OXIDATIVE STRESS MODULATES RESPONSE TO NON-SURGICAL PERIODONTAL THERAPY

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A Master’s thesis submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Masters of Science in the Department of Periodontology

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ABDEL-GHANY ALSAIDI: Oxidative Stress Modulates Response To Non-Surgical Periodontal Therapy

(Under the direction of Dr. Steven Offenbacher)

Oxidative stress is an important physiologic modifier of immune and inflammatory mechanisms. We hypothesized that the elevated total-body oxidative stress, measured by serum levels of 8-isoprostane, impairs: 1- biofilm IgG response to periodontal therapy 2- clinical parameter responses to periodontal therapy. 108 subjects diagnosed with moderate to severe chronic periodontitis were recruited in this single-blinded, delayed treatment, randomized, controlled clinical trial. Subjects were randomly assigned to 2 groups (delayed treatment and combination treatment groups). Both groups were clinically evaluated and biological samples collected at minus 6 weeks (run-in period), 0 weeks (baseline- treatment provided to one group only), 2 weeks, 6 weeks, 3 months and 6 months. In addition to probing depth (PD) and bleeding on probing (BOP) measured at 6 sites per tooth, serum was used for analysis of total 8-isoprostane and IgG antibodies against a panel of 17 common periodontal bacteria. At the completion of the study, 87 patients had a complete data set at the 6 month follow-up. Treatment resulted in a transient increase in total IgG levels at 2-weeks which returned to baseline levels by 6 weeks. This increase in IgG titers in response to
treatment was seen predominantly among subjects with low oxidative stress at baseline (2.2 fold greater than high oxidative stress group). Furthermore, low oxidative stress was associated with much better clinical response (PD and BOP) as compared to subjects with high oxidative stress. These findings suggest that systemic oxidative stress impairs IgG response and the clinical response to non-surgical periodontal therapy.
ACKNOWLEDGMENTS

First of all, praise is due to Allah, The Most Beneficent, The Most Merciful, for all His guidance and giving while I was preparing this master’s thesis.

I would like to express my deep gratitude to my supervisors Drs. Steven Offenbacher, Silvana Barros and James Beck for their guidance, suggestions and invaluable encouragement in my graduate education and throughout the development of this thesis.

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LIST OF ABBREVIATIONS

IRB: Institutional Review Board
GCF: Gingival Crevicular Fluid
ROS: Reactive Oxygen Species
IgG: Immunoglobulin G
BOP: Bleeding on Probing
CAL: Clinical attachment level measured in millimeters
PD: Probing Depth measured in millimeters
PMNs: Polymorphonuclear Leukocytes
SD: Standard Deviation
8-Iso: 8-Isoprostane
Tx: Treatment
ANOVA: ANalysis Of VAriance between groups
APC: Antigen-presenting cell
BMI: Body mass index
LPS: Lipopolysaccharide
COSD: Center for Oral and Systemic Diseases
INTRODUCTION AND BACKGROUND

Periodontitis is a term used to describe an inflammatory process, initiated by the plaque biofilm, that leads to loss of periodontal attachment to the root surface and adjacent alveolar bone and which ultimately results in tooth loss unless treated. The inflammatory and immune responses to the bacteria and also viruses\(^1\) that colonize the periodontal and associated tissues involve the systemic circulation and ultimately the peripheral systems of the body. This creates a complex bi-directional series of host–microbial interactions involving cellular and humoral factors and networks of cytokines, chemokines, and growth factors. It is believed that the primary etiological agent is specific, predominantly gram-negative anaerobic or facultative bacteria within the sub-gingival biofilm.\(^2, 3\)

The majority of periodontal tissue destruction is caused by an inappropriate host response to those microorganisms and their products.\(^3\) More specifically, a loss of homeostatic balance between proteolytic enzymes (e.g. neutrophil elastase) and their inhibitors (e.g. \(\alpha_1\)-antitrypsin) and reactive oxygen species (ROS) and the antioxidant defense systems that protect and repair vital tissue, cell, and molecular components is believed to be responsible.

The basis for such dysregulation is in part genetic (38–82\%),\(^4\) and in part the result of environmental factors (e.g. smoking).\(^5\)
In humans, oxidative stress is thought to be involved in the development of many diseases or may exacerbate their symptoms. These include cancer, Parkinson's disease, Alzheimer's disease, atherosclerosis, heart failure, myocardial infarction, Schizophrenia, Bipolar disorder, fragile X syndrome, Sickle Cell Disease, lichen planus, vitiligo, autism, and chronic fatigue syndrome.\textsuperscript{5}

A paradigm shift in our understanding of the importance of reactive oxygen and antioxidant species to human biology over the last decade came from the realization that vital and ubiquitous transcription factors, such as nuclear factor-κB and activating protein-1 were redox-sensitive. Large, upward shifts in the pro-oxidant/antioxidant ratio intracellularly bring about direct damage to vital biomolecules and structures, cell membrane damage and dysfunction, and cell death (by necrosis or accelerated apoptosis), and extracellularly cause direct connective tissue damage (both mineralized and unmineralized) and damage to extracellular matrices and their components (Fig. 1).\textsuperscript{6}
Figure 1. The biological effects of small and large shifts in the balance of activity between reactive oxygen species (ROS) and antioxidants (AO) – adapted from Chapple et al. Reproduced with permission.

