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A study to evaluate superoxide dismutase levels in chronic periodontitis patients before and after non-surgical periodontal therapy

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Abstract

Background: Periodontitis, the destructive category of periodontal disease, is a chronic nonreversible inflammatory state of the supporting structures. It is initiated by the colonization of the gingiva by bacterial pathogens which stimulate the host cells to release pro-inflammatory cytokines as part of the immune responses.

Objective: To evaluate and compare the levels of periodontal parameters and Superoxide Dismutase (SOD) enzyme in chronic generalized periodontitis patients before and 1 month after non-surgical periodontal therapy (NSPT).

Materials and Method: 60 subjects were equally allocated into: Group I- subjects with clinically healthy

gingiva and Group II- Subjects with chronic generalized periodontitis. Periodontal parameters such as plaque index, gingival index, sulcus bleeding index, probing pocket depth, recession depth and clinical attachment level were recorded and unstimulated saliva and blood samples were collected from all subjects at baseline and 1 month after NSPT in group II only. Samples were assessed for SOD by SOD assay kit and ELISA reader.

Results: At baseline, periodontal parameters were increased and SOD levels were decreased in group II compared to Group I. After 1 month of periodontal therapy, SOD levels increased and periodontal parameters decreased in group II.

Conclusion: Reduction of inflammatory response following periodontal therapy resulted in improved antioxidant profiles in both blood and salivary compartments.

Keywords: Periodontitis, Reactive Oxygen Species, Antioxidants, Superoxide Dismutase, Unstimulated, SOD assay kit.

Introduction

Periodontitis is defined as "an inflammatory disease of supporting tissues of the teeth caused by specific microorganisms or groups of specific microorganisms, resulting in progressive destruction of the periodontal ligament and alveolar bone with pocket formation, recession, or both."[1]

It is a chronic, multifactorial disease with complex etiology and pathogenesis. The gram- negative anaerobic or facultative bacteria within the subgingival plaque biofilm are the primary etiologic agents that stimulate host cells to release various pro-inflammatory cytokines such as interleukins and Tumor Necrosis Factor- Alpha (TNF-α) which in turn attract polymorphonuclear leucocytes (PMNs) to the site of infection.[2] PMNs kill the bacteria by producing reactive oxygen species (ROS)[3], which is an essential protective immune mechanism against diseases associated with phagocytic infiltration. But, as the released ROS is not target-specific, damage to host tissues (i.e. damage to lipids, proteins, DNA etc) also occurs.[4] Therefore, at sites of chronic inflammation (as in periodontitis), there is considerable over production of free radicals (FR) and reactive species[5][7] including superoxide ($O_2^{\bullet-}$), hydroxyl ($OH^{\bullet}$), perhydroxyl ($HO_2^{\bullet-}$), hydroperoxyl ($HOO^{\bullet}$), alkoxyl ($RO^{\bullet}$), aryloxyl ($ArO^{\bullet}$), arylperoxyl ($ArOO^{\bullet}$), peroxyl ($ROO^{\bullet-}$), acyloxyl ($RCOO^{\bullet}$) and acylperoxyl ($RCOOO^{\bullet}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), singlet oxygen (O_2), and ozone (O_3).[2]

Superoxide anion ($O_2^{\bullet-}$), arising either through metabolic processes or following oxygen "activation" by physical irradiation, is considered as the "primary" ROS, and can further interact with other molecules to generate "secondary" ROS, either directly or prevalently through enzyme- or metal-catalysed processes.[6] Therefore, it can be stated that the host reactions that are primarily intended to eliminate the invading bacteria are responsible for the majority of periodontal tissue destruction.[7]

A first line of defense against ROS is protection against their formation, i.e. prevention.[8] All organisms possess an antioxidant (AO) defense system against these harmful effects of ROS.[9] The human body contains an array of antioxidant defenses including both non-enzymatic (Vitamins E, C, reduced glutathione etc) and enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSHPx) to counter this excessive production of harmful ROS as they are formed and to prevent their deleterious effects.[10]

