UNIVERSIDADE FEDERAL DE MINAS GERAIS
Faculdade de Odontologia
Colegiado de Pós-Graduação em Odontologia

Shirlene Barbosa Pimentel Ferreira

AVALIAÇÃO EPIDEMIOLÓGICA E IMUNOLÓGICA DE ALTERAÇÕES PULPO-PERIAPICAIS EM INDIVÍDUOS COM ANEMIA FALCIFORME

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Orientador: Prof. Dr. Antônio Paulino Ribeiro Sobrinho (UFMG).
Coorientador: Prof. Dr. Hercílio Martelli Júnior (UNIMONTES).

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FOLHA DE APROVAÇÃO

"Avaliação epidemiológica e imunológica de alterações pulpo-periapicias em indivíduos com anemia falciforme"

SHIRLENE BARBOSA PIMENTEL FERREIRA

Tese submetida à Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ODONTOLOGIA, como requisito para obtenção do grau de Doutor em ODONTOLOGIA, área de concentração ENDODONTIA.

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Belo Horizonte, 20 de setembro de 2013.

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RESUMO

As periapicopatias se desenvolvem como consequência da resposta imune aos microrganismos e seus subprodutos. Esta resposta ainda não havia sido avaliada em pacientes com anemia falciforme (AF): a doença genética mais prevalente no mundo. Esta hematoglobinopatia resulta na produção de hemoglobina anormal, chamada hemoglobina S (HbS). A HbS leva a rigidez dos eritrócitos e fenômenos de vaso-oclusão, resultando em dano progressivo aos órgãos sistêmicos. Os objetivos deste estudo foram avaliar a necessidade de tratamento endodôntico em uma dada população AF e avaliar a resposta imune periapical à infecção em pacientes com AF. Neste estudo, dados demográficos e médicos (índices hematológicos, testes virais, transfusão sanguínea, medicação recebida, esplenectomia) e informação sobre a necessidade de tratamento endodôntico foram obtidos dos pacientes-AF. Adicionalmente, nos pacientes-AF que necessitavam de tratamento endodôntico avaliou-se, utilizando-se a real time PCR, a expressão gênica das citocinas interferon (IFN)-γ, fator de necrose tumoral (TNF)-α, interleucina (IL)-1β, IL-17A, IL-10, e o ligante do recepetor ativador do fator nuclear kappa beta (RANKL) e as quimiocinas CCL-2/MCP-1 e CCL-5 no fluido intersticial coletado do periápice dos dentes submetidos ao tratamento e comparados com os resultados obtidos de pacientes não-AF. 108 pacientes-AF compuseram a população estudada, e a taxa de necessidade de tratamento endodôntico foi de 10.2%. Entre os dados médicos, observou-se uma diferença significante na contagem de eosinófilos ($p<0.05$) e linfócitos atípicos ($p<0.05$) quando os grupos (com e sem necessidade de tratamento endodôntico) foram comparados. Estas diferenças sugerem que as infecções endodônticas podem ser prejudiciais aos pacientes-AF. Quanto à resposta imune periapical, nenhuma diferença significativa foi observada na expressão gênica de citocinas ou quimiocinas entre indivíduos-AF e não-AF ($p> 0.05$). Entretanto, a expressão gênica das citocinas Th1, IFN-γ, TNF-α e IL-1β foram significativamente maiores nos indivíduos AF que no controle (vitalidade pulpar) ($p<0.05$). Entre as citocinas Th1, apenas o IFN-γ encontrava-se aumentado de maneira significativa nos indivíduos não-AF quando comparados aos pacientes do grupo controle. A expressão gênica da IL-17A estava significativamente aumentada nos pacientes-AF em relação àqueles do grupo controle ($p< 0.05$), enquanto a expressão da IL-10 encontrava-se aumentada nos indivíduos-AF e não-AF quando comparados aos indivíduos do grupo controle ($p<0.05$). Níveis similares de expressão de RANKL, CCL-2 e CCL-5 foram observados em todas as amostras. Os resultados não foram capazes de demonstrar quaisquer diferenças nas respostas imunes periapicais entre os indivíduos-AF e não-AF apesar dos pacientes-AF terem apresentado uma tendência pró-inflamatória, expressando IL-1, TNF-α e IL-17A em níveis elevados e significantes quando comparados àqueles dos pacientes do grupo controle.

Palavras-chave: epidemiologia; imunologia; tratamento endodôntico; anemia falciforme.

ABSTRACT

Epidemiological and immunological evaluation of pulpo-periapical changes in individuals with sickle cell anemia

Periapical diseases develop as a consequence of immune response against microorganisms and their byproducts. This response has not been previously analyzed in patients with sickle cell anaemia (SCA): the most prevalent genetic disease worldwide. This hemoglobinopathy results in the production of abnormal hemoglobin, called hemoglobin S (HbS). HbS leads to erythrocyte rigidity and vaso-occlusion, resulting in progressive damage to systemic organs. The aims of this study were to evaluate the need of endodontic treatment and to do the immunologic analyse of the teeth with the need of endodontic treatment in patients with SCA. In this study, personal information, medical data (haematological indices, virologic testing, blood transfusions, medications received, splenectomy) and information on the need for endodontic treatment were obtained from SCA patients. Moreover, in the SCA patients that needed for endodontic treatment it was evaluated, using *real-time* PCR**,** the mRNA expression levels of the cytokines interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-17A, IL-10, receptor activator for nuclear factor kappa B ligand (RANKL), and the chemokines CCL-2/MCP-1 and CCL-5 in the periapical interstitial fluid and compared with results from non-SCA individuals. One hundred eight SCA patients comprised the studied population, and the rate of the need for endodontic therapy was 10.2%. Among the medical data, a significant difference was observed for eosinophil ($p=0.045$) counts and atypical lymphocyte counts ($p=0.036$) when the groups (with and without the need for endodontic treatment) were compared. These differences suggest that endodontic infections could be harmful to SCA individuals. Concerning to periapical immune response, no significant differences were observed in the expression of cytokine or chemokine mRNA between SCA and non-SCA individuals ($p> 0.05$). However, the expression of mRNA for the Th1-associated cytokines IFN-γ, TNF-α, and IL-1β were significantly higher in SCA individuals than in the control individuals ($p< 0.05$). Among Th1-associated cytokines, only IFN-γ was significantly increased in non-SCA compared with control patients (vital pulp). The expression of IL-17A mRNA was significant higher in SCA cases than in control samples ($p< 0.05$), while the IL-10 mRNA expression was significant increased in SCA and non-SCA individuals when compared with the control group. Similar levels of RANKL, CCL-2, and CCL-5 mRNA expression were observed in all samples. The results were unable to demonstrate any differences in periapical immune responses between SCA and non-SCA individuals despite the fact that SCA patients presented prone proinflammatory ability, expressing IL-1, TNF-α, and IL-17A at a significantly higher level compared to control patients than non-SCA individuals compared to control patients.

Keywords: epidemiology; immunology; endodontic treatment; sickle cell anaemia.

LISTA DE ABREVIATURAS E SIGLAS

AF Anemia Falciforme

CD4 *Cluster of Differenciation* 4

CD8 *Cluster of Differenciation* 8

CCL *Chemokine (C-C motif) Ligand*

CCR *Chemokine (C-C motif) Receptor*

CXCR:C-X-C *Chemokine Receptor Type*

DNA: *Deoxyribonucleic Acid*

GAPDH: *Glyceraldehyde 3-Phosphate Dehydrogenase*

IL-: *Interleukin*

IFN-: Interferon

MCP-1: *Monocyte Chemotactic Protein-1*

PCR: *Polymerase Chain Reaction*

RANKL: *Receptor Activator of Nuclear Factor Kappa-B Ligand*

RANTES: *Regulated upon Activation Normal T-cell Expressed and Secreted*

RNA: *Ribonucleic Acid*

SD: *Standart Deviation*

SCA: *Sickle Cell Anaemia*

TCD4+: *Tcell CD4*

TCD8+: *Tcell CD8*

TGF: Trans*forming Growth Factor*

Th: T *Helper Cell*

TNF: *Tumor Necrosis Factors*

SUMÁRIO

1 INTRODUÇÃO E RELEVÂNCIA

Uma vez estabelecida uma infecção nos sistemas de canais radiculares, as bactérias e seus produtos difundem-se para os tecidos perirradiculares levando à instalação de respostas inflamatórias e imunológicas e consequentemente à formação da lesão periapical (KAKEHASHI *et al*., 1965; STASHENKO *et al*., 1994; TAKAHASHI, 1998; TORABINEJAD, 1994).

As evidências demonstram que os efeitos patogênicos microbianos sobre os tecidos periapicais operam-se de forma indireta, via estimulação de mediadores solúveis derivados do hospedeiro, como as citocinas e quimiocinas (STASHENKO *et al.,* 1998). Nas ultimas décadas, houve um grande interesse em se conhecer esses mediadores e seus efeitos sobre as células imunocompetentes aí presentes (AKAMINE *et al.*, 1994; BRITO *et al*., 2012; COLIC *et al.*, 2009b; SASAKI *et al.*, 2000; SILVA *et al*., 2005).