**OXIDATIVE STRESS**

Oxidative stress was defined by Sies in 1991 as ‘a disturbance in the pro-oxidant–antioxidant balance in favor of the former, leading to potential damage’. This disturbance in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA. Further, some reactive oxidative species act as cellular messengers in redox signaling. Thus, oxidative stress can cause disruptions in normal mechanisms of cellular signaling.

In normal physiology there is a dynamic equilibrium between ROS activity and antioxidant defense capacity, and when that equilibrium shifts in favor of ROS, either by a reduction in antioxidant defenses or an increase in ROS production or activity, oxidative stress is the consequence.
Chemically, oxidative stress is associated with increased production of oxidizing species or a significant decrease in the effectiveness of antioxidant defenses, such as glutathione.\(^9\)

The effects of oxidative stress depend upon the size of these changes, with a cell being able to overcome small perturbations and regain its original state. However, more severe oxidative stress can cause cell death and even moderate oxidation can trigger apoptosis, while more intense stresses may cause necrosis.\(^{10}\)

Production of reactive oxygen species is a particularly destructive aspect of oxidative stress. Such species include free radicals and peroxides. Some of the less reactive of these species (such as superoxide) can be converted by oxidoreduction reactions (Redox) with transition metals or other redox cycling compounds (including quinones) into more aggressive radical species that can cause extensive cellular damage.\(^{11}\)

The major portion of long-term effects is inflicted by damage to DNA.\(^{12}\)

The DNA damage that can be induced by ionizing radiation is similar to oxidative stress, and these lesions have been implicated in aging and cancer. Most of these oxygen-derived species are produced at a low level by normal aerobic metabolism. Normal cellular defense mechanisms destroy most of these ROS and free radicals. Likewise, any damage to cells is constantly repaired. However, under the severe levels of oxidative stress that cause necrosis, the damage causes ATP depletion, preventing controlled apoptotic death and causing the cell to simply fall apart.\(^{13,14}\)
Thus, Oxidative stress is an important physiologic modifier of immune and inflammatory mechanisms. Mediators of oxidative stress or “redox signaling”, or both, have been shown to regulate receptor and transcription factor signaling and kinase dependent signaling pathways, thereby inducing the expression of key cytokines and inflammatory mediators. Oxidative stress can shift the balance of Th1 and Th2 cytokine profiles in in vitro and in vivo model systems. This shift is usually toward increased levels of Th2 cytokines, suggesting that oxidative stress might modify humoral immune responses to commensal and pathogenic microorganisms. Clinical conditions associated with increased oxidative stress in humans (e.g., diabetes, periodontitis, and aging) have been associated with impaired immune function. Smoking, a recognized risk factor for periodontitis has been associated with depressed levels of total serum IgG2, which is a Th1-dependent IgG antibody subtype, and with decreased serum levels of antibody for selected oral bacteria.

**ROS & FREE RADICALS**

Free radicals are defined as ‘any species capable of independent existence that contain one or more unpaired electrons’.
They are, by nature, highly reactive and diverse species, capable of extracting electrons and thereby oxidizing a variety of biomolecules vital to cell and tissue function, which not only include oxygen free radicals, but also nitrogen and chlorine species. ROS is a term that has become more popular because it encompasses other reactive species which are not true radicals but are nevertheless capable of radical formation in the intra- and extracellular environments. ROS are chemically reactive molecules containing oxygen. Examples include oxygen ions and peroxides. ROS form as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signaling and homeostasis. However, during times of environmental stress (e.g., UV or heat exposure), ROS levels can increase dramatically. This may result in significant damage to cell structures. Cumulatively, this is known as oxidative stress. ROS are also generated by exogenous sources such as ionizing radiation, trauma, and smoking.

**ANTIOXIDANTS**

They are defined as “those substances which when present at low concentrations, compared to those of an oxidizable substrate, will significantly delay or inhibit oxidation of that substrate.”
Given that it is estimated that between 1 billion and 3 billion reactive species are generated per cell per day, the importance of antioxidant defense systems to the maintenance of health becomes clear.\textsuperscript{28}

The preventative antioxidants function by enzymatic removal of superoxide and hydrogen peroxide or by sequestration of divalent metal ions, preventing Fenton reactions and subsequent hydroxyl radical formation.\textsuperscript{29}