Superoxide dismutase, a preventative, metal ion sequestrator, antioxidant enzyme has been regarded as the most significant antioxidant within mammalian tissues.[11] SODs are the first line of defense against oxygen free

radicals, and the vast majority of organisms that live in the presence of oxygen express at least one SOD.[12] The enzyme exists in three forms based on the associated co-factored metal ion namely SOD 1: Cu^{2+}/Zn^{2+} found within cytosol, SOD 2: Mn^{2+} found within mitochondria, SOD 3: Extracellular Cu^{2+}/Zn^{2+} SOD.[11] Eukaryotes only express Cu/Zn SODs (in the cytoplasm and extracellularly) and Mn SODs (in the mitochondria).[12] The concentration of SOD differs in various body compartments in health and disease. It has also been localized within human periodontal ligament and may represent an important defense mechanism within gingival fibroblasts against superoxide release.[11]

The main role of SODs in all aerobic organisms is to neutralize the $O_2^{\circ-}$ produced in the cytosol, mitochondria and endoplasmic reticulum of cells.[13] Superoxide anion formed, either dismutates spontaneously (a rapid reaction at pH 7.4) or is actively converted to hydrogen peroxide (H_2O_2) by one of the three superoxide dismutase enzyme systems.[11]

Thus, in normal physiology, there is a dynamic equilibrium between ROS activity and antioxidant defense capacity, and, when that equilibrium shifts in favor of ROS, by either a reduction in antioxidant defense or an increase in ROS production or activity, it results in oxidative stress (OS)[9],[14] which is described as a disturbance in the pro-oxidant-antioxidant balance in favor of the former, leading to potential damage.[9] In periodontitis, PMNs which are naturally capable of producing ROS are thought to be functionally activated and thus, leading to increased ROS production and eventually oxidative stress.[15] Also, it is conceivable that bacteria in the oral cavity and periodontal pockets consume antioxidants and suppress ROS detoxification. This reduced antioxidant defense may enable entry of ROS from the periodontal tissues into the systemic circulation thereby, leading to damage.[4] Therefore, oxidative stress lays at the heart of periodontal tissue damage.

Non-surgical periodontal therapy (NSPT) is the primary therapy in the treatment of chronic periodontitis and aims to accomplish the removal of dental calculus and cementum contaminated with toxins or microorganisms[16] thereby, reducing inflammation in gingiva and enabling pocket reduction/closure.[17]

Currently, there is growing interest in the linkage between reactive oxygen species, antioxidants and periodontal disease. Hence, the present study was planned to evaluate the levels of SOD in subjects with clinically healthy gingiva and chronic generalized periodontitis, to find the association, if any, with periodontal health and disease and to evaluate the effectiveness of non-surgical periodontal therapy on the oxidative burden as well as in restoring physiological SOD levels in patients with chronic generalized periodontitis.

Materials and method

This randomized clinical study was conducted on 60 subjects, visiting the Outpatient Department of Periodontology and Oral Implantology, Sri Guru Ram Das Institute of Dental Sciences and Research, Sri Amritsar. The research protocol was initially submitted to the institutional ethical committee. After approval, all subjects were verbally informed and written consent was taken from all before inclusion in the study.

Criteria for Selection

Inclusion criteria

- Subjects aged between 20-50 years.
- Subjects with clinically healthy gingiva.
- Patients with chronic generalized periodontitis (30% or more of the sites demonstrating bone loss and > 3 mm of clinical attachment loss).

- Co-operative subjects willing to sign the consent form.

Exclusion criteria

- Subjects suffering from any systemic disease that can influence periodontal health.
- Alcoholics, smokers, tobacco chewers and drug addicts.
- Subjects who had received multivitamins, antioxidant supplements, anti-inflammatory agents or antibiotics within 3 months.
- Use of medications such as antibiotics, anticoagulants, steroids, hormonal therapy in last six months.
- Subjects who had undergone periodontal treatment in past six months.
- Pregnant or lactating women.
- Uncooperative subjects.

The subjects who fulfilled the inclusion and exclusion criteria were included in the study and were divided into two groups viz Group I and Group II each:

Group I (Control group): 30 subjects with clinically healthy gingiva.

Group II (Test group): 30 subjects with chronic generalized periodontitis.

After the selection of subjects, periodontal parameters such as plaque index[18], gingival index[18], sulcus bleeding index[19], probing pocket depth[20], recession depth and clinical attachment level[21] were recorded and saliva and blood samples were collected for the analysis of biochemical parameters i.e. salivary and plasma Superoxide Dismutase enzyme levels at baseline for both the groups.