Inúmeras células são detectadas nas lesões perirradiculares humanas, dentre elas os linfócitos $TCD4^+$ e $TCD8^+$, macrófagos, células plasmáticas, mastócitos, eosinófilos, com um predomínio das células T (COLIC *et al.*, 2009b). Os linfócitos $TCD4^+$ e $TCD8^+$, após o contato com antígenos ou após serem estimulados por outras células inflamatórias, podem produzir uma grande variedade de citocinas (MARTON; Kiss KISS, 2000). As células $TCD4^+$ atualmente são subdivididas em vários subgrupos que incluem as células: Th1, Th2, Th17 e T regulatórias (T_{reg}) (CUA; McGEACHY, 2008). A resposta Th1 caracteriza-se pela produção de IFN-γ, IL-12, IL-2, e TNF, estando envolvida na progressão e destruição óssea perirradicular (COLIC *et al.*, 2009b; STASHENCO *et al.*, 1998). A resposta Th2 induz a síntese e atividade das citocinas IL-4, IL-5, IL-6, IL-9, e IL-13, estando relacionada com a cicatrização e regeneração dos tecidos perirradiculares (AKAMINE *et al.*, 1994; KAWASHIMA & STASHENKO, 1999; SASAKI *et al.*, 2000; STASHENKO *et al.*, 1998; TEIXEIRA-SALUM *et al.*, 2010). O subgrupo Th17 produz a IL-17, que é considerada como uma ponte entre a resposta adaptativa e inata (GAFFEN; YU, 2008). Por sua vez, as células T_{reg}, produtoras de TGF-β e IL-10 possuem um efeito inibitório sobre a reabsorção óssea durante a formação e diferenciação dos osteoclastos, além de atuarem na regulação da resposta imune contra a infecção (COLIC *et al.*, 2009a).

As quimiocinas, por sua vez, participam do processo inflamatório gerando

gradientes quimiotáticos que são responsáveis pela migração guiada e manutenção de células inflamatórias nestes locais (MANTOVANI *et al*., 1998, SILVA *et al*., 2005). O aumento da expressão da quimiocina CCL2/MCP-1 está associado ao aumento do número de células recrutadas nos sítios inflamados, tendo evidenciada na região periapical de dentes lesões periapicais crônicas quando comparados aos dentes sadios (SILVA *et al*., 2005). A quimiocina CCL5 atua na quimiotaxia de células T e sua ação na regulação de células efetoras T $CD8^+$ tem sido demonstrada (SZCZEPANEK *et al*., 2012).

A anemia falciforme é a doença hereditária mais comum no Brasil (LOUREIRO; ROZENFELD, 2005) e no mundo (WHO, 2006). A doença resulta de uma mutação pontual no gene que codifica a proteína beta-globulina, originando uma molécula de hemoglobina anormal denominada hemoglobina S (HbS). Essa hemoglobina alterada sofre desoxigenação o que leva à formação de insolúveis polímeros que alteram a morfologia celular dos eritrócitos que assumem a forma de foice. A falcização das hemáceas ocasiona a redução da vida média dessas células, hemólise, fenômenos de vaso-oclusão, episódios de dor e lesão de órgãos (BRASIL, 2007; REES *et al*., 2010). As infecções são as complicações mais comuns nos indivíduos portadores de anemia falciforme, podendo evoluir para a sepse e morte se não forem identificadas e tratadas precocemente (DI NUZZO; FONSECA, 2004; REES *et al.* 2010).

A análise da associação entre necrose pulpar e a anemia falciforme revelou uma ocorrência dessa alteração 8.33 vezes maior nos indivíduos AF que naqueles indivíduos sadios (COSTA *et al*. 2013). Apesar das alterações imunológicas serem um achado frequente nos pacientes falcêmicos (ASAREA *et al*., 2010; BELCHER *et al*., 2000; JISON *et al*., 2004; MUSA *et al*., 2010; VEIGA *et al*., 2013), o papel da infecção endodôntica e as respostas perirradiculares a esta infecção, bem como a correlação entre os dados sistêmicos e a presença de alterações endodônticas não haviam sido ainda avaliados nesses indivíduos.

2 OBJETIVOS

2.1 Objetivo geral

Avaliar a necessidade de tratamento endodôntico em pacientes portadores de anemia falciforme e comparar com seus dados clínicos. Subsequentemente, avaliar a expressão de citocinas no fluido intersticial periapical de dentes submetidos a tratamento endodôntico nos pacientes AF, comparando com a expressão das mesmas citocinas nos tecidos perirradiculares de indivíduos não-AF, com ou sem alterações periapicais.

2.2 Objetivos específicos

1. Avaliar e determinar a necessidade de tratamento endodôntico em pacientes com anemia falciforme;

2. Comparar a necessidade de tratamento endodôntico com os dados demográficos e sistêmicos (índices hematológicos, testes sorológicos, transfusão sanguínea, medicação e esplenectomia) em pacientes com anemia falciforme;

3. Caracterizar a expressão gênica das citocinas inflamatórias IL-1β, IL-17A, IL-10, IFN-γ, TNF-α, RANKL, MCP-1/CCL-2 e CCL-5 no fluido intersticial periapical de dentes com necrose pulpar em pacientes AF e comparar com pacientes não-AF, com ou sem alterações perirradiculares.

3 TRABALHOS CIENTÍFICOS

3.1 Artigo científico 1: “ASSESSMENT OF THE NEEDS OF ENDODONTIC TREATMENT IN BRAZILIAN INDIVIDUALS WITH SICKLE CELL ANAEMIA”.

Assessment of the needs of endodontic treatment in brazilian individuals with sickle cell anaemia.

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Abstract

Aim: The purpose of this study was to evaluate the relationship between sickle cell anaemia (SCA) and endodontic diseases. **Methodology:** Personal information, medical data (haematological indices, virologic testing, blood transfusions, medications received, splenectomy) and information on the need for endodontic treatment were obtained from SCA patients who were registered and followed up by the Hemominas Foundation – Regional Blood Centre of Montes Claros, Minas Gerais, Brazil. These data were compared with the need for root canal treatment in SCA patients. **Results:** One hundred eight patients comprised the studied population, and the rate of the need for endodontic therapy was 10.2%. Among the medical data, a significant difference was observed for eosinophil ($p=0.045$) counts and atypical lymphocyte counts ($p=0.036$) when the groups (with and without the need for endodontic treatment) were compared. **Conclusion:** The differences in statistical medical data observed between the groups with or without the need for root canal treatment suggest that endodontic infections could be harmful to SCA individuals.

Keywords: sickle cell anaemia, Endodontic treatment, haemoglobinopathy.

Introduction

Sickle cell anaemia (SCA) is the most prevalent genetic disease worldwide (WHO 2006), as well as in Brazil (Loureiro & Rozenfeld 2005). SCA is a heterogeneous clinical condition characterised by episodes of vaso-occlusion and infectious events (Kato *et al.* 2009). This haemoglobinopathy is caused by a mutation in the beta globulin gene of the haemoglobin molecule, resulting in the production of abnormal haemoglobin, called haemoglobin S (HbS). The presence of haemoglobin S in homozygosis (HbSHbS) triggers the formation of polymers when it is deoxygenated, leading to erythrocyte rigidity, loosening of the biconcave discoid shape of erythrocytes and changing the cells' shape into a sickle shape, followed by membrane damage and haemolysis. As a consequence of this occurrence, the sickle-shaped red blood cells obstruct the capillaries, restricting blood flow to organs, which results in ischaemia, pain, and often tissue damage (Kato *et al.* 2009, Miller *et al*. 2006). Studies have shown an association between haematological factors and pathologic alterations in SCA (Hayes *et al.* 1981, Miller et al. 2000). The elevated white blood cell counts in sickle cell disease have been reported as being able to predict morbid events in patients with the disease (Jison *at al.* 2004). Moreover, during persistently low oxygen tension these cells are destroyed. The red blood cells of individuals with normal haemoglobin (HbA) survive approximately 120 days, while the red blood cells of Hbs individuals survive only between 15 and 25 days (Costa *et al.* 2013). The accumulation of these distorted cells is responsible for vaso-occlusion and most SCA-related disorders.

Oral facial manifestations are observed in SCA individuals, such as delays in tooth eruption, atrophy of the tongue papillae, impaired dentine mineralisation,

mandibular osteomyelitis, and orofacial pain (Kelleher *et al.* 1996; Mendes *et al*., 2011). Additionally, the occurrence of asymptomatic pulp necrosis has been described in clinically intact permanent teeth, due to vaso-occlusion of the pulp microcirculation (Kaya *et al*. 2004, Costa *et al*. 2013).

The aim of this study was to compare the personal information and need for root canal treatment of SCA patients with their medical and haematological data at the time of initial dental presentation (first appointment).

Materials and methods

A descriptive and cross-sectional study was conducted from October 2010 to August 2012. Data were obtained from 108 evaluated patients with confirmed diagnoses of SCA (HbSS genotype), who were registered and followed up by the Hemominas Foundation – Regional Blood Centre of Montes Claros, Minas Gerais, Brazil. Personal information (sex and age) and information on the need for endodontic treatment were obtained from the patients during dental appointments. Medical data (haematological indices, serologic testing, blood transfusions, medications received, splenectomy) were obtained from medical records. The criteria adopted to determine the need for endodontic treatment were based on clinical and radiographic analyses, along with pulp vitality tests (Brito *et al*. 2009). Correlations among the collected data were calculated. This study was approved by the Research Ethics Committees of the Hemominas Foundation (#283/10), State University of Montes Claros (#1848/10) and Federal University of Minas Gerais (#203/10).

