A COMPARATIVE STUDY ON SERUM LEVELS OF NITRIC OXIDE BEFORE AND AFTER INITIAL PERIODONTAL THERAPY IN HEALTHY AND TYPE 2 DIABETES MELLITUS PATIENTS WITH CHRONIC PERIODONTITIS

DISSERTATION

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CERTIFICATE

Certified that the dissertation entitled **"A COMPARATIVE STUDY ON SERUM LEVELS OF NITRIC OXIDE BEFORE AND AFTER INITIAL PERIODONTAL THERAPY IN HEALTHY AND TYPE 2 DIABETES MELLITUS PATIENTS WITH CHRONIC PERIODONTITIS"** is a bonafide record of the work done by **Dr.Gayathri.S** under our guidance during her post graduate study during the period of 2010-2013 under THE TAMIL NADU Dr. M.G.R. MEDICAL UNIVERSITY, CHENNAI, in partial fulfillment for the degree of MASTER OF DENTAL SURGERY IN PERIODONTICS, BRANCH II. It has not been submitted (partial or full) for the award of any other degree or diploma.

Dr. Elizabeth Koshi (Guide)

Professor and Head

Dr. Elizabeth Koshi M.D.S.
Professor & Head
Dept. of Periodontics
Sree Mookambika Institute of Dental Sciences
Kulashekaram

Dr. Arun Sadasivan (Co-Guide)

Professor

Dr. ARUN SADASIVAN MDS
PROFESSOR, DEP OF PERIODONTICS
SREE MOOKAMBIKA INSTITUTE OF DENTAL SCIENCE
KULASEKHARAM - 629161

Dept. of Periodontics,

Sree Mookambika Institute of Dental Sciences,

Kulasekharam

SREE MOOKAMBIKA INSTITUTE OF DENTAL SCIENCES

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CONTENTS

LIST OF ABBREVIATIONS

ADMA - Asymmetric dimethylarginine

AG - Aminoguanidine

AGE - Advanced glycation end products

ATP - Adenosine triphosphate

BOP - Bleeding on probing

bNOS/ NOS1 - Brain or Neuronal nitric oxide synthase

CAL - Clinical attachment loss

cGMP - Cyclic guanosine monophosphate

cNOS - Constitutive nitric oxide synthase

COX - Cyclooxygenase

DNA - Deoxyribonucleic acid

eNOS/NOS3 - Endothelial nitric oxide synthase

EDRF - Endothelium derived relaxing factor

EPD - Experimental Periodontal Disease

GCF - Gingival crevicular fluid

GI - Gingival index

HbA_1c - Glycated haemoglobin

HDL - High density lipoprotein

HGF - Human gingival fibroblasts

IFN-γ - Interferon gamma

IL-1β - Interleukin-1β

IL-6 - Interleukin-6

IMT - Intima media thickness

iNOS/ NOS2 - Inducible nitric oxide synthase

LDL - Low density lipoprotein

L-NAME - N^{ω}-nitro-L-arginine methyl ester

LPS - Lipopolysaccharide

MEG - Mercaptoethylguanidine

MMP - Matrix metalloproteinase

MPO - Myeloperoxidase

NADPH - Nicotinamide adenine dinucleotide phosphate

NF-κB - Nuclear factor kappa B

nNOS - Neuronal nitric oxide synthase

NO - Nitric oxide

NOS - Nitric Oxide Synthase

NO_2^- - Nitrites

NO_3^- - Nitrates

OC - Osteoclast

$ONOO^-$ - Peroxynitrite

OHI-S - Oral hygiene index- simplified

OPG - Osteoprotegerin

PGE2 - Prostaglandin E2

PMN - Polymorphonuclear leukocyte

PPD - Probing pocket depth

RANK - Receptor activator of nuclear factor kappa B

RNS - Reactive nitrogen species

ROS - Reactive oxygen species

SOD - Superoxide dismutase

SDF-1α - Stromal-cell-derived factor-1α

Th - T lymphocyte helper cells

TNF-α - Tumor necrosis factor alpha

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Abstract

A COMPARATIVE STUDY ON SERUM LEVELS OF NITRIC OXIDE BEFORE AND AFTER INITIAL PERIODONTAL THERAPY IN HEALTHY AND TYPE 2 DIABETES MELLITUS PATIENTS WITH CHRONIC PERIODONTITIS

ABSTRACT

Background

Abnormal nitric oxide (NO) synthesis has been implicated in the pathogenesis of both periodontal disease and diabetes mellitus. Nitric oxide (NO) is a highly reactive, free radical which has complex roles in both health and disease. Nitric oxide (NO) is both harmful and beneficial to the general pathophysiology of tissues. When iNOS is released simultaneously with superoxide it forms the reactive nitrogen species peroxynitrite anion. This peroxynitrite is believed to be responsible for many of the cytotoxic effects.

Aim of the study

To evaluate serum levels of nitric oxide in healthy, chronic periodontitis and chronic periodontitis patients with type 2 diabetes mellitus before and after initial periodontal therapy.

Objectives of the study

1) To find out the effect of initial periodontal therapy on serum levels of nitric oxide in healthy, chronic periodontitis and chronic periodontitis patients with type 2 diabetes mellitus
2) To evaluate whether nitric oxide is an indicator of the inflammatory status of periodontium

Materials and methods

This was an interventional study including a total of 90 (30 healthy controls, 30 chronic periodontitis and 30 chronic periodontitis with type 2 diabetes mellitus) subjects. Clinical parameters (OHI-S, GI, PPD and CAL) were recorded at baseline and 4 weeks after initial periodontal therapy. Initial periodontal therapy included oral hygiene instructions and full mouth subgingival scaling. 5ml of venous blood sample was collected from each subject at baseline and four weeks after initial periodontal therapy. Serum nitric oxide (NO) levels were assayed by Griess colorimetric reaction. Optical density was measured at 545 nm using a spectrophotometer.

Results

The periodontal parameters- Oral Hygiene Index-Simplified(OHI-S), Gingival Index (GI)- in all the three groups showed a marked reduction at the end of four weeks following initial periodontal therapy from its baseline values. No pronounced reduction was observed in the mean values of probing pocket depth (PPD) and clinical attachment level (CAL) from its baseline values at the end of four weeks. However there was a statistically significant reduction in the serum levels of nitric oxide at the end of four weeks in all the three groups. Simultaneous comparison of pre treatment and post treatment periodontal parameters and serum levels of nitric oxide between the three groups showed that the mean values of group II and group III were higher when compared to group I. However comparison of the pretreatment mean value of Gingival Index (GI) alone was statistically significant between group II and group III. Similarly the post treatment mean values of Oral Hygiene Index-Simplified(OHI-S), Gingival

Index (GI) and serum levels of nitric oxide were statistically significant between group II and group III.

Conclusion

Serum levels of NO are elevated in individuals with chronic periodontitis and chronic periodontitis with type 2diabetes mellitus. This study provides evidence that initial periodontal therapy contributes to reduction in serum levels of NO in these patients. Larger controlled trials are needed to confirm these findings.

Introduction

Periodontal diseases are chronic inflammatory diseases of the supporting tissues of the teeth caused by specific microorganisms or groups of specific microorganisms, resulting in progressive destruction of periodontal ligament, alveolar bone with pocket formation, recession or both. The balance between periodontal stability ("health") and periodontal breakdown ("disease") is tipped toward disease by risk factors, excessive production of inflammatory cytokines and enzymes, under activity or over activity of aspects of the immune-inflammatory host response, poor compliance, and a pathogenic microflora.[1]

The concept concerning the etiology of periodontal disease considers three groups of factors which determine whether active periodontal disease will occur: a susceptible host, the presence of pathogenic species, and the absence of so-called "beneficial bacteria". It is generally accepted that the oral biofilm in association with anaerobic bacteria is the main etiological factor in periodontal disease.[2] Bacterial species in plaque biofilm including Aggregatibacter actinomycetemcomitans; Porphyromonas gingivalis, Treponema denticola, Fusobacterium nucleatum and Tannerella forsythia have been strongly associated with periodontitis. They destroy the periodontal tissues by both direct and indirect mechanisms. Once the bacterial virulence factors (lipopolysaccharide cell wall and endotoxins) have overwhelmed the local defense mechanisms, they stimulate a myriad of reactions in the host, resulting in loss of soft connective tissue elements and bone resorption.[3]

A diverse range of endogenous chemical mediators orchestrates the host response and controls the inflammatory response. These chemical signals regulate the traffic of leukocytes and control the leukocyte response. The classic eicosanoids such as prostaglandins and leukotrienes exert a wide range of actions and play a key role in inflammation. The chemical mediators include novel lipid mediators, new cytokines,

chemokines, gases such as nitric oxide and carbon monoxide, reactive oxygen species (ROS) and reactive nitrogen species (RNS) etc. The initial host response is the release of reactive oxygen species (ROS) and reactive nitrogen species (RNS) via the metabolic process of "respiratory burst" in the Polymorphonuclear neutrophils (PMNs), macrophages and monocytes. Excessive production of these reactive species creates oxidative stress in the body. The reactive oxygen species (ROS) include oxygen-derived free radicals (e.g., superoxides, hydroxyl, peroxyl, alkoxyl) and non radical compounds (e.g., hypochlorus acid, ozone, singlet oxygen and hydrogen peroxide). Reactive nitrogen species (RNS) include nitric oxide (NO), peroxynitrite ($ONOO^-$), nitrogen dioxide radicals and products arising from the reaction of NO with oxygen-free radicals.[4]

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia and insufficiency of secretion or action of endogenous insulin and is an established risk factor for periodontitis.[5] There are two main types of diabetes, a) Type I diabetes mellitus - Three interlocking mechanisms are responsible for the islet cell destruction: genetic susceptibility, auto-immunity, environmental insult; b) Type-2 diabetes mellitus - The two metabolic defects characterizing type-2 diabetes mellitus are 1) derangement of insulin secretion that is delayed or is insufficient relative to glucose load and 2. inability of peripheral tissues to respond to insulin - called insulin resistance.[6] Diabetes mellitus is associated with a number of complications directly resulting from hyperglycemia. The hyperglycemic state is also associated with activated innate immunity, which is characterized by higher levels of inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin-1β(IL-1β), and interleukin- 6 (IL-6). Hyperglycemia can affect cell-signaling transduction, in particular, activation of diacylglycerol and protein kinase C, which are important

signaling molecules responsible for cell function. Hyperglycemia is also shown to increase the oxidative stress via protein kinase C-dependent activation of nicotinamide adenine dinucleotide diphosphate (NADPH) oxidase[7,8,9] (an enzyme that catalyzes superoxide and peroxide generation) in neutrophils[7,10] and other phagocytes.[7,11] Oxidative stress is thought to play a causative role in the pathogenesis of diabetes and its complications and has been shown to increase insulin resistance both in animal models and in diabetic patients.[7,12,13,14] It was postulated that high glucose could activate reactive oxygen species via multiple processes, such as enhanced formation of advanced glycation products (AGE), dysfunction of the mitochondrial electron transport chain, and activation of the plasma membrane NADPH oxidase.[7,12,15] Among these possibilities, NADPH oxidase is considered as the most important potential source of reactive oxygen species production in diabetic/hyperglycemic conditions.[7,9]

The physiologic and pathologic changes in the human body depend on free radical and reactive oxygen species interactions to maintain normal cellular activities.[16] Oxygen and nitrogen reactive species are involved in a large number of physiological and pathological processes including periodontal disease and diabetes. Nitro-oxidative stress represents all the oxidative damages induced by reactive oxygen species (ROS) and reactive nitrogen species (RNS) on the cell membranes.[17]

Individuals with periodontal disease have an imbalance between oxidants and antioxidants. Following bacterial antigen stimulation, reactive oxygen species (ROS) or free radical molecular species are generated by polymorphonuclear neutrophils (PMNs), as a result of the inflammatory tissue response mechanisms. These mechanisms have been implicated in reactive oxygen species (ROS) involvement in periodontal disease, including the possible interactions of polymorphonuclear

neutrophils (PMNs) with respect to the level of oxidation products and transition metal ions, neutrophil dysfunction, and antioxidant levels.[16]

In diabetes, free radicals are formed disproportionately by glucose oxidation, nonenzymatic glycation of proteins, and the subsequent oxidative degradation of glycated proteins. Abnormally high levels of free radicals and the simultaneous decline of antioxidant defense mechanisms can lead to damage of cellular organelles and enzymes, increased lipid peroxidation, and development of insulin resistance.[18]

Reactive nitrogen species (RNS) consist of the nitric oxide free radical and its products. Nitric oxide (NO) is an important short-lived, reactive free radical molecule. The arrangement of one atom of nitrogen and one of oxygen leaves an unpaired electron, which makes the molecule a highly reactive free radical. Nitric oxide (NO) readily reacts with superoxide, oxygen and thiol groups (-SH) to produce a number of other products, such as nitrothiols (R-SNO), the toxic molecule- peroxynitrite (ONOO-) and nitrites.[19] NO is synthesized from L-Arginine by a complex family of enzymes called nitric oxide synthases (NOS).[20] NOS is one of the most regulated enzymes in biology. Three different isoforms are known - endothelial NOS (eNOS), brain or neuronal NOS (bNOS), inducible NOS (iNOS). [21]

