Expression of Superoxide Dismutase Isoforms in Inflamed Gingiva

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Ⅰ. Introduction

Periodontal diseases are inflammatory disorders that give rise to tissue damage and loss, as a result of the complex interactions between pathogenic bacteria and the host's immune response[1,2]. Several mechanisms of periodontal tissue destruction have been proposed and include a complex array of factors such as those derived from the immune response, direct bacterial influence and the host system in response to this trauma. The popularist view would hold that a primary etiological influence is the engagement of bacterial and host enzymes, including proteases, metalloproteinases and glycosidases, in the destruction of periodontal tissues[3,4].

Chapple et al.[5] reported that total antioxidant activity is reduced in saliva of patients with periodontitis relative to that in non-periodontitis subjects. It is likely that the role of reactive oxygen species (ROS) is common to both bacterial and host-mediated pathways of tissue damage. A number of studies have considered the effect of an imbalance in the oxidant/antioxidant activity detected in saliva and gingival crevicular fluid (GCF) in periodontitis patients[2,4], predisposing such individuals to the damaging effects of ROS within the periodontium. Studies have indicated that total antioxidant activity within the saliva of patients with periodontal disease remains at the same level[6] or is reduced[2,4], compared with non-periodontitis groups. No alterations in antioxidant levels within GCF samples in response to elevated O_2^- production have been demonstrated in adult periodontitis[4,7] and chronic apical periodontitis[4,8]. Therefore, such imbalances in oxidant/antioxidant con-

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centrations, coupled with sources of iron and copper which exacerbate the production of the highly reactive ·OH species, could potentiate severe periodontal tissue damage. Such events have been noted with periodontal ligament cells and gingival epithelial cells undergoing cellular damage and cell lysis in the presence of ROS[4,9]. Free radical species have been implicated in the pathogenesis of over 100 conditions[1] including rheumatoid arthritis[10], acute respiratory distress syndrome[11], AIDS[12], and more recently periodontal disease[13-16].

Superoxide dismutase (SODs) are one of the antioxidant enzymes in protecting the cells against the deleterious effects of ROS[17]. In humans, three forms of SOD have been identified, each with distinct distribution and metal components: copper/zinc-superoxide dismutase (Cu/Zn-SOD, SOD1), manganese superoxide dismutase (Mn-SOD, SOD2) and extracellular superoxide dismutase (EC-SOD, SOD3). They convert O_2^- into H_2O_2, which is subsequently neutralized to oxygen and water by catalase or glutathione peroxidases[18]. Although EC-SOD contains Cu and Zn, it is very different from intracellular Cu/Zn-SOD that EC-SOD has much higher relative molecular mass and possess attached carbohydrate[17,19]

More recently, ROS have been implicated in the pathogenesis of periodontal disease. Several studies have shown increased generation rate of ROS from peripheral blood polymorphonuclear leukocytes (PMN) in rapidly progressive [17,20], juvenile[17], and chronic adult periodontitis[17,21]. There are very few studies concerning the relationship between SOD and inflammation in the oral cavity or periodontium, and the results are conflicting. A significant reduction of SOD activity was found in gingival tissue adjacent to deep periodontal pockets[17,22]. Similar SOD activities were found in periapical granuloma and healthy gingiva[8,17]. A remarkably higher SOD activity was shown in irreversible pulpitis, suggesting that SOD activity increases with the progression of inflammation[23]. Studies concerning ROS and/or antioxidant enzymes mechanisms in oral fluids are also limited[2,6,17]. In addition, the relationship between antioxidant levels and activities in the gingival tissues have not been systematically studied in patients with periodontitis.

It can be hypothesized that these enzymes have individual distributions and expressions, they may be upregulated in oxidant related inflammatory periodontal disease.

To understand better the mechanisms by which human gingival cells are protected against oxidants, this study was to evaluate the cell-specific protein expression and distribution of the major antioxidant enzymes, Cu/Zn-SOD, Mn-SOD and EC-SOD in healthy and periodontally inflamed human gingiva.

Ⅱ. Materials and Methods

1. Subjects and gingival tissue specimens

The subjects consisted of patients (n=53) who had been referred to the Department of Periodontics, Chonnam University Hospital. Their mean age was 47 years (range, 22-67). Thirty three subjects were diagnosed with

chronic periodontitis based on their clinical and radiographic findings (American Academy of Periodontology, 1999). Twenty subjects were referred to lengthen the clinical crown for esthetic purpose. Gingival tissues were harvested from buccal or lingual sites on teeth during periodontal surgery after initial therapy including motivation, oral hygiene instruction, scaling and root planing. Specimens (n=62) consisted of gingival epithelium and connective tissue. The subjects did not engage in tobacco smoking, or alcohol consumption, and suffer from any systemic disease. It was observed that mild to moderate inflammatory infiltration in the clinically healthy gingival tissue under the microscope. Then all specimens stained with haematoxylin and eosin were divided into four groups by severity of inflammation: healthy, mild, moderate and severe periodontitis.

