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Correlation of the expression of tumor necrosis factor-alpha in chronic periapical lesions with the expression of bacterial chaperonin 60

Korelacija ekspresije faktora nekroze tumora-alfa u hroničnim periapeksnim lezijama sa ekspresijom bakterijskog šaperonina 60

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Abstract

Background/Aim. Chronic microbial infections of the root canal are a common issue. This process very often causes an immune reaction in the root canal system that results in forming of chronic periapical lesions (PLs). The aim of this study was to determine the quantitative expression of the bacterial heat shock protein (HSP), chaperonin 60 (*cpn60*), and the pro-inflammatory and anti-inflammatory cytokines in periapical tissue obtained from individuals with chronic PLs and to determine if there is a correlation between the expression of the bacterial HSP and the expression of these cytokines. **Methods.** The study was performed on 18 PLs and 6 control samples of healthy periapical tissue, taken at the Department of Dentistry, Faculty of Medicine, University of Priština/Kosovska Mitrovica. The levels of messenger ribonucleic acid (mRNA) expression of pro- and anti-inflammatory cytokines and bacterial HSP were determined by quantitative real-time polymerase chain reaction (RT-PCR) and quantified by comparing to the internal control gene for glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). **Results.** Analysis revealed significantly higher mRNA levels of tumor necrosis factor-alpha (*TNF-a*) and *cpn60* in the tissue of PLs compared with normal periapical tissue ($p < 0.05$). Contrary to these results, the mRNA expression of anti-inflammatory interleukin-10 (*IL-10*) was significantly higher in the samples of normal periapical tissue compared with the mRNA levels of this cytokine in the tissue of PLs ($p < 0.001$). Expression of *cpn60* is in strong correlation with *TNF-a* expression in PLs. **Conclusion.** *cpn60* released from bacteria in periapical tissue could be a strong stimulator of inflammatory response and one of the important players in the pathogenesis of PLs.

Key words:
bacterial outer membrane proteins; chaperonin 60; cytokines; gene expression; heat-shock proteins; interleukin 10; periapical diseases; tumor necrosis factor-alpha.

Apstrakt

Uvod/Cilj. Hronične infekcije korenskog kanala su uobičajen problem. Ovaj proces veoma često izaziva imunsku reakciju u sistemu korenskog kanala, a kao rezultat toga se formiraju hronične periapikalne lezije (PL). Cilj studije bio je da se utvrdi kvantitativna ekspresija bakterijskog proteina „toplotnog udara“ (HSP), šaperonina 60 (*cpn60*) i pro- i anti-inflamacijskih citokina u periapeksnom tkivu dobijenom od osoba sa hroničnim PL i utvrdi da li postoji korelacija između ekspresije bakterijskog HSP i ekspresije ovih citokina. **Metode.** Istraživanje je sprovedeno na 18 PL i 6 kontrolnih uzoraka zdravog periapeksnog tkiva koji su uzeti na Odeljenju za stomatologiju Medicinskog fakulteta Univerziteta u Prištini/Kosovskoj Mitrovici. Nivoi ekspresije informacione ribonukleinske kiseline (iRNK) pro-inflamacijskih i anti-inflamacijskih citokina i bakterijskog HSP određeni su metodom lančane reakcije polimeraze u realnom vremenu (RT-PCR) i kvantifikovani su poređenjem sa internim kontrolnim genom *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*). **Rezultati.** Analize su pokazale značajno više nivoe iRNK faktora nekroze tumora-alfa (*TNF-a*) i *cpn60* u tkivu PL u poređenju sa normalnim periapeksnim tkivom ($p < 0,05$). Suprotno ovim rezultatima, ekspresija iRNK anti-inflamacijskog citokina interleukina-10 (*IL-10*) bila je značajno viša u uzorcima normalnog periapeksnog tkiva u poređenju sa nivoima iRNK ovog citokina u tkivu PL ($p <$

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0,001). Ekspresija *cpn60* je u značajnoj korelaciji sa ekspresijom *TNF-a* u PL. **Zaključak.** *cpn60* oslobođen iz bakterija u periapeksnom tkivu mogao bi biti snažan stimulator zapaljenskog odgovora i jedan od važnih aktera u patogenezi PL.