The etiology of Alzheimer's disease progression is still debated; however, increased oxidative stress is an early and sustained event that underlies much of the neurotoxicity and consequent neuronal loss. Amyloid beta is a metal binding protein and copper, zinc and iron promote amyloid beta oligomer formation. Additionally, copper and iron are redox active and can generate reactive oxygen species via Fenton (and Fenton-like chemistry) and the Haber-Weiss reaction. Copper, zinc and iron are naturally abundant in the brain but Alzheimer's disease brain contains elevated concentrations of these metals in areas of amyloid plaque pathology. Amyloid beta can become pro-oxidant and when complexed to copper or iron it can generate hydrogen peroxide. Accumulating evidence suggests that copper, zinc, and iron homeostasis may become perturbed in Alzheimer's disease and could underlie an increased oxidative stress burden.
Lactoferrin is probably more important than transferrin within the periodontal tissues, given the dominance of the neutrophil infiltrate\textsuperscript{29} and the recognition of high levels of lactoferrin within gingival crevicular fluid.\textsuperscript{30}

Antioxidants neutralize free radicals by donating one of their own electrons, ending the electron-"stealing" reaction. The antioxidant nutrients themselves don't become free radicals by donating an electron because they are stable in either form. They act as scavengers, helping to prevent cell and tissue damage that could lead to cellular damage and disease.

Vitamin E (α-tocopherol) the most abundant fat-soluble antioxidant in the body. One of the most efficient chain-breaking antioxidants available. Primary defender against oxidation. Primary defender against lipid peroxidation (creation of unstable molecules containing more oxygen than is usual).\textsuperscript{31}

Vitamin C ( L-ascorbic acid, or simply ascorbate ) The most abundant water-soluble antioxidant in the body. Acts primarily in cellular fluid. Of particular note in combating free-radical formation caused by pollution and cigarette smoke. Also helps return vitamin E to its active form.
**Figure 2: Major cellular sources of ROS in living non-photosynthetic cells**

**8-ISOPROSTANES**

The Isoprostanes are prostaglandin-like compounds formed *in vivo* from the free radical catalyzed peroxidation of essential fatty acids (primarily arachidonic acid) without the
direct action of cyclooxygenase (COX) enzyme. The isoprostanes possess potent biological activity, and their potential role in mediating certain aspects of the detrimental effects of oxidant stress is then examined. In addition, evidence showed that these biological effects may be mediated through interaction with a unique receptor. A considerable portion of this commentary deals with the utility of measuring isoprostanes as markers of oxidant injury both in vitro and in vivo. A number of studies have shown these compounds to be extremely accurate markers of lipid peroxidation in animal models of oxidative stress and have illuminated the role of oxidant injury in association with several human diseases.  

Lipid peroxidation refers to the oxidative degradation of lipids. It is the process in which free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage. Isoprostanes and their metabolites have also been shown to be elevated in the urine of cigarette smokers, and have been suggested as biomarkers of oxidative stress in smokers.

Direct-8-iso-Prostaglandin F2a (d-8-iso PGF2a) is a stable end product of both specific inflammatory enzymatic pathways and nonspecific mechanisms and reflects total lipid peroxidation, representing an excellent in vivo marker for oxidative stress.

**OXIDATIVE STRESS & PERIODONTAL DISEASE**

The idea that ROS are associated with the pathogenesis of a variety of inflammatory diseases and have a role (direct or indirect) in tissue damage has become a major area of research over the last decade as demonstrated by electronic searches of the
literature. However, supporting evidence for their role in tissue damage is often indirect and circumstantial.\(^6\)

The relationship to periodontal disease could be derived from understanding the mechanism in which ROS can affect tissue damage\(^5\)

1- Protein damage

2- Lipid peroxidation

3- DNA damage

**Bleeding on probing**

BOP is a term used referring to bleeding that is induced by gentle manipulation of the tissue at the depth of the gingival sulcus or pocket, or interface between the gingiva and a tooth. BOP is a sign of inflammation and indicates some sort of destruction and erosion.\(^{35}\)

Also, since \(P.\) gingivalis, a very important periodontal pathogen, has an absolute requirement for heme (from blood, and cultivated using blood agar plates in vitro) it is an intriguing relationship between BOP and periodontal disease. Longitudinal studies on BOP are available. At present, bleeding on probing is widely used as an indication for needed treatment. However, bleeding on probing alone is not a predictor of elevated risk for future loss of clinical attachment. On the other hand, a lack of bleeding on probing, especially on two or more occasions, is an excellent indicator of periodontal health, with a predictive value of 0.972.\(^{36}\)
The general hypothesis of this study is that elevated levels of oxidative stress at baseline (measured by serum 8-isoprostane) will have a detrimental effect on the antibody and clinical response to periodontal therapy.

