Gingival tissue heat shock protein expression in aggressive and chronic periodontitis

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Abstract

Aims: The purpose of the study was to examine the gingival tissue mRNA and protein expression of heat shock protein (HSP) 60 and HSP70 in generalized chronic periodontitis (GCP), generalized aggressive periodontitis (G-AgP) and individuals with periodontal health (PH).

Materials and methods: Gingival tissue biopsies were acquired from 39 patients with G-CP (n=39), G-AgP (n=37) and PH (n=15). Localization and mRNA expression of HSP60 and HSP70 were evaluated by immunohistochemistry and quantitative real-time PCR. Probing depth (PD), clinical attachment level (CAL), plaque index (PI) and bleeding on probing (BOP) were assessed. Non-parametric statistical methods were used.

Results: The mRNA expression of HSP60 was significantly higher in the G-AgP (1.7-fold, $P<0.001$) and G-CP (2.5-fold, $P<0.001$) than the PH group. The mRNA expression of HSP60 was positively correlated with the CAL, PI and BOP. HSP70 mRNA expression did not significantly differ among the study groups ($P>0.05$). In gingival tissues of G-CP and G-AgP strong positive staining of HSP60 and HSP70 was observed both in the epithelium and lamina propria.

Conclusion: The higher mRNA expression of HSP60 in both aggressive and chronic periodontitis points a relationship of this molecule with the pathogenesis of periodontitis. HSP70 mRNA expression levels did not show any contribution to periodontal disease.

Keywords: Chronic periodontitis, aggressive periodontitis, heat shock proteins, gingiva

Introduction

Periodontitis is a common infectious disease characterized by inflammation-mediated destruction of tooth-supporting tissues. The presence of periodontopathogens, the primary etiologic agent, is required but not sufficient, for disease initiation (Graves, 2008). The initiation and progress of periodontal disease depend on complicated interactions between periodontopathic bacteria and the host immune system. The antigens, lipopolysaccharides and other virulent factors of microorganism are known to be a potent stimulator of host inflammatory mediators (AAP, 1999; AAP, 2002). Chronic (G-CP) and aggressive periodontitis (G-AgP) are two principal forms of periodontitis and differ in their clinical phenotypes (Armitage and Cullinan, 2010). These two forms do not exhibit adequately different histopathological characteristics or microbiological features (Armitage, 2010) but there is evidence of immunological differences, including the existence of neutrophil abnormalities in G-AgP (Ryder, 2010).

Heat shock proteins (HSPs) are present in all living organisms from bacteria to humans and rapidly upregulated when the cells exposed to environmental stress such as elevated temperatures, infection and inflammation (Kaufmann, 1990). Bacterial and human HSPs show extensive homology (up-to 50–60%) (Kiessling *et al.*, 1991; Honda *et al.*, 2006). A variety of cell types express HSPs, including neuronal cells, monocytes, macrophages, B cells and tumour cells of epithelial origin (Robinson *et al.*, 2005; Clayton *et al.*, 2005; Davies *et al.*, 2006). HSPs mainly act as chaperones and have

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function in protein folding, protecting proteins from denaturation or aggregation and protein transport through membrane channels (Ellis *et al.*, 1996; Hartl, 1996). Moreover, bacterial HSPs are known to generate a strong pro-inflammatory response in human cells of the innate immune system, such as macrophages (Ueki *et al.*, 2002; Yamazaki *et al.*, 2002).

HSPs are clustered into several groups based to their molecular weight. HSP60 and HSP70 are among the small HSPs. HSP60 has been implicated in regulation of cell signaling and inflammatory responses (Pockley, 2003). The HSP70 family comprises several members, the best known of which is the heat inducible HSP72 and the constitutively expressed HSP73/HSP73 (Mayer, 2010; Bethke *et al.*, 2002). HSP70 was shown to be an activator of the innate immune system and has antigen presenting function, which is important as an initiator of immune defence (Mayer, 2010; Fincato *et al.*, 1991). Although, the role of bacterial HSPs in periodontal disease pathogenesis is still not well established, there is some evidence that periodontal pathogens including *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, *Prevotella intermedia* express HSPs (Hotokezaka *et al.*, 1994; Nakano *et al.*, 1995; Vayssier *et al.*, 1994; Tabeta *et al.*, 2001). Furthermore, it has been demonstrated that gingival tissue lysates and serum obtained from patients with chronic periodontitis had a positive response to human HSP60 (Tabeta *et al.*, 2000). Moreover, subgingival biofilms can enhance HSP 60 and HSP70 expression in gingival fibroblasts (Ando *et al.*, 1995; Belibasakis, 2014).

Although these findings suggest that HSPs may have a role in the pathogenesis of chronic forms of periodontitis, the gingival tissue expression and localization of HSP60 and HSP70 in healthy periodontium, aggressive and chronic forms of periodontal disease are not clear. We hypothesized that HSP60 and HSP70 play a role in periodontal lesions and their expression patterns in gingiva may differ among the different clinical forms of periodontitis. Therefore, the aim of this study was to evaluate HSP60 and HSP70 expression and localization in gingival tissues obtained from generalized chronic periodontitis (G-CP), generalized aggressive periodontitis (G-AgP) and periodontally healthy volunteers by quantitative real time polymerase chain reaction (qPCR) and immunohistochemical methods.

Materials and Methods

Study population

Ninety-one patients including G-AgP (n=37), G-CP (n=39) and PH (n=15) were recruited from the Department of Periodontology, School of Dentistry, Ege University, İzmir. Patient recruitment was performed between December 2012 and December 2014. The study protocol was approved by the Ethics Committee of the School of Medicine, Ege University (ethics approval number:11–12.1/11). The aim of the study was explained to each participant before entering the study and written informed consent in accordance with Helsinki declaration was obtained from each participant. Medical and dental histories were taken from all participants.

None of the participants had a history of systemic disease, which could impair immune responses, and none had used antibiotics or other medicines that could affect their periodontal status or periodontal treatment in the last 3 months. All participants received radiographic examination upon entering to the study. Subjects were included if they had a minimum 16 teeth.