Group II subjects with chronic generalized periodontitis were treated with scaling and root planing (SRP) using hand scalers (Hu-Friedy, Chicago, USA), ultrasonic scaler (Satelec's Suprasson P5 Booster) and Gracey curettes (Hu-Friedy, Chicago, USA). Oral hygiene instructions for home care procedures were given and patients were reassessed for periodontal parameters and levels of salivary and plasma superoxide dismutase enzyme at an interval of four weeks post-treatment.

Collection of saliva and blood samples and superoxide dismutase enzyme determination

Saliva collection (Figure 1)

Samples of unstimulated whole saliva (2 ml) were collected from all subjects at baseline and at an interval of four weeks post-treatment from the chronic periodontitis patients. Subjects were instructed to void the mouth of saliva prior to collection, by rinsing the mouth thoroughly with water to wash out any debris or exfoliated cells and they were instructed not to eat, drink, chew gum or brush teeth for atleast 30 minutes before sampling. They were seated comfortably with head tilted slightly forward. Saliva was allowed to accumulate in the floor of mouth and they were asked to spit it out into sterile plastic containers. The samples were then transferred to Department of Biochemistry, Sri Guru Ram Das University of Medical Sciences and Research, Amritsar in sterile storage containers maintaining the sample chain. They were then centrifuged at 3,000 rpm for 17 minutes at room temperature. The supernatant was removed and stored in small aliquots at -80°C until analysis.

Blood collection (Figure 2)

2 ml of venous blood was collected from anticubital vein in tubes with anticoagulants (EDTA) at baseline from all the subjects and 1 month after treatment from chronic periodontitis patients. Plasma was separated from blood by centrifugation at the speed of 3000 rpm for 30 minutes and was collected in small aliquots. Samples were then transferred to the Department of Biochemistry, Sri Guru Ram Das University of Medical Sciences and Research, Amritsar maintaining the sample chain. They were then stored at -80°C until analysis.

Superoxide Dismutase Enzyme levels were determined in the plasma and saliva samples by SOD Assay Kit (Qayee-Bio) **(Figure 3)** according to manufacturer's instructions and Enzyme-Linked Immunosorbent Assay (ELISA) system at 450 nm.

Statistical Analysis

The recorded data was collected, compiled and put to statistical analysis to arrive at results. The analysis was performed with a **statistical software package for social sciences version 17.0 (SPSS, Version 17.0, Inc, Chicago).**

The mean values, standard deviation and percentage change in the values of periodontal parameters and salivary and plasma superoxide dismutase levels were analysed using **t-test.** Unpaired **t-test** was applied to evaluate the comparison between control and test groups and paired **t-test** was applied in test group to analyse the change in parameters from baseline to 4th week post-treatment.

Results

The baseline mean values of Plaque index, Gingival index, Sulcus bleeding index, Probing pocket depth were higher in Group II compared to Group I as shown in **Table 1 and Figure 4** whereas baseline mean values of biochemical parameters (Plasma and Salivary SOD) were lower in Group II than Group I as shown in **Table 1 and Figure 5.** The p-value between Group I and Group II was highly significant ($p<0.001$) at baseline for all the parameters.

Table 2 and Figure 6 show highly significant ($p<0.001$) reduction in mean values of plaque index, gingival index, sulcus bleeding index, probing pocket depth and clinical attachment level from baseline to 4th week within group II. However, the mean recession depth value showed a statistically non-significant ($p>0.05$) increase after NSPT in Group II.

The mean plasma and salivary SOD levels showed statistically highly significant ($p<0.001$) increase from baseline to 4-weeks after treatment in group II patients **(Table 2 and Figure 7).**

Discussion

Periodontitis is characterized by an abnormal host response to the subgingival plaque biofilm comprising of hyper-inflammatory response involving excessive release of oxygen radicals and proteolytic enzymes by neutrophils.[22] More specifically, loss of homeostatic balance between proteolytic enzymes & their inhibitors & ROS & antioxidant defenses (e.g. SODs etc) that protect as well as repair vital tissue, cell & molecular components is believed to be responsible.[23] Therefore, there is a need for careful assessment of SOD levels and their association with periodontitis.