Ključne reči:
protein spoljne membrane bakterija; šaperonin 60; citokini; geni, ekspresija; protein toplotnog udara; interleukin 10; periapikalne bolesti; faktor nekroze tumora alfa.

Introduction

Periapical lesions (PLs) represent a very common pathology in humans that occurs as a consequence of an immune reaction to the microbial infection in the root canal system [1]. The composition of the infiltrating periapical cells (PCs) in PLs includes various cells of innate and acquired immunity, such as neutrophilic granulocytes, macrophages, T lymphocytes, and plasma cells. In addition to these cells, the cells that make up the normal structure of periapical tissue: fibroblasts, osteoblasts, and the epithelial rests of Malassez, play an important role in the pathogenesis of periapical changes, as well [2].

The primary causes of untreated PLs are the most common bacteria that belong to the genera *Campylobacter*, *Enterococcus*, *Eubacterium*, *Fusobacterium*, *Peptostreptococcus*, *Porphyromonas*, *Prevotella*, *Propionibacterium*, and *Streptococcus* [3]. Bacterial antigens in periapical tissue trigger cellular and humoral immune response [4].

Heat shock proteins (HSPs) are synthesized in both prokaryotic and eukaryotic cells and represent the basic cellular defense response to various stressful situations such as fever, bacterial and viral infections, ischemia, hypoxia, radiation, and malignant transformation [5]. Despite having a protective role, HSPs can be strong stimulators of immune cells due to the high homology with bacterial HSPs and the mechanism of molecular mimicry and could contribute to the development of chronic inflammatory processes [6]. The housekeeping gene that encodes the bacterial protein chaperonin 60 [*cpn60* (synonyms are *groEL* and *hsp60*)] assists proper protein folding in bacterial cells and is ubiquitously distributed among bacteria [7]. Gene *cpn60* is marked as a DNA barcode for bacteria [8]. It has been shown that Cpn60 proteins isolated from different bacteria stimulate immune response and contribute to the activation of lymphocytes [9]. Moreover, in several different studies, it has been shown that Cpn60 proteins stimulate the production of cytokines that play the main role in tissue destruction and bone resorption in periodontal disease [10].

Pro-inflammatory and anti-inflammatory cytokines are present in PLs, and the development of PLs depends on their mutual relationship [11, 12]. Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine whose expression is increased in PLs and has a stimulating effect on PL progression and bone destruction [13]. It is produced by macrophages, fibroblasts, monocytes, T and B lymphocytes, and large amounts of this cytokine are released under the influence of bacterial products [13]. By contrast, interleukin-10 (IL-10) belongs to the group of anti-inflammatory cytokines and has a suppressive effect on the development of PLs and bone destruction [14]. In PLs, the main source of IL-10 is B and T lymphocytes, fibroblasts, and macrophages [15]. During the acute inflammatory response, IL-10, in combination with transforming growth factor-beta (TGF-β), suppresses the immune response and promotes tissue repair [14, 15].

The aim of this study was to evaluate the quantitative mRNA expression of the bacterial HSP *cpn60* and pro-inflammatory and anti-inflammatory cytokines in the periapical tissue obtained from individuals with chronic PLs and to evaluate the correlation between the expression of bacterial HSP and the expression of these cytokines.

