

## Influence of the Etiological Factors for Gingival Enlargement on some Angiogenic and Inflammatory Mediators: An immunohistochemical study

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**Abstract:** Inflammatory gingival enlargement is the most common inflammatory gingival disease and is associated with multiple factors including inflammation due to bacterial plaque colonization, as aside-effect of systemic medications, prolonged orthodontic treatment, and other etiological factors. This study investigated the effect of different etiological causes of gingival enlargement; including the treatment with cyclosporine A, the plaque, and the orthodontic treatment; on the angiogenic inflammatory mediators such as vascular endothelial growth factor (VEGF), cyclooxygenase-2 (COX-2), 5-lipoxygenase (5-LO), and tumor necrosis factor- (TNF- ), using histopathological and immune-histochemical analysis. The results of the immune-histochemical analysis of different angiogenic inflammatory mediators in the gingival enlargement samples indicated that in cyclosporine A-induced enlargement neither of VEGF, COX-2, 5-LO, nor TNF- were affected, while there is a remarkable general over-expression of VEGF, COX-2, and 5-LO in the parakeratinized epithelial surface, the epithelial layer, connective tissue and in the fiber bundles regions of plaque-induced enlargement gingival. Additionally, orthodontic treatment samples indicated that there is a very high expression of VEGF in the epithelial layer of gingival but not in the connective tissue nor in the fiber bundles regions with no change in COX-2, 5-LO, nor TNF- expression. In conclusion, this report indicated that the expression of different angiogenic and inflammatory mediators in gingival enlargement is influenced by the etiological factor that initially induced this enlargement.

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### 1.Introduction

The inflammatory gingival enlargement is caused by bacterial plaque colonization on tooth surfaces and subsequent invasion of the micro-organisms. Clinically detectable fibrotic overgrowth of gingiva is caused by a variety of etiological factors and is aggravated by local bacterial plaque accumulation. Affected gingival tissues are red, oedematous, soft in consistency, and may bleed when gently probed Clocheret et al.,(2003). In some pathological conditions, gingivitis caused by plaque accumulation can be more severe. Gingival enlargement can be inherited, or is of idiopathic origin, or is sometimes associated with other systemic diseases. The majority of cases, however, occur as a side-effect of systemic medications. There is now general agreement that gingival overgrowth lesions all contain fibrotic or expanded connective tissues with various levels of inflammation and an enlarged gingival epithelium Clocheret et al.,(2003). The degrees of inflammation,

fibrosis, and cellularity depend on the duration, dose, and identity of the drug, on the quality of oral hygiene, and on individual susceptibility that stems from genetic factors and environmental influences Trackman and Kantarci (2004).

The clinical presentation of gingival overgrowth varies from a non-inflamed, firm and fibrous condition to an edematous, hemorrhagic appearance with a tendency to spontaneous bleeding. It is usually begins at the interdental papilla in a lobular form, while at later stages it affects the entire gingival and extends coronally, leading to functional, esthetic and phonetic complication, Costa et al.,(2007). A patient history helps establish the inflammatory enlargement as acute or chronic, localized or generalized. Chronic enlargements are generally painless and slow to progress, whereas acute enlargements are characterized by a painful, rapid onset, Lundergan (2003). Chronic inflammatory gingival enlargements are generally associated with identifiable systemic or

local factors. The primary local factor associated with these enlargements is plaque. Secondary local factors can include calculus, poor dental restorations, prolonged orthodontic treatment, orthodontic braces, caries, tooth crowding or misalignment, open contacts with food impaction, mouth breathing, and removable appliances. A gingival enlargement that is inflammatory and fibrotic can represent an enlargement that was initially fibrotic with secondary inflammation or an enlargement that was initially inflammatory but has become secondarily fibrotic, Lundergan (2003)..

During the past few years, the list of the medications causing a similar gingival overgrowth condition has increased. These medications include the anti-seizure drug phenytoin, the immune suppressor cyclosporin A, and certain anti-hypertensive dihydropyridine calcium-channel-blockers, most notably nifedipine, Trackman and Kantarci (2004).