The subjects were selected according to the clinical criteria proposed by the 1999 International World Workshop for a Classification of Periodontal Disease (Armitage, 1999) and intra-oral radiographs were used to determine the alveolar bone loss.

The patients in the G-AgP group exhibited a generalized pattern of severe destruction with clinical attachment loss (CAL) ≥ 5 mm, probing depth (PD) ≥6 mm on 8 or more teeth (minimum 3 of these were other than central incisors or first molars) and radiographic bone loss ≥ 30% of the root length on the affected teeth. G-AgP patients had CAL that was not commensurate with the presence of local factors. The patients in the G-CP group had moderate to severe alveolar bone loss present in radiography and CAL of ≥ 5 mm and PD of ≥ 6 mm in multiple sites of all four quadrants of the mouth. G-CP patients had CAL that was commensurate with the presence of local factors. The patients in the periodontally healthy group exhibited full-mouth PD ≤3 mm, no gingival recession due to periodontitis (due to mechanical forces i.e tooth brushing) and full-mouth CAL ≤ 2 mm at more than or equal to 90% of the measured tooth sites as well as full-mouth bleeding on probing in less than 10% of the probing sites at examination. These patients presented no radiographic evidence of alveolar bone loss.

The PD, CAL, plaque index (PI) (Quigley and Hein, 1962) and dichotomous measurement of bleeding on probing (BOP) were recorded at six sites around each tooth (mesiobuccal,mid-buccal, disto-buccal, mesio-lingual, mid-lingual and disto-lingual locations). A manual Williams probe was used for the PD and CAL measurements. Clinical measurements were performed by two calibrated examiners (S.B. and V.Ö.Ö.). The intra-examiner reliability was high as shown by an intra-class correlation coefficient of 0.88 (S.B.) and 0.90 (V.Ö.Ö.) for PD measurements. The inter-examiner agreement for the measurement of PD was reliable, with an inter-examiner reliability coefficient of 0.82. Patients were recalled for tissue sampling procedures two days after the periodontal index was recorded.

Gingival tissue sampling

The healthy gingival tissue samples were acquired from teeth extracted for orthodontic reasons or crown-lengthening surgeries of healthy volunteers. The healthy tissues were obtained from sites with PD of 2 mm or less and the absence of BOP. The tissue samples in G-AgP and G-CP groups were collected from sites with a PD of ≥6 mm, CAL of ≥5 mm and presence of BOP under local anesthesia before nonsurgical periodontal therapy. Gingival tissue samples involving the inter-proximal papilla and the periodontal sulcus/pocket with approximate thickness of 2–3 mm and height of 1.5 mm were obtained from periodontal patients and healthy volunteers. Gingival tissue biopsies included both epithelium and connective tissue and were obtained from the single-rooted teeth by internal bevel and sulcular incisions using 15c blade. The biopsies obtained were separated into two portions; one portion was used for tissue mRNA expression analysis while the other part was used for immunohistochemistry.

Analysis of HSP60 and HSP70 mRNA levels

The gingival tissue samples were immersed in RNA stabilization reagent (RNA Later, Ambion, CA, USA) according to the manufacturer's instructions and then frozen at -80°C. The AllPrep Kit (Qiagen, Basel, Switzerland) was used in the extraction of total RNA. One microgram of total RNA was reverse transcribed into single- stranded cDNA using M-MLV Reverse Transcriptase, Oligo(dT)15 Primers and PCR Nucleotide Mix (Promega, Manheim, Germany) in accordance with the manufacturer's protocol. StepOne Plus Real Time PCR System, the TaqMan Gene Expression Master Mix and Gene Expression Assay kits (Applied Biosystems, Life Technologies, Basel, Switzerland) were used in the HSP60 (Assay ID: Hs01036753) and HSP70 (Assay ID: Hs00359163) gene expression analysis. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Assay ID: Hs99999905) was used as a house keeping gene. The expression levels of HSP60 and HSP70 transcripts in each sample were calculated using the $2^{-\Delta CT}$ method.

HSP60 and HSP70 protein expression in gingival tissues

Gingival tissue samples were stored in 4% paraformaldehyde (Sigma Chemical Co., St Louis, MO, USA.) for 24 hours and were embedded in paraffin wax. Serial coronal sections were obtained by cutting from paraffin blocks on a microtome and sections were placed on glass slides. According to the routine protocols the sections were deparaffinized and rehydrated by embedding in graded ethanol series. The goat ImmunoCruz Staining System (Santa Cruz Biotechnology, Inc., Dallas, TX, USA.) was used for immunostaining of sections in accordance with the manufacturer's directions with several adjustments. A citrate buffer-based antigen retrieval protocol was recycled and the antigenic epitope was unmasked. The slides were embedded in 10 mM sodium citrate buffer (pH 6.0) and heated for 10 min. The slides were left and cooled in buffer for 20 min then washed with deionized water. All sections were embedded in 3% hydrogen peroxide for 20 min at room temperature in order to block endogenous peroxidases completely. The sections were pretreated with blocking buffer for prevention of nonspecific reaction with primary antibody. After three washes with PBS, the slides were incubated for overnight in a fridge on a shaker with monoclonal mouse anti-human antibody against HSP60 (Catalog number:MA1-35434, Concentration: 1mg/ml), (Thermo Scientific Pierce, Rockford, IL, USA.) and or HSP70 (Catalog number: LF-MA0101, Concentration: 1mg/ml) (Thermo Scientific Pierce.) or a control mouse IgG (Santa Cruz Biotechnology, Inc.). Then, the slides were incubated with biotinylated anti-mouse secondary antibody (Santa Cruz Biotechnology, Inc.) which was flowed by three washes for 5 minutes with deionized water. The antibody doses were optimized starting from suggested working dilutions in each antibody product data sheets. The optimal dilution was 1:5. HRP-streptavidin complex was added to the slides for 60 minutes in dark, and following a rinsing step, the DAB substrate was added to the slides for 10 minutes. The nuclei of the cells were counterstained with hematoxylin, and subsequently mounted and covered with glass coverslip. The slides were examined using x10, x20, x40 magnifications. Negative Control Mouse IgG was used in place of a primary mouse monoclonal antibody with to evaluate nonspecific staining. The respective images are provided as supplementary files (Supplementary Files 1, 2 & 3).

