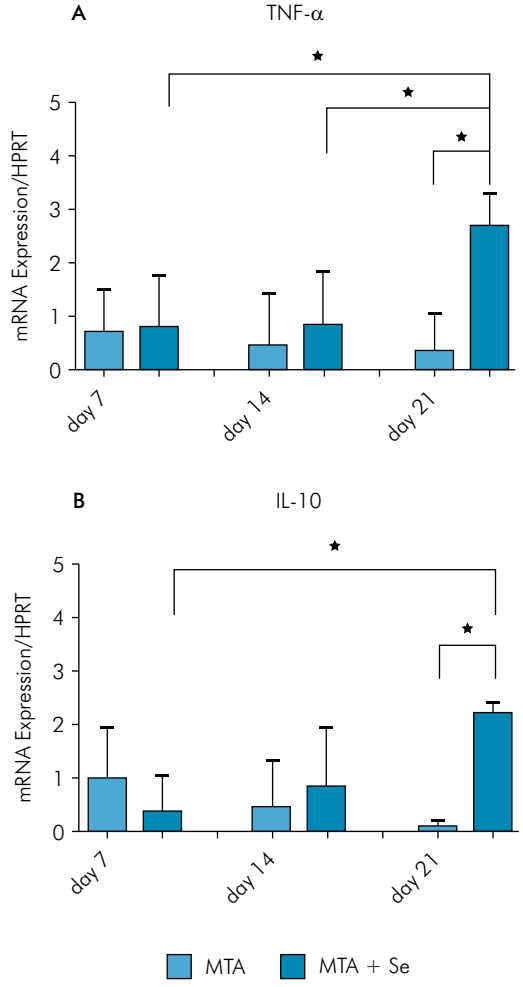

Figure 2. mRNA expression of TNF-α and IL-10 in the furcal area of germ free mouse subjected to experimental furcal perforation sealed with MTA or MTA+Se at 21 days. The expression levels were determined by real time PCR and quantified by comparison with a housekeeping gene (HPRT). Bars represent the average of five experiments performed in duplicate, while horizontal lines represent the standard error.

higher compared to that after sealing with MTA alone (control group) at the same time (Figure 2). Additionally, TNF-α expression at 21 days was significantly higher than that at 7 and 14 days in the experimental group ($p < 0.05$). IL-10 expression presented a similar profile, where it was significantly higher at 21 days in the group treated with MTA/Se, compared to the control group treated with MTA ($p < 0.05$). In the experimental group (MTA/Se), IL-10 expression was significantly higher at day 21 than at day 7 ($p < 0.05$; Figure 2).

Discussion

Gnotobiotic animals are good models for the study of dental materials in vivo.

These animal models are beneficial for determining the real effects of dental materials since the absence of indigenous bacterial microbiota allows for analysis of the relationship between their properties and the host immune system free from bacterial interference.[3] In this study, a well tested experimental furcal perforation was used.[25]

Furcal perforation is mechanical or pathological communication between the root canals and the supportive dental tissues.[1] After tooth perforation, a biocompatible material is chosen to seal the cavity to avoid bacterial invasion and periradicular inflammation.[28,29] In this regard, MTA has been shown to have excellent potential for endodontic applications.[2,8] In this study, the expression of cytokines was longitudinally assayed to associate the specific genotype-first response to the biocompatible material used to seal the furcal perforation. This approach can prevent initial phenotypic bias and can allow for the identification of cytokine genes that significantly contribute to the immune response.[30]

In agreement with previous studies[9,31] MTA induces a host-protective proinflammatory response, increasing the expression of proinflammatory cytokines in the initial phase after its placement (7 to 14 days) and favouring the expression of immunoregulatory cytokines in the late phase (21 days). In this study, with the intention of potentiating the immune response, inorganic Se was added to MTA to affect, the proliferation, differentiation, and function of immunological cells.[18,19,20,21,22,23,32]

The expression of the proinflammatory cytokines IL-1α, IFN-γ, RANK, RANKL, and IL-17 and the anti-inflammatory cytokines TGF-β and IL-4 was at basal levels at 7, 14, and 21 days in the control and experimental groups. Similar to the outcomes demonstrated, MTA/Se does not interfere with IFN-γ expression.[24] Conversely, we reported an increase in TNF-α, IL-4, and RANKL expression by macrophages in vitro on days 7 and 14, followed by a decrease on day 21.[9]

Some inflammatory cytokines play essential roles in the innate immune response. IL-1 and TNF-α induce the expressions of adhesion molecules by endothelial cells, increasing the trafficking of phagocytes during inflammation.[23,31,32,33,34] Moreover, these cytokines induce pulpal inflammation[23] and bone resorption in inflamed periradicular tissues.[33] In this study, when comparing the expression of TNF-α between the control and experimental groups, a significant increase in its expression was observed on day 21 in the experimental group. On the other hand, MTA did not interfere with the production of TNF-α by M1 and M2 macrophages.[1] However, an increase in the production of TNF-α by T-memory lymphocytes was observed on days 7 and 14, followed by a reduction on day 21 in the presence of MTA.[35] Previously, it was demonstrated that macrophages stimulated with lipopolysaccharide in Se-free cultures secrete significantly lower levels of TNF-α, IL-1β, and IL-6 compared to cultures stimulated with LPS in the presence of Se.[36] Accordingly, in this study, the increase in TNF-α expression on day 21 was found only in perforations treated with MTA/Se. Moreover, TNF-α has numerous biological activities, including the ability to stimulate neoangiogenesis.[37] This finding suggests that the increased expression of TNF-α in the perforations treated with MTA/Se may stimulate the initiation and progression of angioproliferative processes that will culminate in furcal perforation healing, as demonstrated elsewhere.[38]

IL-10 is a regulatory cytokine that inhibits proinflammatory responses.[39] IL-4 and IL-10 expression is generally reduced in the initial phase and increased in the late chronic phase of the immune response.[40] Similar to what has been reported in the literature, a significant increase in IL-10 production was detected on day 21 in the experimental group compared to the control group in this study.[9,23,33] This higher Se-related IL-10 expression modulates the proinflammatory response that occur during perforation, which is helpful for the perforation healing process, as previously demonstrated.[41,42] Appropriate intake of Se contributes to the balance of the differentiation of Th1 and Th2 lymphocytes, thus promoting a balance between the production of proinflammatory and anti-inflammatory cytokines.[36]

Conclusion

The results of this study support our hypothesis that Se may indeed improve the ability of MTA to treat root canal perforation. Se not only maintained the same cytokine profile induced by MTA but also led to higher late expression levels of TNF-α and IL-10. TNF-α stimulates angioproliferative processes that culminate in furcal perforation healing. IL-10 inhibits proinflammatory responses and stimulates the beginning of wound healing in a given site. Nonetheless, further experimental studies should be performed to demonstrate whether the addition of Se to MTA definitively contributes to the improvement of its biological properties.

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