2. Immunohistochemistry of SOD isoforms

The obtained specimens were fixed in 10% buffered formalin, rinsed in absolute ethanol, rehydrated to 60% ethanol and embedded in paraffin. Paraffin-embedded tissue samples were cut into 4 ㎛ sections and mounted on poly-L-lysine-coated glass slides. The sections were deparaffinized in xylene and rehydrated in a descending ethanol series prior to immunohistochemistry.

The endogenous peroxidase activity was blocked with 3% hydrogen peroxide in distilled water for 20 minutes. The sections were incubated with rabbit polyclonal antibodies (StressGen Biotechnologies Corp, Victoria BC, Canada) to Cu/Zn-SOD (SOD-100), Mn-SOD (SOD-110), and EC-SOD (SOD- 105) at a dilution of 1:800, 1:400 and 1:100, respectively overnight at 4℃. The sections were rinsed thoroughly in Tris-buffered saline (TBS, pH 7.5) and subsequently incubated with biotinylated secondary antibody (Goat anti-rabbit IgG, LSAB kit, DAKO, Carpinteria, CA, USA) at a 1:200 dilution with streptavidin conjugated with horseradish peroxidase for 1 hour at room temperature. Sections were stained by incubation in 3-amino-9 ethylcarbazole (AEC, DAKO, Carpinteria, CA, USA) as the chromogenic substrate resulting in a red staining product for 1~10 minutes at room temperature. Finally, the sections were lightly counterstained in Mayer's haematoxylin. For the control sections, the primary antibodies were replaced by nonimmunized serum.

3. Scoring of the immunoreactivity

All sections were examined under a light microscope (Nikon, Tokyo, Japan). Among sixty-two specimens, it was selected only 27 specimens with complete epithelium and connective tissue for the analysis. The extent of the immunoreactivity for SOD was evaluated semiquantitatively as the relative intensities with scale ranging from '-' for negative staining to '+++' for the most intense.

Ⅲ. Results

1. Microscopic features and Expression of SODs

Mild to moderate inflammatory cell infiltration was observed in the clinically healthy gingiva under the microscope (Figure 1, 2). One specimen showed diverse intensity of inflammation (Figure 1).

Inflammatory cell infiltration and immunoreactivity of SOD isoforms were absent or mild in healthy gingiva (Figure 3), while there were heavy inflammatory cell infiltration and prominent immunoreactivity in severe periodontitis specimen (Figure 4).

1) Cu/Zn-SOD

In healthy gingiva, Cu/Zn-SOD was slightly expressed diffusely in the basal layer of epithelium and connective tissue, especially near the rete peg (Figure 5A). In higher magnification it was expressed in the cytoplasm and nucleus of epithelial cells (Figure 5B and 5C).

In inflammatory gingiva, immunostaining for Cu/Zn-SOD showed a diffuse and granular cytoplasmic pattern in the basal layer of epithelium and connective tissue near the rete peg (Figure 5D, 5E and 5F). Immunoreactivity was increased with severity of inflammation.

2) Mn-SOD

In healthy gingiva, Mn-SOD was expressed in the granular and keratinized layer of epithelium as well as connective tissue, particularly near the rete peg (Figure 6A). It showed granular pattern, and especially cytoplasm of the epithelial cells was strongly immunopositive for Mn-SOD (Figure 6B and 6C).

In inflammatory gingiva, immunoreactivity was markedly increased in the granular and keratinized layer of epithelium as well as connective tissue near the rete peg with severity of inflammation (Figure 6D, 6E and 6F).

3) EC-SOD

In both healthy and inflammatory gingiva, expression of EC-SOD was limited or nearly absent and was mainly localized diffusely around the vessel walls of small capillaries (Figure 7). Immunostaining for EC-SOD showed a diffuse pattern. The vessel wall of capillaries was thickened and the expression of EC-SOD was strong with severity of inflammation.

2. Immunoreactivity pattern of SOD isoforms with severity of inflammation

1) Cu/Zn-SOD

Cu/Zn-SOD was expressed similarly in the epithelium and connective tissue with severity of inflammation. It was also observed in the vessel wall of the small capillaries (Table 1).