Statistical analysis

Normality of the data was assessed by D'Agostino-Pearson omnibus normality test. Statistical analysis was performed using non-parametric tests and the patient was used as the unit of the observation. Comparisons between the study groups were performed using the Kruskal- Wallis test. Dunn's test was used to correct multiple comparisons and p-values $p < 0.05$ were considered to be statistically significant. Spearman's rank correlation analysis was used to correlate clinical parameters of the sampling sites and HSP60 or HSP70 tissue levels and $p < 0.05$ was considered as significant. Correlation between age and HSP60 or HSP70 tissue levels was also detected with Spearman's rank correlation analysis and $p < 0.05$ was considered as significant.

Results

Clinical and demographical findings

The clinical parameters of the sampling sites was presented in Table 1. The PD, CAL, presence of BOP and PI of the sampling sites were significantly higher in G-AgP and G-CP groups when compared with the control group ($p<0.05$). The G-AgP and G-CP groups had similar PD, CAL, BOP and PI ($p > 0.05$). The only difference in ages among the study groups was between G-CP and G-AgP groups ($p <0.05$).

mRNA expressions of HSP60 and HSP70 in gingival tissues

mRNA expression of HSP60 was shown in all samples studied (Figure 1). HSP60 mRNA was significantly elevated in G-CP group when compared to healthy group (2.5 fold, $p <0.001$). G-AgP group also had significantly higher HSP60 gene expression levels compared to the control group (1.7-fold, $p < 0.001$). No significant difference was detected in HSP60 expression levels between the G-CP and G-AgP groups ($p >0.05$). mRNA expression of HSP60 was positively correlated with the CAL (R=0.274, p =0.043*), PI (R=0.264, p =0.049*) and BOP (R=0.338, p =0.012*) of the sampling sites. No significant correlation was observed between mRNA expression of HSP60 and age of the participants.

All samples showed ubiquitous mRNA expression of HSP70 (Figure 2). HSP70 mRNA expression was higher in both G-CP and G-AgP groups when compared to healthy group (1.3 fold and 1.1 fold, respectively), but the difference was not significant for both groups ($p >0.05$). The mRNA expression levels of HSP70 did not show a correlation with the clinical periodontal parameters of the sampling sites and age of the participants.

Immunohistochemical detection of HSP60 and HSP70 in gingival tissues

Expression of HSP60 protein in human gingival tissues of patients with G-AgP and G-CP, as well as in clinically healthy individuals, was assessed using immunohistochemistry. Immunohistochemical expression of HSP60 was detected in all samples studied including gingival tissues of periodontally healthy volunteers (Figure 3: A1, A2, A3). Various amounts of inflammatory cell infiltration and granulation tissue were present in tissues with chronic and aggressive forms of the disease. In gingival tissues with G-CP (Figure 3: B1, B2, B3) and G-AgP (Figure 3: C1, C2, C3), strong staining of HSP60 was observed in the connective tissue infiltrate, the cells infiltrating epithelium and also the endothelial cells of blood vessels. HSP60 positive cells were mainly localized nearby the blood vessels. HSP60 was expressed mainly on the surface and/or nucleus of polymorphonuclear neutrophils, macrophages and lymphocytes as well as fibroblast and endothelial cells in G-CP and G-AgP groups.

The protein expression pattern of HSP70 in gingival tissue was also evaluated (Figure 4). All tested gingival tissues had immunopositivity for HSP70. The epithelial cells had a dense HSP70 expression. The connective tissue infiltrate and the endothelial cells of blood vessels were also positively stained for HSP70. HSP70 was expressed mainly on the surface and/or nucleus of polymorphonuclear neutrophils, macrophages and lymphocytes as well as fibroblast and endothelial cells in G-CP and G-AgP groups.

Discussion

In the present study, HSP60 mRNA expression was found to be higher in gingival tissues from patients with G-CP and G-AgP than those of the healthy controls, while no difference was detected in HSP70 mRNA expression.

Table 1. Clinical periodontal parameters of the sampling sites in the study groups [mean ±SD].

	Healthy (N=15)	Chronic Periodontitis (N=39)	Aggressive Periodontitis (N=37)
Female/Male	7/8	21/18	20/17
Age (years)			
Mean±std	38.95 ±13.31	46.32 ± 7.9	34.8 ± 5.76†
Min-max	30-63	35-52	26-40
PD (mm)	1.32 ± 0.63	6.84 ± 1.75*	6.58 ± 1.93*
CAL (mm)	1.32 ± 0.63	7.86 ± 2.64*	7.76 ± 2.28*
PI	0	2.30 ± 0.50*	2.36 ± 0.34*
BOP	0	90.3 ± 28.35*	91.0 ± 63.36*

*= Significantly different from the healthy group. (P<0.001) †= Significantly different from the Chronic periodontitis group. (P<0.05)