NO can readily diffuse through cytoplasm and plasma membrane due to its solubility in both aqueous and lipid environments.[22] NO does not bind to receptors and its effects are transient. It gets readily oxidized and remains stored in tissues as nitrates (NO_3^-) or nitrites (NO_2^-).[23] NO in low concentration helps in the normal physiological process. It mainly acts as a biological messenger and takes important roles in vasodilatation, immunomodulation, regulation of mineralized tissue function, neurotransmission, platelet aggregation and antimicrobial action.[23, 24, 25, 26] Nitric

oxide (NO) produced in high concentrations proves to be crucial in non-specific host defense and is cytotoxic.[23]

When iNOS is released simultaneously with superoxide, it forms the reactive nitrogen species peroxynitrite anion (ONOO-). This peroxynitrite is believed to be responsible for many of the cytotoxic effects previously attributed to nitric oxide and superoxide. These activities include: lipid peroxidation; glutathione depletion by oxidation; nitrotyrosine formation which may inhibit superoxide dismutase activity; DNA damage by nitrosilation, deamination and oxidation; high concentrations cause rapid cellular necrosis.[27]

Estimation of nitrate and nitrite, the stable end products of nitric oxide production, is a common indirect method used to monitor nitric oxide levels in various biological fluids.[28,29] Endogenous nitric oxide (NO) production is highly correlated with nitrite and nitrate levels in serum and plasma.[28]

Abnormal nitric oxide synthesis has been implicated in the pathogenesis of both periodontal disease and diabetes mellitus.[30] Till date, there are only limited data concerning the influence of serum levels of Nitric oxide (NO) in the inflammatory reactions of the periodontium in chronic periodontitis patients with type 2 diabetes mellitus after initial periodontal therapy. Hence an attempt has been made in this study to evaluate and compare the serum levels of nitric oxide in healthy, chronic periodontitis and chronic periodontitis patients with type 2 diabetes mellitus before and after initial periodontal therapy.

Aims & Objectives

Aim of the study

To evaluate serum levels of nitric oxide in healthy, chronic periodontitis and chronic periodontitis patients with type 2 diabetes mellitus before and after initial periodontal therapy.

Objectives of the study

1) To find out the effect of initial periodontal therapy on serum levels of nitric oxide in healthy, chronic periodontitis and chronic periodontitis patients with type 2 diabetes mellitus
2) To evaluate whether nitric oxide is an indicator of the inflammatory status of periodontium

Review of Literature

Periodontal disease and diabetes are strongly interrelated and have common pathobiology. Inflammatory events during periodontal disease may play an important role in development of diabetes and insulin resistance probably facilitates the progress of periodontal disease. Both diseases can modulate host immune response, such as upregulation of inflammatory cell phenotype, elevation of proinflammatory cytokines, and initiation of tissue damage. The outcome of activation of the inflammatory response in both diseases is similar in many aspects. The inflammatory response in diabetes is diverse, complicated, and affects a multitude of tissues and organs. The pathways of vascular tissue complications in diabetes are mediated through the formation of advanced glycation end products (AGE), and increased production of reactive oxygen species by both activation of the diacylglycerol–protein kinase C pathway [7,31] or increased activation of the polyol pathway.[7,32] The interaction between these mechanisms in the periodontium with pre-existing periodontal disease provides insight into the exacerbated periodontal destruction in diabetics, and also may explain why diabetic patients are at greater risk for periodontitis.

Oxidative stress is one of the main factors studied to explain the pathophysiological mechanisms of inflammatory conditions, such as diabetes and periodontitis. It seems that peripheral blood neutrophil hyperactivity in chronic and aggressive periodontitis exists as a constitutional element, rather than being entirely the result of peripheral priming by cytokines or plaque bacterial LPS. In addition, there may be possible baseline hyperactivity, with low-level extracellular ROS release in the absence of any exogenous stimulus in persons with periodontitis. Increased pro-oxidative state and decreased anti-oxidant capacity in persons with periodontitis could facilitate the onset of a decrease in insulin sensitivity. The presence of a high RAGE

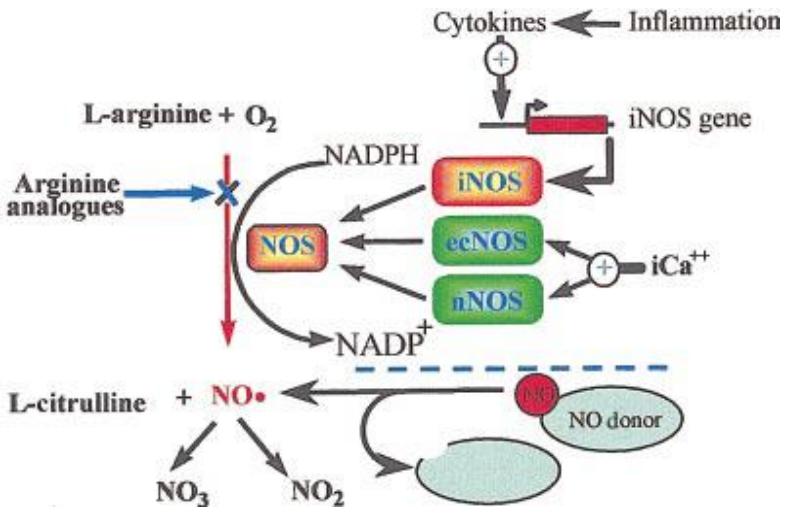
expression in periodontal tissues is an important finding supporting the sensitivity of these tissues to products derived from oxidative damage. Moreover, AGE may promote apoptosis in osteoblasts and fibroblasts, and this might have an influence on alveolar bone homeostasis and the progression of periodontitis.[33] This way, it is plausible to draw a patho-physiological picture in which a bidirectional influence exists between both conditions, with oxidative stress as a common link.

Nitric oxide: A multifaceted molecule - An overview

In 1980, Furchgott and Zawadski[34,35] discovered that the endothelium releases a factor that relaxes the underlying vascular smooth muscle. They termed this substance endothelium derived relaxing factor (EDRF). EDRF was identified as nitric oxide (NO) by Palmer et al[34, 24] and Ignarro et al. [34,36]

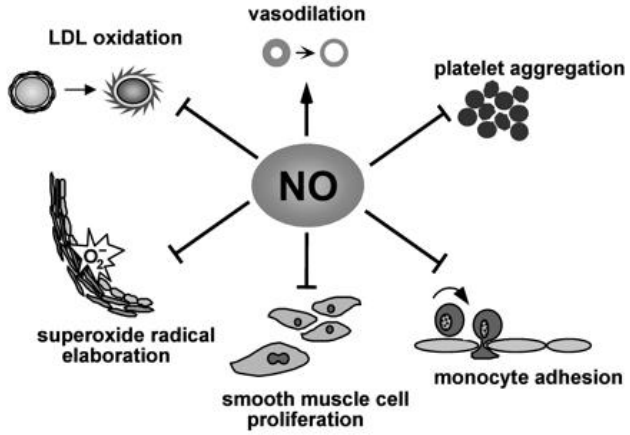
Nitric oxide (NO) is a free radical produced from L-arginine through the action of isoenzymes, globally named nitric oxide synthases (NOS). Three different isoforms are known - endothelial NOS (eNOS), brain or neuronal NOS (bNOS), inducible NOS (iNOS).[21]These isoforms have historically been categorized based upon differences in their primary locations in the body, inducibility, levels of nitric oxide production upon activation, and calcium dependence. Neuronal NOS and endothelial NOS are expressed constitutively, are calcium dependent and produce low levels of NO upon stimulation. Conversely, iNOS is regulated at the transcriptional level and is shown to be induced in multiple cell types upon bacterial or cytokine stimulation. Inducible NOS produces high levels of NO upon stimulation.[37]

The L-arginine nitric oxide pathway



NO appears to be an important regulator of various physiologic processes in both animals and humans. The cytotoxic effects of nitric oxide are mediated by peroxynitrite, a highly reactive oxidant formed from the reaction of nitric oxide with superoxide .[38,39] Formation of peroxynitrite can result in both oxidative and nitrosative stress since it consists of both a reactive oxygen and a reactive nitrogen species.[37]

Pleiotropic effects of NO



NO is regulated by feedback inhibition of the presence of endogenous inhibitors such as asymmetric dimethylarginine (ADMA), and enzymes which compete for the substrate L-Arginine.[37,40] Moreover, the availability of L-Arginine and necessary NOS cofactors, the presence of compounds which readily bind NO (heme,glutathione), and the modulation of transcription and translation of NOS by deoxyribonucleic acid (DNA) methylation or by actions of various cytokines and mediators also are involved in the regulation of NO production.[41]

Nitric oxide and diabetes mellitus

Oxidative stress and changes in nitric oxide formation or action play major roles in the onset of diabetic complications. Diabetes is usually accompanied by increased production of free radicals or impaired antioxidant defenses. Hyperglycemia is also found to promote lipid peroxidation of low density lipoprotein by a superoxide-dependent pathway resulting in the generation of free radicals. Another important source of free radicals in diabetes is the interaction of glucose with proteins leading to the formation of an Amadori product and then advanced glycation end products (AGEs). These AGEs, via their receptors (RAGEs), inactivate enzymes and alter their structures and functions, promote free radical formation, and quench and block antiproliferative effects of nitric oxide. By increasing intracellular oxidative stress, AGEs activate the transcription factor NF-kB, thus promoting up-regulation of various NF-kB controlled target genes. NF-kB enhances production of nitric oxide, which is believed to be a mediator of islet beta cell damage.[18] Nitric oxide may react with superoxide anion radical to form reactive peroxynitrite radicals.

1) In vitro studies

Lindsey et al. (1996)[42] investigated the effect of (a) insulin-treated diabetes, and (b) chronic in vivo administration of N^{ω}-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor, on mean arterial pressure and in vitro vascular reactivity to noradrenaline in mesenteric arterial bed preparations from spontaneously diabetic, insulin-dependent and treated BB rats, the best animal model of insulin-dependent mellitus. They reported that following chronic L-NAME treatment, diabetic BB/E rats exhibited attenuated hypertension and an absence of enhanced vascular responsiveness to noradrenaline in vitro compared to similarly treated non-diabetic rats. These results, together with the significantly impaired endothelium-dependent vasodilatation and unchanged endothelium-independent vasodilatation in vitro of preparations from diabetic BB/E rats, were consistent with the hypothesis that functional changes in the synthesis and metabolism of NO occur in diabetes. Their results indicate that good glycaemic control alone is insufficient to prevent these abnormalities in NO availability.

S.N. Zykova et al. (2000)[43] studied peritoneal macrophages from diabetic type 2–like db/db mice. They found that the release of TNF-α and IL-1from lipopolysaccharide plus IFN-γ –stimulated macrophages and vascular endothelial growth factor from both stimulated and nonstimulated macrophages was significantly reduced in diabetic animals compared with nondiabetic controls. Nitric oxide production from the stimulated db/db macrophages was significantly higher than that in the db/+ cultures, whereas there was no difference in their ability to generate reactive oxygen species. When studied both at light and electron microscopic levels, macrophages in diabetic animals had an altered morphological appearance compared

with those of normal controls. They concluded that the function and morphology of the macrophages were disturbed in db/db mice due to the mechanisms underlying common inflammatory and degenerative manifestations in diabetes.

Daghigh F. et al. (20002)[44] assessed NO production under basal conditions or following incubation with TNF-α, IL-1β, and IFN-γ by measurement of stable NO metabolites, nitrite and nitrate, in a microplate adaptation of the Griess assay. Total RNA was isolated from HGF for determination of iNOS mRNA levels. NO production was elevated in HGF that were stimulated simultaneously by TNF-α, IL-1β, and IFN-γ. They found that addition of Mercaptoethylguanidine (MEG), a specific inhibitor of iNOS, profoundly reduced the production of NO in HGF. They concluded that NOS2 inhibitors reduced gingival fibroblast NO production and it was postulated that pharmacological inhibition of NO might be therapeutically valuable in the management of periodontal disease.

2) Animal studies

Mohan I.K. et al. (1998)[45] reported that nitric oxide levels in plasma were decreased in alloxan-diabetic rats, an effect that could be abrogated by prior and simultaneous administration of L-arginine, a precursor of nitric oxide. When N monomethyl- L-arginine, a specific inhibitor of nitric oxide synthase, was given along with alloxan, the beneficial actions of L-arginine in diabetes were blocked. When sodium nitroprusside and L-arginine were administered simultaneously with alloxan for 5 days, nitric oxide production remained at control levels. These results suggest that both L-arginine and sodium nitroprusside, with the capacity to enhance nitric oxide levels in alloxan-diabetic animals, could prevent alloxan induced islet beta-cell damage and the development of diabetes as well as restore the antioxidant status.