2) Mn-SOD

Immunoreactivity of Mn-SOD was markedly increased with severity of inflammation and in particular, expression tended to concentrate in the border of epithelium and connective tissue. It was also expressed in the capillary wall (Table 2).

Table 1. The immunoreactivity pattern of Cu/Zn-SOD in human gingiva according to severity of inflammation

Area \ Severity	Healthy(n=7)				Periodontitis(n=20)											
					Mild(n=7)				Moderate(n=4)				Severe(n=9)			
	-	+	++	+++	-	+	++	+++	-	+	++	+++	-	+	++	+++
Epithelium	6	1				5	2			2	2		1	4	4	
Connective tissue	6	1				5	2				4		1	2	6	
Wall of vessels	5	2			1	5	1				4		1	4	2	2

Table 2. The immunoreactivity pattern of Mn-SOD in human gingiva according to severity of inflammation

Area \ Severity	Healthy(n=7)				Periodontitis(n=20)											
					Mild(n=7)				Moderate(n=4)				Severe(n=9)			
	-	+	++	+++	-	+	++	+++	-	+	++	+++	-	+	++	+++
Epithelium	4	1	2			4	3				4			2	3	4
Connective tissue	4	1	2			5	2				2	2			4	4
Wall of vessels	3	3	1			5	2				3	1	1	2	2	4

Table 3. The immunoreactivity pattern of EC-SOD in human gingiva according to severity of inflammation

Area \ Severity	Healthy(n=7)				Periodontitis(n=20)											
					Mild(n=7)				Moderate(n=4)				Severe(n=9)			
	-	+	++	+++	-	+	++	+++	-	+	++	+++	-	+	++	+++
Epithelium	7				7				4				9			
Connective tissue	7				7				4				9			
Wall of vessels	4	2	1			6	1			2	2		2	5	2	

3) EC-SOD

EC-SOD was not expressed in the epithelium and connective tissue and only expressed around the small capillaries and it showed similar distribution in immunoreactivity regardless of severity of inflammation (Table 3).

Ⅳ. Discussion

Soon after the discovery of SOD activity by McCord and Fridovich in 1969, it became clear that this enzyme is absolutely necessary to maintain life in aerobic organisms[24,25]. SODs detoxify O_2^- by converting it

to H2O2 in what appears to be the fastest enzyme-catalysed reaction known. In the consequence, loss of SOD function might induce cellular oxidative toxicity through an increase in O_2^- levels or, more importantly, in its downstream metabolites, such as ·OH or peroxynitrite. In humans, three forms of SOD have been identified, each with distinct distribution and metal components[24,25].

Cu/Zn-SOD is diffusely located throughout the cytoplasm and, to a lesser extent, in the nucleus, but is absent in mitochondria. It is by far the most abundant SOD isoform, constituting approximately 70% of the total SOD activity, and can be found in the epithelium and all types of phagocytes in most organs.

Mn-SOD is exclusively located in the mitochondria. It constitutes approximately 15% of the total SOD activity in most tissues, where it has been detected in epithelial cells as well as in phagocytes. The importance of this particular SOD isoform and the potential toxicity of mitochondrially produced O_2^- have been effectively illustrated in Mn-SOD knockout mice, which die within several days after birth. Cu/Zn-SOD knockouts and EC-SOD knockouts, on the contrary, survive quite well until they are stressed.

EC-SOD is the third, and most recently described, SOD family member. It is the dominant SOD isoform in the plasma and the interstitium, and is the only known extracellular enzyme to scavenge O_2^-. It is a secretory, tetrameric, copper- and zinc-containing glycoprotein with a high affinity for glycosaminoglycans, such as heparin. Due to this heparin affinity, it may exist at very high concentrations in unique extracellular compartments, whereas it is estimated to make up only 0.5~17% of the total SOD activity. It has been speculated to contribute to the protection of collagen matrix elements against ROS, or to be involved in the modulation of vascular tone by the prevention of NO conversion to peroxynitrite.

PMN are widely believed to be the initial and predominant defense cell produced during the host response against bacterial pathogens in a variety of pathological conditions, including periodontal disease. Following stimulation by bacterial antigen, PMN produce O_2^- via the metabolic pathway of the respiratory burst, catalysed by NADPH oxidase, during phagocytosis and lead to an increase in ROS levels. Two studies had demonstrated that PMN present in the GCF and blood of patients with adult periodontitis showed an enhancement in the production of O_2^- following stimulation, compared with healthy group[7,16]. The circulating PMN of such patients within the adult periodontitis group also produced low levels of O_2^- spontaneously, whilst the healthy group appeared to exhibit no spontaneous O_2^- production[7]. It is also well accepted that various PMN dysfunctions play a key role in the progression of early-onset forms of periodontitis, with impairment in leukocyte chemotaxis and adherence mechanisms. And a pathogenic link between the PMN cells and the disease was recognized in Åsman's study showing the increased release of free oxygen radicals from activated PMN cells in juvenile periodontitis[15]. However, the subjects

of this study were in chronic periodontitis and feature of the disease is related to not PMN but plasma cell and lymphocyte, therefore further study is needed about the association between SODs and plasma cell and lymphocyte.