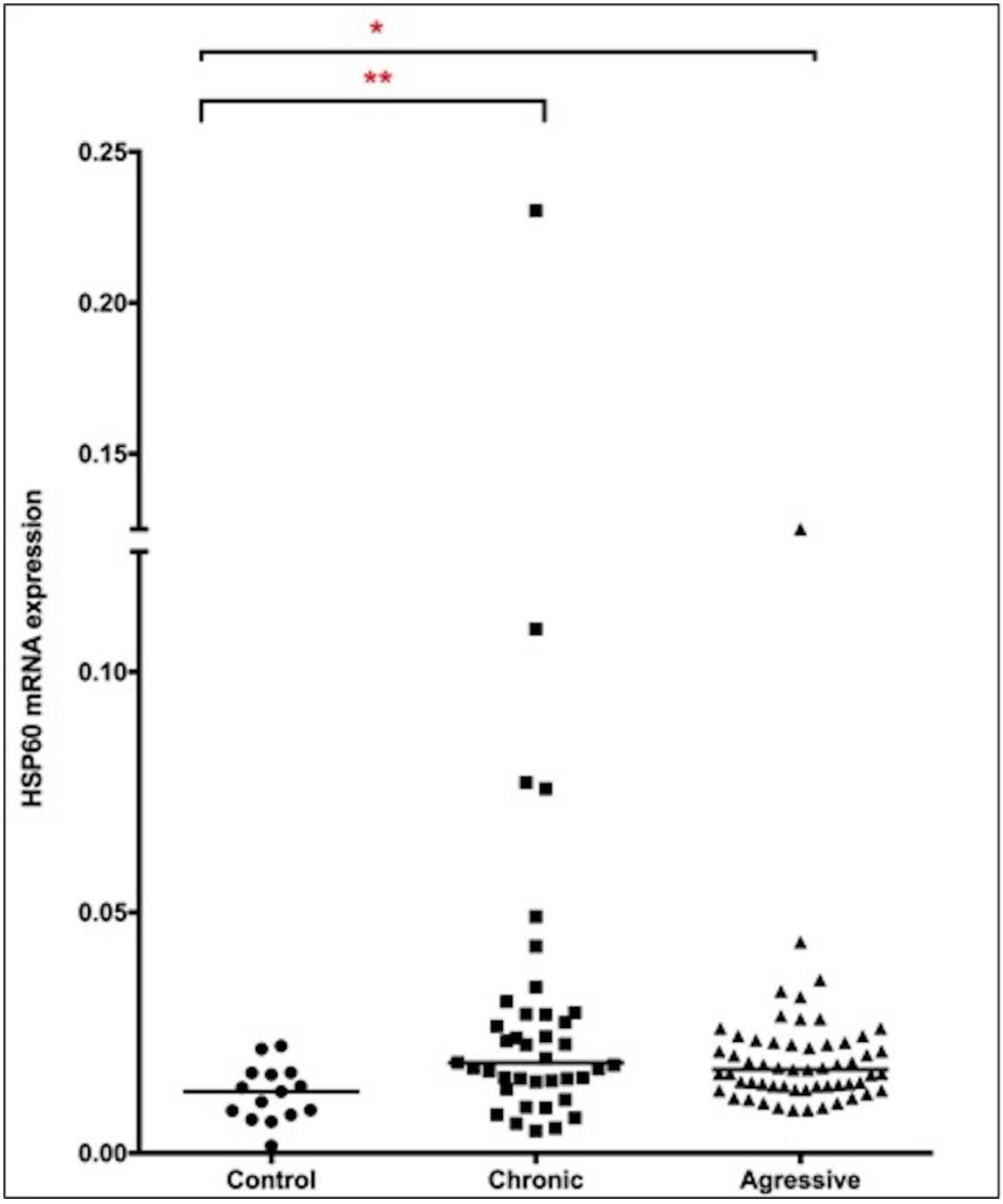

Figure 1: The mRNA expression of HSP60 in the study groups. The mRNA expression levels were measured using quantitative PCR (qPCR) analysis, normalized against the expression levels of the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase. *: mRNA expression of HSP60 in chronic periodontitis group is higher than the H group (P<0.001). **: mRNA expression of HSP60 in agressive periodontitis group is higher than the H group (P<0.001).

HSP60 levels positively correlated with BOP, PI and CAL indicating an association with sites specific inflammation and bacterial load. Immunohistochemistry analysis showed that HSP60 expression was distributed in the connective tissue infiltrate as well as in the gingival epithelium.

HSPs primarily function in the chaperoning of intracellular proteins during protein folding at the ribosome. During cellular stress, rapid up-regulation of HSP expression helps prevent extensive protein degradation and reinstate cellular homeostasis (Hartl and Hayer-Hartl, 2002; Meunier *et al*., 2002). Host immune response to microbial HSP 60 has been shown to initiate chronic inflammatory diseases in which autoimmune responses to human HSP60 may be central to the pathogenesis of chronic disease (Kiessling *et al*., 1991). The incidence of seropositivity and the antibody titre to human HSP60 and *P. gingivalis* GroEL has been shown to be significantly higher in patients with periodontitis than in periodontally healthy controls (Tabeta *et al*., 2000). It was suggested that an immune response originating from