Komers R. et al. (2000)[46] assessed systemic and renal hemodynamics before and after acute inhibition of iNOS enzyme with a specific inhibitor, S-methyl-L-thiocitrulline, in control and diabetic rats. The interaction of this pathway and the renin-angiotensin system was studied in separate groups of rats pretreated with the angiotensin II receptor blocker losartan. They reported that kidneys of 3 week diabetic rats could be further enhanced by S-methyl-L-thiocitrulline treatment, whereas administration of losartan along with S-L-thiocitrulline for 3–5 weeks could normalize the nitric oxide levels implying that angiotensin II is an important modulator of nitric oxide pathway in diabetes.

3) Human studies

Kedziora-Kornatowska K.Z. (1999)[47] studied the production of the superoxide radical anion O_2^-. and the nitric oxide radical NO^-. by granulocytes in type 2 diabetes patients with/without diabetic nephropathy. They reported that O_2^-. Production by both resting and stimulated granulocytes in type 2 diabetes patients without nephropathy was increased but decreased in type 2 diabetes patients with nephropathy, compared with healthy subjects. NO^-. generation was highly augmented in type 2 diabetes patients without nephropathy by both resting and stimulated cells; values for type 2 diabetes patients with nephropathy were intermediate between the type 2 diabetes patients without nephropathy and the healthy subjects. Their findings point to granulocytes as one of possible sources of oxidative stress in type 2 diabetes.

Yilmaz et al. (2000)[48] determined nitric oxide levels in the vitreous of patients with proliferative diabetic retinopathy, using the spectrophotometric method based on Griess reaction. They found that vitreous nitric oxide levels were elevated in patients

with proliferative diabetic retinopathy with tractional retinal detachment. Hence nitric oxide might play a role in the pathogenesis of proliferative diabetic retinopathy.

Maejima K. et al. (2001)[49] assessed the underlying mechanisms of decreased endothelial function and advanced vascular complications in patients with Type 2 diabetes, by determining the basal levels of plasma nitric oxide (NOx: NO_2^- and NO_3^- using a newly developed high-performance liquid chromatography (HPLC)-Griess method. Serum lipid peroxide and advanced glycation end products (AGEs) as markers of oxidative stress were also measured, and intima-media thickness (IMT) of the carotid artery was evaluated as a marker of atherosclerosis. In diabetic subjects, microvascular complications were newly evaluated during their admission. There was no difference in basal plasma NO_2^- levels between the two groups, the basal levels of plasma NO_3^- in diabetic subjects were significantly higher than those in nondiabetic subjects. Plasma NOx levels in neither diabetic nor nondiabetic subjects correlated with serum lipids, HbA1c, or IMT. In diabetic subjects, plasma NO_3^- levels were related not only to the presence of hypertension but also to advanced microvascular complications. Plasma NO_3^- levels were positively correlated with both serum lipid peroxide and AGEs. Their findings were found to be consistent with the hypothesis that decreased endothelium-dependent vasodilation in diabetic subjects is associated with the impaired action of NO secondary to its inactivation resulting from increased oxidative stress, rather than decreased NO production from vascular endothelium, and that abnormal NO metabolism is related to advanced diabetic microvascular complications.

M. Kawaktsu et al. (2002)[50] analyzed nitrite /nitrate (NOx) in healthy mass population and investigated the relationship with gender, aging and some diabetic

complications. At the fasting stage, the mean value of NOx in the healthy subjects was 47.5±26 (μM/L), and the ratio of nitrite to nitrate was approximately 1:6. After an intake of celery containing rich nitric compounds, NOx markedly increased to 2.5 times, mainly because of an increase in nitrate. In the male subjects, NOx gradually increased with aging, whereas in the females, it tended to decrease until the menopausal stage but turned upward after that. NOx in the group without any diabetic complication was much lower than that in the control group but the group with coronary artery disease showed a higher value. They concluded that NOx was influenced by exogenous factors, aging, and difference of gender, and showed some correlations with hyperglycemic vascular impairments**.**

Dogany N. et al. (2002)[51] suggested that serum NO, sIL-2R, IL-8 and TNF-alpha might play important roles in the pathophysiology and progression of diabetic retinopathy and these potentially inflammatory cytokines and NO with their endothelial implications might act together during the course and progression of diabetic retinopathy. These molecules could be served as therapeutic targets for the treatment and/or prevention of diabetes with its systemic and ocular microvascular complications.

Nogueira-Machado J.A. et al. (2002)[52] performed a study with granulocytes from non-diabetic subjects and from type II -Non Insulin Dependent Diabetes mellitus (NIDDM) patients. The NO generation was comparatively determined by the nitrite concentration (micromolar of nitrite) after cell incubation in the presence of cyclic nucleotide-elevating agents. Their results showed an inverse reactivity for granulocytes from diabetic patients when compared to non-diabetic subjects. They

also found that both cyclic AMP and cyclic GMP were able to modulate nitric oxide production in human granulocytes and that cell reactivity in diabetic patients.

Ozden S. et al. (2003)[53] demonstrated both elevated levels of serum NOx in diabetic patients as compared with nondiabetic controls and a relationship between NOx and diabetic retinopathy severity. They concluded that abnormal NO metabolism might have a role in the pathogenesis of diabetic retinopathy.

Torres et al. (2004)[54] assessed nitric oxide generation in skeletal muscle in type 2 diabetic patients. They found that iNOS is increased in skeletal muscle samples of type 2 diabetic patients. Although iNOS was induced in muscle fibres and endothelial cells, the presence of macrophages and the increased levels of TNF and CD154 were evidence of an inflammatory process with macrophage and T-cell activation. The moderate increase of the NO products NO_2^- and NO_3^-, contrasting with the markedly augmented levels of nitrotyrosine, indicates that NO reacted with O2 to produce $ONOO^-$, which finally nitrated tyrosine residues. They suggested that superoxide ion necessary to react with NO for the production of $ONOO^-$ might have been generated in relation to AGE formation.

S.R. Kashyap et al. (2005)[55] determined nitric oxide synthase (NOS) activity in skeletal muscle of 10 type 2 diabetics (HbA_{1C} = 6.8 ± 0.1%). They reported that basal and insulin-stimulated muscle NOS activity is impaired in well-controlled type 2 diabetic subjects, and the defect in insulin-stimulated NOS activity correlated closely with the severity of insulin resistance and suggested that impaired NOS activity may play an important role in the insulin resistance in type 2 diabetic individuals.

Izumi et al. (2006)[56] estimated the levels of plasma NO_x (nitrite and nitrate), the stable metabolites of NO, by high-performance liquid chromatography with the Griess method. They concluded that NO might be associated with the pathogenesis of diabetic retinopathy.

M.H. Mahfouz et al. (2009)[57] conducted a study to determine two cardiovascular risk factors (ADMA- asymmetric dimethylarginine and NO) in type 2 diabetic patients with and without cardiovascular disease and to evaluate the association between ADMA and HbA1c on the one hand and nitric oxide on the other hand. Fasting and postprandial serum glucose, HbA1c, lipid profile (total cholesterol, triacylglycerol, HDL-c and LDL-c), ADMA and serum NO metabolite level, were determined. Serum glucose (fasting and postprandial), HbA1c and ADMA levels showed significant increase in diabetic patients type 2 with and without cardiovascular complications compared to healthy normal control. Total cholesterol, triacylglycerol and LDL-c manifested significant elevations, while HDL-c level showed insignificant change in both groups in compared to non diabetic healthy subjects. Serum NO metabolite level was significantly reduced in the both diabetic patient groups compared with controls. No correlation between ADMA level and studied parameters in diabetic patients without evidence of cardiovascular complications, whereas in cardiovascular complications group, the ADMA level was positively correlated with both postprandial serum glucose and HbA1c, but there was a negative correlation between ADMA levels and NO. Also, NO was negatively correlated with postprandial serum glucose and HbA1c. They concluded that ADMA and NO might serve as predictors for future cardiovascular events in type 2 diabetic patients

P. Tessari et al. (2010)[58] measured nitrite/nitrate (NOx) fractional (FSR) and absolute (ASR) synthesis rates in type 2 diabetic patients with diabetic nephropathy and in control subjects, after L-[15N2-guanidino]-arginine infusion, and use of precursor– product relationships. They reported that type 2 diabetic patients with nephropathy, intravascular NOx synthesis from arginine is decreased under both basal and hyperinsulinemic states. This defect might extend the concept of insulin resistance to NO metabolism.

A.Ghosh et al. (2011)[59] compared serum nitric oxide level among type 2 diabetic patients along with other biochemical parameters. Griess reaction was used for indirect assay of stable decomposition products in serum (serum nitrite and nitrate levels) as an index of NO generation. Serum NO was observed significantly low in diabetic participants as compared to control, along with difference in other biochemical parameters.

Nitric oxide and periodontal disease

Periodontal diseases are chronic inflammatory infections accompanied by destruction of surrounding connective tissue and alveolar bone. Cytokines and other bacterial products stimulate the expression of iNOS and interfere with periodontal disease progression. The primary causative agents are gram-negative bacteria, which stimulate cells like macrophages, fibroblasts to generate NO. More specifically, bacterial lipopolysaccharides stimulate NO expression in bone as well as other tissues. The presence of NO in periodontal disease may reflect the participation of an additional mediator contributing to periodontal tissue damage and bone resorption responsible for progression of periodontitis. The evaluation of involvement of nitric

oxide in the periodontal disease will enable us to understand the complexity of periodontal disease progression.

1) In vitro studies

Shapira L. et al. (2000)[60] designed a study to examine whether the functional response to mouse macrophages stimulated by porphyromonas gingivalis lipopolysaccharide is affected by experimental stress, and to investigate the role of corticosterone (CS) in the stress related effects. Their results showed that experimental stress might modulate the response of macrophages to inflammatory stimulants and that CS is not the sole mediator involved. The presence of IFN-γ in the culture may mask the functional differences induced by stress. The stress induced Upregulation of NO secretion might be involved in the accelerated periodontal destruction in stressed subjects.

2) Animal studies

Z. Lohinai et al. (2001)[61] investigated the potential role of nitric-oxide-derived nitrating species (such as peroxynitrite) in a rat model of ligature-induced periodontitis. Formation of 3-nitrotyrosine, the stable product formed from tyrosine reacting with nitric-oxide-derived nitrating species, was detected in the gingivomucosal tissue. 3-Nitrotyrosine immunohistochemical analysis revealed a significant elevation in the number of immunopositive leukocytes, and higher immunoreactivity of the gingival ligaments and epithelium in the ligated than in the contralateral (control) side. They suggested that resident bacteria of the gingivomucosal tissue induce an increase in reactive nitrogen species, which might be greatly enhanced by plaque formation in periodontitis.

R.F.C. Leitao et al .(2005)[62] conducted a study in Winstar rats subjected to a ligature placement around the second upper left molars and were sacrificed at 11 days. Alveolar bone loss was evaluated by the sum of distances between the cusp tips and the alveolar bone along the axis of each molar root, subtracting from the contralateral side. Histopathological analysis was based on cell influx, alveolar bone and cementum integrity. Leukogram was performed at 6 hours and 1,7,and 11 days after the EPD induction. Groups were treated with the NOS inhibitors, aminoguanidine (AG) or L-arginine methyl ester intraperitoneally, 1hour before the EPD induction and daily for 11 days. Results showed that both these inhibitors significantly and dose –dependently inhibited the alveolar bone loss . L-NAME (20mg/kg/d) reduced the alveolar bone loss by 50%, whereas AG (5mg/kg/d) reduced it by 47% compared to EPD group.

S.Y. Fukada et al. (2008)[63] evaluated the role of NO in bone loss in bacterial infection-induced apical periodontitis by using iNOS-deficient mice ($iNOS^{-/-}$). The $iNOS^{-/-}$ mice developed greater inflammatory cell recruitment and osteolytic lesions than WT mice. Moreover, tartrate-resistant acid-phosphatase-positive ($TRAP^{+}$) osteoclasts were significantly more numerous in $iNOS^{-/-}$ mice. Furthermore, the increased bone resorption in $iNOS^{-/-}$ mice also correlated with the increased expression of receptor activator NF-kB (RANK), stromal-cell-derived factor-1α (SDF-1α/CXCL12), and reduced expression of osteoprotegerin (OPG). Their results showed that NO deficiency was associated with an imbalance of bone-resorption-modulating factors, leading to severe infection-stimulated bone loss.