The results of this study showed similar differential expression and distribution. Cu/Zn-SOD and Mn-SOD were expressed in the epithelial cells but, EC-SOD was only observed in the vessel wall of small capillaries. Markedly increased levels of Mn-SOD, primarily localized in the border of epithelium and connective tissue, were found in inflammatory gingival tissue, compared to healthy gingiva. Cu/Zn-SOD was also prominently expressed near the border, but its expression level was not as prominent as Mn-SOD. EC-SOD was relatively highly immunopositive in the inflammatory gingival connective tissue. That was because the number of small capillary was increased and the vessel wall was thickened due to the inflammation. And Cu/Zn-SOD and EC-SOD were expressed as diffuse pattern, while Mn-SOD was granular pattern. These results indicated that SOD activity was increased in severely inflamed gingiva.

Several attempts with antioxidant have been carried out to eliminate oxidative stress in the medical field. Attenuating oxidative stress in inflammatory bowel disease patients has already been a therapeutic strategy for 50 years. In addition, there have been attempts to specifically prevent or attenuate intestinal oxidative stress through either the inhibition of ROS-producing enzymes or the direct scavenging of ROS. Specific antioxidant trials have been carried out in animal models, however most of them have been withdrawn[25,27,28]. Antioxidants, including the SOD mimetic tempol, vitamin E, and vitamin C, have been used to treat hypertension[29,30]. These antioxidants have multiple effects and require continuous and usually long-term administration of a narrow range of doses to produce an antihypertensive effect, and vitamin C has not proven to be effective in the treatment of hypertension in patients[30,31]. Although the therapeutic applicability of natural SODs has its limitations in terms of its limited cell permeability, short circulating half-life, immunogenicity and cost of production, SOD-based clinical trials have been performed. And several innovative antioxidant agents have recently been developed that have overcome these limitations, and these await further clinical evaluation[32]. This study may have implications for the alternative of SOD-based treatment strategies in inflammatory periodontal disease.

In this study to know the association of inflammation with SODs, it was recognized Cu/Zn-SOD and Mn-SOD were increased with severity of inflammation. And further study by quantitative measure and confirmation in cellular level may be valuable in implicating a significant role for SODs in the complex pathological events occurring during periodontal disease.

Ⅴ. Conclusion

This study was to analyze the cell-specific protein expression and distribution of the

major antioxidant enzymes, Cu/Zn-SOD, Mn-SOD and EC-SOD in healthy and periodontally inflamed human gingiva. Gingival specimens stained with haematoxylin and eosin were divided into four groups, healthy, mild, moderate and severe periodontitis, with severity of inflammation under the microscope.

From this study, following results were obtained.

1. All SODs were expressed around vessel wall but had differential expression and distribution: Cu/Zn-SOD was expressed in diffuse and granular pattern in the basal epithelium and connective tissue near the rete peg, Mn-SOD showed granular pattern in the granular and keratinized layer of epithelium as well as connective tissue near the rete peg, and EC-SOD showed diffuse pattern around vessel wall of small capillaries.

2. Mn-SOD was markedly increased in the inflammatory gingival tissue with severity of inflammation compared to healthy gingiva. And Cu/Zn-SOD was increased but not as much as Mn-SOD. EC-SOD was increased in the inflammatory gingival connective tissue where the number of small capillary and thickness of capillary wall were increased due to the inflammation.

From these results, it was suggested that SOD had differential expression and distribution and there could be relationship between SOD isoforms and periodontal gingival inflammation.

Ⅵ. References

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사진 부도 설명

Figure 1. Clinically healthy gingival tissue. Although it was clinically healthy, mild to moderate inflammatory cell infiltration was observed. (A) x 20, (B), (C), (D) x 40, Asterisk (*) : Normal gingiva, Arrow : Moderate inflammatory cell infiltration

Figure 2. Clinically severe inflammatory gingival tissue. Severe inflammatory cell infiltration was observed. (A) x 20, (B) x 40, Asterick (*) : Severe inflammatory cell infiltration

Figure 3. Healthy gingival tissue (x40). Inflammatory cell infiltration was absent or limited and immunoreactivity was very low. (A) H & E, (B) Cu/Zn-SOD, (C) Mn-SOD, (D) EC-SOD, (E) Negative