We could divide the hypothesis into

1- Systemic oxidative stress, as measured by increasing serum levels of 8-isoprostane, are a significant negative modifier of the serum-antibody responses to mucosal biofilm microorganisms. Specifically, we hypothesized that increased oxidative stress would be associated with suppression of the systemic IgG antibody responses to the indigenous oral biofilm microorganism

2- Systemic oxidative stress, as measured by increasing serum levels of 8-isoprostane, are a significant negative modifier of the clinical response to non-surgical periodontal therapy as measured of Bleeding on Probing (BOP) and Probing Depth (PD).
MATERIALS AND METHODS

**Study Population and Power Analyses**

This study was approved by the Institutional Review Board at the University of North Carolina at Chapel Hill (IRB project #2019). A total of 108 systemically healthy subjects aged between 23-63 years, diagnosed with moderate to severe chronic Periodontitis were recruited. The sample size estimate was based upon the anticipated decrease in markers of serum inflammatory response, using CRP as the primary effect size estimator. (The original investigators anticipated having 90% power to detect a 95% decrease in serum markers, using CRP estimates as previously reported by D'Aiuto et al.).

This report is a secondary analysis of the investigation to assess the relationship between oxidative stress, serum IgG and clinical outcomes.

**Inclusion and Exclusion Criteria**

108 subjects were recruited; all were diagnosed with moderate to severe periodontitis based on the 1999 International Workshop for a Classification of Periodontal Diseases and Conditions.
Subjects were excluded if they were diabetic, had any acute infection, history of antibiotic use in the month prior to the baseline assessments, needed prophylactic antibiotic coverage or had a history of active periodontal treatment during the year prior to the clinical evaluation.

**Study Design**

Subjects were recruited into this single-blinded, delayed treatment, controlled randomized clinical trial. They were randomized to one of two treatment arms

1- **Group 1: Combined therapy (scaling and root planing plus Oral Hygiene Instructions)**

   Subjects (Combination Treatment) received scaling and root planing at baseline (0 week) along with oral hygiene instructions and supportive therapy continued through the 6 month interval consisting of 1- a dentifrice with sodium fluoride, with a choice of Crest Cavity Protection or Crest Whitening 2- Daily flossing using Glide dental floss, 3- use of Crest SpinBrush® and 4- Scope mouthwash (non-market FDA Monograph-compliant Scope® cetylpyridinium chloride (CPC) Mouthwash for twice daily rinsing. 53 subjects were randomly assigned to the this treatment arm and 42 completed the 24 week study.

2- **Group 2: Delayed treatment. Subjects were followed for 6 months.**
At baseline group 2 (Delayed treatment) received examinations and biological sampling only with no oral hygiene instructions or scaling or root planing. Upon completion at 6 months they received the same full treatments as above with the exception of the mouthwash. 53 subjects were randomly assigned to this treatment arm and 45 completed the 24 week study.

![Study timeline](image)

**Figure 3. Study timeline**

<table>
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<th>VISIT 2 0</th>
<th>VISIT 3 2</th>
<th>VISIT 4 6</th>
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</table>

**Table 1. Study timeline procedures**
Run-in Period & Randomization of subjects

The run-in period in this study was the 6 weeks period before the clinical trial commenced (when no treatment was given). It was employed to ensure that participants were in a stable condition and compliant. It was also used as a washout period if treatments that participants were using before entering the clinical trial were discontinued.

Subjects were randomly assigned to each treatment arm, adjusting for sex, age, smoking status and BMI.

Calibration & Blinding of examiners

In our study, 3 examiners performed the clinical measurements and harvested the blood samples at the appropriate time-points. The examiners were calibrated for probing depth, clinical attachment loss measurements and bleeding on probing detection. 98%, 80% and 92% agreements were noted for PD, CAL and BOP respectively in a pre-study calibration exercise.

The analysis aimed to determine whether periodontal treatment modified the serum IgG response to oral organisms and the relationship of that response to levels of serum 8-isoprostane.

We hypothesized that individuals with high oxidative stress would have an impaired IgG response and would potentially have an impaired wound healing response. The clinical component of the study began in spring of 2002 and was completed in early 2005.
**Serum IgG and 8-isoprostane Analyses**

Following a 6 week run-in period during which no treatment was provided, blood samples and periodontal parameters were collected from both study groups at 0, 2, 6, 12 and 24 weeks.

Blood samples were collected and processed for measurement of serum IgG levels for specific oral organisms as described by Singer et al.\textsuperscript{39}

Total IgG directed against oral organisms was computed by summing across all IgG specific organism which included *Porphyromonas gingivalis; Prevotella intermedia; Prevotella nigrescens; Tannerella forsythensis; Treponema denticola; Fusobacterium nucleatum; Aggregatibacter actinomycetemcomitans; Campylobacter rectus; Eikenella corrodens; Parvimonas micra; Veillonella parvula; Capnocytophaga ochracea; Selemonas noxia; Actinomyces viscosus; Streptococcus intermedius; Streptococcus sanguis; and Streptococcus oralis.*

**Total 8-isoprostane**

8-isoprostane is a stable end product of both specific inflammatory enzymatic pathways and nonspecific mechanisms and reflects total lipid peroxidation, representing an excellent *in vivo* marker for total body oxidative stress.\textsuperscript{39}