Cyclosporine A is a lipophilic, hydrophobic, cyclic undecapeptide used to prevent rejection of transplanted organs and to treat various autoimmune diseases, Seymour RA, Jacobs(1992). A common side effect of cyclosporine is gingival overgrowth which is characterized by epithelial hyperplasia, interstitial fibrosis, changes in blood vessel profile, and focal inflammatory cell infiltrate, Chiu et al.,(2008). A diagnosis of drug induced gingival enlargement can be made if the development of the fibrotic enlargement coincided with the administration of one of these medications. The gingival tissues are enlarged, oedematous, soft, and tender to touch, with a tendency to bleed easily. The gingiva is bluish-red with some pseudo-membrane plaques covering ulcerated surfaces. In some conditions, gingival enlargement can progress rapidly into destructive periodontal diseases, as a result of the altered immune response of the gingiva to the bacterial plaque, Trackman and Kantarci (2004).

Orthodontic treatment may initiate oral clinical manifestations, such as labial desquamation, Lindsten and Kurol (1997) gingival enlargement, Bishara et al.,(1993); Genelhu et al.,(2005); Kouraki et al.,(2005) and multifactorial erythema, Starkjaer and Menne(1990) gingivitis Shelley (1981). Such manifestations are usually associated with the inflammatory response induced by the corrosion of orthodontic appliances, and major emphasis has been placed on nickel, Eliades et al.,(2003). Inflammatory response to nickel is considered as type IV hypersensitivity and is manifested as nickel allergic contact stomatitis, Holmstrup(1999); Vanarsdall(2000).

Gingival enlargement is a more common sequela of orthodontic treatment than other manifestations, Genelhu et al.,(2005); Genelhu et al.,(2005); Gursoy

et al.,(2007). Fibrous gingival enlargements associated with fixed orthodontic appliances seem to be transitory, and it is generally thought that enlargement resolves after orthodontic therapy, Carranza (2006). However, there are also studies reporting that this resolution is not complete Ramadan (2004). Orthodontic treatment-induced gingival overgrowth shows a specific fibrous and thickened gingival appearance, different from fragile gingiva with marginal gingival redness, which is seen in allergic or inflammatory gingival lesions, Ramadan (2004).; Gursoy et al.,(2007).

This study investigated the effect of different etiological causes of gingival enlargement; including the treatment with cyclosporine A, the plaque, and the orthodontic treatment; on the angiogenic inflammatory mediators such as vascular endothelial growth factor (VEGF), cyclooxygenase-2 (COX-2), 5-lipoxygenase (5-LO), and tumor necrosis factor- (TNF- ), using histopathological and immunohistochemical analysis.

## 2. Material and methods

### 2.1. Materials:

#### 2.1.1. Study population

A total of 30 subjects (24 males and 6 females aged 22- 45 years) were submitted to this study. They were attending the Oral Medicine, Periodontology, Oral Diagnosis and Radiology Department, Faculty of Dental Medicine Girls' Al-Azhar University. They included 10 renal transplant patients receiving cyclosporine A therapy for at least 6 months without any other drugs reported to cause drug- induced gingival overgrowth. Ten healthy subjects with teeth plaque and had no systemic disease and none of them had taken medications that could affect their periodontal status for at least 3 months prior to the study. The last 10 subjects were orthodontically treated with fixed orthodontic appliance for a period of 2 years suffering from hyperplastic enlargements covering one third to two thirds of the clinical crown. Smokers, pregnant and post menopausal women were excluded. The patients signed approval consents and samples were collected under the approval of the ethical Committee of the National Research Centre, Cairo, Egypt.