Figure 4. Severe periodontitis gingival tissue (x40). Heavy inflammatory cell infiltration and severe immunoreactivity was observed especially in Mn-SOD. EC-SOD was expressed only in the vessel wall of small capillaries. (A) H & E, (B) Cu/Zn-SOD, (C) Mn-SOD, (D) EC-SOD, (E) Negative, Arrow : Severe immunoreactivity of Mn-SOD

Figure 5. Immunohistochemical staining for Cu/Zn-SOD in healthy and severe periodontitis gingival tissue. Cu/Zn-SOD was expressed in the basal layer of epithelium and connective tissue near the rete peg (A, B, C) and increased in the severe periodontitis gingiva (D, E, F) as a diffuse or granular pattern. (A), (B), (C) healthy gingiva (x 40, 100, 200, separately), (D), (E), (F) severe periodontitis gingiva (x 40, 100, 200, separately)

Figure 6. Immunohistochemical staining for Mn-SOD in healthy and severe periodontitis gingival tissue. Mn-SOD was expressed in the granular and keratinized layer of epithelium as well as connective tissue near the rete peg (A, B, C) and markedly increased with severity of inflammation as a granular pattern (D, E, F). (A), (B), (C) healthy gingiva (x 40, 100, 200, separately), (D), (E), (F) severe periodontitis gingiva (x 40, 100, 200, separately)

Figure 7. Immunohistochemical staining for EC-SOD in healthy and severe periodontitis gingival tissue. EC-SOD was expressed in the vessel wall of small capillaries and expression was increased in the severe periodontitis gingival tissue where the number of small vessel was increased and vessel wall was thickened (G, H). (A), (B), (C) healthy gingiva (x 40, 100, 200, separately), (D), (E), (F) severe periodontitis gingiva (x 40, 100, 200, separately), (G), (H) vessel wall of small capillaries in the severe periodontitis gingiva (x100, 200), Arrow : Thickenld vessel wall

사진부도 (Ⅰ)

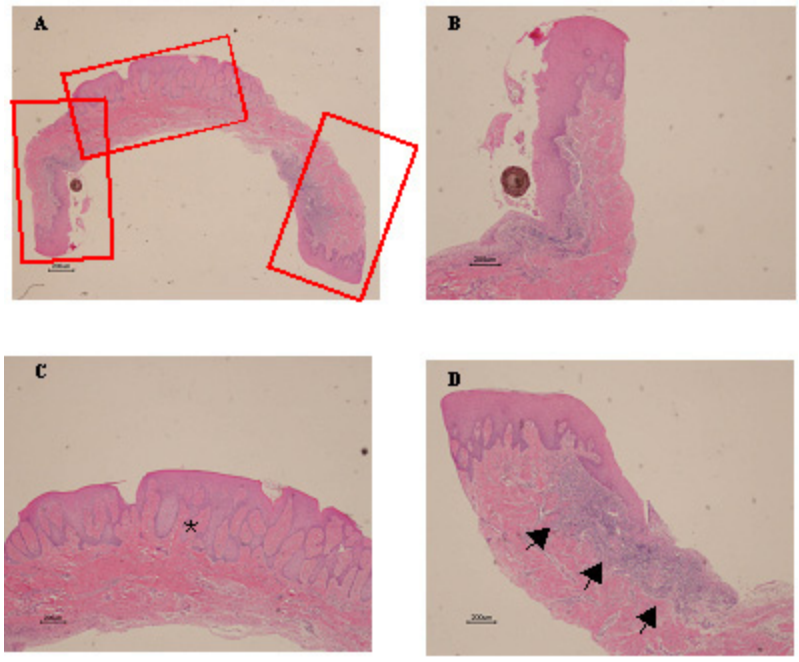

Figure 1.

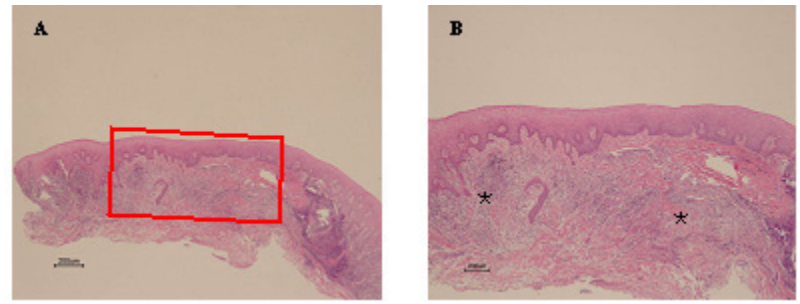

Figure 2.