Since SOD is released as a homeostatic mechanism to protect the tissues, it can be detected in extra- and intracellular compartments.[24] Thus, the enzyme is present in all body tissues and fluids such as saliva, lymph, gingival crevicular fluid (GCF), plasma etc.

Saliva is the first biological medium confronted by external materials taken into our body as part of food, drink, or inhaled volatile ingredients.[25] Thus, it constitutes the first line of defense against the free radical mediated oxidative stress in oral cavity[11] and is a perfect medium to be explored for health and disease surveillance. With the progression of periodontitis, ROS produced by periodontal inflammation diffuse into the blood stream causing oxidation of various molecules in blood leading to circulating oxidative stress which may gradually injure multiple organs. Therefore, the increase in circulating oxidative stress induced by periodontitis may negatively affect systemic health.[4]

In the present study, Group II had significantly higher baseline values of PI, GI & SBI compared to Group I

which explains the role of periodontal pathogens in the development and severity of periodontal disease.[26] Increase in plaque accumulation results in more gingival inflammation and inflamed gingival tissues bleed when gently probed because of minute ulcerations in the pocket epithelium and the fragility of the underlying vasculature.[21]

Higher mean probing pocket depth in Group II at baseline could be due to increase in bacterial invasion and the amount of plaque that triggered destruction of sulcular epithelium, junctional epithelium and surrounding alveolar bone.[26] Another reason could be that the probe tip penetrates apically through the junctional epithelium into the connective tissue when gingiva is inflamed.[27]

On the contrary, Group II patients showed significantly lower mean plasma and salivary SOD levels compared to Group I at baseline as a consequence of ongoing free radical activity and breakdown of protective antioxidant species indicating a compromised oxidant-antioxidant balance in chronic periodontitis group. Another possible reason for the reduced SOD levels could be that, during CP, the body itself, in response to free radical generation, induces the production of antioxidants as a compensatory mechanism for bacterial killing, but because it is a chronic condition, the enzymes are used up, and the ratio of production/use decreases.[9]

NSPT results in significant reduction in PI, GI, SBI in group II. This can be explained by the fact that the major cause of gingival inflammation is accumulation of microbial plaque in and around the dentogingival complex, which when removed, results in the complete resolution of the inflammatory lesion.[28] SRP also effectively decreases the population of gram-negative microbes while concomitantly allowing for an increase in the population of gram-positive rods and cocci.[29] This shift is usually associated with gingival health.

Highly significant reduction in probing pocket depth from baseline to 4 weeks post-treatment in Group II is due to "Biphasic response": a component due to gingival recession and a component due to gain in clinical attachment.[30],[31] Additionally, resolution of inflammatory state subsequently results in the reorganization of the connective tissue and as a result, the probe stops coronal to its original position.[27] Besides, histological observations indicate that the attachment between tooth and the gingival tissues after periodic root planing, soft tissue curettage and plaque control is primarily accomplished by the formation of a long junctional epithelium.[32] Hence, these results indicate that with removal of supra-and subgingival plaque, control of retention factors and with strict personal hygiene regime, healing periodontal pockets of patients with moderate-severe chronic periodontitis led to a coronary positioning of the level of the epithelial attachment i.e. attachment gain.[33]

Statistically highly significant increase was seen in the values of plasma and salivary SOD levels in group II from baseline to 4 weeks post-treatment. This change in the SOD levels signifies the evidence that NSPT reduces oxidative stress both locally and systemically. This can be attributed to decreased levels of periodontal pathogens post NSPT, thereby decreasing the prevailing inflammation, which in turn reduces the reactive oxygen species production and enables the body defense to restore the physiologic proantioxidant balance.[34] Thus, successful NSPT is highly effective in restoring superoxide dismutase level, hence defending its badge of the gold standard periodontal therapy.

The results of present study are in accordance with **Kim** SC et al. (2010)[35], Karim S et al. (2012)[36], Siqueira MA et al. (2013)[37], Singh N et al. (2014)[38], Daiya S et al. (2014)[39], Fenol A et al. (2015)[34], Elavarasu S et al.