The d-8-iso PGF2a assay is based on the competition between sample 8-iso-PGF2a and a fixed amount of alkaline phosphatase (AP)-labeled 8-iso-PGF2a for sites on a rabbit polyclonal anti-8-iso-PGF2a (Assay Designs, Ann Arbor, MI). During the incubation, the
polyclonal antibody becomes bound to the goat anti-rabbit antibody coated onto the microplate. After a wash to remove excess conjugate and unbound sample, a substrate solution is added to the wells to determine the bound enzyme activity. Color development is stopped, and absorbance read at 405 nm. The intensity of the color is proportional to the amount of AP-PGF2a bound to the well, which is inversely proportional to the concentration of total 8-iso-PGF2a originally present in the sample. Standard curves ranged from 100,000 down to 32 pg/ml, and serum samples were diluted 1:5 to yield values within the working range. Deviations of standard duplicates ranged from 1.0 to 18.8%, with a mean deviation of 5.9%. Sensitivity limit of detection was estimated by the manufacturer as 103.2 pg/ml for the 2-h incubation format.

Participants were ranked on the serum concentration of d-8-iso PGF2a. With this ranking, participants in the top half (above the median) of concentration of d-8-iso were considered “High oxidative stress” and those in the remaining lower half of concentration of d-8-iso were considered “Low oxidative stress”.

**Statistical Analysis**

Statistical analyses and data management were performed using SAS (SAS Institute, Cary, NC); statistical significance was set at p≤0.05. Frequency distributions, means, functions, and standard errors were determined to describe the data. Bivariate relations
were investigated by using t tests for continuous variables. Multivariable modeling was performed by using SAS Procedure GLM to calculate least-squared means. 2-way ANOVA and Graphs were performed and generated using GraphPad Prism™ (2013 GraphPad Software, Inc)
RESULTS

Study Population Demographics

We enrolled and randomized 108 participants which included 55 delayed treatment control subjects and 53 treatment subjects. The characteristics of this population appear in Table 2. At enrollment there were no significant differences in the treatment or delayed treatment groups with regards to age, sex, ethnicity, smoking status and BMI among the two study groups (table 2), suggesting that randomization was effective in matching the two groups at baseline. The mean participant age was 46.1 (for the control group) and 45.3 (for the treatment group) years old with a standard deviation ranged from 4.4 to 10.3 (Table 3). The majority of the participants were Caucasians (48.6%), and more female participants were recruited (60.2%). BMI was recorded for all participants and ranged from 15.8 to 58.3 kg/m2. Statistically significant differences were not found between the treatment and control arms for the 87 subjects who successfully completed the study.
<table>
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<th>DELAYED TREATMENT (CONTROLS) (N=55)</th>
<th>OVERALL (N=108)</th>
<th>TWO-SIDED P-VALUE</th>
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<td>11 (20.0%)</td>
<td>23 (21.3%)</td>
<td></td>
</tr>
<tr>
<td>BMI [lbs/m2]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>29.8 (7.7)</td>
<td>28.5 (6.0)</td>
<td>29.1 (6.9)</td>
<td>0.35</td>
</tr>
<tr>
<td>Min.-Max.</td>
<td>15.8-58.3</td>
<td>18.2-47.4</td>
<td>15.8-58.3</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Study Demographics
Table 3. Summary of evaluable subject population

**Total Serum Biofilm IgG Antibody Response**

Figure 4 demonstrates the levels of total IgG specific for the oral biofilm at baseline, 2, 6, 12 and 24 weeks for both the treated and untreated groups. In the delayed treatment group there were no changes in total IgG levels over time. However, in the group that received scaling and root planing plus OHI there was a significant increase in IgG titer at 2 weeks, (p<0.01) as compared to the delayed treatment group, but the levels returned to baseline by 6 weeks.
Thus, periodontal therapy was associated with a temporal increase in serum IgG probably directed against the oral biofilm development.

Thus, the combined therapy was a potent inducer of the acquired immune response leading to increased titers of immunoglobulins, which is consistent with a protective role of IgG serum titer.

Figure 4. Total IgG for Treatment and control groups indicating a statistically significant increase in total IgG noted at 2 weeks for the Tx group (*P<0.01)

There were no significant time or treatment-based changes in levels of 8-isoprostane, comparing the treatment to the delayed treatment group over time (data not shown).
IgG Response Stratified on levels of Oxidative Stress

Studies in a large cross-sectional population cohort demonstrated that high oxidative stress was strongly associated with low total IgG levels.\textsuperscript{39}

We compared antibody changes stratified upon baseline levels of 8-isoprostane dichotomized at the median (high or low). First, we examined the effect of the baseline oxidative stress levels on the IgG response to non-surgical periodontal therapy. The IgG titers for all subjects with 8-iso values above the median (table 2) were included in the high-oxidative stress sub-group and all the subjects with 8-iso values below the median (table 2) were included in the low-oxidative stress sub-group, to divide our cohort to 4 groups for statistical analysis