### 2.2. Methods:

#### 2.2.1. Clinical procedures

Clinical recordings including plaque index O'leary et al.,(1993) gingival index Loe and Silness (1963) and probing depth were performed as shown in table 1. All selected subjects did not have any evidence of clinical attachment loss or bone resorption as diagnosed by radiographs and had not undergone any periodontal therapy. Gingival biopsies were obtained from diseased gingival during gingivectomy for

cyclosporine A treated group as well as in orthodontically treated group as a part of their routine clinical management, which also included intensive plaque control, while the samples were taken during pre-prosthetic crown lengthening procedure for plaque induced gingival enlargement subjects.

### 2.2.2. Histopathology

The biopsies were immersed in 10% formalin and decalcified in multiple baths of 10% trichloroacetic acid. After decalcification, the blocks were immersed in paraffin, and semi-serial 4 µm histologic sections were stained with haematoxylin and eosin (HE).

### 2.2.3. Immunohistochemistry

Paraffin sections of the 4 µm of biopsies were collected on Superfrost Plus slides (Menzel-Gläser), the paraffin was removed and the sections were rehydrated again. Before staining, the slides were treated with 0.1 % trypsin 250 (DIFCO Laboratories, Detroit, USA) for 10 minutes and rinsed with PBS. Thereafter, the slides were treated with 3 % H<sub>2</sub>O<sub>2</sub> in PBS for 30 minutes to block endogenous peroxidase and rinsed in PBS. All sections were pre-incubated with 5 % bovine serum albumin (PBSA; Sigma Chemical Co., St Louis, Missouri, USA) in PBS buffer. VEGF, COX-2, 5-LO, and TNF- were detected using their corresponding rabbit antibodies (Abcam, Cambridge, UK). After being washed, bound antibody was detected using goat anti-rabbit antibody linked to horseradish peroxidase (Cambridge, UK) and bound complexes were detected using O-phenylenediaminedihydrochloride (OPD) (Sigma Aldrich, VA, USA). Representative sections were photographed on a Leitz DMRD Microscope (Leica, Wetzlar, Germany).

## 3. Results:

### 3.1. Histopathological examination :

#### 3.1.1. The gingival specimens

Histopathological examination of the gingival specimens in cyclosporin of the treated group revealed a parakeratinized surface epithelium of the attached gingiva of variable thickness in different parts of the attached gingiva of variable thickness in different parts, connective tissue stroma formed of longitudinal bundles of hyalinized delicate collagen fibrils which showed various distribution, either dense or finely arranged. They were interspersed with a heavy chronic inflammatory infiltrate (mainly lymphocytes and plasma cells) and quite a noticeable number of dilated blood vessels (Fig. 1).

#### 3.1.2. In plaque induced gingival enlargement group:

Histopathological examination in plaque induced gingival enlargement group showed a dense thick

band of collagenous fibrous tissue arranged subepithelially demarcating a heavy zone of chronic inflammatory cell infiltrate packed with plasma cells and lymphocytes with fewer dilated blood vessels were found (Fig. 1).

#### 3.2.3. In orthodontic group specimens :

Histopathological examination In orthodontic group specimens showed thinner parakeratinized surface epithelium covering a connective tissue stroma with contentious dense organized collagen bundles with chronic inflammatory infiltrate and a fewer dilated blood vessels were observed (Fig. 1).

### 3.2. The effect of the immune suppressor drug; cyclosporine A

This effect on the gingival expression of different angiogenic inflammatory mediators using immunohistochemical analysis revealed that neither of VEGF (Fig. 2), COX-2 (Fig. 3), 5-LO (Fig. 4), nor TNF- (Fig. 5) were affected (Table 2), as indicated by negatively stained gingival enlargement samples. Despite extensive research, Results from table (1) indicated that there was a remarkable general overexpression of VEGF (Fig. 2), COX-2 (Fig. 3), and 5-LO (Fig. 4) in the parakeratinized epithelial surface, the epithelial layer, connective tissue and in the fiber bundles regions of gingiva. Additionally, plaque was found to no influence of the gingival content of TNF- (Fig. 5).