(2016)[40], Bansal N et al. (2017)[41], Narendra S et al (2018).[42]

Conclusion

The present study suggests that significant oxidative stress may occur in periodontitis which leads to excessive production of free radicals like superoxide anions that disturb the subtle balance in favor of free radicals. Periodontal tissue depends on natural antioxidants to overcome this oxidative stress and maintain homeostasis. So, in order to detoxify these superoxide free radicals, large amounts of SOD are utilized. Hence, the levels of these antioxidant enzymes are reduced in cells, tissues and fluids of chronic periodontitis patients in comparison to periodontally healthy controls. When the antioxidants are depleted, the ability of gingival tissues to overcome oxidative stress, maintain normal tissue and control the bacterial damage appears to be compromised. However, non-surgical periodontal therapy with improvements in clinical parameters can increase the antioxidant defense in chronic periodontitis patients.

More longitudinal studies are needed which will further enhance our knowledge on the role of SOD in periodontal health and disease and effect of non-surgical periodontal therapy in restoring the body's antioxidant defenses.

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Legend Tables and Figures

Table 1: Baseline Readings of Clinical And Biochemical Parameters of Group I And Group II

Parameters	Group I	Group II	Grp I VS Grp II (p- value)
Plaque Index (PI)	0.14 ± 0.018	2.39 ± 0.163	<0.001**
Gingival Index (GI)	0.14 ± 0.031	2.41 ± 0.24	<0.001**
Sulcus BleedingIndex (SBI)	0.15 ± 0.03	2.57 ± 0.14	<0.001**
Probing Pocket Depth (PD)	1.22 ± 0.16	5.39 ± 0.39	<0.001**
Plasma Superoxide Dismutase (SOD)	219.89 ± 83.40	79.82 ± 37.28	<0.001**
Salivary Superoxide Dismutase (SOD)	250.30 ± 78.14	121.19 ± 44.29	<0.001**

** P<0.001:Highly Significant, * P<0.05: Significant, P> 0.05: Not significant

Table 2: Comparison of Clinical And Viochemical Parameters At Baseline And After 4 Weeks In Group II

Clinical Parameters	Baseline Score	4^{th} week Score	Baseline - 4^{th} week reduction		
			Mean ± SD	t value	P Value
Plaque Index	2.39 ± 0.163	1.65 ± 0.12	0.74 ± 0.15	26.63	<0.001**
Gingival Index	2.41 ± 0.24	1.23 ± 0.17	1.18 ± 0.17	37.80	<0.001**
Sulcus Bleeding Index	2.57 ± 0.14	1.23 ± 0.20	1.34 ± 0.16	44.94	<0.001**
Probing Pocket Depth	5.39 ± 0.39	4.52 ± 0.36	0.87 ± 0.22	21.38	<0.001**
Recession Depth (RD)	0.85 ± 0.25	0.87 ± 0.25	-0.02 ± 0.06	-1.73	0.095
Clinical Attachment Level	6.25 ± 0.46	5.40 ± 0.41	0.86 ± 0.23	20.80	<0.001**
Biochemical Parameters					
Plasma SOD	79.82 ± 37.28	127.62 ± 40.97	47.79 ± 32.35	8.090	<0.001**
Salivary SOD	121.19 ± 44.29	184.54 ± 58.79	63.35 ± 53.04	6.543	<0.001**