Methods

Human subjects

Samples of PLs (n = 18) were taken from individuals aged 18–65 years who had their diagnosis of chronic PLs made based on the anamnestic data, clinical manifestations, and X-ray findings. Samples were taken during dental extraction or apical surgery. The control sample (n = 6), obtained from the healthy periapical tissue, was taken during the extraction of healthy teeth for orthodontic purposes. All participants were free of systemic diseases and free of antibiotic therapy during the month that preceded the intervention compared to the sampling period. Participants with PLs were included in the study from the total population of patients treated at Department of Dentistry, Faculty of Medicine in Kosovska Mitrovica, after being examined by an oral surgeon. The control group was selected after the orthodontist's check-ups and after defining the indications for tooth extraction. This study was approved by the Ethics Committee of the Faculty of Medicine, University of Priština/Kosovska Mitrovica (No: 09-537-1). All patients signed a written informed consent form prior to all the procedures. The study was conducted in full accordance with ethical principles. The inclusion of all samples was carried out successively, according to the principle of the natural sample.

Sample collection

After tooth extraction, PLs were removed from the root tip with a sterile scalpel, while during an apicotomy procedure, a sample of the PL was taken by removing the PL of the bone with a curette [12]. The samples were soaked in sterile saline and dried with sterile gauze. The samples were stored in sterile Eppendorf tubes containing RNAlater™ Stabilization Solution (Thermo Fisher Scientific) at -20 °C until RNA extraction.

RNA extraction

Total RNA was extracted from 100 mg of each sample with TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's recommendations. Briefly, periapical tissues were homogenized in 1 mL of TRIzol reagent, centrifuged at 12,000 xg at 4 °C for 10 min, and supernatants collected. Bromochloropropane (Sigma Aldrich) was added to supernatants, and after incubation at room temperature, samples were centrifuged at 12,000 g at 4 °C for 20 min. The aqueous phase was collected, and RNA was precipitated by isopropanol. Samples were washed twice with cold 70% ethanol. The samples were dried and dissolved in RNase-free water.

Real-time Polymerase Chain Reaction

Complementary DNA was synthesized using 1 mg RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) in the presence of oligo (dT) primers. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using Power SYBR MasterMix (Applied Biosystems) and mRNA-specific primers for *TNF-a, IL-10,* and bacterial *cpn60,* while glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as a housekeeping gene for normalization (Table 1). qRT-PCR reactions were initiated with a 10-minute incubation time at 95 °C followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s in a Mastercycler® ep realplex (Eppendorf, Hamburg, Germany). For each sample relative amount of mRNA was normalized to an endogenous reference gene *GAPDH*. Results were obtained as threshold cycle (CT) values. Fold expression changes were calculated with the ΔΔCT method [16]. The expression levels of mRNAs were expressed as the ratio and mean ± standard error (SE) of each specific primer to *GAPDH* expression.

Statistical Analysis

Data analysis was performed using SPSS Statistics for Windows software (version 22.0; SPSS, Chicago, IL). Data were subjected to the Shapiro-Wilk test to characterize their normality, and statistical significance was determined by the Mann-Whitney *U* test. All *p*-values less than 0.05 were considered significant.

Results

The levels of mRNA expression of pro- and anti-inflammatory cytokines and bacterial HSP were determined by RT-PCR and quantified by comparison with the internal control gene *GAPDH*. Analysis revealed significantly higher mRNA levels of *TNF-a* and bacterial HSP, *cpn60,* in the tissue of PLs compared with normal periapical tissue ($p < 0.05$). Contrary to these results, the mRNA expression of anti-inflammatory *IL-10* was significantly higher in the samples of normal periapical tissue compared with the mRNA levels of this cytokine in the tissue of PLs ($p < 0.001$) (Figure 1).