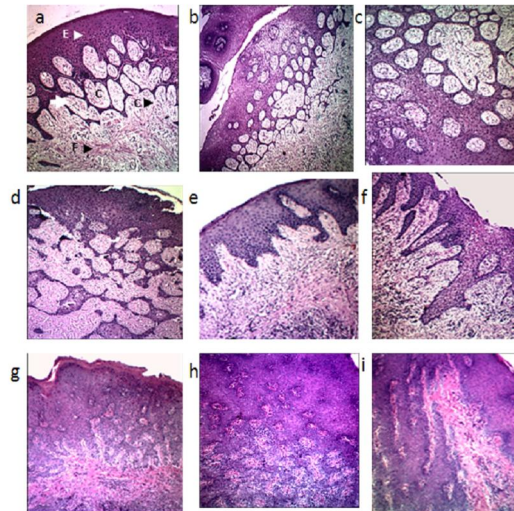


Fig. 1: Histochemical analysis using HE stain of sections represent different gingival enlargement causes including cyclosporine A-induced enlargement (A-C), plaque-induced enlargement (D-F), and the orthodontic treatment-induced enlargement (G-I). The photos labels are E= epithelium, C= connective tissue, F= fibre bundles. Microscopic magnification was X100.



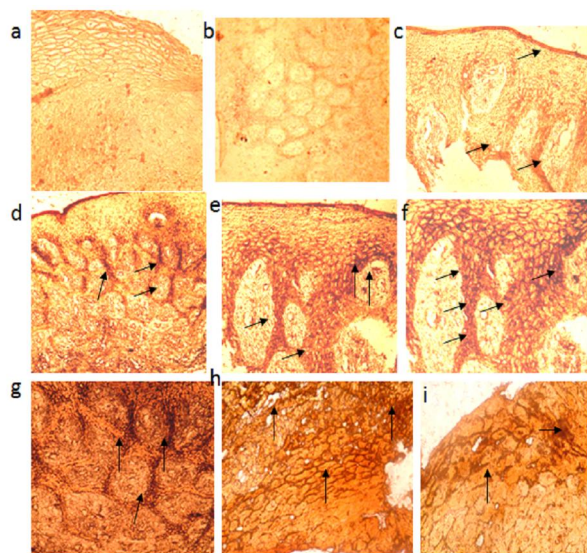


Fig. 2: Immunohistochemical analysis of VEGF expression in sections represent different gingival enlargement causes including cyclosporine A-induced enlargement (A, B), plaque-induced enlargement (C-G), and the orthodontic treatment-induced enlargement (H-I). Microscopic magnification was X100 (A-E) and X400 (F-I) Representative regions of high VEGF expression were marked by black arrows.

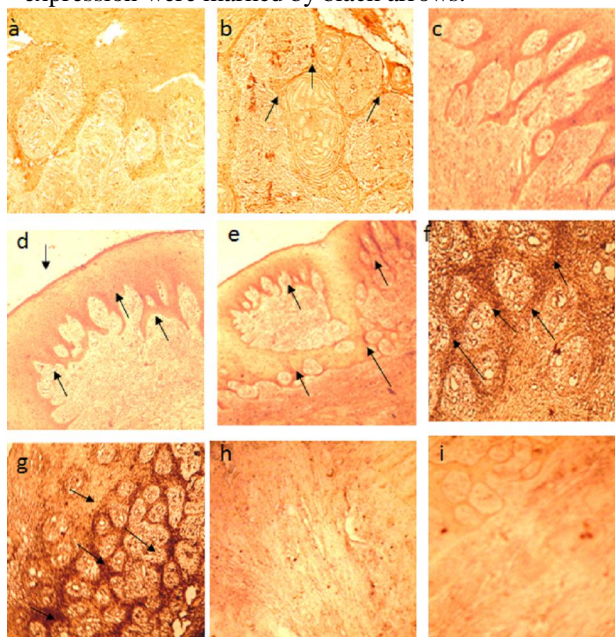


Fig.3:Immunohistochemical analysis of COX-2 expression in sections represent different gingival enlargement causes including cyclosporine A-induced enlargement (A, B), plaque-induced enlargement (C-G), and the orthodontic treatment-induced enlargement (H, I). All microscopic magnification was X100, except in (F, G) it was X400. Representative regions of high COX-2 expression were marked by black arrows.