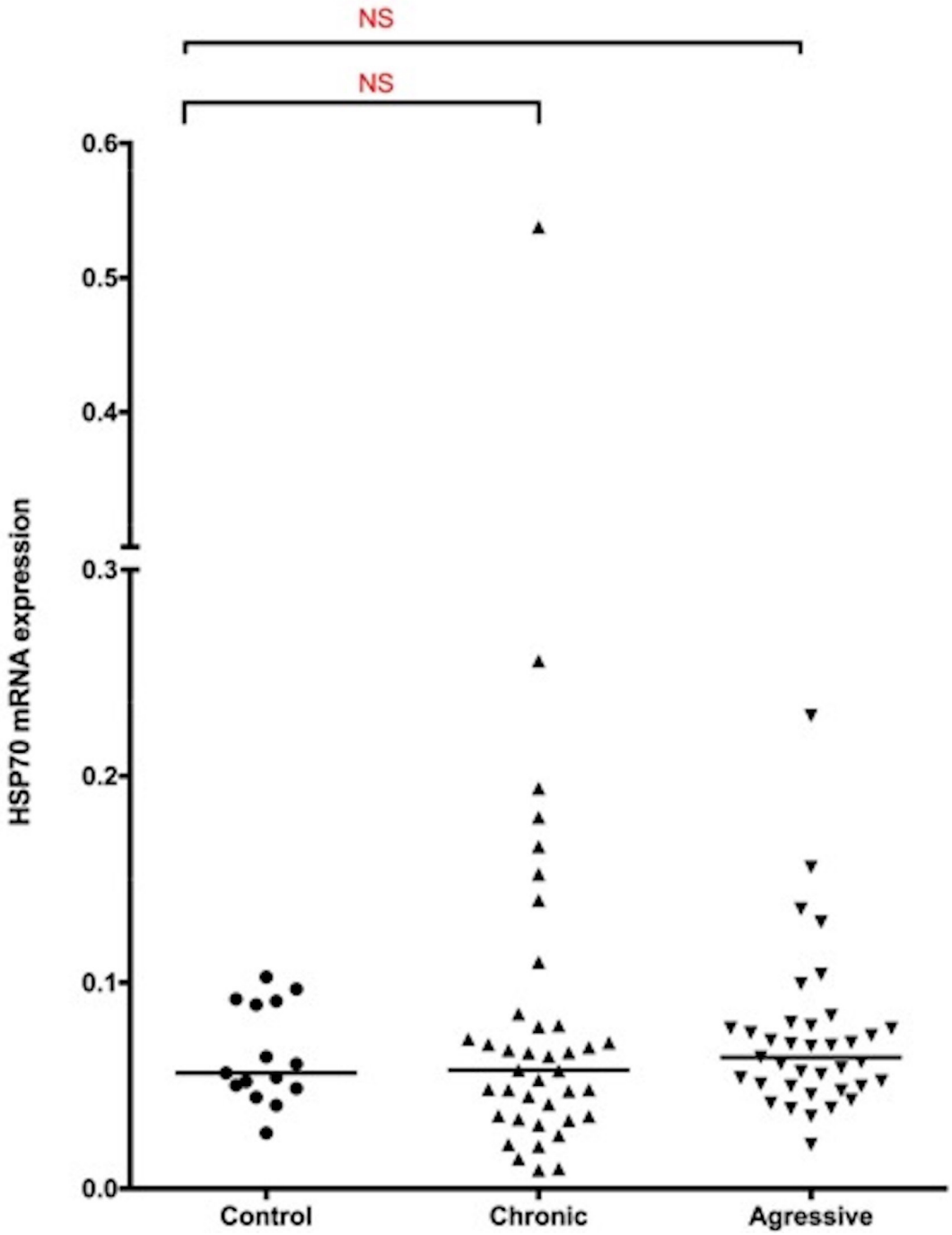

Figure 2: The mRNA expression of HSP 70 in the study groups. The mRNA expression levels were measured using quantitative PCR (qPCR) analysis, normalized against the expression levels of the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase.

the molecular mimicry between *P. gingivalis* GroEL and human HSP60 might have a role in the pathogenesis of periodontitis (Ueki *et al.*, 2002).

In both periodontitis and gingivitis lesions, mainly B cells express HSP60 and HSP70 (Domon *et al.*, 2009). Furthermore gingival homogenate samples from patients with adult periodontitis react with anti-human HSP60 and bovine brain HSP70 monoclonal antibodies and it has been concluded that infiltrating inflammatory cells and endothelial cells increase their expression of endogenous HSP antigens during the development of periodontitis (Ando *et al.*, 1995). In another study, HSP60 was found to be abundantly expressed in periodontitis lesions (Ueki *et al.*, 2002). Immunohistochemical analysis of HSP60 expression demonstrated that basal cells of both oral and pocket epithelium were positive and many mononuclear cells in the inflammatory cell infiltrate beneath the pocket epithelium also stained positively (Ueki *et al.*, 2002). In line with these previous studies, HSP60 mRNA expression was found to be higher in gingival tissue of patients with G-CP and G-AgP than those of healthy controls (Ueki *et al.*, 2002; Pleguezuelos