Statistical analysis

After data collection, statistical analysis was performed using SPSS statistical software for Windows (version 17.0. Chicago: SPSS Inc.). Descriptive analyses were performed to verify the patients' characteristics and the prevalence of oral and systemic conditions. Bivariate analysis was performed to examine the relationship between the needs for endodontic treatment and for blood cells. The significant differences between categorical variables were examined with the Chi-square test and with Fischer's exact test. Statistical significance was defined as a *P*-value of 0.05 or less.

Results

Patient characteristics

One hundred eight patients (n=108) were included in this study. A total of 61 female patients (56.5%) and 47 male patients (43.5%), 5 to 59 years old (mean, 18.16 years; standard deviation, 12.353) comprised the eligible individuals. The patients who needed to receive endodontic care corresponded to 10.2% (n=11) of the study subjects.

Blood transfusions

Among all of the patients, 68.5% received blood transfusions in the course of the disease. Of the patients who required root canal therapy, 63.6% were submitted to blood transfusions, whereas 69.1% of the patients without the need for endodontic treatment were not transfused. When the two groups (with and without the need for endodontic therapy) were compared, no statistically significant differences were observed (p=0.739).

Serologic testing

Of the eligible population, positive serologic test results for syphilis (0.9%), Chagas disease (3.7%), hepatitis (anti-HBc) (3.7%), hepatitis (anti-HCV) (2.8%) and HTLV I/II (0.9%) were detected. However, in the group of patients who needed root canal treatment, the HTLV I/II test results were negative (Table 1).

SCA regimen

Folic acid and hydroxyurea were the most commonly prescribed agents for the SCA patients in this study. Of the eligible population, 99.1% received folic acid therapy, and 21.3% received hydroxyurea therapy. All of the patients who required endodontic treatment received folic acid therapy, and in the same group, 9.1% received hydroxyurea therapy. When the two groups (with and without the need for endodontic treatment) were compared, no significant differences were observed for folic acid administration (p=1.000) or hydroxyurea administration (p=0.451). Table 2 shows the medications, including different drugs, according to the regimen and the proportion of patients who received each therapy.

Splenectomy

Splenectomised patients represented 1.9% of the SCA patients included in this study. Of the patients who required root canal treatment, 0% was submitted to splenectomy, and 2.1% of the patients in the group without the need for root canal treatment were splenectomised. No significant difference was observed when these data were compared between the groups of patients with and without the need for root canal treatment (p=1.000)

Haematological indices

In this study, 43.5% of all of the patients presented leucocyte (white blood cells) counts greater than 11.000 cells/mm^3, whereas 56.5% presented leucocyte counts between 4.000 cells/mm^3 and 11.000 cells/mm^3 (Table 3). In addition, 99.1% of SCA patients presented basophil counts between 0 cells/mm^3 and 200 cells/mm^3 (Table 4). The eosinophil counts were greater than 500 cells/mm^3 for 20.4% of the population and for 45.5% and 17.5% of the patients with and without the need for root canal therapy, respectively. The SCA patients presented banded neutrophil counts < 700 cells/mm^3 (100%) and segmented neutrophils count between 1.800 cells/mm^3 and 7000 cells/mm^3 (69.4%). In the groups that required and did not require endodontic therapy, 81.8% and 81.4% exhibited lymphocyte counts between 1000 cells/mm^3 and 4.500 cells/mm^3, respectively. Concerning atypical lymphocyte counts, 45.5% and 16.5%, respectively, of the patients who required or did not require endodontic treatment presented counts > 1 cell/mm^3. The percentages of SCA patients who presented metamyelocyte counts and myelocyte counts of 0 cells/mm^3 were 98.1% and 100%, respectively. When the white blood cell counts were compared between the patients with and without the need for root canal therapy, a statistically significant difference was detected only for the eosinophil ($p=0.045$) counts and atypical lymphocyte counts ($p=0.036$) (figure 1).

Table 5 shows the erythrocytes counts: they were < 4.0 X 10^6 cells/mm^3 for female subjects and < 4.5 X 10^6 cells/mm^3 for male patients in all of the patients in the group requiring endodontic treatment and in 86.6% of the group not requiring endodontic care. Haemoglobin concentrations < 12.8 (g/dl) for male patients and < 11.6 (g/dl) for female patients was observed in 100% and 95.9% of the patients with

and without the requirement for endodontic therapy, respectively. In this study, the mean corpuscular volume (MCV) levels were > 98% in 26.8% of the patients who did not require endodontic therapy and in 9.1% of the patients who required root canal treatment. Among all of the patients of this study, 19.4% showed mean corpuscular haemoglobin (MCH) less than 27 (pg). Of those patients who did not require endodontic treatment, 19.6% presented MCH less than 27 (pg), and 35.1% presented MCH greater 34 (pg). However, in 18.2% of patients with the need for root canal therapy, MCH was greater than 34 (pg). The mean corpuscular haemoglobin concentration (MCHC) was < 31.5 (g/dl) for 15.7% of the eligible patients. An MCHC < 31.5 (g/dl) was observed in 9.1% and 16.5% of the groups with and without the need for endodontic treatment, respectively. Herein, 96.3% of the patients presented reticulocyte counts > 2.3%. Regarding the need or not for endodontic treatment, 100% and 95.9% of these patients presented reticulocyte counts > 2.3%, respectively. The majority of the SCA patients (96.3%) presented haematocrit < 36% for female patients and < 38% for male patients. In the groups with and without the need for endodontic treatment, 100% and 95.9% of the patients, respectively, presented haematocrit < 36%. No significant differences were observed when comparing the groups with and without the need for endodontic treatment, taking into consideration the red blood cell counts ($p>0.05$).

Finally, platelet counts > 360 x 10^3 cells/mm^3 were observed in 72.7% and 62.9% of those patients who required and did not require root canal treatment, respectively. None (0%) of the patients with the need for endodontic treatment exhibited platelets counts < 140 x 10^3 cells/mm^3. No significant difference ($p=0.769$) was observed when the two groups (with and without the need for endodontic treatment) were compared.

Discussion

A recent study attempted to assess the epidemiological aspects of root canal diseases, defining the prevalence of the need for endodontic care in a given population (Brito *et al.* 2009). This study reported a rate of 14.5% of HIV-infected patients requiring endodontic treatment (Brito *et al.* 2009). In this study, we detected a rate of 10.2% of SCA patients requiring endodontic therapy. Despite being slightly lower than the rate in the earlier study, this rate could reflect lower budgets and less policy support for dental programs in public dental health services, which offer basic dental assistance mostly to lower socioeconomic groups in Brazil. Moreover, it is important to note that the true prevalence of the need for endodontic treatment in SCA patients in Brazil would only be possible to determine via multicentre studies performed in different states.

Sickle cell anaemia affects between 0.1% and 0.3% of Afro-Brazilian-descended people (Ramalho *et al*. 2003). In 2001 in the state of Minas Gerais, 87 of 128.326 newborns were carriers of haemoglobin S (HbS), 46 of whom had the disease (Paixão *et al*. 2001). The Hemominas Foundation - Regional Blood Centre of Montes Claros, Minas Gerais, Brazil, is responsible for monitoring SCA patients referred from different cities, and this centre is located in northern of the state of Minas Gerais.

The prognosis of people living with SCA depends on the SCA regimen. Herein, the most commonly prescribed agents were folic acid and hydroxyurea. The hydroxyurea provides therapeutic benefit through multiple mechanisms of action (Ware 2010). The efficacy of hydroxyurea in the treatment of SCA-related disease has generally been attributed to its ability to increase fetal haemoglobin ($\alpha 2\gamma 2$) and

consequently to decrease haemoglobin S (Hbs) as a compensatory mechanism that prevents the polymerisation of intercellular haemoglobin. Hydroxyurea administration leads to greater survival of erythrocytes, resulting in a decrease in the frequency and severity of vaso-occlusive episodes and diminishing the severe course of SCA, thus bringing clinical benefits (Lanzkron *et al.* 2008). Folic acid reduces the risk of endothelial damage. Its prescription is justified by its improvement of the production and maturation of red blood cells (Dijs *et al.* 2002, Hyacinth *et al.* 2010). All of the SCA patients in this study who required endodontic treatment were under folic acid regimens, but only one of them was taking hydroxyurea. When the two groups (with and without the need for endodontic treatment) were compared, no significant difference was observed for the two agents. Costa *et al.* (2013) reported that HbS individuals presented more pulp necrosis and, as expected, in this study this rate remained significant after folic acid prescriptions.