Table 1

Primer sequences of analyzed genes

Gene	Sense and antisense primers
TNF-a	5′-CCAGGCAGTCAGATCATCTTC-3′ 5′-GTTATCTCTCAGCTCCACGC-3′
IL-10	5′-ATG CCC CAA GCT GAG AAC CAA GAC CCA-3′ 5′-TCT CAA GGG GCT GGG TCA GCT ATC CCA-3′
cpn60	H279: GAIIIIGCIGGIGAYGGIACIACIAC H280: YKIYKITCICCRAAICCIGGIGCYTT
GAPDH	5′-TGGAAGGACTCATGACCACA-3′ 5′-AGGGGTCTACATGGCAACTG-3′

***TNF-a* – tumor necrosis factor-alpha; *IL-10* – interleukin 10; *cpn60* – chaperonin 60; *GAPDH* – glyceraldehyde 3-phosphate dehydrogenase.**

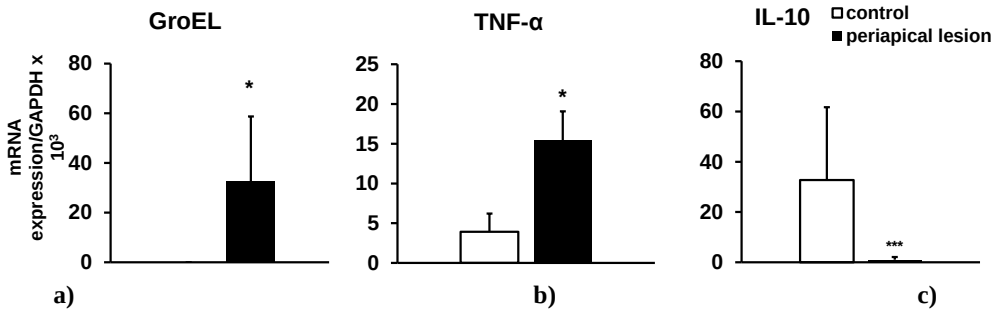


Fig. 1 – Relative mRNA expression levels for: bacterial HSP, *groEL* (*cpn60)* (a), *TNF-a* (b) and *IL-10* (c) in healthy periapical tissue (control) and tissue of periapical lesions determined by qRT-PCR. Data were normalized to the expression of the endogenous control gene, *GAPDH*. Relative expression is calculated by using the ΔΔCT method and presented as mean ± SE. **p* < 0.05, ****p* < 0.001. mRNA – messenger RNA; *TNF-a* – tumor necrosis factor-alpha; *IL-10* – interleukin 10; HSP – heat shock protein; *cpn60* – chaperonin 60; qRT-PCR – quantitative real-time polymerase chain reaction; *GAPDH* – glyceraldehyde 3-phosphate dehydrogenase; SE – standard error.

Table 2

Correlation analysis between relative gene expression levels of the analyzed genes in periapical tissue lesions

Gene		*TNF-α*	*IL-10*	*cpn60*
TNF-a	r	-	0.195	0.632**
IL-10	r	0.195	-	0.099
cpn60	r	0.632**	0.099	-

***TNF-a* – tumor necrosis factor-alpha; *IL-10* – interleukin 10; *cpn60* – chaperonin 60; r – Pearson correlation coefficient.**

****Positive correlation, significant.**

A significant positive correlation was observed between the expression levels of pro-inflammatory cytokine *TNF-a* and mRNA expression level of bacterial HSP, *cpn60* (Table 2), a well-known marker of bacterial contamination [17].

Discussion

This is the first study investigating the quantitative expression of bacterial HSP *cpn60* in the tissue of chronic PLs. Here we have shown for the first time that bacterial *cpn60*, detected by the universal target for bacterial HSP detection, degenerate PCR primers for the amplification of a 549-567 bp region of *cpn60* corresponding to nucleotides 274-828 of the *Escherichia coli* [18, 19], that it is highly expressed in PLs, and that its expression highly correlates with expression of the pro-inflammatory cytokine *TNF-a*.

In apical periodontitis, microorganisms release various molecules that stimulate the innate immune response and thus cause inflammation and tissue damage, regardless of their virulence and tissue invasiveness [20]. HSPs, in addition to their protective role, are also known to have potential to significantly influence the development of the immune response [9].