Herrera B.S. et al. (2011)[64] evaluated the alveolar bone loss in rats with periodontitis under long-term iNOS inhibition, and the differentiation and activity of

osteoclast (OC) from iNOS-knockout (KO) mice in vitro. Oral aminoguanidine (an iNOS inhibitor) or water treatment was started 2 weeks before induction of periodontitis. Rats were sacrificed 3, 7, or 14 days after ligature placement, and alveolar bone loss was evaluated. In vitro osteoclast culture experiments were also performed to study the differentiation of freshly isolated bone marrow cells from both iNOS KO and wild-type C57BL/6 mice. Osteoclast were counted 6 days later after tartrate-resistant acid phosphatase staining (a marker of osteoclast identity), and bone resorption activity was assessed by counting the number of resorption pits on dentin disks. Results demonstrated that iNOS inhibition prevents alveolar bone loss in a rat model of ligature-induced periodontitis. Thus they confirmed that iNOS-derived NO might play a crucial role in the pathogenesis of periodontitis, probably by stimulating osteoclast differentiation and activity.

Herrera B.S. et al. (2011)[65] assessed the renal and cardiac consequences of ligature-induced periodontitis in both normotensive and NO-deficient (L-NAME-treated) hypertensive rats. In NO-deficient hypertensive rats, periodontitis-induced alveolar bone loss was significantly diminished. In addition, periodontitis-induced cardiac NT elevation was completely prevented by L-NAME treatment. L-NAME treatment enhanced MPO production in both heart and kidneys of rats with periodontitis. No changes due to periodontitis were observed in cardiac or renal thiobarbituriuc acid reactive substances (TBARS) content. They concluded that in addition to mediating alveolar bone loss, NO could contribute to systemic effects of periodontitis in the heart and kidney.

3) Human studies

Kankanian and Akapov (1996)[66] have shown that saliva from healthy subjects stimulates NO synthesis in PMNL, while the saliva from patients with gingivitis or periodontitis does not or even suppresses it. Furthermore, PMNL from gingival fluid or blood of patients with periodontitis, in contrast to PMNL from healthy subjects, inhibits NO-induced cGMP accumulation in cultured fibroblasts.

Aurer A. et al. (2001)[67] analysed salivary NO_2^- concentrations in patients with rapidly progressive periodontitis (RPP). The concentrations of NO_2^- were determined by the Griess reaction in microtitration plates. Periodontal tissue destruction was determined by measuring the attachment level loss using standard methods. Results showed that subjects with periodontitis had significantly less $NO2^-$ in saliva than healthy subjects. Subjects with RPP had lower $NO2^-$ concentrations. Parotid gland saliva contained less $NO2^-$ than sublingual gland or total saliva. They concluded that local NO production is decreased in patients with periodontitis. This effect would be more pronounced in those with severe types of disease.

Carossa et al. (2001)[68] reported that oral NO production and salivary nitrite levels increased during de novo plaque deposition in periodontally healthy subjects. They proposed that this may be an early defense mechanism against bacterial proliferation in the dental plaque. They further demonstrated that this response was inhibited in smokers and was associated with an elevation in plaque bacterial counts as compared to non-smokers.

M. Hirose et al. (2001)[69] determined the mRNA expression of cytokines and iNOS in inflamed and healthy gingival tissue using polymerase chain reaction (RT-PCR), and the relationship between their profiles and the detection of specific bacteria

was analyzed. They concluded that the expression of IL-6 may reflect inflammation in gingival tissue , and iNOS may be involved in the inflammatory process in periodontitis. The presence of Aggregatibacter actinomycetemcomitans or Porphyromonas gingivalis might relate to the different cytokine profiles of IL-1α,IL-6,IL-8 and IL-10.

K. **Shibata et al. (2001)**[70] examined the involvement of NOS in chemotaxis of normal neutrophils and NOS activity in neutrophils from normal subjects and patients with localized aggressive periodontitis (LAgP). Membrane associated –NOS and soluble NOS were extracted from cells with or without N-formyl-methionyl-leucyl-phenylalanine (fMPLP) stimulation. NOS activity was measured using the radiolabeled L-arginine to L-citrulline conversion assay. They found that N^{ω}-Nitro-L-arginine methyl ester (L-NAME) and inhibitor of NOS, significantly attenuated the inhibition by L-NAME. Unstimulated MA-NOS activity in LAgP neutrophils was statistically higher than that in normal neutrophils. They suggested that NOS is present in human neutrophils and may be involved in fMLP- induced chemotaxis in neutrophils.NOS activity is increased in LAsP and is negatively correlated to chemotaxis response.

In order to further characterize the presence of NO in human periodontal disease, **Batista A.C. et al. (2002)**[21] undertook a quantitative study of iNOS positive cells in samples of clinically healthy gingival tissues, plaque-induced gingivitis and localized chronic periodontitis using immunohistochemistry. The results indicated that iNOS increases in the presence of periodontal disease and they suggested that polymorphonuclear cells present an additional activation pathway in periodontal

disease, expressing significant iNOS and probably representing an important source of NO in human periodontal disease.

Sosreseno et al. (2004)[71] determined nitric oxide (NO) production of a murine macrophage cell line (RAW 264.7 cells) when stimulated with Porphyromonas gingivalis lipopolysaccharides (Pg-LPS). RAW264.7 cells were incubated with i) various concentrations of Pg-LPS or Salmonella typhosa LPS (St-LPS), ii) Pg-LPS with or without L-arginine and/or N^G-monomethyl-L-arginine (NMMA), an arginine analog or iii) Pg-LPS and IFN-γ with or without anti-IFN-γ antibodies or interleukin-10 (IL-10). Tissue culture supernatants were assayed for NO levels after 24 h in culture. NO was not observed in tissue culture supernatants of RAW 264.7 cells following stimulation with Pg-LPS, but was observed after stimulation with St-LPS. Exogenous L-arginine restored the ability of Pg-LPS to induce NO production; however, the increase in NO levels of cells stimulated with Pg-LPS with exogenous L-arginine was abolished by NMMA. IFN-γ induced independent NO production by Pg-LPS-stimulated macrophages and this stimulatory effect of IFN-γ could be completely suppressed by anti-IFN-γ antibodies and IL-10. Their results suggest that Pg-LPS is able to stimulate NO production in the RAW264.7 macrophage cell model in an L-arginine-dependent mechanism which is itself independent of the action of IFN-γ.

Skaleric et al. (2006)[30] evaluated the expression of NO in gingivae of type I diabetic patients presenting with periodontal disease and to correlate the level of NO with P. Intermedia infection. They suggested that in diabetic patients, increased inducible NO synthase in inflamed gingiva correlated with NO in gingival crevicular fluid. Although increased NO reflected more-severe inflammation, it was associated

with reductions in colony forming units (CFU) of Prevotella intermedia, a major periodontopathogen, highlighting dual roles for NO.

A. Berdeli et al. (2006)[72] evaluated genotype distribution and genotype-phenotype association in periodontal disease regarding Glu298Asp polymorphism of the eNOS gene. They showed that eNOS Glu298Asp polymorphism is associated with BOP in GAgP patients. Moreover, the 298Asp allele of the eNOS gene might be related to CP in the Turkish population.

K.B. Menaka et al. (2009)[23] assessed the level of NO in serum in chronic periodontitis, and correlated these levels with the severity of periodontal disease. Results showed that subjects with periodontitis had significantly high nitrite in serum than healthy subjects. NO production was increased in periodontal disease. This finding could help to understand its role in disease progression and selective inhibition of NO might be of therapeutic utility in limiting the progression of periodontitis.

Zeynep Pan et al. (2010)[73] evaluated the expression of inducible nitric oxide synthase (iNOS) in the gingival tissues of periodontitis patients with and without type 2 diabetes to assess whether NO plays a role in the severity of periodontitis in patients with diabetes. They reported that inflammation and iNOS expression were more prominent in the gingiva of the patients with both diabetes and periodontitis. However, iNOS expression did not seem to have an additional detrimental effect on the course of periodontitis in patients with diabetes compared to those with periodontitis alone.

Ozer et al. (2011)[74] examined the arginine-NO-polyamine pathway alteration in saliva and gingival biopsy samples of patients with gingivitis or periodontitis and

healthy controls and evaluated the response to periodontal treatment. Periodontal clinical measurements, gingival biopsies, and saliva samples were obtained before treatment and 1 month after periodontal treatment. Arginase and ornithine de carboxylase (ODC) activities and NO levels were determined spectrophotometrically. They reported that regarding arginine-NO-polyamine metabolism, gingival tissue would be more informative about periodontal pathogenesis than saliva. At early phase of periodontal inflammation, NO arginase and ODC levels were measured as higher than at an established lesion of periodontitis.

S.R. Parwani et al. (2012)[75] conducted a study to estimate salivary nitric oxide levels in inflammatory periodontal diseases. They found that NO levels were increased significantly in gingivitis and periodontitis subjects as compared with controls. They noted a statistically significant decrease in the nitric oxide levels after the healing period(curresponding to the reduced clinical signs of inflammation). They also found a positive correlation between probing pocket depths with salivary nitric oxide levels. They concluded that salivary nitric oxide levels could be utilized as a good indicator of the inflammatory status of the periodontium.

D.H. Han et al. (2012)[76] investigated the relationship between salivary nitric oxide and periodontitis in an elderly Korean population. Periodontitis was determined by a clinical attachment loss of over 6mm at 6 probe points in 12 index teeth. NO was measured in unstimulated saliva via the Griess reaction. After controlling for age, gender, education, salivary flow rate, number of teeth, smoking status, physical activity, hypertension, and diabetes, three metabolites of salivary NO (total NO, nitrite, and nitrate) were independently associated with the percentage of probe points exhibiting periodontitis. Total NO was found to have the strongest correlation with

periodontitis. These associations were most pronounced in females, non-smokers (except for nitrate), those without hypertension and those without diabetes.

I.S. Choi et al. (2012)[77] attempted to investigate the effect of kaempferol on the production of NO by murine macrophage-like RAW264.7 cells stimulated with LPS from Prevotella intermedia, a pathogen implicated in periodontal disease, and to determine the underlying mechanisms of action. NO production was assayed by measuring the accumulation of nitrite in culture supernatants. They reported that Kaempferol inhibited NO production and iNOS protein expression in P. intermedia LPS-stimulated RAW264.7 cells at the translational level via HO-1-mediated ROS reduction and could be an efficient modulator of host response in the treatment of periodontal disease.

F.G. Amorim et al. (2012)[78] investigated the role of G894T polymorphism in the eNOS gene as a predisposing factor to periodontal disease. They observed that the GG geno-type was associated with the progression of periodontal disease as indicated by an increase in frequency of approximately 18% in the moderate and 26% in the severe groups compared to the Healthy control group. Their finding indicated that patients carrying the GG genotype have a greater chance of developing periodontal disease compared with those carrying the T allele, and it reinforces the notion that genetic factors contribute to the development and aggravation of periodontal disease.

F.S. Mariano et al. (2012)[79] compared the production of antimicrobial peptides (s LL-37, human neutrophil peptides (HNP) 1-3) and NO by LPS-stimulated neutrophils isolated from healthy subjects and from patients with periodontitis. Peripheral blood neutrophils were cultured with or without Aggregatibacter actinomycetemcomitans-LPS (Aa-LPS), Porphyromonas gingivalis-LPS (Pg-LPS)

and Escherichia coli-LPS (Ec-LPS). qRT-PCR was used to determine quantities of HNP 1-3 and LL-37 mRNA in neutrophils. Amounts of HNP 1-3 and LL-37 proteins in the cell culture supernatants were also determined by ELISA. NO levels in neutrophil culture supernatants were quantitated by the Griess reaction. Neutrophils from periodontitis patients cultured with Aa-LPS, Pg-LPS and Ec-LPS expressed higher HNP 1-3 mRNA than neutrophils from healthy subjects. LL-37 mRNA expression was higher in neutrophils from patients stimulated with Aa-LPS. Neutrophils from periodontitis patients produced significantly higher LL-37 protein levels than neutrophils from healthy subjects when stimulated with Pg-LPS and Ec-LPS, but no difference was observed in HNP 1-3 production. Neutrophils from periodontitis patients cultured or not with Pg-LPS and Ec-LPS produced significantly lower NO levels than neutrophils from healthy subjects. The significant differences in the production of LL-37 and NO between neutrophils from healthy and periodontitis subjects indicate that production of these molecules might influence individual susceptibility to important periodontal pathogens.

Very few studies have shown the influence of levels of NO on subjects with both periodontal disease and diabetes mellitus.[30, 73]

Nitric oxide and other oral diseases

Liu R.H. et al. (1995)[80] found that salivary nitrite (implying that NO had been produced) was significantly increased in patients with OLP (Oral lichen planus) compared to healthy controls. It is clearly not possible to speculate whether salivary NO production was increased as a result of OLP, or whether increased salivary NO has a detrimental effect on the oral mucosa, and might be responsible in part for the

development of OLP. Increased salivary nitrite has also been found in patients with recurrent oral apthous ulceration. These findings suggest that excessive salivary NO has a potential role in modifying oral mucosal diseases as a patho-physiological regulator.

Bentz et al. (1999)[81] assessed NOS3 expression in a variety of benign and malignant salivary tumours and found increased NOS3 expression in all 48 tumours studied relative to normal salivary tissue where little NOS3 was found outside blood vessel endothelium.