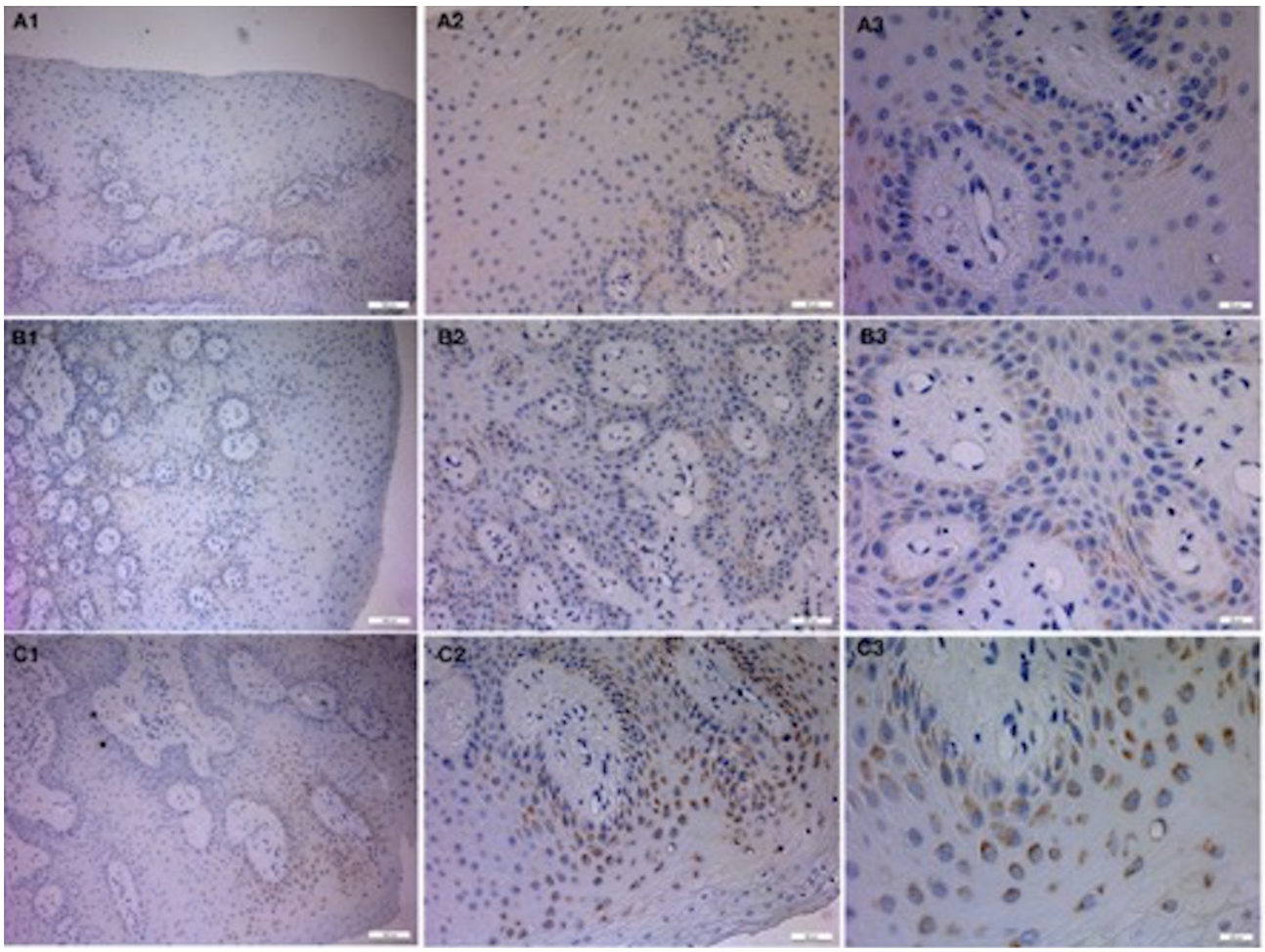

Figure 3: Localization of HSP 60 in gingival tissues
A:Stanining for HSP 60 in healthy group (A1 with x10, A2 with x20 and A3 with x40 magnifications).
B:Stanining for HSP 60 in G-CP group (B1 with x10, B2 with x20 and B3 with x40 magnifications).
C:Stanining for HSP 60 in G-AgP group (C1 with x10, C2 with x20 and C3 with x40 magnifications).

et al., 2005). Interestingly, HSP60 expression did not differ among the two forms of periodontitis. Although, the earlier studies have indicated that HSP expression may be altered with age (Njemini *et al*., 2011), we could not find a significant association between age and HSP expression in our cohort.

Periodontopathic bacteria stimulate the expression of HSP60 by cells in the periodontium, which may enhance the production of proinflammatory cytokines by macrophages and possibly other cells, leading to the chronic inflammation and tissue destruction in chronic periodontitis (Ueki *et al*., 2002). Furthermore, oral keratinocytes respond to LPS from oral pathogens by increasing the secretion of self-HSP60 and treatment of oral keratinocyte cells with rhHSP60 also resulted in increased gene expression of the inflammatory cytokine IL-1b and increased IL-1b protein secretion (Pleguezuelos *et al*., 2005).

Taken together our results also point to the possible role of HSP60 in chronic and aggressive forms of periodontal disease while no significant difference in the tissue expression of HSP70 was shown. In a recent study, it was shown that HSP70 family proteins, specifically HSPA1B, HSPA2 and HSPA4 were markedly down-regulated in inflamed periodontal tissues (Seo *et al*., 2016). In the study by Seo *et al*., tissue specimens were used from patients with gingivitis or periodontitis and grouped according to the presence of inflammation determined by light microscopy. The difference in findings between the present study and the study by Seo *et al*., (2016) might originate from the differences in the study group designs.

Our study has some potential limitations. The first is the significant difference in mean ages of the CP and AgP groups which is almost inevitable due to the classification criteria used to diagnose the disease. Age correlation analysis was performed to see the effect of age and no significant correlation was observed between age and tissue expression of HSP60 and HSP70. The second limitation concerns the absence of a gingivitis group. The presence and localization of HSP60 and HSP70 was qualitatively shown by immunohistochemistry but quantification of these data could not be performed, which might be another limitation of the present study. Quantification of protein expression in gingival tissue