The expression of human HSPs [21], which are thought to play a role in the pathogenesis of PLs and affect the course of various odontogenic lesions [22, 23], has been established in PLs, but the expression level of bacterial HSPs in PL tissue, has not been examined so far. The negative finding of HSP60 in the epithelial rests of Malassez, and the increased level of HSP60 expression in the epithelium of radicular cysts, also indicate the connection of HSPs with inflammatory processes in PLs [24]. There are also grounds for determining the connection between the expression of certain HSPs and the genetic susceptibility for the development of PLs [25]. The proliferating and cytotoxic activities of *Aggregatibacter actinomycetemcomitans's* GroEL protein on epithelial cells were also reported [26]. In this study, we found *cpn60* expression in all samples of PLs and all samples of the control group, but this expression is significantly higher in PLs than in healthy control tissue.

Increased expression of human Cpn60 in periodontitis has been reported suggesting its significant role in immune-related events during the development of periodontal diseases and in the course of the diseases [27]. Hasan et al. [28] reported that bacterial HSP65 induces a stronger proliferation of peripheral blood T lymphocytes in patients with periodontitis compared with the human HSPs. Yamazaki et al. [29] proved a stronger proliferative response of mononuclear cells isolated from peripheral blood of patients with periodontal disease to the human HSP60 compared to the response of mononuclear cells isolated from the blood of healthy individuals, while the proliferative response of peripheral blood cells to the bacterial HSP, GroEL, was not detected.

Literature data indicate that bacterial HSP (GroEL, Cpn60), originating from different bacteria, induces the expression of cytokines in cells of the host's immune system [30, 31]. It is known that chaperonin Cpn60 from *Mycobacterium tuberculosis* induces the production of TNF-α in the human monocyte cell line, THP-1 [32, 33]. TNF-α is a mediator of osteoclastic activity in states of inflammatory osteolysis such as PLs [34]. The expression of pro-inflammatory cytokine TNF-α in PLs has been demonstrated [13]. Bacterial chaperonins are also known as strong activators of osteolytic activity, similar to TNF-α [35].

In addition, cpn60 from *Mycobacterium tuberculosis*, after the stimulation of toll-like receptor 2 (TLR2) on macrophages, induces the production of the anti-inflammatory cytokine IL-10 [36]. The anti-inflammatory activity of IL-10 is well known and is crucial in the control of periodontal diseases and inhibition of bone resorption [37].

Hence, our goal was to explore the correlation between the expression of bacterial chaperonin *cpn60* and two cytokines, *TNF-a* and *IL-10*, both expressed in the tissue of PL but with opposite effects on PL development and progression.

In this study, we have shown a strong correlation between the expression of *cpn60* and *TNF-a* in all samples of PLs. These results correlate with studies that examined the effect of bacterial HSPs on the TNF-α expression [30, 38, 39].

The higher expression of the immunoregulatory cytokine *IL-10* was found in samples of healthy periapical tissue relative to PLs. Although it has been previously shown that *Actinobacillus actinomycetemcomitans's* GroEL protein can induce the production of interferon-gamma (IFN-γ) and IL-10 in T-bet expressing $CD4^+$ T lymphocytes [31] and those human monocytes stimulated with GroEL have increased expression of IL-10 [30], no correlation in expression of *IL-10* and *groEL (cpn60)* in the tissue of PLs was found in this study. Instead, we have found higher expression of IL-10 in healthy tissue in comparison with the tissue of PLs. Recently, an increased expression of IL-10, 7 days after the reduction of the intracanal microbial load, has been observed [14]. Thus it could be possible that lower expression of *IL-10* in the tissue of PLs and the absence of correlation with the expression of *cpn60* is associated with the clinical characteristics of these lesions.

Conclusion

Based on the results obtained from a small number of patients, we can conclude that the expression of the bacterial HSP *cpn60* in the tissue of PLs strongly correlates with the expression of the pro-inflammatory cytokine *TNF-a*, imposing *cpn60* as a stimulator of inflammatory response in periapical tissue and one of the players in the pathogenesis of PLs.

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