Brennan P.A. et al. (2000)[82] found that NOS2 was expressed both in pleomorphic adenoma and normal salivary ducts, and postulated that its expression in this tumour was due to its origin from myoepithelial cells.

J.B. Patel et al. (2009)[83] evaluated nitric oxide and antioxidant enzyme levels in healthy individual without tobacco habits ,healthy individuals with tobacco habits, patients with oral precancers and oral cancer patients. They found that plasma NO_2^- $+NO_3^-$ levels were elevated in patients with oral precancers and oral cancer patients. The erythrocyte SOD and catalase activities were lower in oral cancer patients than patients with oral precancers. But erythrocyte SOD activity was higher in advanced oral cancer than the early disease. They concluded that the alterations in antioxidant activities were associated with production of nitric oxide in oral cancer, which may have significant role in oral carcinogenesis.

Nitric oxide and other systemic diseases

Nitric oxide is an important gas molecule that plays pivotal role in physiology and pathology in various systems. There is a large body of evidence that NO is involved in several inflammatory disorders.

1) **In vitro studies**

Sancak B. et al. (2003)[84] investigated the role of NO in Behcet's disease**.** Behcet's disease (BD) is a chronic multisystemic disorder which is characterized by a relapsing systemic inflammatory process. They measured serum nitrate + nitrite levels, by using an enzymatic one-step methodology based on the reduction of nitrate to nitrite by nitrate reductase from Aspergillus species, in the presence of beta-NADPH. When compared to healthy controls, serum nitrate + nitrite levels were found to be higher in active periods of Behcet's disease patients. It was concluded that increased NO production in patients with BD might have critical biological activities relevant to vasculitic events in the active period of disease.

2) **Animal studies**

K. E. Armour et al. (1999)[85] investigated the pathogenic role of NO in an animal model of inflammation-induced osteoporosis (IMO). NO production was increased in IMO when compared with controls, and this was accompanied by activation of inducible NOS (iNOS) in the bone marrow space. Bone mineral density (BMD) was reduced in IMO when compared with controls ,and this was found to be associated with reduced osteoblast numbers and increased osteoclast numbers . The NOS inhibitor L-NMMA reversed the deleterious effects of IMO on bone mass and bone turnover, but L-NMMA had no effect on bone mass in control animals. They

suggested that iNOS activation and increased NO production contribute to the pathogenesis of osteoporosis in these situations, but that NOS inhibitors could be of therapeutic value in the prevention and treatment of such bone loss.

K. J. Armour et al. (2001)[86] investigated the role of the inducible NO synthase (iNOS) pathway in the pathogenesis of inflammation-mediated osteoporosis (IMO) by studying mice with targeted inactivation of the iNOS gene iNOS knockout (iNOS KO) mice. IMO was induced in wild-type (WT) and iNOS KO mice by subcutaneous injections of magnesium silicate. The skeletal response was assessed at the tibial metaphysis by measurements of bone mineral density (BMD), using peripheral quantitative computed tomography, by bone histomorphometry, and by measurements of bone cell apoptosis. NO production increased 2.5-fold in WT mice with IMO, but did not change significantly in iNOS KO mice. Total BMD values decreased by a mean SEM of 14.4 ± 2.0% in WT mice with IMO, compared with a decrease of 8.6 ± 1.2% in iNOS KO mice with IMO. Trabecular bone volume was lower in WT mice with IMO and showed that IMO was associated with reduced bone formation and a 320% increase in osteoblast apoptosis in WT mice. iNOS KO mice with IMO showed less inhibition of bone formation than WT mice and showed no significant increase in osteoblast apoptosis.

Pilichos et al. (2004)[87] assessed N^{ω}-nitro-L-arginine methyl ester (L-NAME) and aminoguanidine (AMG), the most studied inhibitors of nitric oxide synthases, with regard to their effectiveness as modulators of inflammation in trinitrobenzene sulfonic acid (TNBS)-induced colitis in the rat. Over expression of nitric oxide (NO) has been implicated in the pathogenesis of experimental and clinical inflammatory bowel disease. They reported that administration of both nitric oxide synthases

inhibitors L-NAME and AMG were beneficial in all the examined parameters (clinical (body weight), haematological (hematocrit and erythrocytes sedimentation rate-ESR) and morphological (gross and microscopic) criteria) compared with the control group. They concluded that NOS inhibitors might be promising agents in preventing the onset, or mediating the symptoms, of inflammatory bowel disease.

3) Human studies

N. Valero et al. (2002)[88] carried out a study in dengue fever patients. Dengue sera were taken 1 to 8 days after onset of the disease. Virus dengue is capable of inducing increased levels of NO when cocultured with human Kupffer and spleen cells. Because platelets can generate NO through stimulation of NO synthase, they performed experiments to determine the production of NO after coculture of human platelets with active and inactive forms of dengue virus type 2. Virus-platelet interaction did not contribute to increased levels of NO in the culture supernatants. They suggested that platelets are not a source of NO during the course of DF. Levels of NO in the severe form of illness (DHF) were not found to be increased; they were similar to the normal serum levels. Hence NO could play a role in the course of the disease.

Y. Ersoy et al. (2002)[89] conducted a study to assess and compare serum nitrate and nitrite levels in patients with ankylosing spondylitis, rheumatoid arthritis, and osteoarthritis. Concentrations of nitrate and nitrite in serum were determined by direct and indirect Griess reactions. C reactive protein and erythrocyte sedimentation rate levels were determined as markers of systemic activity of disease (SAD) in rheumatoid arthritis and ankylosing spondylitis groups. Their findings suggested that nitrate and nitrite production is enhanced in patients with inflammatory arthritis

compared with osteoarthritis. Serum nitrate and nitrite levels were enhanced in patients with rheumatoid arthritis, ankylosing spondylitis, and osteoarthritis compared with healthy subjects. A correlation was found between the SAD variables and serum nitrate and nitrite levels in patients with RA and AS.

J. Brice Weinberg et al. (2006)[90] assessed NO production in RA patients and compared levels of serum, urine, and salivary nitrite and nitrate (NOx) in patients with RA and normal subjects, and they examined the relationships of these measures to disease activity. They found that RA patients had lower renal NOx clearance and lower renal NOx fractional excretion.

Taffi R. et al. (2008)[91] investigated changes in nitric oxide metabolism and oxidative stress status by measuring plasma nitric oxide (NO) and peroxynitrite (ONOO (-)) levels. Findings show that changes in NO metabolism may be considered as markers of brain injury in patients with ischemic stroke.

A. Kulkarni et al. (2008)[92] reported that serum nitric oxide and protein carbonyl levels were concomitantly increased and positively correlated with each other in patients with pulmonary tuberculosis and extra-pulmonary tuberculosis. The changes in the level of nitric oxide and protein carbonyl are a reflection of increased defence mechanism and free radical activity in tuberculosis.

Oates et al. (2008)[93] demonstrated longitudinal associations between serum nitric oxide levels and markers of systemic lupus erythematosus and lupus nephritis disease activity. They found that NOx levels were associated with serum levels of C3 and creatinine and the urinary protein, creatinine ratio. Among patients with lupus nephritis, those with proliferative lesions had higher NOx levels, and higher NOx

levels were associated with accumulation of renal damage and lack of response to therapy.

Wimalawansa S.J. (2008)[94] suggested that postmenopausal NO deficiency could be rectified with hormone replacement therapy, which enhance local production of NO. Declining local NO production secondary to estrogen deficiency in postmenopausal women, and perhaps in older men, could be one of the key reasons for age-related increased incidences of cardiovascular events, sexual dysfunction as well as osteoporosis. Thus, in addition to supplementation of NO compounds in acute situations such as alleviating angina and erectile dysfunction, it could be a valuable addition to the armamentarium of therapies for chronic conditions such as osteoporosis

P. Hussain et al. (2008)[95] used a genetic strategy to test the hypothesis that an inflammatory microenvironment with an enhanced level of NO. accelerates spontaneous tumor development. C. parvum– induced inflammation and increased NOS2 expression coincided with accelerated spontaneous tumor development, mostly lymphomas, in p53_/_NOS2+/+ C57BL6 mice when compared with the controls. In C. parvum–treated p53_/_NOS2+/+ mice, tumor development was preceded by a higher expression of NOS2 and phosphorylated Akt-Ser ^{473} (pAkt-Ser473) in spleen, increased cell proliferation measured by Ki-67 IHC in spleen and thymus, and a lower apoptotic index and CD95-L expression in spleen and thymus. C. parvum–treated p53_/_NOS2+/+ mice showed an increase in the number of Foxp3(+) T-reg cells, dendritic cells (DC), as well as increased CD80+, CD86+, CD40+, and CD83+ on DC in the spleen. Regulatory T-cells (T-reg) and the maturation of DCma y modulate tumorigenesis. An increase in the FoxP3 (+)T-reg cells in C. parvum–treated

p53_/_NOS2+/+ mice indicates a role of NO. in the regulation of T-reg cells that may contribute to a protumor shift of the immune environment favoring an accelerated tumor development. These data provided genetic and mechanistic evidence that an inflammatory microenvironment and an increased level of NO can accelerate tumor development.

Kumar et al. (2009)[96] have studied NO levels of patients with ankylosing spondylitis (AS) using nitrite as a surrogate marker for NO. Levels of patients with ankylosing spondylitis were significantly higher than that of controls. Only one patient had levels comparable to those of controls. However, clinical assessment of disease activity , individual Assessment in Ankylosing Spondylitis domains-physical function (BASFI), pain, patient global assessment and inflammation did not show a good correlation with serum nitrite levels.

B. Bayram et al. (2009)[97] investigated plasma nitric oxide synthesis activity at acute phase of stroke and stroke subtypes. The plasma nitric oxide synthesis activity was determined by colorimetric assay in conformity with the kit procedure. Plasma nitrite+nitrate levels were determined as 0.83 (0.83-0.83) μM and 0.89 (0.76-1.12) μM for the patient group and the control group respectively. They asserted that plasma nitric oxide synthesis activity does not constitute an important criterion for stroke and stroke subtypes.

E. M. Mahdy et al. (2011)[98] reported that serum determination of HGF, Bcl-2 or NO may help in diagnosis of the breast cancer and may aid in disease prognosis. They suggested that elevated nitrate and nitrite levels in the patients may be a result of increased NOS II activity, which is stimulated by a host defense system against tumor growth. NO might promote tumor growth by modulating the production of

prostaglandins and activate cyclooxygenase-2 (COX-2), by generating prostaglandins, promotes angiogenesis and inhibits apoptosis.

M. Miletic et al. (2012)[99] tested the serum levels of IL-17 and nitric oxide (as possible IL-17-induced product), in patients with primary Sjogren's syndrome , an intricate and complex chronic autoimmune disorder of exocrine glands and suggested that they have increased IL-17 and NO production, especially if they had associated elevated rheumatoid factor and antinuclear antibody values.

P. Samant et al. (2012)[100] performed a study to determine whether hyperuricemia depletes serum NO level in diabetic patients and to find out correlation between the levels of NO in study group. The elevated level of uric acid is a risk factor in diabetes mellitus. Hyperuricemia if not corrected can inactivate NO which leads to hyperlipidemia & increases the risk of cardiac diseases by affecting endothelial function & arterial stiffness. The values of fasting sugar, lipid profile, and serum uric acid were estimated by auto analyzer using transasia kits. Serum NO was estimated by Cortas & Wakid method. Diabetic patients with hyperlipidemia & hyperuricemia showed highly significant decreased levels of serum NO. Their results suggest that diabetic patients with hyperlipidemia & hyperuricemia shows low levels of serum NO & indicate that hyperuricemia inactivates serum NO and decreases its bioavailability.

E. Profumo et al. (2012)[101] investigated a possible association of oxidized low-density lipoproteins (ox-LDLs), nitric oxide (NO), 3-nitrotyrosine, vitamin A, vitamin E, and β-carotene serum levels with subclinical atherosclerosis in rheumatoid arthritis and psoriatic arthritis. By the use of ELISA, they observed higher ox-LDL levels in patients with intima-media thickness (IMT) > 1 than in patients with

IMT ≤ 1 and a negative correlation between NO levels and IMT values. By the use of high-performance liquid chromatography, we determined higher levels of vitamin A in patients with psoriatic arthritis and IMT ≤ 1 than in controls and lower levels of β-carotene in patients with rheumatoid arthritis and psoriatic arthritis than in controls. They confirmed that ox-LDLs and NO may be markers of accelerated atherosclerosis in rheumatoid arthritis and psoriatic arthritis.

Materials & Methods

The subjects in this study were selected from the outpatients at the department of Periodontics, Sree Mookambika Institute of Dental Sciences, Kulasekharam, from September 2012 to December 2012. Subjects who fulfilled the following inclusion/exclusion criteria were included in the study.