** P<0.001:Highly Significant, * P<0.05: Significant, P> 0.05: Not significant

Figure 1: Unstimulated saliva collection by spitting method

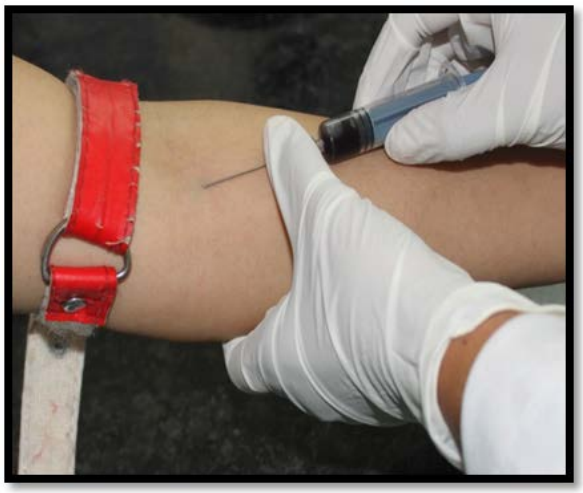

Figure 2: Collection of blood sample

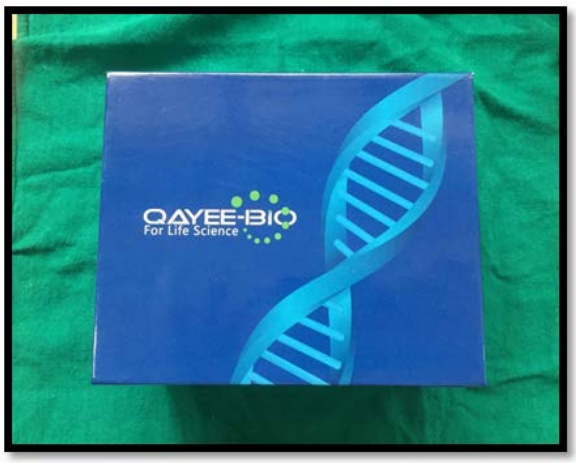

Figure 3: Superoxide dismutase assay kit (QAYEE-BIO)