사진부도 (Ⅱ)

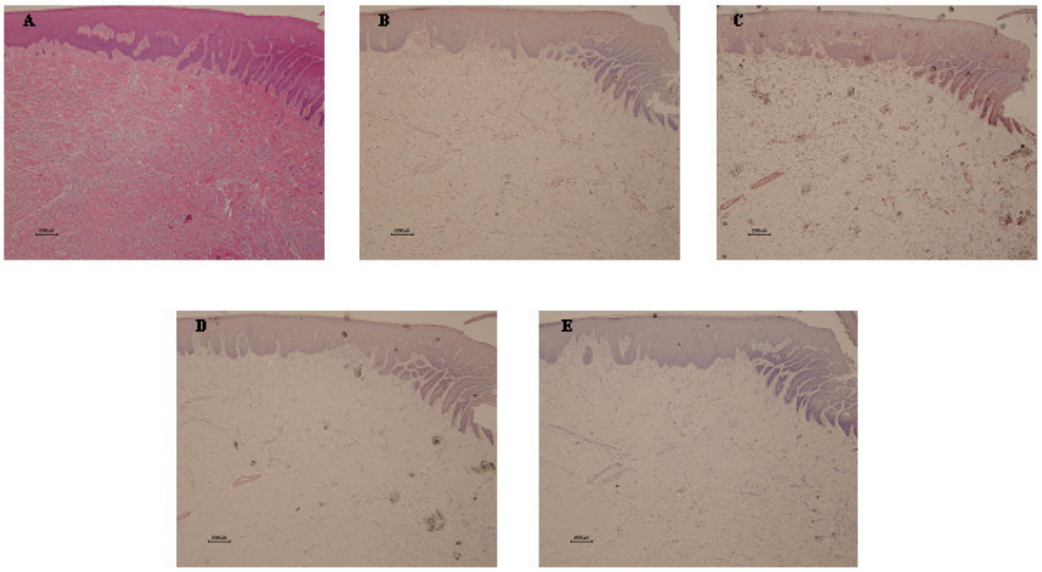

Figure 3.

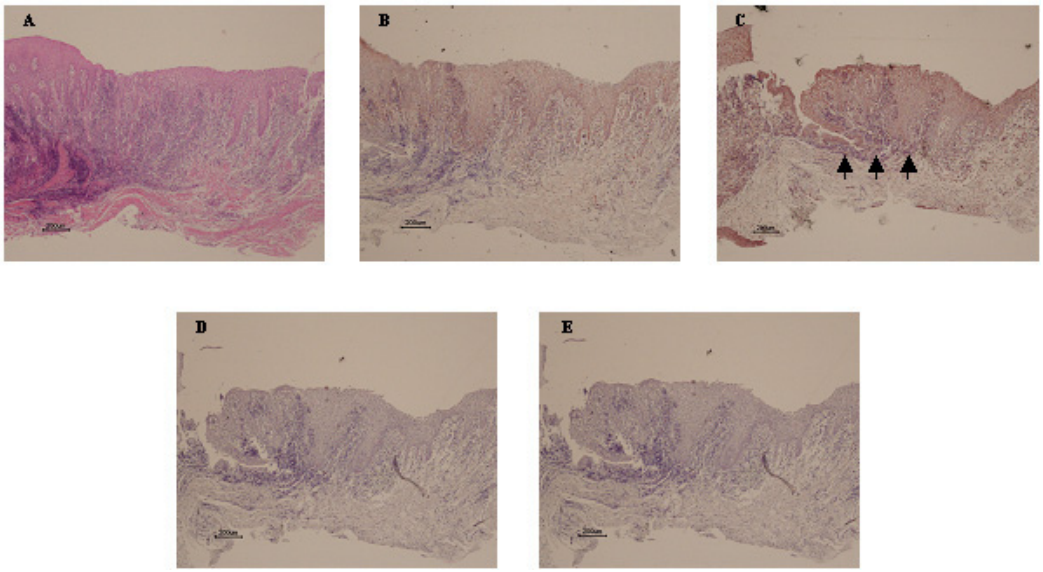

Figure 4.