Blood transfusions were required in 68.5% of the patients as an ordinary procedure in the management of the SCA (Verduzco & Nathan 2009). Continual transfusions of red blood cells greatly decrease disease severity. Their use is supported by transfusions decreasing the percentage of HbS, suppressing HbS synthesis, and reducing haemolysis (Davies & Roberts-Harewood 1997, Rees *et al*. 2010). However, blood transfusions are considered a risk factor for infections (Davies & Roberts-Harewood 1997). Today, the exclusion of high-risk donors, along with increases in technology, has reduced during procedures of the risk of the transmission of diseases, such as viral hepatitis and human immunodeficiency viruses (HIV I and HIV II), in northern Europe and the United States (Davies & Roberts-Harewood 1997). In this study, positive serology for syphilis, Chagas disease, hepatitis (anti-HBc), hepatitis (anti-HCV) and human lymphotropic virus

(HTLV) was assayed for the eligible individuals. However, in the group of patients who required endodontic treatment, the latter test (HTLV I/II) was negative. Positive serology for viral diseases is an important date for endodontic diseases since they cause immunological impacts that may interfere in pulp and periapical responses to infection (Kakehashi *et al*.1965; Stashenko &Yu 1989; Brito *et al*. 2009).

Neutrophilia is a useful marker of infection in many clinical settings, as well as in SCA (Ahmed 2011). Furthermore, the prevalence and intensity of neutrophilia are higher in SCA patients with bacterial infections than in those without infections. In endodontic infections neutrophil plays a role in the defense against bacteria (Nakamura *et al*. 2002), in spite of the role of systemic neutrophilia on pulp inflammation is not well known. Moreover, neutrophilia in SCA individuals can be accompanied by eosinopoenia (Ahmed 2011). Our findings showed that only one SCA patient presented with neutrophilia. Despite the significant difference (p=0.045) observed in the eosinophil counts when the groups with and without the need for endodontic treatment were compared, eosinopoenia was not observed among the eligible patients.

Leucocytosis is a risk factor for haemorrhagic stroke in children and adults, and, additionally, increased white blood cell counts can predict morbid events in sickle cell disease (Jison *et al*. 2004). Repeated vaso-oclusive events in SCA patients can lead to numerous end-organ complications (Jison *et al*. 2004, Verduzco *et al*. 2009, Rees *et al*. 2010). In pulp tissues the restricted blood flow promoted by obstructed capillaries may contribute to bacterial proliferation, which could culminate with tissue necrosis (Almeida & Roberts 2005). In this study, leucocytosis was observed in 43.5% of the SCA patients included in this study. Moreover, it was observed in 45.4% and 27.3% of the patients in the groups with and without the need

for endodontic treatment, respectively. Interesting, significant result was observed when comparing the patients with and without the need for root canal therapy concerned atypical lymphocyte counts. The atypical lymphocyte has more cytoplasm contents and thus grows larger in size than a normal lymphocyte as a reaction to infection or other factors that influence the immune system. In agreement, it was shown that the maintenance of root canal infection in lesion refractory to endodontic treatment or in human inflammatory periapical lesions contribute for the progression of immune system activation (Henriques *et al*. 2011; de Brito *et al*. 2012).

Sickle cell disease is a disorder that affects the red blood cells, which use a protein called hemoglobin to transport oxygen from the lungs to the rest of the body. In these individuals erythrocytes adhere to endothelium and may cause vaso-occlusion (Zennadi *et al.* 2008, Verduzco & Nathan 2009). Several reports have associated this phenomenon to pulp necrosis (Kaya *et al*. 2004; Costa *et al.* 2013). In this study, 96.3% of the SCA patients presented haematocrit <39% among male patients and < 36% among female patients. Accordingly to previous results in SCA patients (Kato *et al*. 2009), 88% of eligible patients presented red blood cells <4.500 (X 10^6/mm^3) cells and < 4.000 (X 10^6/mm^3) cells for male and female patients, respectively.

Platelets contribute to vascular inflammation by activating neutrophils in SCA patients, in addition to their pro-coagulant role (Polanowska-Grabowska *et al.* 2010). The platelet counts were > 360x10^3 cells/mm^3 in 63.9% of the patients. Despite the increased platelet counts detected in the patients in this study, platelet counts have not been associated with severe SCA disease (Miller *et al.* 2000). In contrast, increased platelet count activation has been recognised in the vasculopathy of SCA

disease, but its clinical relevance and pathogenesis remain uncertain (Villagra *et al.* 2007).

This study assessed the need for endodontic treatment in a controlled population of SCA patients assisted at a public dental health service in Brazil, and it attempted to determine the relationships between SCA and endodontic diseases. The outcomes of this study not only provide knowledge of the epidemiological root canal requirements of the SCA population, but also the statistical relevance when comparing the patients with and without the need for root canal therapy concerned eosinophil counts and atypical lymphocyte counts. Both parameters are naturally connected to the stimulation of the immune system that can occur in the presence of root canal infections and that can be harmful to SCA individuals.

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Legend to Figure

Percentage and number of patients with SCA eosinophil counts above and below 500 cells/mm^3, and atypical lymphocyte counts equal 0 and below 1 cell/mm^3, in the group that needed root canal treatment (yes) and in the group that did not need the treatment (no). *$p < 0.05$ by the Fisher exact test.

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Table 1: Description of positive serologic testing in HbSS patients with and without the need for endodontic treatment

Serologic testing	HbSS patients	Endodontic treatment	
		Yes	No
Syphilis	0.9%	9.0%	0
Chagas disease	3.7%	9.0%	27.3%
Hepatitis (Hbs Ag)	0	0	0
Hepatitis (Anti-HBc)	3.7%	9.0%	27.3%
Hepatitis (Anti-HCV)	2.8%	9.0%	18.18%
HIV 1	0	0	0
HIV 2	0	0	0
HTLV I/II	0.9%	0	9.09%

Table 2: Percentage of patients according to each prescribed medication

Percentage/Number		Medication
SCA suffers (n=108)		
71.3	77	Folic acid
18.5	20	Folic acid, hydroxyurea
0.9	1	Folic acid, captopril
0.9	1	Folic acid, caverdilol
0.9	1	Folic acid, captopril, caverdilol
0.9	1	Folic acid, captopril, furosemida
0.9	1	Folic acid, exjade
0.9	1	Folic acid, fenitoína
0.9	1	Folic acid, hidroxyurea, exjade
0.9	1	Folic acid, hidroxyurea, paracetamol
0.9	1	Folic acid, paracetamol
0.9	1	Folic acid, vitamin D
0.9	1	Carbamazepina, captopril, hidroxyurea

Table 3: Reference values for blood cells and haemathological indices for adults

Blood cells and haemathological indices

Leucocyte counts (cell/mm^3) 3.600 -11.000

	Basophil	Eosinophils	Metamielocyte	Banded	Segmentaded	Limphocyte	Monocyte	Mielocyte	Atipic limphocyte
Adults	0 a 200	0 a 500	0	0 a 700	1.800 a 7.000	1.000 -4.500	100 a 1.000	0	0

	Erithrocyte (x10^6/mm3)	Hemoglobin (g/dl)	Hematocrit (%)	H.C.M (µµg)	C.H.C.M (g/dl)	V.C.M (µ3)	Reticulocyte (%)	Platelet (X10^3/mm^3)
Adults ♂	5,3 ± 0,8*	15,3 ± 2,5*	46 ± 7*	27,0 - 34,0	31,5 - 36,0	89 ± 9*	1,43% Limits: 0,5 - 2,3	140-360
Adults♀	4,7 ± 0,7*	13,6 ± 2,0*	42 ± 6*	27,0 - 34,0	31,5 - 36,0	89 ± 9*	1,43% Limits: 0,5 - 2,3	140-360

* mean ± standard deviation; ♂ male; ♀ female.

** Reference values according to Brasil (2004).

Table 4: White blood cell scores in HbSS patients with and without the need for endodontic treatment