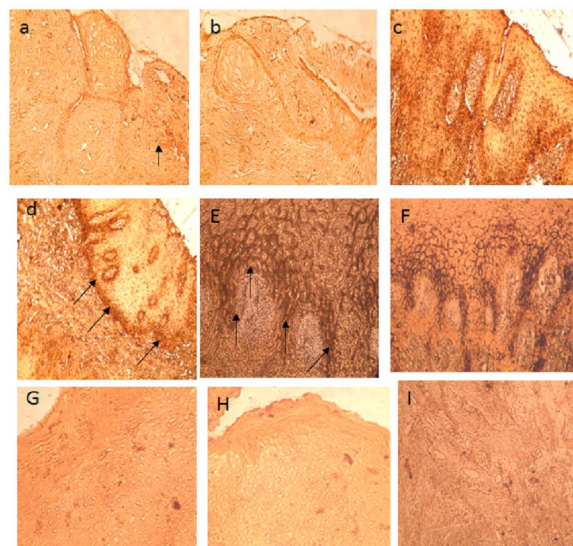
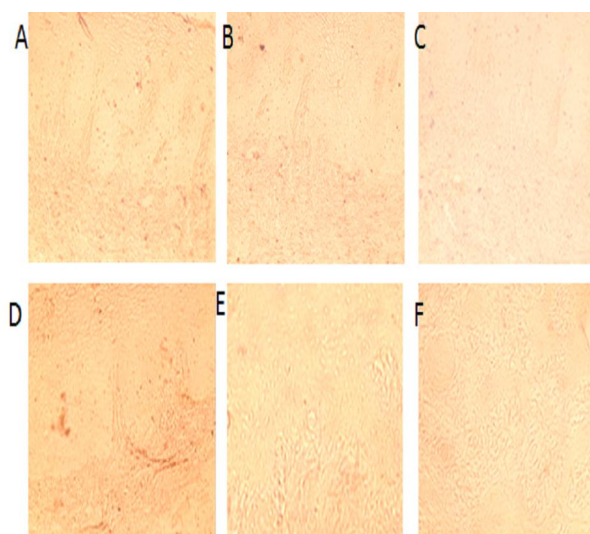


Fig. 4: Immunohistochemical analysis of 5-LO expression in sections represent different gingival enlargement causes including cyclosporine A-induced enlargement (A, B), plaque-induced enlargement (C-F), and the orthodontic treatment-induced enlargement (G- I). All microscopic magnification was X100, except in (E, F) it was X400. Representative regions of high 5-LO expression were marked by black arrows.



Fig(5):Immunohistochemical analysis of TNF-expression in sections represent different gingival enlargement causes including cyclosporine A-induced enlargement (A, B), plaque-induced enlargement (C, D), and the orthodontic treatment-induced enlargement (E, F). All microscopic magnification was X100, except in (F) it was X400.

### 3.3. Immunohistochemical analysis of plaque-induced gingival enlargement

Table 1: Clinical characteristics of the gingival enlargement groups that was induced by different etiological factors including Cyclosporine A treatment (n = 10), Plaque (n = 10), and Orthodontic treatment (n = 10); (mean  $\pm$  SD).

Marker	Cyclosporine A group	Plaque group	Orthodontic group
VEGF	0/10	10/10	8/10
COX-2	0/10	10/10	2/10
5-LO	0/10	8/10	2/10
TNF-	0/10	0/10	0/10

Table 2: Number of patients with positively high expression of different angiogenic inflammatory markers, as assayed by immunohistochemical analysis, of the gingival enlargement groups that was induced by different etiological factors including Cyclosporine A treatment (n = 10), Plaque (n = 10), and Orthodontic treatment (n = 10).