Inclusion criteria

- Age group: 20-70 years
- Subjects with a diagnosis of chronic generalized periodontitis based on the clinical criteria proposed by the 1999 world workshop for classification of periodontal diseases and conditions.[102] Patients diagnosed with chronic periodontitis should have atleast 15 natural teeth, more than 30%sites with clinical attachment loss ≥4mm and probing pocket depth ≥ 5mm at baseline.
- Well-controlled type 2 diabetes mellitus patients were selected and classified based on criteria given by American Diabetic Association in 2008 and HbA1c levels ≤7% .[73]
- Healthy subjects without chronic periodontitis and other systemic diseases as control group.
- Subjects who have not received periodontal therapy within the last 6 months.

Exclusion criteria

- Subjects with any other systemic disease (except type2 diabetes)
- Subjects who have used anti-inflammatory drugs or antimicrobial drugs within a 3 month period before the study began
- Subjects who have used mouthwashes within previous 3 months

- Subjects who have used vitamin supplements within previous 3 months
- Pregnant or lactating females
- Subjects who are smokers or alcoholics
- Subjects who have special dietary requirements

Informed consent was obtained from all subjects after the screening. Before obtaining consent , information on the the nature and potential benefits of their participation in the study, was also explained. The study protocol was approved by Ethics Committee of Sree Mookambika Institute of Medical Sciences, Kulasekharam.

The selected patients were assigned into 3 groups; each group consist of 30 patients:

Group I Healthy subjects without chronic periodontitis and other systemic diseases

Group II Chronic periodontitis patients

Group III Patients with chronic periodontitis and type2 diabetes mellitus (Well controlled – HbA_1c levels $\leq 7\%$)

Clinical parameters

Periodontal examination was conducted at the department of Periodontics, Sree Mookambika Institute of Dental Sciences, Kulasekharam. All investigations were performed by single examiner.

The following clinical parameters were assessed at six sites of all teeth (Mesiobuccal, mid- buccal, disto buccal, mesio-lingual, mid lingual and disto-lingual) using William's graduated periodontal probe. Clinical parameters were assessed at baseline and 4 weeks after initial periodontal therapy. [**Refer colorplate. 1 & 2]**

Initial periodontal therapy includes oral hygiene instructions and full mouth subgingival scaling.

1. Oral Hygiene- Simplified Index(OHI-S) –Greene and Vermillion – 1964)
2. Gingival Index (GI)- Loe and Silness-1964)
3. Probing pocket depth (PPD)
4. Clinical attachment level (CAL)

Collection of sample

A fasting blood sample (5ml of venous blood) was collected from the antecubital fossa by venipuncture using a 20 guage needle with 5ml syringe, before periodontal examination at baseline and 4 weeks after initial periodontal therapy. **[Refer colorplate. 3a & 3b]** Serum was separated from blood by centrifuging the sample at 3000*xg* for 10 min. and stored till the assay procedures**. [Refer colorplate. 4&5]**

Biochemical assay

NO is a highly reactive free radical gas and remains stored in tissues as nitrates (NO_3^-) or nitrites (NO_2^-). Thus , NO concentration can be estimated by measuring concentrations of nitrates (NO_3^-) and nitrites (NO_2^-) in combination.[23] The reduction of nitrate to nitrite by copper coated cadmium granules followed by Griess colorimetric reaction can be used to measure the nitrite levels (NO_2^-)[28,103].All the reagents used in this study were of analytical grade.

Reagents

1. Cadmium granules- Cadmium granules are obtained from Fluka Chemische Fabrik AG, Buchs Switzerland. Using a wire cutter, cadmium granules are

broken down into smaller pieces (20 to 40 mg) and stored in 0.1 mol/L H_2S0_4, they are stable for at least nine months**. [Refer colorplate.6]**

2. Glycine-NaOH buffer- It is prepared by dissolving 15.0 g of glycine (Sigma Chemical Co., St. LOUIS, MO) in de-ionized distilled water, adjust pH to 9.7 with 2 mol/L NaOH solution, and make up to 1 L.

3. Sulfanilamide- It is prepared by dissolving 5 g of sulphanilamide in 500 mL of warm 3 mol/L HCI solution, then let cool. This is stable for one year at room temperature.

4. N-Naphthylethylene diamine- It is prepared by dissolving 50mg of N-Naphthylethylene diamine in 250mL of distilled water. This is stable for two months at 0 to8°C.

5. Standards- Working standards are prepared by diluting stock 0.1mol/L solutions of $NaNO_2$ or KNO_3 on the day of use. Working standards are stable for three days.

Methods

Activation of cadmium granules:

Rinse the acid from the granules three times with de-ionized distilled water**. [Refer color plate.7]** Swirl the granules for 1 to 2 minutes in a 5 mmol/L CuSO4 solution in glycine-NaOH buffer**. [Refer colorplate. 8]** Drain, and then rinse the $CuS0_4$ three times with glycine buffer. Drain and use the copper-coated granules within 10 minutes. **[Refer colorplate.9]** Prolonged exposure of the granules to air diminishes their reductive ability. After use, rinse the granules and store them in 0.1 mol/L H_2SO_4 solution.

Deproteinization of sample:

Add 0.5ml of serum to 2.0ml of 75mmol/L zinc sulphate ($ZnSO_{4)}$ solution. Then add, with mixing, 2.5ml of 55mmol/L NaOH reagent. The final pH should be between 7.0 and 7.5. **[Refer colorplate.10]** Let stand for 10 minutes. From this after centrifugation 1 ml supernatant taken for nitrite estimation.

Nitrite assay:

Label test tubes - blank, sample and standard. Add 1ml of glycine-NaOH buffer to each. Then add 1ml of the clear supernatant of the deproteinized sample into the sample tubes. Adjust the volume in all the test tubes to 4ml by adding de-ionized distilled water.

	Blank	**Sample**	**Standard**
Glycine- NaOH buffer	1ml	1ml	1ml
Deproteinized sample	-	1ml	-
Working standard	-	-	3ml
Distilled water	3ml	2ml	-

Start the reaction by adding 2.5 to 3gm of freshly activated cadmium granules and stir once by swirling. Exactly 90 minutes later transfer 2.0ml from each test tube to an appropriately labelled test tube for nitrite determination. Add 0.5ml of distilled water to all tubes to yield a volume of 2.5ml. . Then add 1ml of sulphanilamide solution, followed by 1ml of N- naphthylethylenediamine. Mix well. **[Refer colorplate.11]** Take sample in a cuvette.**[Refer colorplate. 12]** Then read absorbances at 545 nm

after 20-60 minutes using a spectrophotometer **[Refer colorplate. 13]** , against the blank containing reagents but no biological sample.

Formula for measurement of nitrite levels

$$\frac{\text{OD of sample} \times \text{Concentration of standard } (\mu g) \times \text{total volume (ml)} \times 1000 * \text{ml}}{\text{OD of standard} \times 1 \text{ ml supernatant} \times 0.5 \text{ ml serum}} = \text{Concentration of sample}(\mu g)$$

Molecular weight of NO_2 = 46

Therefore, $$\frac{\text{Concentration of sample } (\mu g)}{46} = \text{Level of nitrite } (\mu M/L)$$

OD - Optical density

1000* = Multiplying with 1000 is for calculating the NO2 value to litre of serum so, final value is in micromol/ litre (µM/L)

COLOR PLATES

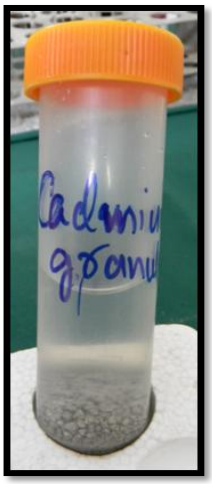
CP.1 Diagnostic instruments

CP. 2 Recording of clinical parameters

CP. 3a and 3b Collection of venous blood sample

CP.4 Kemi C-48 Table top centrifuge **CP.5 Separation of serum**

Activation of cadmium granules



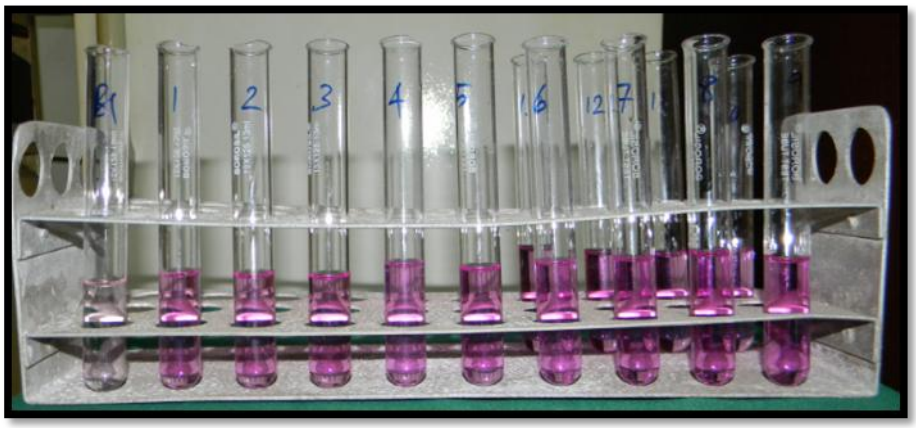
CP. 6 Cadmium granules in rinsed 0.1 M H_2SO_4

CP. 7 Cadmium granules with distilled water

CP. 8 Swirl the granules in $CuSO_4$ solution

CP. 9 Copper coated granules

CP.10 Deproteinization of sample



CP.11 Griess colorimetric reaction

CP.12 Sample taken in a cuvet

CP.13 Spectrophotometer

Results and Observations

The purpose of this study was to compare the serum levels of NO before and after initial periodontal therapy in healthy and chronic periodontitis with and without Type II Diabetes Mellitus patients. This study included a total number of 90 patients of both sexes and satisfying the inclusion criteria. The patients were categorized into 3 groups of 30 patients each:

Group I Healthy subjects without chronic periodontitis and other systemic diseases

Group II Chronic periodontitis patients

Group III Patients with chronic periodontitis and type2 diabetes mellitus

Baseline data for OHI-S, GI , PPD, CAL were recorded for all the patients in each group. Venous blood was collected from all the patients in each group to estimate the serum levels of NO. Serum nitrite levels were estimated by Spectrophotometric method using the Griess colorimetric reaction. After initial periodontal therapy, at the end of 4 weeks all the periodontal parameters were recorded and serum levels of NO was estimated.

Statistical analysis

The data was analysed by a software SPSS (version 16.0). Paired t test was used for statistical significance within the group comparison (pre and post treatment). ANOVA was used for multiple comparisons between groups. Post Hoc Test followed by Dunnet t test was applied to find the statistical significance at 95% confidence interval. $P < 0.05$ considered statistically significant.

Interpretation of results

Table. 1: Demographic characteristics of the study population

Groups	Gender		Number of subjects in different age groups				
	Male	Female	21-30 Y	31-40 Y	41-50 Y	51-60 Y	61-70 Y
Group-I	13	17	27	3	0	0	0
Group-II	13	17	0	9	12	7	2
Group-III	22	8	0	7	8	12	3

Group I consisted of 13 males and 17 females with maximum number of subjects between 21-30 years of age group. Group II consisted of 13 males and 17 females with maximum number of subjects between 41-50 years of age group. Group III consisted of 22 males and 8 females with maximum number of subjects between 51 and 60 years of age group.

Table. 2 & 3 shows the mean values of pre–treatment and post treatment periodontal parameters and serum levels of NO of the 3 groups.

Table. 2 : Pre treatment

Groups	OHI-S (Mean±SD)	Gingival Index (Mean±SD)	PPD (mm) (Mean±SD)	CAL (mm) (Mean±SD)	Nitric oxide (µM/L) (Mean±SD)
Group-I	1.95±0.52	1.09±0.46	1.85±0.19	1.85±0.18	65.23±1.57
Group-II	3.64±0.53	1.93±0.30	3.29±0.42	3.60±0.42	89.21±1.46
Group-III	3.66±0.44	2.03±1.32	3.57±0.40	3.65±0.45	85.54±1.54

Table. 3: Post treatment

Groups	OHI-S (Mean±SD)	Gingival Index (Mean±SD)	PPD (mm) (Mean±SD)	CAL (mm) (Mean±SD)	Nitric oxide (µM/L) (Mean±SD)
Group-I	0.72±0.27	0.24±0.15	1.76±0.22	1.76±0.18	56.29±1.24
Group-II	1.73±0.42	1.27±0.23	3.16±0.46	3.47±0.58	70.91±1.68
Group-III	2.39±0.49	1.71±0.33	3.44±0.42	3.61±0.48	68.69±1.17

Table. 4: Comparison of pre and post treatment periodontal parameters and serum levels of nitric oxide in group-I

Treatment	**OHI-S (Mean±SD)**	**Gingival Index (Mean±SD)**	**PPD (mm) (Mean±SD)**	**CAL (mm) (Mean±SD)**	**Nitric oxide (µM/L) (Mean±SD)**
Pre-treatment	1.95±0.52	1.09±0.46	1.85±0.19	1.85±0.18	65.23±1.57
Post-treatment	0.72±0.27*	0.24±0.15*	1.76±0.22	1.76±0.18	56.29±1.24*

(*P<0.05 significant compared pre-treatment with post treatment)

At the start of the trial, the mean value for OHI-S was 1.95±0.52. At the end of 4 weeks following therapy the mean value for OHI–S was reduced to 0.72±0.27 which was significant statistically ($p < 0.05$).