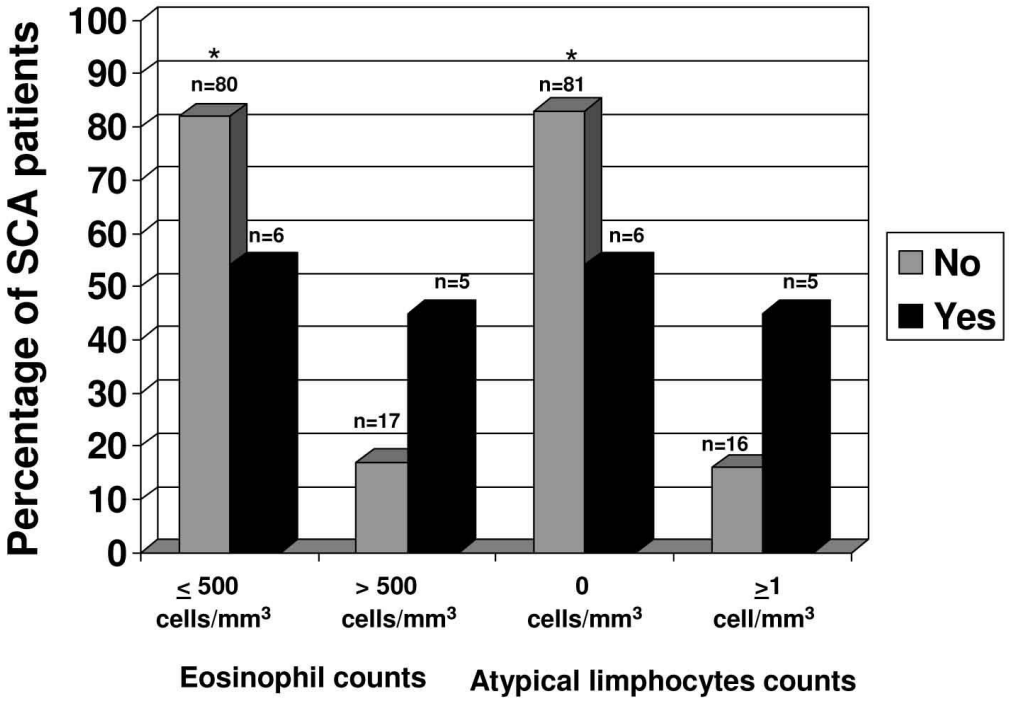
White blood cells (cells/mm^3)	Endodontic treatment							
	Yes				No			
	Mean	Median	SD	Min/Max	Mean	Median	SD	Min/Max
Basophil	82.82	69.00	119,765	0/402	49,98	0,00	73,856	0/348
Eosinophil	570.73	163,00	576,756	69,1582	601,91	360,00	826,732	0/5989
Metamielocyte	0.00	0,00	0,000	0/0	0,00	0,00	0,000	0/0
Banded neutrophil	128,55	0,00	244,068	0/815	102,38	40,00	133,385	0/656
Segmented neutrophil	4251,18	3955,00	1492,078	1789/7335	5195,24	4880,00	2352,76	345/12672
Limphocyte	4543,45	4154,00	1480,140	2760/7342	4723,71	4715,00	1687,80	763/8855
Monocyte	635,64	472,00	361,384	180/1340	581,55	578,00	382,405	0/2132
Mielocyte	0,00	0,00	0,000	0/0	0,00	0,00	0,000	0/0
Atypical limphocyte	59,75	40,00	67,724	0/163	29,78	0,00	70,081	0/374

SD, standard deviation; Min, minimum; Max, maximum.

Table 5: Haematological scores in HbSS patients with and without the need for endodontic treatment

Haematological data	**Endodontic treatment**							
	Yes				**No**			
	Mean	Median	SD	Min/Max	Mean	Median	SD	Min/Max
Erythrocyte ($x10^6/mm^3$)	2,448	2,600	0,4006	2,0/3,1	8,484	8,300	1,7182	6,0/15,7
Hemoglobin (g/dl)	7,400	7,300	1,3387	5,2/9,2	25,779	24,800	5,9009	16,5/47,0
Hematocrit (%)	22,545	22,400	3,2235	18,0/28,0	91,852	92,200	11,4578	70,2/122,7
MCV (fl)	92,491	92,600	12,4037	74,6/122,0	30,733	31,200	5,1010	20,4/44,2
MCH (pg)	31,000	31,800	4,9651	23,8/41,6	33,259	33,200	2,2544	28,9/41,4
MCHC (g/dl)	33,482	33,500	1,5151	31,3/36,6	8,656	7,000	7,0266	1.0/55,0
Reticulocyte (%)	9,318	10,00	3,3337	5,0/14,0	429,61	414,00	148,083	117/960
Platelet ($x10^3/mm^3$)	434,18	434,00	114,707	204/620	8,484	8,300	1,7182	6,0/15,7

MCV, mean corpuscular volume; MCH, Mean corpuscular hemoglobin; MCHC, Mean corpuscular hemoglobin concentration; SD, standard deviation; Min, minimum; Max, maximum.


Percentage of SCA patients
100
90
80
70
60
50
40
30
20
10
0
*
n=80
n=6
n=17
n=5
*
n=81
n=6
n=16
n=5
No
Yes
≤ 500 cells/mm3
> 500 cells/mm3
0 cells/mm3
≥1 cell/mm3
Eosinophil counts
Atypical limphocytes counts

3.2 Artigo científico 2: “PERIAPICAL CYTOKINE EXPRESSION IN SICKLE CELL DISEASE”

Periapical Cytokine Expression in Sickle Cell Disease

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Abstract

Patients with sickle cell anemia (SCA) exhibit increased levels of proinflammatory mediators as part of a permanently activated immunoinflammatory status. The aim of this study was to evaluate the mRNA expression levels of the cytokines interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-17A, IL-10, receptor activator for nuclear factor kappa B ligand (RANKL), and the chemokines CCL2/MCP-1 and CCL5 in the periapical interstitial fluid from SCA individuals compared with healthy individuals. **Methods:** Samples were collected from 12 teeth of SCA, and 12 non-SCA patients with apical periodontitis. Additionally, twelve teeth were sampled from the periapical region of healthy patients with vital pulp (control). The expression of cytokine mRNA was detected using real-time PCR. **Results:** No significant differences were observed in the expression of cytokine or chemokine mRNA between SCA and non-SCA individuals ($p > 0.05$). However, the expression of mRNA for the Th1-associated cytokines IFN-γ, TNF-α, and IL-1β were significantly higher in SCA individuals than in the control individuals ($p < 0.05$). Among Th1-associated cytokines, only IFN-γ was significantly increased in non-SCA compared with control patients (vital pulp). The expression of IL-17A mRNA was significant higher in SCA cases than in control samples ($p < 0.05$), while the IL-10 mRNA expression was significant increased in SCA and non-SCA individuals when compared with the control group. Similar levels of RANKL, CCL-2, and CCL-5 mRNA expression were observed in all samples. **Conclusions:** The results were unable to demonstrate any differences in periapical immune responses between SCA and non-SCA individuals despite the fact that SCA patients presented prone proinflammatory ability, expressing IL-1, TNF-α, and IL-17A at a significantly higher level compared to control patients than non-SCA individuals compared to control patients.

Key words: Chemokines, cytokines, apical periodontitis, sickle cell anemia

Introduction

Periapical lesions develop when the human body attempts to control the bacteria and bacterial byproducts that are present in infected root canals (1). This reaction is characterized by immunological mechanisms involving inflammatory cells and the production of cytokines and chemokines (1, 2), which ultimately lead to bone resorption (3). The CD4+ cells differentiate into two subtypes, Th1 and Th2 cells, according to the cytokines produced (4, 5). Both types of effector responses are regulated by a heterogeneous family of cells known as regulatory T (Treg) cells, which form a subset of 5 - 10% of all CD4+ T cells and regulate the periapical immune response (6, 7). Recently, a new subset of Th cells has been discovered; termed Th17 cells, these cells predominantly produce IL-17 (8, 9, 10). T cells also express chemokines that mediate leukocyte recruitment to periapical lesions (7).

Sickle cell anemia (SCA) is the most prevalent genetic disease worldwide (11). This hemoglobinopathy is caused by a mutation in the beta globulin gene of the hemoglobin molecule, resulting in the production of abnormal hemoglobin, called hemoglobin S (HbS). HbS leads to erythrocyte rigidity and vaso-occlusion, resulting in progressive damage to systemic organs. Such damage subsequently leads to avascular necrosis of the bones, osteopenia, osteoporosis, retinal infarction, stroke, pulmonary hypertension and skin ulcerations (12, 13). Moreover, patients with SCA have an increased susceptibility to infections, which is partly due to autosplenectomy resulting from recurrent spleen vaso-occlusive infarcts (12, 14, 15, 16). Although impaired immunological responses have been shown in SCA individuals (17, 18, 19), periapical immune responses to root canal infections have not been previously analyzed in these individuals. The aim of this research was to assay the SCA periapical immune response in comparison to this response in healthy individuals.

The mRNA expression levels for interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-17A, IL-10, and RANKL, as well as the chemokines CCL5 and CCL2/MCP-1, were assayed using real-time polymerase chain reaction (PCR) and samples collected from the periapical interstitial fluid of both groups of individuals.

Materials and Methods

Subjects

The subjects consisted of 12 SCA patients (SCA group) and 12 healthy patients presenting with periapical lesions (non-SCA experimental group). An additional 12 healthy patients presenting with pulp vitality and referral for endodontic treatment with prosthetic indications were included as controls (control group). Both groups of healthy individuals were drawn from patients who were referred to the School of Dentistry at the Universidade Federal de Minas Gerais (Belo Horizonte, MG, Brazil). SCA (HbSS genotype) individuals were registered and followed by the Hemominas Foundation (Regional Blood Center of Montes Claros, Minas Gerais, Brazil). Those SCA patients requiring endodontic care were treated at the Associação Brasileira de Odontologia (ABO)/Montes Claros. Patients were excluded from this study if they had taken antibiotics within the 3 months prior to the initiation of endodontic therapy. All participants signed the Free Agreement Formulary. This study was approved by the Research Ethics Committees of the Hemominas Foundation (ETIC 283/10), the State University of Montes Claros (ETIC 1848/10) and the Federal University of Minas Gerais (ETIC 203/10).