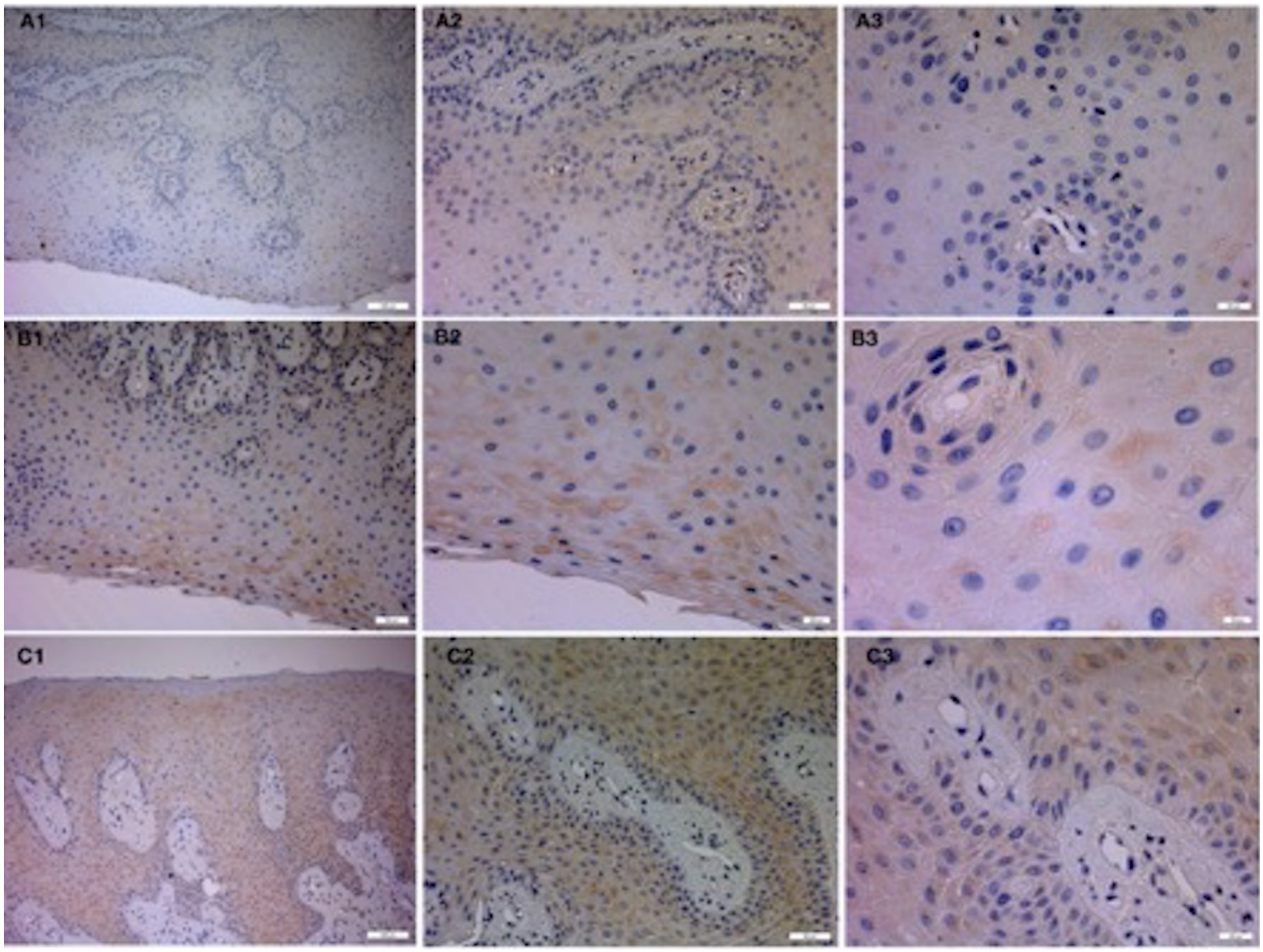

Figure 4: Localization of HSP 70 in gingival tissues . (A): Stanining for HSP 70 in healthy group (A1 with x10, A2 with x20 and A3 with x40 magnifications). (B): Stanining for HSP 70 in G-CP group (B1 with x10, B2 with x20 and B3 with x40 magnifications). (C): Stanining for HSP 70 in G-AgP group (C1 with x10, C2 with x20 and C3 with x40 magnifications).

samples by immunohistochemistry would provide important information, but non-quantified immunohistochemistry studies are also available in the literature (Ueki *et al.*, 2002; Nezu *et al.*, 2017).

In conclusion, the elevated gingival tissue expression level of HSP60 but not HSP70 in G-CP and G-AgP indicates an association of HSP60 with periodontitis. HSP60 expression did not differ among the two forms of periodontitis. The pronounced expression of HSP60 in both chronic and aggressive forms of periodontal tissues needs further investigation to clarify the exact roles of HSP60 in periodontal tissue destruction.

Acknowledgments and conflict of interest

No conflicts of interest exist for any person or institution involved in this study. Veli Özgen Öztürk contributed to collection of clinical data and samples and edited the manuscript. Sema Becerik contributed to the study design, data collection, data analysis and drafted the manuscript. Nagihan Bostanci contributed to the study design, data analysis and edited the manuscript. Gülnur Emingil contributed to the study design, data analysis and editing of the manuscript.