The mean value for Gingival index showed a decrease from the initial mean value of 1.09±0.46 to 0.24±0.15 which was significant statistically ($p < 0.05$).

The probing pocket depth showed an initial mean value of 1.85±0.19mm. At the end of 4 weeks it reduced to 1.76±0.22mm which was not significant statistically.

The mean value for CAL showed a decrease from the initial mean value of 1.85±0.18mm to 1.76±0.18mm which was not significant statistically.

The mean value of serum levels of NO at baseline was 65.23±1.57µM/L and it was reduced to 56.29±1.24 µM/L at the end of 4 weeks which was significant statistically ($p < 0.05$).

Table. 5: Comparison of pre and post treatment periodontal parameters and serum levels of nitric oxide in group-II

Treatment	**OHI-S (Mean±SD)**	**Gingival Index (Mean±SD)**	**PPD (mm) (Mean±SD)**	**CAL (mm) (Mean±SD)**	**Nitric oxide (µM/L) (Mean±SD)**
Pre-treatment	3.64±0.53	1.93±0.30	3.29±0.42	3.60±0.42	89.21±1.46
Post-treatment	1.73±0.42*	1.27±0.23	3.16±0.46	3.47±0.58	70.91±1.68*

(*P<0.05 significant compared pre-treatment with post treatment)

The OHI-S scores showed an initial mean value of 3.64±0.53. At the end of 4 weeks it reduced to 1.73±0.42 which was significant statistically ($p < 0.05$).

The mean value for gingival index showed a decrease from the initial mean value of 1.93±0.30 to 1.27±0.23 which was not significant statistically.

There was no much reduction in the probing pocket depth from the initial mean value of 3.29±0.42mm to 3.16±0.46mm which was not significant statistically.

The mean value for CAL showed a decrease from the initial mean value of 3.60±0.42mm to 3.47±0.58mm which was not significant statistically.

The mean value of serum levels of NO at baseline was 89.21±1.46 µM/L and it reduced to 70.91±1.68 µM/L at the end of 4 weeks which was significant statistically($p < 0.05$).

Table. 6: Comparison of pre and post treatment periodontal parameters and serum levels of nitric oxide in group-III

Treatment	**OHI-S (Mean±SD)**	**Gingival Index (Mean±SD)**	**PPD (mm) (Mean±SD)**	**CAL (mm) (Mean±SD)**	**Nitric oxide (µM/L) (Mean±SD)**
Pre-treatment	3.66±0.44	2.03±1.32	3.57±0.40	3.65±0.45	85.54±1.54
Post-treatment	2.39±0.49*	1.71±0.33	3.44±0.42	3.61±0.48	68.69±1.17*

(*P<0.05 significant compared pre-treatment with post treatment)

At the start of the trial the mean value for OHI-S was 3.66±0.44 . At the end of 4 weeks following therapy the mean value for OHI–S reduced to 2.39±0.49 which was significant statistically ($p < 0.05$).

The mean value for gingival index showed a decrease from the initial mean value of 2.03±1.32 to 1.71±0.33 which was not statistically significant.

The probing pocket depth showed an initial mean value of 3.57±0.40mm . At the end of 4 weeks it reduced to 3.44±0.42mm which was not significant statistically.

There was no much reduction in the CAL from the initial mean value of 3.65±0.45mm to 3.61±0.48mm which was not significant statistically.

The mean value of serum levels of NO at baseline was 85.54±1.54 µM/L and it reduced to 68.69±1.17 µM/L at the end of 4 weeks which was significant statistically($p < 0.05$).

Table. 7: Comparison of pre treatment periodontal parameters and serum levels of nitric oxide between groups

Groups	**OHI-S (Mean±SD)**	**Gingival Index (Mean±SD)**	**PPD (mm) (Mean±SD)**	**CAL (mm) (Mean±SD)**	**Nitric oxide (µM/L) (Mean±SD)**
Group-I	1.95±0.52	1.09±0.46	1.85±0.19	1.85±0.18	65.23±1.57
Group-II	3.64±0.53*	1.93±0.30*	3.29±0.42*	3.60±0.42*	89.21±1.46*
Group-III	3.66±0.44*	2.03±1.32*,#	3.57±0.40*	3.65±0.45*	85.54±1.54*

(*P<0.05 significant compared group-I with other groups, # P<0.05 significant compared group-II with other groups)

The mean value of OHI-S scores of group II and group III was markedly higher when compared to group I which was significant statistically. However, no statistically significant difference was observed between group II& III.

When the mean value of GI scores of the 3 groups was compared, group II and group III showed higher values when compared to group I which was statistically significant. Similarly a statistically significant difference was observed when group II& III were compared ($p < 0.05$).

The mean value of probing pocket depth of group II and group III were markedly higher when compared to group I which was significant statistically. However, when group II& III were compared, no statistically significant difference was observed.

When the mean values of CAL of group II and group III were compared with group I , higher mean values was observed with respect to group II &III which was significant statistically. However, comparison between group II& III showed no statistically significant difference.

When comparison of the mean value of serum levels of NO at baseline for 3 groups was done , higher values was observed for group II & III when compared to group I which was statistically significant . However when the values of group II &III were compared it was not significant statistically.

Table. 8: Comparison of post treatment periodontal parameters and serum levels of nitric oxide between groups

Groups	**OHI-S (Mean±SD)**	**Gingival Index (Mean±SD)**	**PPD (mm) (Mean±SD)**	**CAL (mm) (Mean±SD)**	**Nitric oxide (µM/L) (Mean±SD)**
Group-I	0.72±0.27	0.24±0.15	1.76±0.22	1.76±0.18	56.29±1.24
Group-II	1.73±0.4*	1.27±0.23*	3.16±0.46*	3.47±0.58*	70.91±1.68*
Group-III	2.39±0.49*#	1.71±0.33*,#	3.44±0.42*	3.61±0.48*	68.69±1.17*#

(*P<0.05 significant compared group-I with other groups, # P<0.05 significant compared group-II with other groups)

The mean value of OHI-S scores of group II and group III was markedly higher when compared to group I which was significant statistically. However, a statistically significant difference was observed when comparison between group II& III was done ($p < 0.05$).

When the mean value of GI scores of the 3 groups was compared, group II and group III showed higher values when compared to group I which was statistically significant. When comparison between group II& III was done, a statistically significant difference was observed ($p < 0.05$) .

The mean value of probing pocket depth of group II and group III was markedly higher when compared to group I which was significant statistically.

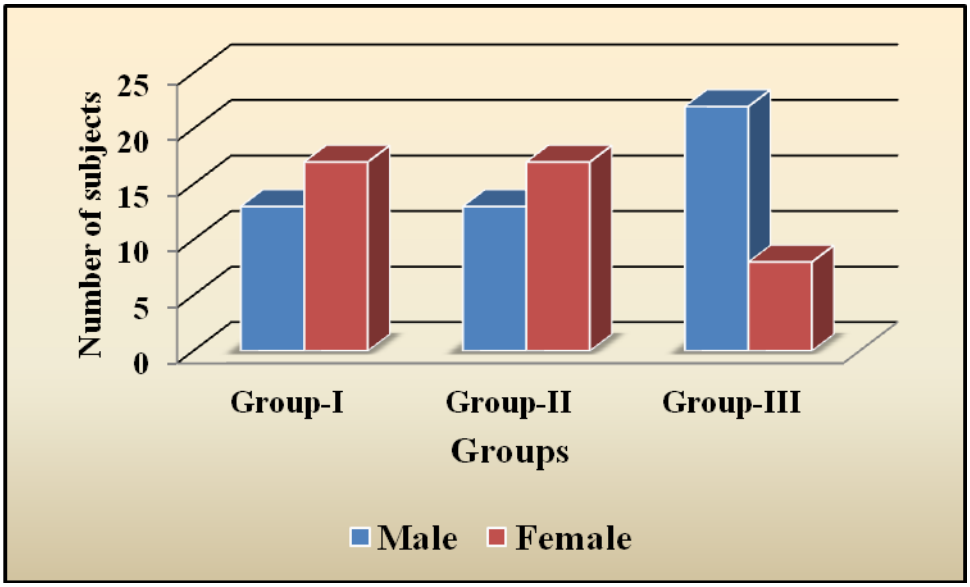
However, comparison between group II& III showed no statistically significant difference.

When the mean values of CAL of group II and group III was compared with group I , higher mean values was observed with respect to group II &III which was significant statistically . However, no statistically significant difference was observed when comparison between group II& III was done.

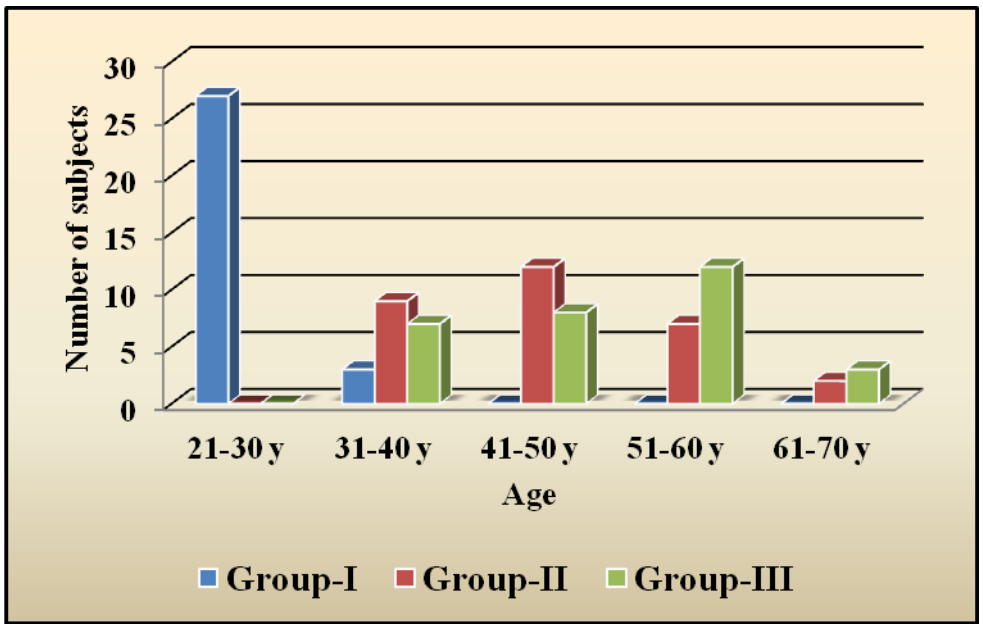
When comparison of the mean value of serum levels of NO for 3 groups was done , higher values was observed for group II & III when compared to group I which was statistically significant . However, a statistically significant difference was observed when comparison between group II& III was done ($p < 0.05$).