Sample Collection

Clinical samples were taken from all of the teeth of each individual in each of the groups that comprised this study, as described above. Each patient was diagnosed based on clinical and radiographic analyses in addition to pulp sensibility tests. Teeth did not present acute periapical symptoms at the time of appointment. The sampling procedures were performed as previously described (10, 20, 21, 22). Briefly, each tooth was isolated, and the root canals were cleaned and shaped using ProTaper nickel-titanium files (Dentsply Maillefer, Ballaigues, Switzerland) and 5.2% sodium hypochlorite. The samples were collected immediately after the root canal cleaning to characterize the cytokine/chemokine expression profile. After cleaning and drying, 3 paper points were introduced into the root canal, passing through the root apex (2 mm), for 1 minute. After withdrawal, the paper points were cut 4 mm from the tip and dropped into a microcentrifuge tube, and the samples were stored at -70°C. RNA was then extracted from the periapical interstitial fluid. At the time of sample collection, no teeth presented clinical signs or symptoms, and the root canals were filled using the lateral condensation technique.

Sample Preparation

Total RNA was extracted from each sample using the TRIzol reagent (GIBCO/BRL Laboratories, Grand Island, NY, USA), as described elsewhere (10, 21, 22). The RNA was then stored at -70°C.

Real-time Polymerase Chain Reaction

Complementary DNA was synthesized using 1 µg of RNA and the reverse transcription reaction described by Barbosa Silva et al (23). Primer sequences were designed using the PRIMER EXPRESS software (Applied Biosystems, Foster City,

CA, USA) based on the nucleotide sequences available in the GenBank database. The real-time PCR assay was performed using an ABI Prism 7900 HT Real-time PCR System (Applied Biosystems). The primer sequences used for the quantitative PCR analysis of IFN-γ, TNF-α, IL-1β, IL-17A, IL-10, RANKL, CCL5, and CCL2/MCP-1 mRNA expression are shown in Table 1. PCR was performed under standard conditions as follows: a holding stage at 95°C (10 minutes); a cycling stage of 40 cycles at 95°C (15 seconds) followed by 60°C (1 minute); and a melting curve stage at 95°C (15 seconds), 60°C (1 minute), and 95ºC (15 seconds). A SYBR-Green detection system (Applied Biosystems) was used to assay primer amplification. Glyceraldehyde-3 phosphate dehydrogenase (GAPDH) was used as a housekeeping gene for normalization and was run with each set of reactions. All samples were run in duplicate. Each reaction was performed in a 25 µL volume containing 1 µg of cDNA. The Sequence Detection Software version 2.4 (Applied Biosystems) was used to analyze data after amplification. The results were obtained as threshold cycle (Ct) values, and the expression levels were calculated using the comparative $2^{-\Delta\Delta C}{}_{T}$ method (24, 25). The values were calculated as the mean value of the duplicates for each patient, and the mRNA expression levels in all samples were defined as the ratio of each specific primer to GAPDH expression.

Statistical Analysis

Data analysis was performed using SPSS for Windows (version 15.0; SPSS Inc, Chicago, IL, USA). Data were subjected to the Shapiro-Wilk test to characterize their normality. Because the samples did not present a normal distribution, the Wilcoxon test was used to determine significant differences in samples from the

same groups ($p < 0.05$). The Mann-Whitney test was used to compare the differences between the groups ($P < .05$).

Results

When comparing the expression of cytokine and chemokine mRNA between SCA group and non-SCA experimental group, no significant differences were observed ($p > 0.05$). However, the mRNA levels for Th1-associated cytokines (IFN-γ, TNF-α, and IL-1β) were significantly higher in SCA individuals than in the vital/healthy (control) individuals ($p < 0.05$) (Fig. 1), while of the Th1-associated cytokines, only IFN-γ was significantly increased in non-SCA experimental group compared with control group (Fig. 1). The expression of IL-17A was significantly increased in SCA cases compared with vital/healthy samples ($p < 0.05$), but no difference was observed when comparing non-SCA experimental group with control group (Fig. 2). Moreover, the expression of IL-10 mRNA was significantly higher in SCA and non-SCA individuals compared with the control group (Fig. 2). Finally, similar expression levels for RANKL (Fig. 2) and the chemokines CCL-2 and CCL-5 (data not shown) were observed among the samples collected from SCA, non-SCA and control groups.

Discussion

Cytokines and chemokines are frequently found in periapical inflammatory tissues (26, 27, 10, 25), but their function is not yet fully understood. In this study, the mRNA expression levels of IFN-γ, TNF-α, IL-1β, IL-17A, IL-10, and RANKL, as well as the chemokines CCL-5 and CCL-2/MCP-1, were assayed in periapical interstitial fluid collected from SCA individuals and compared with healthy individuals with and without periapical lesions. The rationale for this research stems from the fact that

SCA individuals have an increased susceptibility to infections (12, 14, 15, 16, 17, 18, 19), which may interfere in periapical lesion responses. Moreover, in sickle cell anemia, immune cells, such as monocytes, neutrophils and endothelial cells, are in a permanently activated status (28).

In this study, the Th1-associated cytokines IFN-γ, TNF-α, and IL-1β were significantly higher in SCA individuals than in the vital/healthy (control) individuals. Bacterial byproducts released from infected root canals stimulate macrophages and CD4+ T cells to secrete proinflammatory cytokines (29, 30). TNF-α promotes several immune system functions (31, 32), but it has been reported that TNF-α also stimulates bone resorption in periapical lesions (33), despite been 500-fold less potent than IL-1 (34). IL-1 has been identified as a central mediator of periapical and pulpal inflammation (35), increasing leukocyte adhesion, stimulating lymphocytes, and, together with TNF-α, enhancing periapical bone resorption while inhibiting bone formation (36). IFN-γ activates macrophages, reduces macrophage-suppressive activity, and induces IL-1, NO synthesis and O_2^- production (37).

The results of this study show that in the presence of root canal infections, SCA individuals were statistically able to express mRNA for Th1-associated cytokines compared with the control. In contrast, the non-SCA individuals did not over-express IL-1β, as demonstrated by others in primary infections (21, 22, 25), as well as in individuals presenting refractory endodontic treatments (10). Despite the antibacterial effects of IL-1β, which may protect individuals against infection, its strong effects, together with the statistical over-expression of TNF-α, on bone resorption could interfere in the progression and healing of periapical lesions in SCA patients.

The proinflammatory mediator IL-17 induces proinflammatory cytokines such

as IL-1, IL-6 and TNF (38, 39) that are involved in periapical lesion responses (10, 40). Th17 cells, possibly acting through the IL-17-mediated induction of RANKL on osteoclastogenesis supporting cells, also regulate osteoclastogenesis (7). RANKL binds to its receptor, RANK, a cell-surface protein present on osteoclast precursor cells and, when activated, promotes osteoclast maturation by increasing the expression of specific genes (41). In our study, the expression of IL-17A was significantly higher in SCA individuals than in control individuals. In agreement with other periapical analyses, these results demonstrate a positive correlation between IFN-γ and IL-17, suggesting that both cytokines are important for the exacerbation of inflammation (10, 42). However, the correlation between the expression of proinflammatory cytokines and the induction of increased RANKL expression was not observed here because similar levels of RANKL mRNA expression were observed among the samples collected from SCA, non-SCA and control individuals. Conversely, we have previously shown a direct correlation between the decrease in proinflammatory cytokines in non-SCA individuals and the under-expression of RANKL mRNA when the bacterial load of the infected root canal was reduced (25).

Chemokines are proteins that regulate and determine the nature of immune responses and control immune cell trafficking. Here, despite detectable levels of CCL-2/MCP-1 and CCL-5 in the periapical samples collected from SCA, non-SCA and control individuals, no significant difference was observed between the groups. Conversely, we have found that MCP-1 mRNA expression was increased in lesions refractory to endodontic treatment compared with control teeth (10), similar to what was reported in periapical granulomas and cysts (43). Although an association between CCL-5 and Th1-type cellular responses was suggested by the findings that the neutralization of CCL-5 reduced type 1 granuloma formation (44) and that CCL-5

expression, together with other Th1 cytokines, was reduced after endodontic procedures (25), the *in vivo* roles of CCL-5 must be better clarified.

Treg cells, which comprise a minor population of CD4+ T cells, uniquely express Foxp3, which is essential for Treg differentiation (45). These effects were attributed to the IL-10 and TGF-β regulatory phenotype (7), and it was demonstrated in periapical lesions that $CD4^{+}CD25^{+}Foxp3^{+}$ cells (Treg) expressed both IL-10 and TGF-b in higher levels than in peripheral blood (39). Here, IL-10 mRNA expression was significantly higher in the experimental groups (SCA and non-SCA) than in the control group. The increased expression of IL-10 mRNA could represent the triggering of a compensatory anti-inflammatory mechanism to effectively downregulate the periapical inflammatory response longer. Conversely, this decreased expression could be related to the fact that the presence or absence of proinflammatory cytokines determines whether the immune response will be Th17 or Treg (43, 46).

A recent study was unable to demonstrate the direct immunological relationship between SCA and periodontal inflammation (18). Similar outcomes were found in this study, which demonstrated similar cytokine and chemokine mRNA expression in SCA and non-SCA individuals. Nevertheless, SCA individuals presented prone proinflammatory ability, expressing IL-1, TNF-α, and IL-17A at a significantly higher level compared to control patients than non-SCA compared to control individuals. Together, these outcomes suggest that further immunological and prospective studies are necessary to understand the periapical lesion responses in patients presenting with sickle cell disease.