1- High oxidative stress in the combination treatment group

2- Low Oxidative stress in the combination treatment group

3- High Oxidative stress in the delayed treatment group

4- Low Oxidative stress in the delayed treatment group

The changes in total oral biofilm IgG levels for the four subgroups are shown in figure 5. These data demonstrate that the overall increase in total IgG seen in Table 3 above can be entirely attributable to the increase in IgG titer seen among treated subjects with low 8-isoprostane at baseline. The elevation in IgG titer at 2 weeks within the low oxidative stress subgroup is approximately 2.2 fold greater, as compared to the high oxidative stress group, which does not change significantly from baseline levels.
Figure 6 highlights the two-week change in IgG titer. Since serum IgG is likely to be protective and a reflection of activation of the acquired protective Th2 response, the effects of low oxidative stress improving clinical outcomes is logically of interest.
Table 4. Summary statistics for the changes in serum 8-isoprostane (in ng/ml) throughout the study for both groups, means are measured in ng/ml all others pg/ml.

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>Visit</th>
<th>Combination Treatment Group</th>
<th>Delayed Treatment Group (Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Mean (SD)</td>
<td>Median</td>
</tr>
<tr>
<td>Week -6</td>
<td>46</td>
<td>57.5 (99.7)</td>
<td>1061</td>
</tr>
<tr>
<td>Week 0</td>
<td>39</td>
<td>96.7 (12.8)</td>
<td>1415</td>
</tr>
<tr>
<td>Week -6 &amp; 0 Mean</td>
<td>50</td>
<td>78.0 (90.9)</td>
<td>4072</td>
</tr>
<tr>
<td>Week 2</td>
<td>40</td>
<td>88.1 (120.3)</td>
<td>1374</td>
</tr>
<tr>
<td>Week 6</td>
<td>40</td>
<td>75.6 (115.6)</td>
<td>1020</td>
</tr>
<tr>
<td>Week 12</td>
<td>40</td>
<td>57.1 (105.3)</td>
<td>912</td>
</tr>
<tr>
<td>Week 24</td>
<td>38</td>
<td>63.2 (195.7)</td>
<td>916</td>
</tr>
</tbody>
</table>

Figure 5. Total bacterial IgG in means, divided into 4 groups (Tx high oxidative stress, Control high oxidative stress, Tx low oxidative stress and Control low oxidative stress) at all 5 time points.

**shows a statistically significant increase in total IgG count (P<0.01)
Figure 6. Total IgG change between 2 weeks and 0 time-point for the Tx and Control groups divided based on oxidative stress levels, a statistically significant increase in total IgG for the low oxidative stress Tx group as compared to the high oxidative stress. (*P<0.03)

**Changes in Clinical Signs**

We examined the effects of treatment over time (in PD, BOP and AL). There were no significant changes in CAL, but there were improvements in the Bleeding on Probing (BOP) and Probing Depth (PD). Further explanation of the choice of PD over CAL for this study can be found in the discussion section.

It is important to note that no adverse events were reported during the 6 weeks run-in period.
Bleeding on probing Stratified on Levels of Oxidative Stress and treatment

Baseline values and changes in response to therapy in BOP scores are shown in figure 7. Subjects with low oxidative stress exhibit less BOP at baseline as compared to the controls with high oxidative stress; this finding was statistically significant (P<0.04). Also, a significant decrease in mean percentage of BOP was noted in the low oxidative stress Tx group at 6 weeks as compared to the high oxidative stress Tx group. This difference persists at 12 and 24 weeks.

Figure 7. Mean extent bleeding on probing for all 4 groups in percentages. Shows a statistically significant decrease in the percentage of BOP between Tx groups with high ox stress and Tx group with low at baseline (P< 0.04). Similar findings between the control groups.
Probing depth Stratified on Levels of Oxidative Stress and treatment

The mean probing depth was analyzed using 2-way ANOVA was performed to determine the statistical significance. A significant decrease in mean PD was noted for the low oxidative stress group at 12 and 24 weeks (but not at 6 weeks) as compared to low oxidative stress controls at base line (Figure 8)

Figure 8. Mean probing depth in millimeters for all 4 groups at 0, 6, 12 and 24-week time points, show a statistically significant decrease of PD for the low oxidative stress Tx group at 12 and 24 weeks when compared to the low oxidative stress controls at baseline (P<0.05)

The systemic oxidative stress level is dynamic and likely changes for individuals during the 24 week monitoring period. Although we demonstrated that low levels of oxidative stress was associated with improved responses to treatment (BOP and PD changes), we
examined whether those individuals with persistently high 8-isoprostane (above the median) as evaluated at each timepoint differed significantly in the healing response as compared to those that were consistently low in 8-isoprostane levels, below the median, as assessed at baseline, 2, 6, 12 and 24 weeks.

Figure 9 shows the clinical changes in BOP for subjects that have consistently low, consistently high and mixed levels of 8-isoprostane. It can be observed that all subgroups responded with no statistically significant difference observed.