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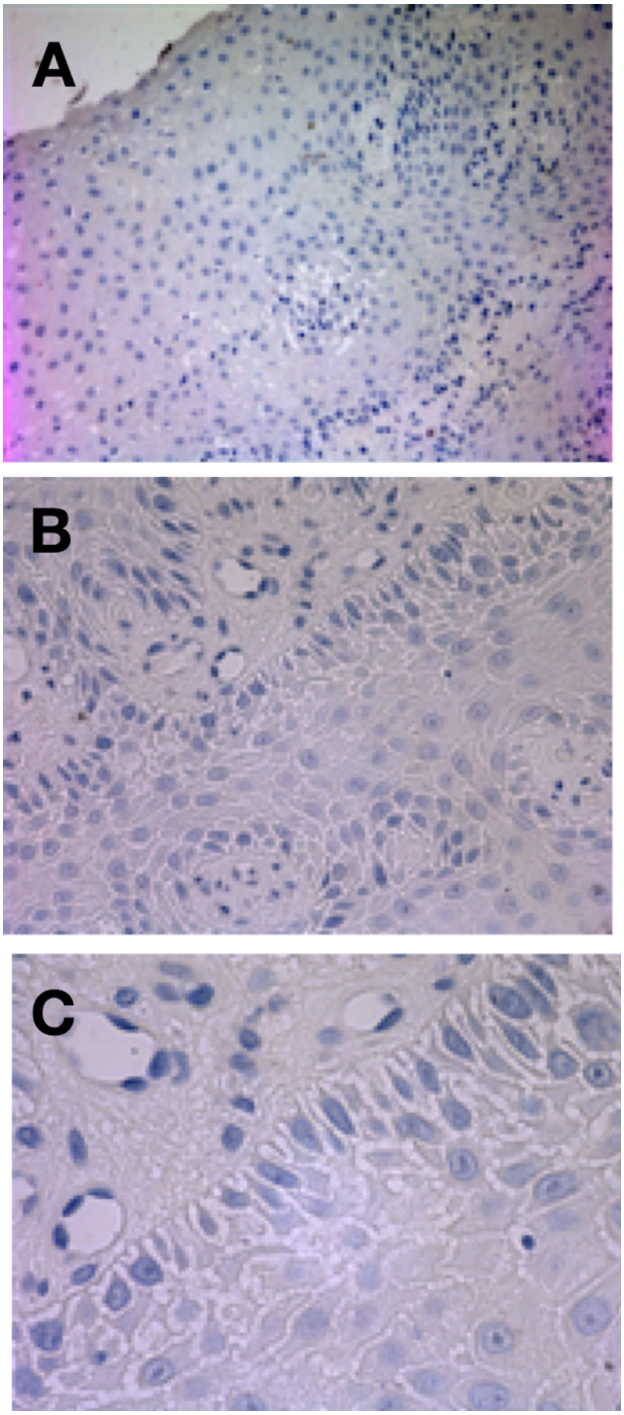
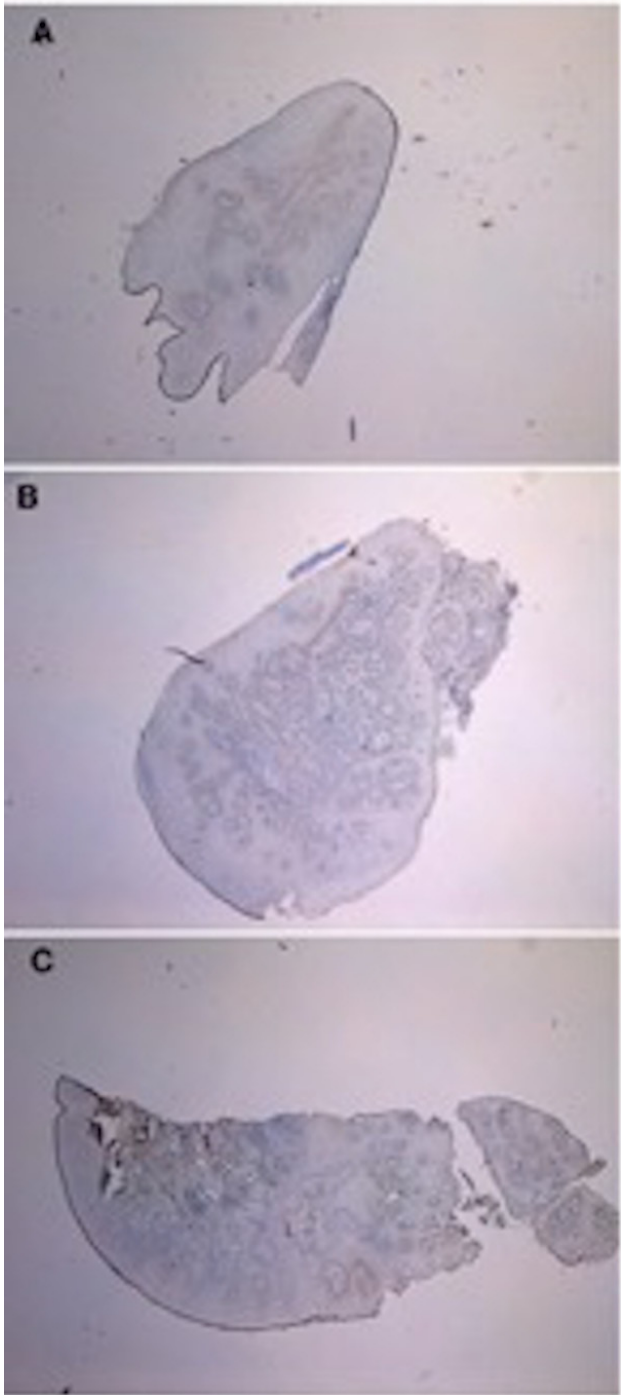
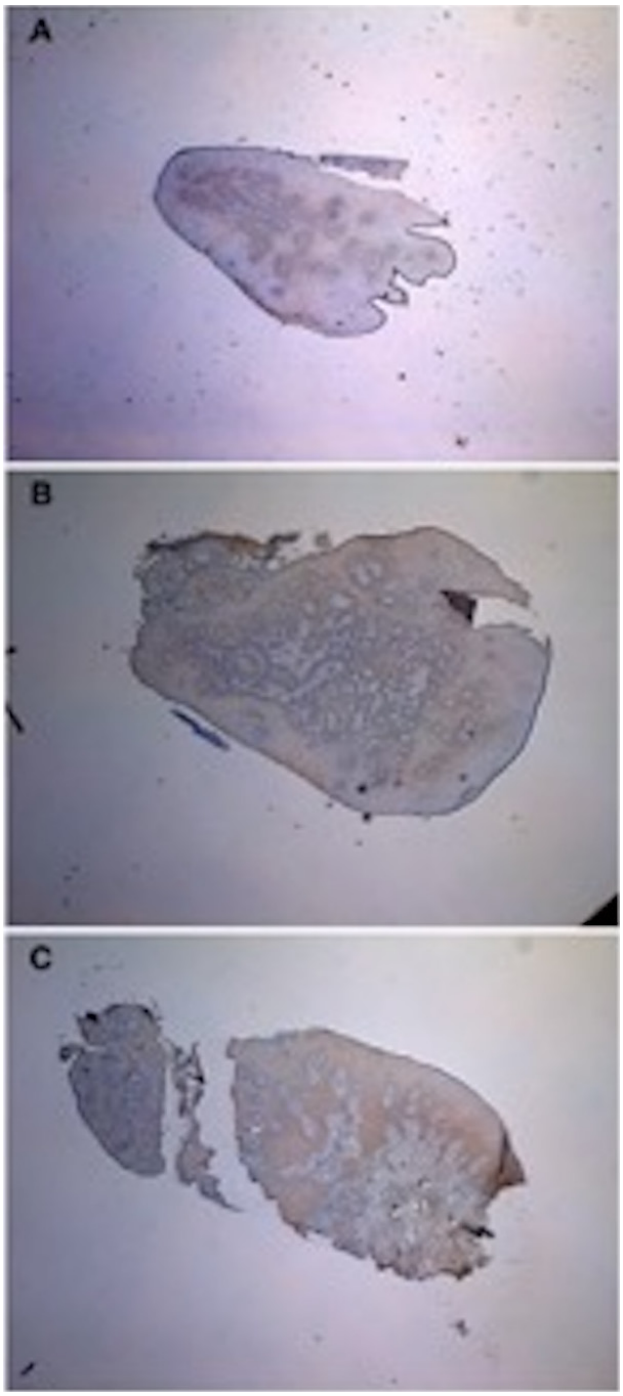
Supplementary files



Supplementary File 1: No negative/non-immune staining of tissues for IHC. (A: x16, B: x32, C: x64 magnifications)



Supplementary File 2: Localization of HSP 60 in gingival tissues. A: Stanining for HSP 60 in healthy group with x10 magnification. B: Stanining for HSP 60 in G-CP group with x10 magnification. C: Stanining for HSP 60 in G-AgP group with x10 magnification



Supplementary File 3: Localization of HSP 70 in gingival tissues. A: Stanining for HSP 70 in healthy group with x10 magnification. B: Stanining for HSP 70 in G-CP group with x10 magnification. C: Stanining for HSP 70 in G-AgP group with x10 magnification