Legends to figures

Figure 1. Expression of IFN-γ, TNF-α and IL-1β genes in periradicular tissues of SCA, non-SCA, and control patients. Expression levels were determined by real-time PCR and quantified by comparison with an internal control (GAPDH). Bars represent the mean values of samples recovered from 12 teeth of SCA patients with pulp necrosis, 12 teeth of healthy patients with pulp necrosis and 12 teeth of healthy patients with pulp vitality; lines represent the standard error of the mean. *P < .05 according to the Wilcoxon or Mann-Whitney tests.

Figure 2. Expression of RANKL, IL-10 and IL-17A genes in periradicular tissues of SCA, non-SCA, and control patients. Expression levels were determined by real-time PCR and quantified by comparison with an internal control (GAPDH). Bars represent the mean values of samples recovered from 12 teeth of SCA patients with pulp necrosis, 12 teeth of healthy patients with pulp necrosis and 12 teeth of healthy patients with pulp vitality; lines represent the standard error of the mean. *P < .05 according to the Wilcoxon or Mann-Whitney tests.

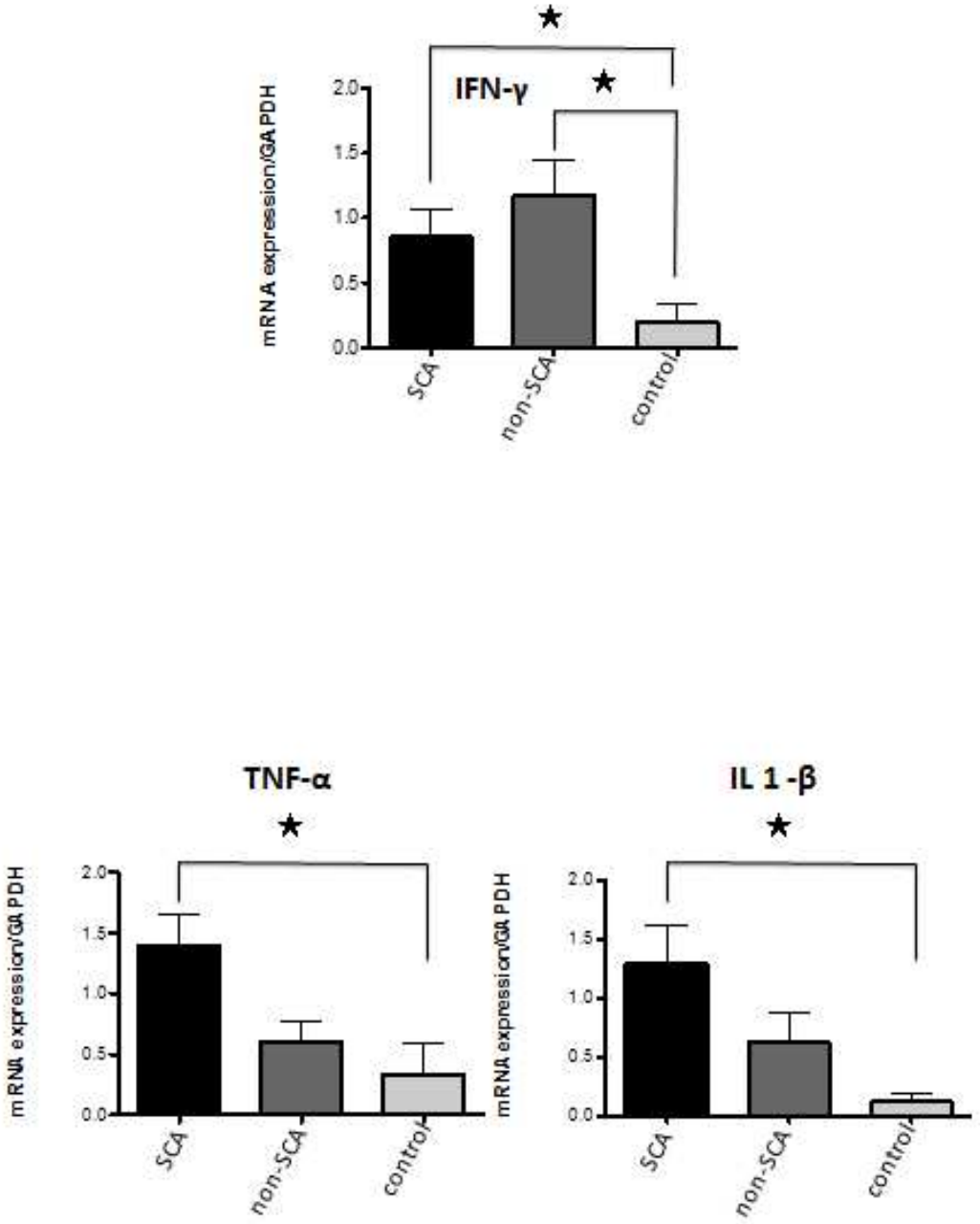
IFN-γ
mRNA expression/GAPDH
2.0
1.5
1.0
0.5
0.0
SCA
non-SCA
control
TNF-α
mRNA expression/GAPDH
IL 1 -β
mRNA expression/GAPDH

FIGURE 1

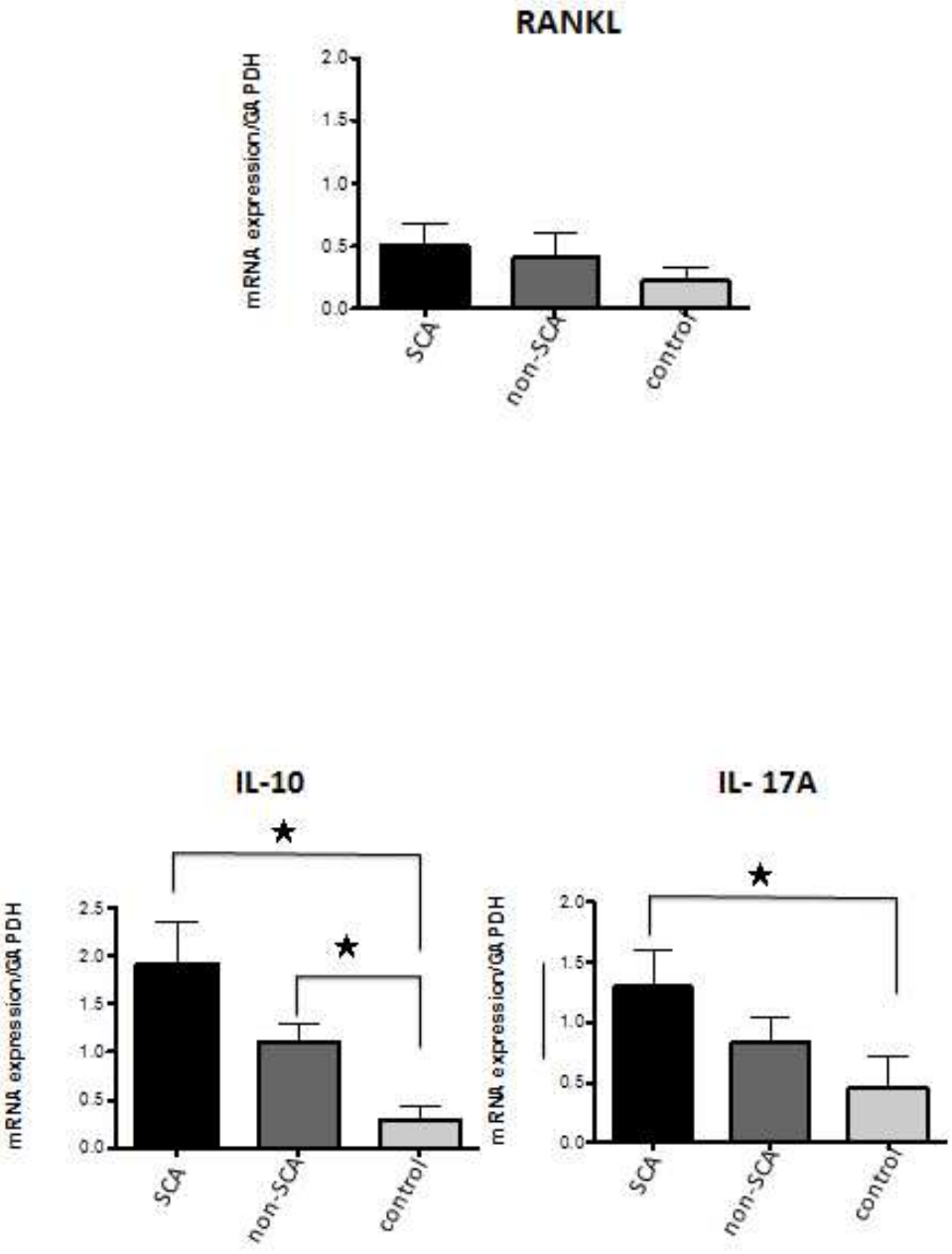
RANKL
mRNA expression/GAPDH
2.0
1.5
1.0
0.5
0.0
SCA
non-SCA
control
IL-10
IL- 17A
mRNA expression/GAPDH
2.5
2.0
1.5
1.0
0.5
0.0
SCA
non-SCA
control
★

FIGURE 2

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4 CONCLUSÕES

Os resultados deste estudo nos permitiram observar que:

A taxa de necessidade de tratamento endodôntico nos indivíduos com anemia falciforme foi de 10.2%;

Houve diferença significativa na contagem de eosinófilos e linfócitos atípicos ao se comparar os pacientes com necessidade de tratamento endodôntico àqueles sem a necessidade do tratamento;

Não houve diferença significativa na expressão gênica das citocinas avaliadas ao se comparar os dados de indivíduos-AF com os de indivíduos não-AF apresentando necrose pulpar e periapicopatia;

Houve um aumento na expressão gênica das citocinas IFN-γ, TNF-α, IL-1β e IL-17A nos indivíduos-AF quando comparada àquela observada no grupo controle.