![Figure 9. Mean Bleeding on Probing extent (percentage) in Response to Treatment throughout the study](image)

6 Weeks: L=Low OS at Baseline, H=High OS at Baseline; p-value 0.95, 12 weeks: LL=Low OS at Baseline & at 6 weeks (Reference), HH=High OS at Baseline & at 6 weeks. MIX=combination of low or high at baseline or 6 weeks.
p-values are 0.78 and 0.95 for Mix and HH respectively. 24 weeks: LLL=Low OS at Baseline, 6 weeks and 3 months, HHH=High OS all previous time points. MIX=combination of low or high OS at baseline, 6 weeks and 12 weeks. p-values are 0.76 and 0.56 for Mix and HH respectively.

Figure 10 shows the clinical changes in PD for subjects that have consistently low, consistently high and mixed levels of 8-isoprostane at 6, 12 and 24 weeks. It can be observed that LLL group had a better reduction in probing depth mean at 6 months as compared to the MIX and HHH matched groups P< 0.03 and 0.002 for Mix and HHH respectively.
Figure 10. Mean Pocket Depth (in millimeters) in Response to Treatment throughout the study

6 Weeks: L=Low OS at Baseline, H=High OS at Baseline; p-value 0.3.

12 weeks: LL=Low OS at Baseline & at 6 weeks, HH=High OS at Baseline & at 6 weeks, MIX=combination of low or high at baseline or 6 weeks. p-values are 0.41 and 0.19 for Mix and HH respectively.

24 weeks: LLL=Low OS at Baseline, 6 weeks and 12 weeks, HHH=High OS all previous time points. MIX=combination of low or high OS at baseline, 6 weeks and 12 weeks. (*)= P=0.02
DISCUSSION

Several studies have shown that periodontal treatment improves the systemic levels of antibodies specific to periopathogens.\textsuperscript{40-42} Also improves the periodontal clinical parameters used in the diagnosis of periodontal disease.

It was demonstrated throughout multiple reports in the medical literature that therapies designed to reduce oxidative stress have been related to improved outcomes in viral infections and improved cell-mediated immune function in animal models and human clinical trials.\textsuperscript{43, 44}

Thus our goal is to determine the association of systemic levels of oxidative stress with serum levels of IgG antibody to commensal and pathogenic microorganisms’ response/alteration to periodontal therapy within the periodontal patient population.

The loss of homeostatic balance between proteolytic enzymes (e.g. neutrophil elastase) and their inhibitors (e.g. a1-antitrypsin) and reactive oxygen species (ROS) and the antioxidant defense systems to protect and repair vital tissue, cell, and molecular components is believed to be responsible for periodontal tissue destruction. The basis for such dysregulation is in part genetic and in part the result of environmental factors (e.g. smoking).\textsuperscript{6}
Our findings demonstrate that serum concentrations of 8-isoprostane above the median are related to decreased response of the serum IgG antibodies to oral bacteria. These findings were controlled for smoking status and other known contributors to oxidative stress and periodontal severity (e.g., age and BMI) (Table 1). These findings are in exact accordance with the Singer, Beck and Offenbacher report which was the first to demonstrate an association between serum 8-isoprostane concentrations and serum IgG antibody levels to mucosal microbes. Thus, this investigation demonstrates that systemic oxidative stress consistently suppresses serum IgG antibody responses to total oral biofilm microbes.

In our study population which could be considered a large representative population (108 subjects), extremes of oxidative stress have been related to changes in a variety of immune-response mechanisms. The bacteria in our panel of antibodies included gram-negative and gram-positive bacteria, strict anaerobes, aerobes, and facultative anaerobes, as well as species associated with periodontal disease and oral health.

Singer et al. showed that serum IgG antibody responses to the oral biofilm microflora are suppressed in the presence of systemic oxidative stress. Additional studies are needed to clarify the role of IgG subtype and avidity to these findings. Singer et al. suggested possible studies to explore the clinical impact of oxidative stress in mucosal disease progression, in the response to therapy, and in the potential use of antioxidant therapies as modifiers.
Our study investigated the response to non-surgical periodontal therapy in subjects with high oxidative stress as compared to low oxidative stress subjects; the findings supported our initial hypothesis that periodontal subjects with high oxidative stress at baseline will respond less favorably to treatment, as shown on the immunological level by investigating the total oral biofilm serum IgG titers response.

Although these titers reflect whole-bacterial titers, a high degree of specificity occurs in organism-specific IgG without significant cross-reactivity across various microbes under the stringency conditions of the immunobinding. 47

Thus, the effect of oxidative stress on IgG responses appears to reflect a suppression of IgG across a wide range of potential antigenic stimuli.