사진부도 (Ⅲ)

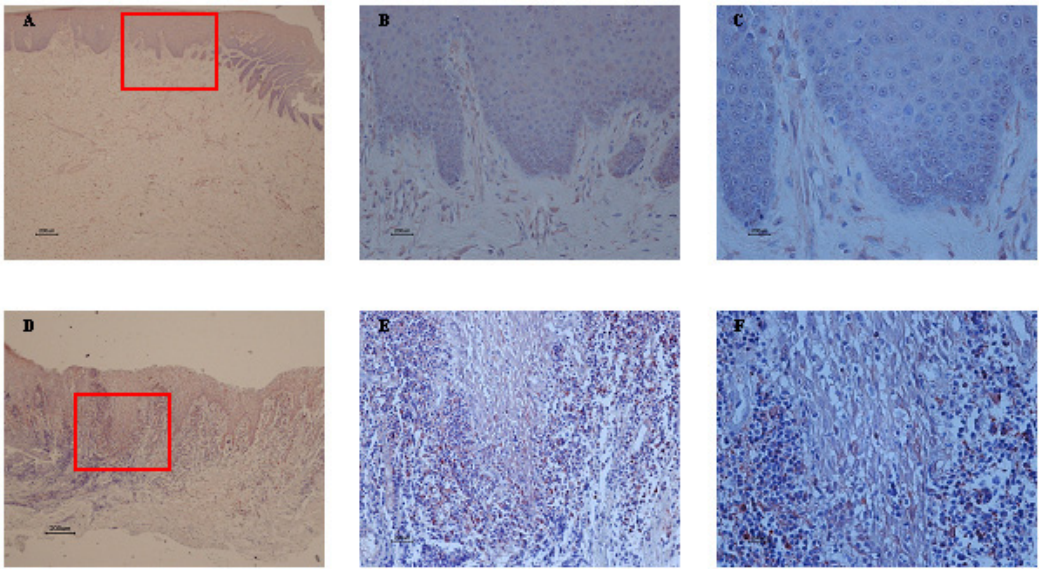

Figure 5.

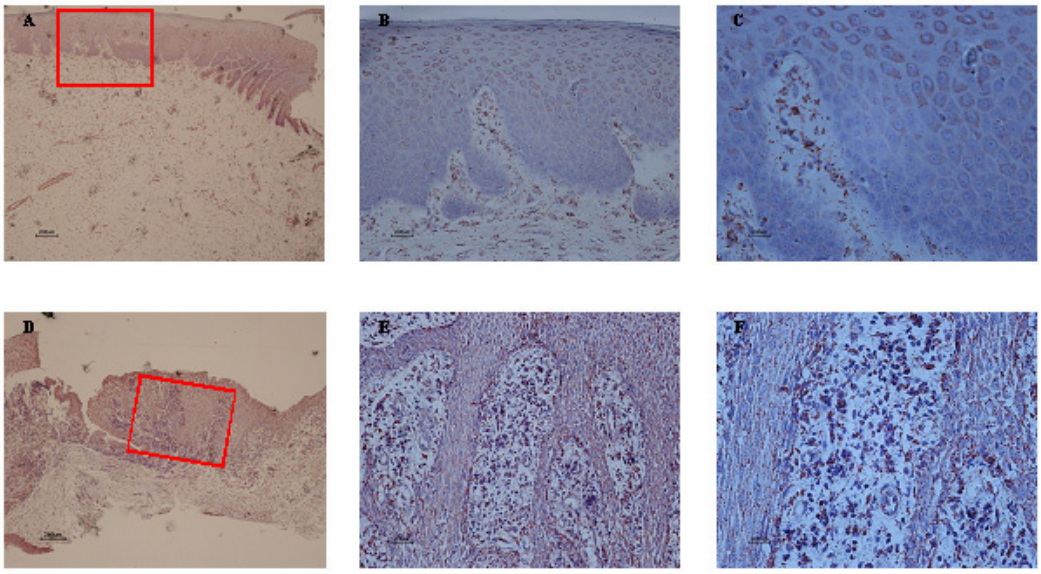

Figure 6.

사진부도 (Ⅲ)