Pode-se concluir que apesar das diferenças hematológicas e imunológicas associadas às alterações endodônticas nos indivíduos portadores de anemia falciforme, estudos prospectivos serão necessários para uma melhor compreensão das respostas pulpo-perirradiculares nesses indivíduos. Por sua vez, os resultados obtidos neste estudo podem subsidiar novas avaliações, bem como gerar estratégias de cuidados ou de educação em saúde para os indivíduos AF.

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ANEXO A – Parecer consubstanciado UFMG

UNIVERSIDADE FEDERAL DE MINAS GERAIS
COMITÊ DE ÉTICA EM PESQUISA - COEP

Parecer nº. ETIC 0011.0.215.203-10

Interessado(a): Prof. Antonio Paulino Ribeiro Sobrinho
Departamento de Odontologia Restauradora
Faculdade de Odontologia - UFMG

DECISÃO

O Comitê de Ética em Pesquisa da UFMG – COEP aprovou, no dia 27 de outubro de 2010, após atendidas as solicitações de diligência, o projeto de pesquisa intitulado **"Avaliação microbiológica de canais radiculares e de citocinas inflamatórias periapicais em pacientes com anemia falciforme"** bem como o Termo de Consentimento Livre e Esclarecido.

O relatório final ou parcial deverá ser encaminhado ao COEP um ano após o início do projeto.

Profa. Maria Teresa Marques Amaral
Coordenadora do COEP-UFMG

Av. Pres. Antonio Carlos, 6627 – Unidade Administrativa II - 2º andar – Sala 2005 – Cep:31270-901 – BH-MG
Telefax: (031) 3409-4592 - e-mail: coep@prpq.ufmg.br

ANEXO B – Parecer consubstanciado UNIMONTES

UNIVERSIDADE ESTADUAL DE MONTES CLAROS
COMITÊ DE ÉTICA
PARECER CONSUBSTANCIADO

Unimontes

Montes Claros, 19 de março de 2010

Processo N.º 1848/10.
Título do Projeto: AVALIAÇÃO MICROBIOLÓGICA DE CANAIS RADICULARES E DE CITOCINAS INFLAMATÓRIAS PERIAPICAIS EM PACIENTES COM ANEMIA FALCIFORME

Coordenador: **Prof. Dr. Antônio Paulino Ribeiro Sobrinho**
Relatora: Profª. Ms. Simone de Melo Costa

Histórico

A reação inflamatória no periápice do dente é considerada conseqüência da extensão da inflamação pulpar. Considera-se que qualquer infecção bacteriana no indivíduo com anemia falciforme tem grande potencial de evoluir para sepse, muitas vezes com êxito letal, se não identificada e tratada precocemente. Este trabalho tem o objetivo avaliar a microbiota dos canais radiculares e de citocinas inflamatórias da região periapical em indivíduos com anemia falciforme, na presença de lesões periapicais. Tata-se de estudo descritivo, transversal e experimental. A amostra será de conveniência sendo formada por pessoas com necessidade de tratamento endodôntico e dividida em dois grupos: grupo experimental (indivíduos com anemia falciforme cadastrados no Hemocentro de Montes Claros, MG, apresentando dente com necrose pulpar e lesão periapical) e grupo controle (indivíduos sadios atendidos nas Clínicas Odontológicas da Universidade Estadual de Montes Claros e na Clínica da Associação Brasileira de Odontologia de Montes Claros, apresentando dente com necrose pulpar e lesão periapical). Serão realizadas as análises microbiológica e imunológica dos dentes selecionados.

Mérito

Considera-se relevante a avaliação microbiológica dos canais radiculares e de citocinas inflamatórias da região periapical em portadores de anemia falciforme uma vez que as infecções bacterianas representam risco de alterações clínicas graves podendo levar à morte do indivíduo portador da doença.

Parecer

O Comitê de Ética da Unimontes analisou o processo **1848**, e entende que o mesmo está completo e dentro das normas do Comitê e das Resoluções do Conselho Nacional da Saúde/Ministério da Saúde. Sendo assim, somos pela **APROVAÇÃO** do projeto de pesquisa.

Profª Vânia Silva Vilas Boas Vieira Lopes
Presidente do Comitê de Ética em Pesquisa da Unimontes

ANEXO C – Parecer consubstanciado HEMOMINAS

MINISTÉRIO DA SAÚDE
Conselho Nacional de Saúde
Comissão Nacional de Ética em Pesquisa - CONEP
Comitê de Ética em Pesquisa da Fundação Hemominas

PARECER CONSUBSTANCIADO

1. Título do Projeto de Pesquisa: Avaliação mirobiológica de canais radiculares e de citocinas inflamatórias periapicais em pacientes com anemia falciforme.
SUJEITOS DA PESQUISA
2. Número de sujeitos No Centro: 60 Total: 60 — 3. Grupos Especiais: (X) Menor de 18 anos; ( ) Portador de deficiência mental ( ) Embrião/feto; ( ) Relação de dependência (militares, presidiários, funcionários...) ( ) Outros; ( ) Não se aplica
PESQUISADOR RESPONSÁVEL
4. Nome: Antônio Paulino Ribeiro Sobrinho
5. Instituição a que pertence: Faculdade de Odontologia, UFMG
INSTITUIÇÃO (ÕES) ONDE SERÁ REALIZADO
6. Nome: Fundação Hemominas
7. Unidade/Órgão: Hemocentro Regional de Montes Claros
8. Participação Estrangeira: Sim ( ) Não (x)
9. Projeto Multicêntrico: Sim ( ) Não (x) Nacional ( ) Internacional ( )
PATROCINADOR Não se aplica ( )
10. Nome:
COMITÊ DE ÉTICA EM PESQUISA - CEP
11. Data de Entrada: 01/07/2010 — 12.Registro no CEP: 283
13. Objetivos: - Objetivo Geral: Avaliar a microbiota dos canais radiculares e de citocinas inflamatórias da região periapical em indivíduos com anemia falciforme, na presença de lesões periapicais. - Objetivos Específicos: 1. Enumerar as espécies microbianas, presentes em canais radiculares de pacientes com anemia falciforme, na presença de necrose pulpar e lesões periapicais. 2. Quantificar as espécies microbianas presentes em canais radiculares de pacientes com anemia falciforme, na presença de necrose pulpar e lesões periapicais. 3. Identificar as citocinas inflamatórias presentes na região periapical de pacientes com anemia falciforme, na presença de necrose pulpar e lesões periapicais. 4. Quantificar a expressão das citocinas inflamatórias presentes na região periapical de pacientes com anemia falciforme, na presença de necrose pulpar e lesões periapicais.
14. Sumário do Projeto: Estudo descritivo para avaliar o conteúdo microbiano em canais radiculares e o perfil de citocinas expressas nos tecidos periapicais adjacentes, na presença de lesões periapicais em indivíduos com anemia falciforme. Os pacientes serão encaminhados pelo Hemocentro Regional de Montes claros após assinatura do TCLE, e as amostras serão coletadas nas Clínicas Odontológicas da UNIMONTES e na Clínica da Associação Brasileira de Odontologia de Montes Claros - ABO/MOC. Serão realizadas uma triagem clínica e anamnese e os dados clínicos de interesse serão registrados em ficha padrão. Outros dados clínicos e hematológicos serão retirados do prontuário dos pacientes com anemia falciforme. A coleta das amostras será realizada utilizando uma lima endodôntica nos canais radiculares seguido de um cone de papel absorvente. A determinação das espécies microbianas será realizada pela técnica de MDA (Multiple Displacemnet Amplification). Primeiramente, as amostras serão crescidas em câmara de anaerobiose durante 3 a 7 dias. Após este período as células serão lisadas, o DNA purificado e as amostras amplificadas pela técnica de MDA. Após amplificação, será feita hibridização com as sondas de DNA, e posterior identificação das espécies microbianas. A quantificação das citocinas será realizada pela técnica de Real Time PCR. Os resultados obtidos serão submetidos à analise estatística pelos métodos não paramétricos Mann-Whitney e Kruskal-Wallis.

15. Comentário dos Relatores: Após adequações, o projeto foi considerado aprovado.		
16. Parecer: Aprovado (x) Data: 02/09/10	Pendência ( ) Data:	Não Aprovado ( ) Data:
17. Cronograma de execução: Início: setembro 2010 Fim: dezembro 2012	18. Enviar relatórios em: setembro de 2011, setembro 2012 e dezembro 2012	
19. Coordenador Coordenadora do Comitê de Ética em pesquisa Fundação Hemominas Assinatura		