8-Isoprostane is a stable product of the oxidative metabolism of arachidonic acid, 47 and 8-isoprostane concentrations in serum and urine have been used as markers of systemic oxidative stress, 48 and are related to the accumulation of oxidized-LDL. 49

The serum concentration of 8-isoprostane has been reported to be related to body mass index and race. 50

To understand the mechanism for the association of 8-isoprostane concentrations with decreased IgG antibody levels, it will be necessary to define better the specific mediators of lipid-based redox signaling, specific cellular and molecular targets, and correlations of IgG subclass antibody levels (and antibody isotypes) to serum 8-isoprostane concentrations. 39
It is expected that clinical and mechanistic insights from these future investigations will enable interventions that better target populations and mechanisms likely to have impact on the systemic diseases that have been associated with systemic exposure to the mucosal biofilm microflora.

8-isoprostane concentrations reflect whole-body lipid oxidation, including enzymatic and nonspecific free-radical lipid oxidative pathways; it is likely that these variables and physiologic or pathologic factors (or both) that influence oxidative lipid metabolism contributed to the serum 8-isoprostane concentrations. Singer et al.\(^{39}\) further indicated that an increased prevalence of deep probing depths (periodontal disease) is associated with increased (1.6-fold) serum concentrations of 8-isoprostane. The theory that was proposed to explain this elevation was that the epithelial lining of the periodontal pocket is adjacent to the subgingival biofilm and is structurally unique without tight junctions or keratin. Even in health, the epithelial attachment to the teeth is a site that has a natural IL-8 chemotactic gradient, which brings neutrophils into the periodontal pocket such that they flush around the teeth and appear in the saliva. Activated neutrophils within the crevice and saliva may serve to increase salivary 8-isoprostane, as recently reported by Wolfram et al.\(^{51}\)

Thus, the periodontal tissues may represent a source of systemic oxidative stress because of the presence of high numbers of activated neutrophils.\(^{39}\)
It is known that the presence of periodontitis is associated with lower serum concentrations of vitamin C, even after adjusting for smoking, suggesting that periodontal disease may pose an oxidative stress that consumes ascorbate. Also, the mucosal tissue wounding associated with deep periodontal pockets has been associated with an increased occurrence of bacteremia. Herzenberg et al. found that extreme high levels of oxidative stress have been related to changes in a variety of immune-response mechanisms. It was hypothesized that infiltrating neutrophils or macrophages, or both, capable of mounting an oxidative burst might control the periodontal microflora and thereby limit consequent serum IgG antibody responses, leading to an inverse relation between serum 8-isoprostane and IgG antibody levels. An increased presence of periodontal pockets of PD greater than 5mm would provide a greater reservoir of activated periodontal PMNs and macrophages and was associated here with increased serum 8-isoprostane levels; however, the greater number of deep pockets also was associated with significant increases in levels of serum IgG antibodies. The second part of this study was to look at the clinical response for periodontal subjects with high oxidative stress to periodontal therapy as evaluated by BOP and PD; the present study suggests that the outcome in high oxidative stress subjects is less favorable than in low oxidative stress periodontal subjects. In this study we mainly looked at the changes in PD even though clinical attachment level (CAL) has been more frequently used than PD to evaluate the effect of periodontal
treatment. However, even if teeth have high CAL, their PD may be less than 3 mm. IgG titer levels, transient in nature, must be influenced more by the size of the area of current infection than by the history of tissue destruction. Therefore, we preferred PD to CAL as a measure of periodontal severity, and particularly as a predictor of systemic immune response. As expected based on previous reports (Alexander et al., 1996; Behle et al., 2009), the IgG titers against total oral biofilm bacteria were significantly decreased by periodontal treatment, corresponding to improvement in periodontal condition.\textsuperscript{54}

The results suggested that this test is useful for evaluating treatment effects from the perspective of infection levels, and the test would be useful as a self-evaluation system for the effects of periodontal treatment.

In our study, both study sub-groups (High and Low oxidative stress with treatment) showed improvement in PD and BOP after treatment. The pattern, however, was that subjects with high oxidative stress exhibited less improvement in both parameters than those with low oxidative stress. The reduction in whole-mouth mean PD was 0.33 mm vs. 0.50 mm (P<0.01) in high vs. low oxidative stress subjects.

To conclude the major findings in this study are

1- Scaling and root planing results in an increase in 2 week total oral biofilm IgG levels in low oxidative stress subjects.
2- High oxidative stress at baseline was associated with lower IgG antibody at 2 weeks after treatment in subjects with moderate to severe periodontitis.

3- At baseline, subjects assigned to the treatment group who had low oxidative stress exhibited less BOP as compared to controls with high oxidative stress.

4- Subjects with low oxidative stress responded better to non-surgical periodontal therapy as measured by mean percentage of BOP reduction at 6, 12 and 24-week time-points and mean PD reduction at 12 and 24 weeks as compared to controls with high oxidative stress.

5- Subjects who presented with consistently low oxidative stress throughout the study responded better to non-surgical periodontal therapy by measure of PD reduction observed at 6 months.

This report suggests further studies to investigate the role of oxidative stress as a modifier of periodontal therapy response in subjects with periodontitis.

These findings suggest possible studies to explore the potential impact of antioxidative therapy as a modifier and possible enhancer of the response to periodontal therapy.
REFERENCES


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