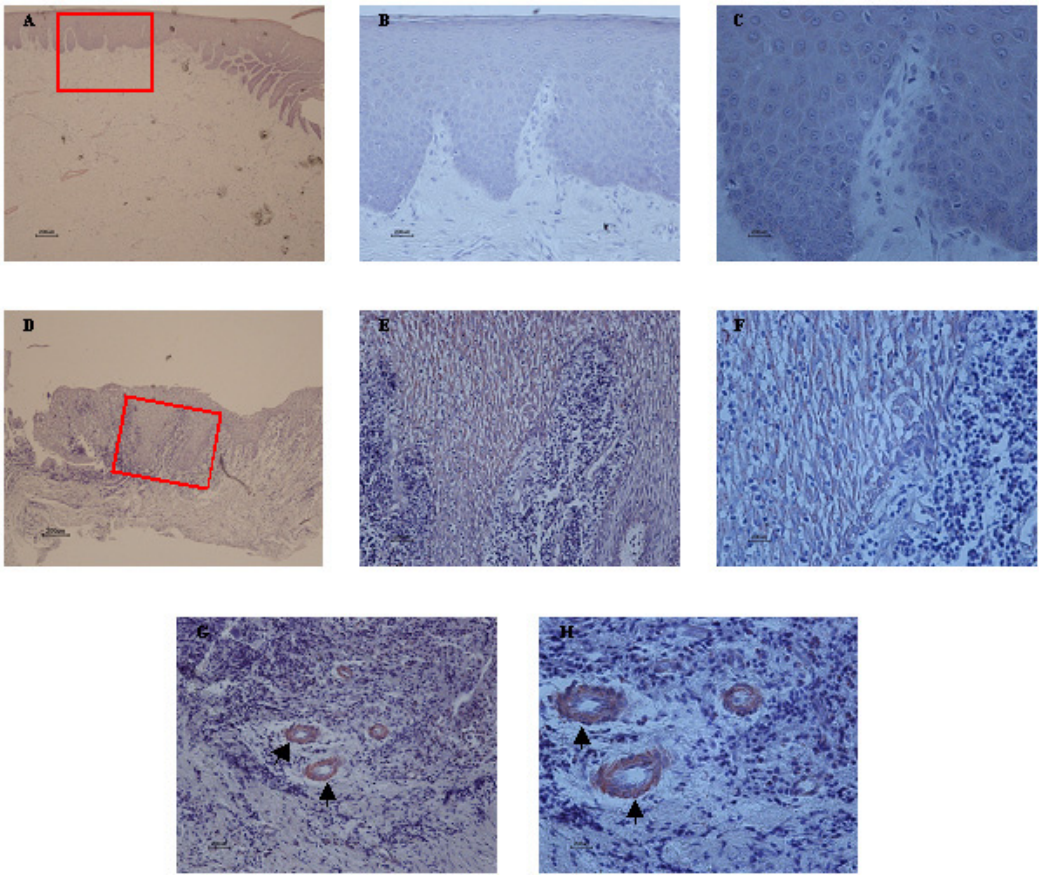

Figure 7.

-Abstract-

염증성 치은에서 superoxide dismutase isoform의 발현에 대한 연구

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유리 라디칼과 활성 산소종, 산화방지제 간의 불균형이 염증성 구강내 질환의 발생과 진행에 있어 중요한 역할을 한다는 주장이 제기되었고 최근에는 만성 염증성 치주질환에서도 산화에 의한 소실이 관찰되었다. 다양한 내적인 항산화 방어 기전 중 superoxide dismutase가 O_2를 H_2O_2로 효과적으로 전환시킴으로써 활성산소종에 대한 일차적인 방어를 맡고 있다. 현재까지 인간에서 발견된 superoxide dismutase는 cytoplasmic copper-zinc SOD와 mitochondrial manganase SOD, extracellular SOD의 3가지 아형이다. 이번 연구는 만성 치주질환을 가진 환자의 치주조직에서 효소 항산화제인 SOD의 발현정도를 알아봄으로써 질환조직 내의 산화자극 정도를 평가해 보고자하였다.

전남대학교 치주과에 내원한 33명의 만성 치주질환자와 20명의 임상적으로 건강한 대상으로부터 조직을 얻어 Cu/Zn-SOD와 Mn-SOD, EC-SOD를 이용한 면역조직화학 염색을 시행하였다. 임상적 소견과 조직학적 소견이 일치하지 않아 조직학적 소견을 기준으로 건강한 조직, 경도, 중등도, 중도 치주질환 조직으로 그룹을 나누고 완전한 상피와 결합조직을 가진 27개의 표본에 대한 분석을 시행하였다.

치주질환 조직에서 건강한 조직에 비해 Cu/Zn-SOD가 상피의 기저층과 상피에 근접한 결합조직에서 발현되고 Mn-SOD는 염증이 증가함에 따라 크게 상피의 과립층과 각화층, 그리고 상피에 근접한 결합조직에서 발현됨으로써 활성산소종이 치주조직 파괴에 관여한다는 것을 알 수 있었다. 세 아형 모두 혈관주위에서 발현되었고 특히 EC-SOD는 작은 모세혈관주위에서만 발현되었으나 염증에 의해 혈관벽이 두꺼워지고 혈관 수가 증가한 곳에서 뚜렷하게 염색되었다.

이번 연구는 염증성 치주조직내 증가된 SOD의 활성이 치주질환자의 산화자극 정도와 관련되어 있음을 시사하였다.

주요어 : periodontitis, Superoxide dismutase(SOD), anti-oxidants, reactive oxygen species(ROS)