	Cyclosporine A group	Plaque group	Orthodontic group
Plaque index	1.45 $\pm$ 0.67	1.58 $\pm$ 0.64	1.08 $\pm$ 0.47
Gingival index	1.24 $\pm$ 0.36	1.38 $\pm$ 0.29	1.02 $\pm$ 0.21
Probing depth	4.17 $\pm$ 0.85	3.38 $\pm$ 0.47	3.29 $\pm$ 0.67

Data was represented as: (number of patients with positive high expression) / (total number of patients in the group).

### 3.4. Immunohistochemical analysis of gingival enlargement samples from orthodontic treatment :

Patients indicated that there was a very high expression of VEGF in the epithelial layer of gingival but not in the connective tissue nor in the fibre bundles regions. On the other hand, as shown in table 2, orthodontic treatment was found to have no influence of the gingival content of COX-2 (fig 3), 5-LO (fig. 4), nor TNF- (Fig. 5).

### 4. Discussion

Gingival tissues are generally in a state of injury and repair that involves repetitive cycles of production of chemotactic factors, angiogenic factors, inflammatory cell recruitment, and tissue resorption, replacement, and remodelling, Clark (1998) . Collagen turnover is unusually high in periodontal tissues, Trackman and Kantarci (2004).

Wound healing and connective tissue turnover are largely controlled by chemokines and cytokines secreted by inflammatory cells such as macrophages and lymphocytes and, to a lesser degree, by fibroblasts. Proliferation and differentiation of connective tissue cells and production of extracellular matrix are controlled by cytokines that initiate signalling cascades mediated by specific receptors, Trackman and Kantarci (2004).

In this regard, the histopathological examination in the present work of the gingival specimens in cyclosporin A treated group revealed a parakeratinized surface epithelium of the attached gingiva of variable thickness in different parts of the attached gingival connective tissue stroma formed of longitudinal bundles of hyalinized delicate collagen fibrils which showed various distribution, either dense or finely arranged. They were interspersed with a heavy chronic inflammatory infiltrate (mainly lymphocytes and plasma cells) and quit a noticeable number of dilated blood vessels. In plaque induced gingival enlargement group a dense thick band of collagenous fibrous tissue arranged subepithelially demarcating a heavy zone of chronic inflammatory cell infiltrate packed with plasma cells and lymphocytes with fewer dilated blood vessels. In orthodontic treated group specimens thinner parakeratinized surface epithelium covering a connective tissue stroma with contentious dense organized collagen bundles with chronic inflammatory infiltrate and a fewer dilated blood vessels were present.

Gingival hyperplasia is a common side-effect of immunosuppression with cyclosporine A. Exploring the effect of the immune suppressor drug; cyclosporine A on the gingival expression of different angiogenic inflammatory mediators using immunohistochemical analysis revealed that neither of VEGF, COX-2 ,5-LO, nor TNF- were affected .Despite extensive research, the mechanism leading to the accumulation of abnormal amounts of gingival tissue in cyclosporine A-induced gingival overgrowth is unclear. Fibroblasts are the main cell type residing in the gingival connective tissue, and are responsible for the formation and turnover of the extracellular matrix. Studies on the effect of cyclosporine A on gingival fibroblast activity have reported conflicting



findings, and it is uncertain if cyclosporine A can induce gingival overgrowth by directly altering the function of fibroblasts Voulgari and Drosos(2002). Recently cyclosporine A was reported to increase both IL-6 and TGF-beta1 levels Chae et al.,(2006)and to inhibit COX-2 Chiang et al.,(2007).