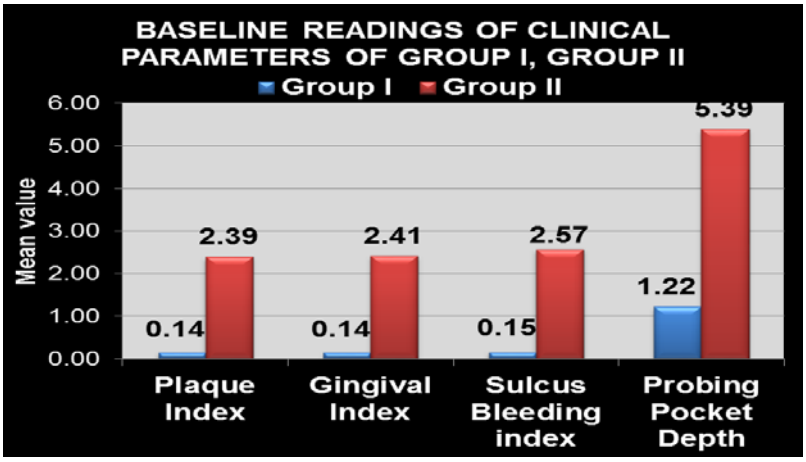

Figure 4: Baseline readings of clinical parameters of group I and II

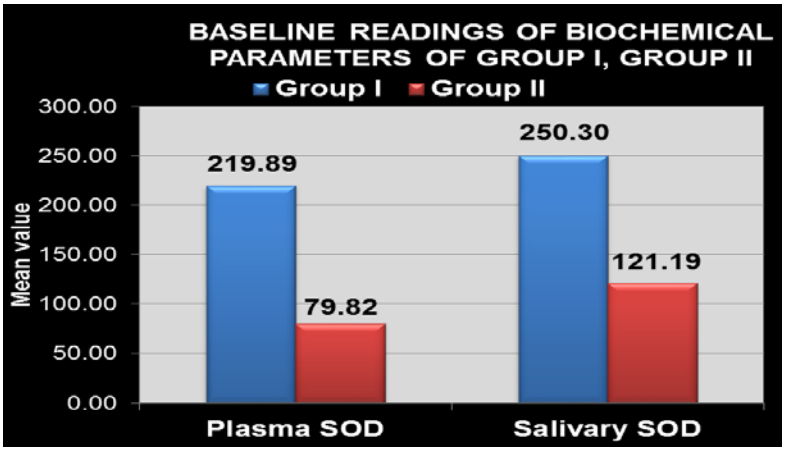

Figure 5: Baseline readings of biochemical parameters of group I and II

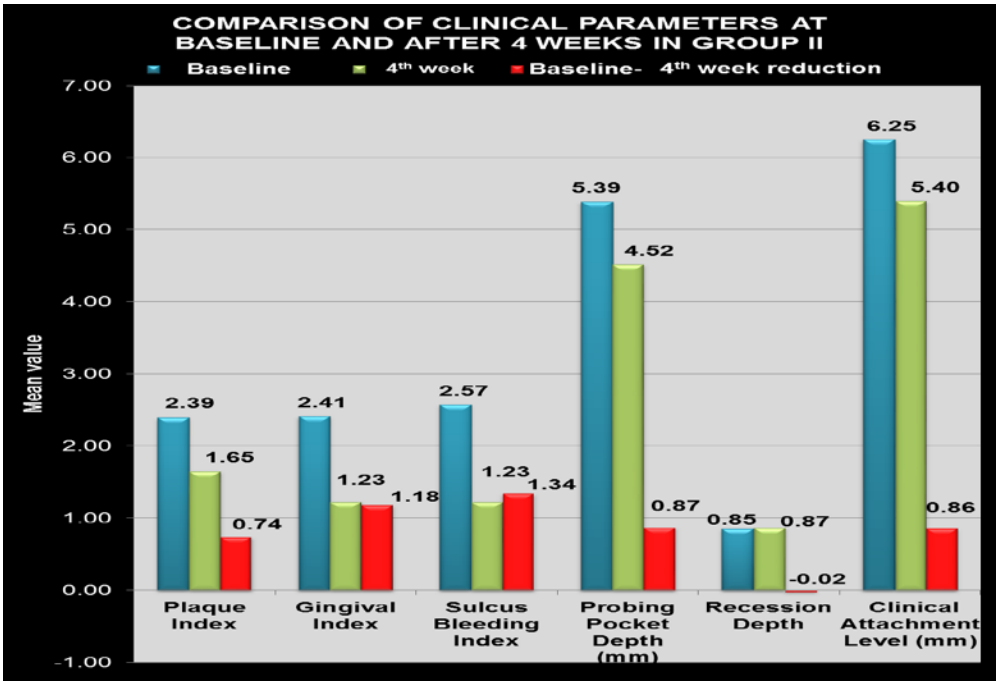

Figure 6: Comparison of clinical parameters at baseline and after 4 weeks in group II

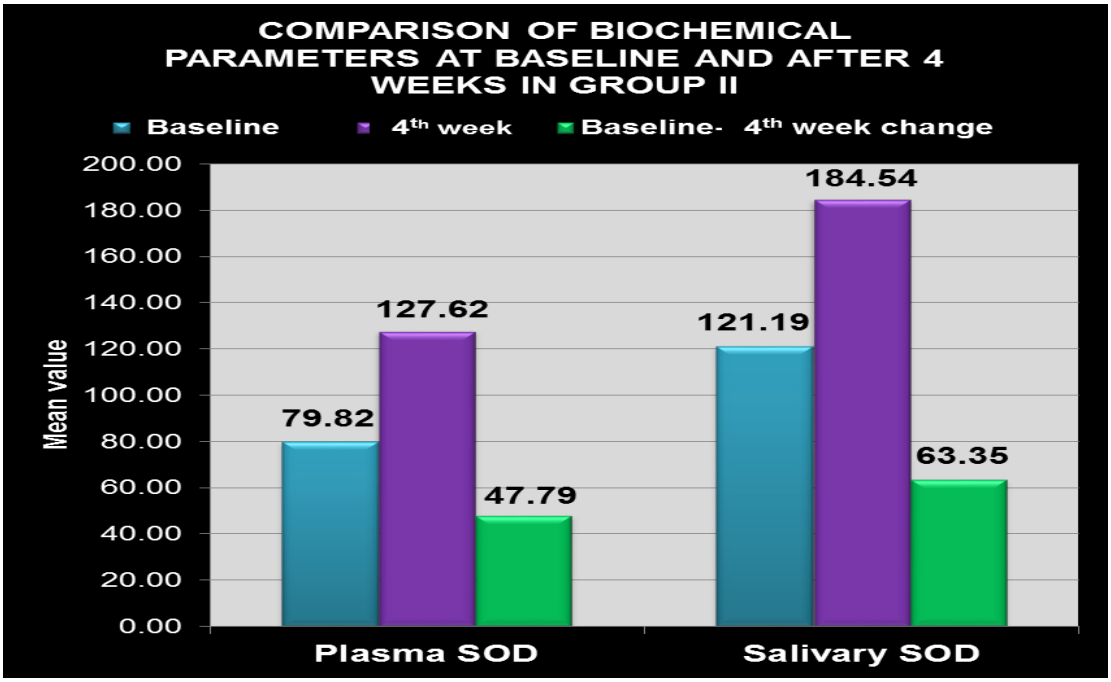

Figure 7: Comparison of biochemical parameters at baseline and after 4 weeks in group II