Plaque induced gingival enlargement can progress rapidly into destructive periodontal diseases, as a result of the altered immune response of the gingiva to the bacterial plaque. In present study, the immunohistochemical analysis of plaque-induced gingival enlargement patients indicated that there was a remarkable general over-expression of VEGF, COX-2, and 5-LO in the parakeratinized epithelial surface, the epithelial layer, connective tissue and in the fiber bundles regions of gingival. Additionally, plaque was found to no influence of the gingival content of TNF- $\alpha$ . Gursoy et al.,(2007).

Nickel, the most common metal used in orthodontic appliances, may activate monocytes and epithelial cells, suppressing or promoting the expression of intracellular adhesion molecule 1 by endothelial cells, mostly depending on its concentration. Nickel ions can also intracellularly accumulate in human oral mucosal cells and human HaCaT keratinocytes, Ermolli et al.,(2001); Faccioni et al.,(2003).

Nickel concentrations, which do not significantly modify oral epithelial cell viability and inflammatory cytokines release (<1.3 mM) can stimulate apoptosis in vitro Trombetta et al.,(2005.)On the contrary, human primary cultured keratinocytes and HaCaT cells also proliferate in response to nickel ions Jia et al.,(1999).Nickel-containing orthodontic wires can reduce cell viability and stimulate apoptosis in three-dimensional cell culture models Vannet et al.,(2005). Even though nickel is related to allergic response seen in orthodontic therapy, Holmstrup (1999); Vanarsdall (2000), its influence of angiogenic inflammatory mediators in gingival overgrowth has not been studied yet.

In the present study, the immunohistochemical analysis of gingival enlargement samples from orthodontic treatment patients indicated that there was a very high expression of VEGF in the epithelial layer of gingival but not in the connective tissue nor in the fibre bundles regions. On the other hand, orthodontic treatment was found to have no influence of the gingival content of COX-2, 5-LO nor TNF- $\alpha$ . TNF- $\alpha$  was expected to be secreted from the immune cell in the intracellular infiltrate within the inflammatory gingiva, but this was not noticed in all of different gingival enlargement samples. Although there was highly noticed inflammatory cell infiltrate in orthodontic treatment samples, no positively stained TNF- $\alpha$  was noticed. As a positive control, samples of bacterial lipopolysaccharide-treated

human macrophages were stained positively for TNF- $\alpha$ .

Nickel has been stated to be corrosive in the oral cavity, Jia et al.,(1999).An average release of 40  $\mu$ g nickel per day from a stimulated full-mouth fixed appliance has been reported, Park and Shearer (1983) and also nickel accumulation was found to be higher in dental plaque samples of patients receiving orthodontic therapy in comparison with untreated subjects, Fors and Persson(2006). However, it is also suggested that the release of nickel is not necessarily proportional to the nickel content of the alloy, Grimsdottir et al.,(1992).The in vivo and in vitro results of a previous study suggested that low-dose continuing nickel release from orthodontic appliances might be the initiating factor for gingival overgrowth, as it has the capability of increasing epithelial cell proliferation, Gursoy et al.,(2007). Depending on the induced VEGF results of the present study, nickel might be responsible for such induction that may subsequently lead to neo-vascularization, which in turn may support nourishment of overgrown tissue.

In conclusion, the immunohistochemical analysis of different angiogenic inflammatory mediators in gingival enlargement samples indicated that in cyclosporine A-induced enlargement neither of VEGF, COX-2, 5-LO, nor TNF- $\alpha$  were affected, while there was a remarkable general over-expression of VEGF, COX-2, and 5-LO in the parakeratinized epithelial surface, the epithelial layer, connective tissue and in the fiber bundles regions of plaque-induced enlargement gingival. Additionally, orthodontic treatment samples indicated that there was a very high expression of VEGF in the epithelial layer of gingival but not in the connective tissue nor in the fiber bundles regions with no change in COX-2, 5-LO, nor TNF- $\alpha$  expression. Taken together, this report indicated that the expression of different angiogenic and inflammatory mediators in gingival enlargement is influenced by the etiological factor that initially induced this enlargement.

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