GRAPHS

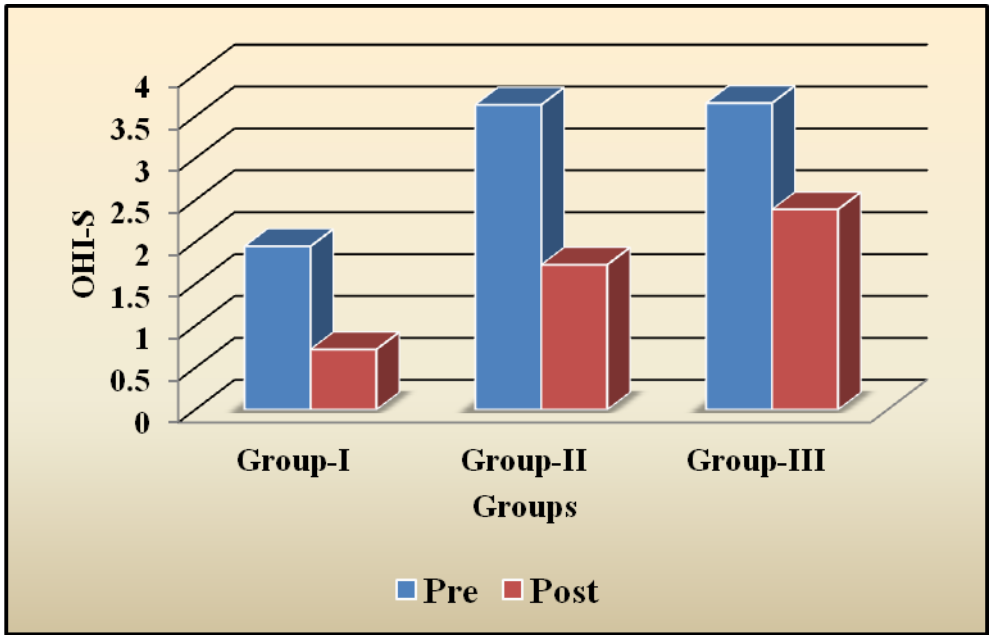
Graph. 1: Demographic data of number of male and female in different groups



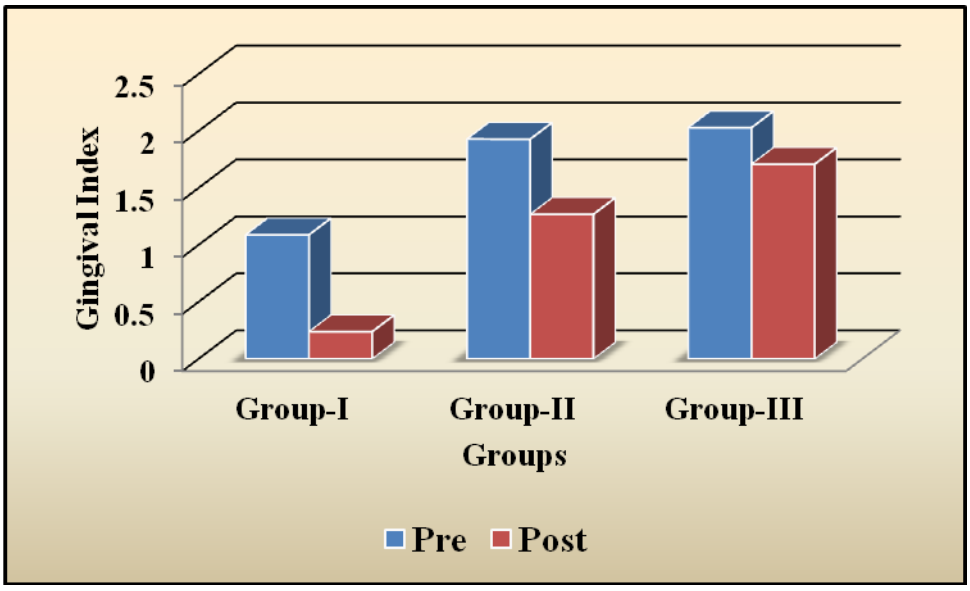
Graph-2: Demographic data of the subjects in different age groups



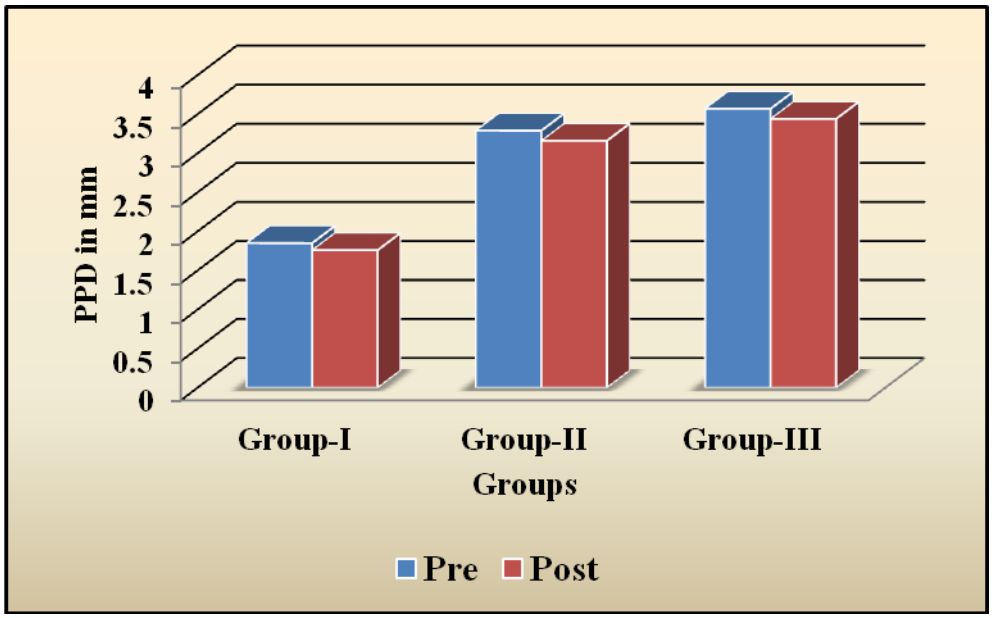
Graph-3: Comparison of pretreatment and post treatment Oral hygiene index-simplified (OHI-S) values



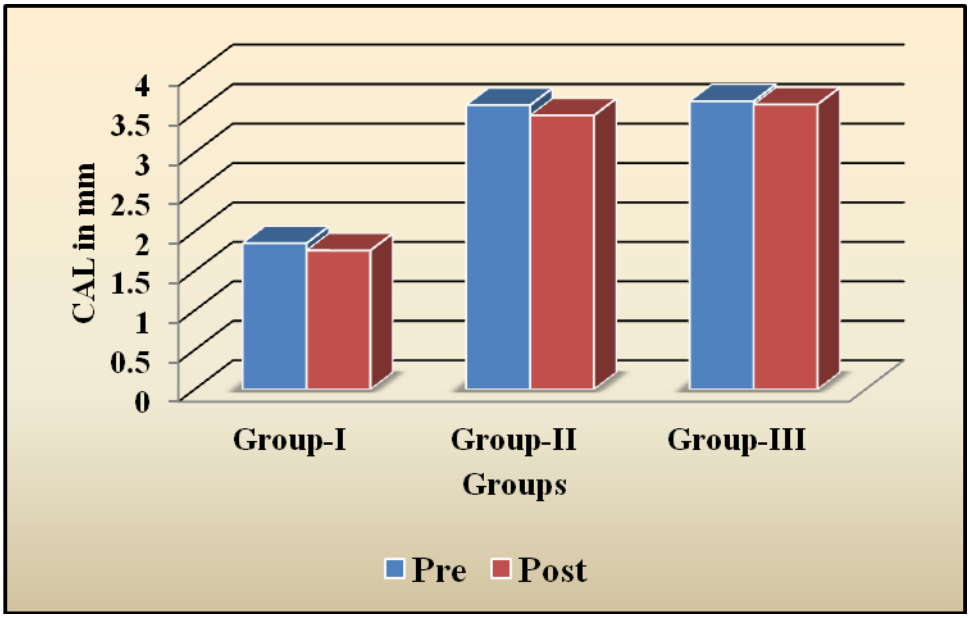
Graph-4: Comparison of pretreatment and post treatment Gingival Index (GI) values



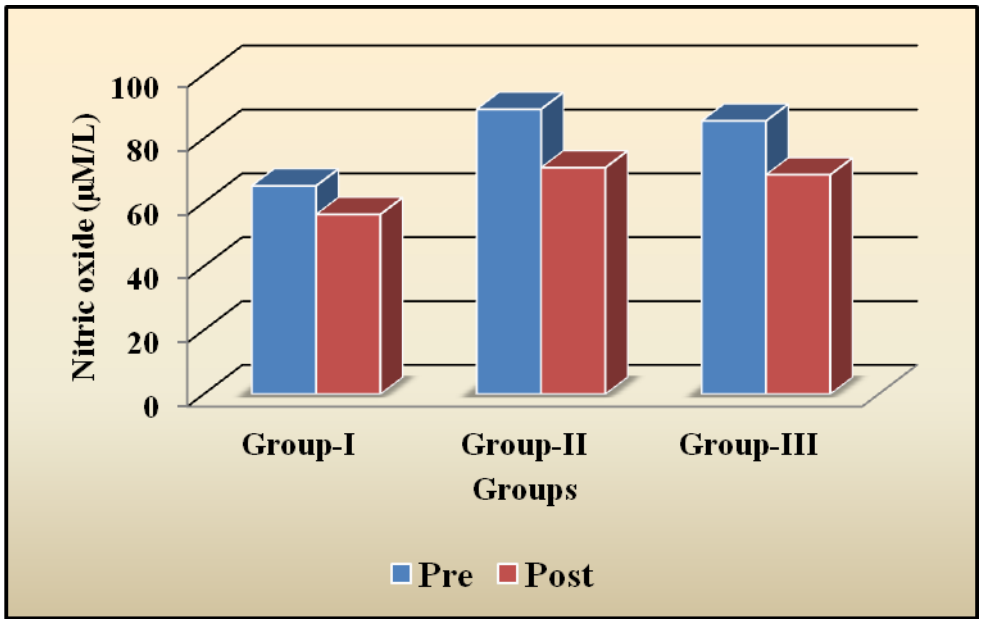
Grpah-5: Comparison of pretreatment and post treatment Probing Pocket Depth (PPD) values in (mm)



Graph-6: Comparison of pre treatment and post treatment clinical attachment level (CAL) values in (mm)



Graph-7: Comparison of pre treatment and post treatment serum level of nitric oxide (NO) values in (µM/L)



Discussion

Diabetes mellitus and periodontal disease are common chronic diseases that have long been considered to be biologically linked that contribute to each other's severity and worsen each other's prognosis. A plethora of epidemiologic studies have demonstrated that periodontitis is more prevalent in type I and type II diabetes mellitus compared to non-diabetics.[104] The incidence of periodontal disease increased by nearly threefold in patients with type 2 diabetes as compared to type 1 diabetes mellitus. The odd's ratio for periodontal disease among subjects with type2 diabetes mellitus ranges from 2to 4.[105,106,107] In the present study, a total of 90 subjects were selected and categorized into 3 groups, each group comprising of 30 subjects - group I (Healthy controls), group II (chronic periodontitis) and group III (chronic periodontitis patients with type2 diabetes mellitus).

Several studies investigated the relationship between diabetes mellitus and periodontitis clinically, outcomes were often controversial.[73,108,109,110,111] Diagnostic parameters and methodologies are not universally defined, which makes comparison of the available evidence difficult[73]. In the present study, the periodontal parameters like OHI-S, GI, PPD and CAL values were higher in group III at baseline with mean values: 3.66±0.44, 2.03±1.32,3.57±0.40mm,3.65±0.45mm respectively as compared to group I and group II. At baseline, there was no significant difference for the periodontal parameters between patients in group II and group III except for GI with mean value of 2.03±1.32 ($p<0.05$) in group III when compared to 1.93±0.30 in group II. This was in agreement with the study of Nowak et al[112] where clinical parameters such as PI, GI, PD and CAL values were higher in patients with diabetes and periodontitis compared to patients with only chronic periodontitis. Several mechanisms have been proposed to explain the greater incidence and severity of

periodontal disease in patients with diabetes. These include polymorphonuclear leukocyte changes, deregulated cytokine dysfunction, vascular changes, altered collagen and glycosaminoglycans synthesis, and the formation of AGEs.[73] AGEs form a critical link between numerous diabetic complications, because they induce marked changes in cells and extracellular matrix components. These changes, including abnormal endothelial cell function, capillary growth and vessel proliferation, also occur in the periodontium of some people with diabetes.[113] AGEs with markers for increased oxidative stress have been demonstrated in human gingiva of subjects with chronic periodontitis and diabetes mellitus.[114] It has been postulated that AGE-RAGE interaction induces an oxidant stress that may be responsible for monocytic upregulation, activation of NF-kB and subsequent expression of mRNA and secretion of proinflammatory cytokines such as IL-1β, TNF –α and IL-6by monocytic phagocytes involved in periodontal tissue inflammation and destruction.[115,116,117]

Increasing evidences suggest that oxidative stress and changes in nitric oxide formation or action play a major role in chronic inflammatory diseases such as periodontitis and diabetes mellitus.[30] In the present study we measured serum nitric oxide levels indirectly by estimating nitrite levels the stable end products of nitric oxide oxidation by Griess reaction. It has been reported that endogenous nitric oxide production is highly correlated with nitrate and nitrite levels in serum and plasma. [28] Hence estimation of nitrite and nitrate is a relative measure of nitric oxide production in vivo. [28,29]

NO is a gaseous, free radical, which can readily diffuse through cytoplasm and plasma membrane due to its solubility in both aqueous and lipid environments.[44,118] It has a short biological half life[44]. NO is generated enzymatically from L- Arginine by

a family of nitric oxide synthase (NOS) isoforms.[44] NO is a ubiquitous molecule essential for many biologic functions, namely neurotransmission[119], vasodilation[24], cytotoxicity[25] and immuno regulation[26], all of which are likely to be relevant to the pathogenesis of periodontal disease. In periodontitis lesion considerable quantities of NO may be generated, for prolonged duration, most likely by macrophages, PMNs, lymphocytes and fibroblasts following cytokines and LPS induction. [120]

The results of our study showed a higher value in the serum levels of NO in group II when compared to group III and group I with mean values 89.21±1.46 μM/L, 85.54±1.54 μM/L, 65.23±1.57 μM/L respectively at baseline. This was in accordance to the previous study[23] where increased NO levels were found in saliva and GCF of subjects with chronic periodontitis as compared to healthy subjects. Studies have shown that oral *de novo* nitric oxide production increases during deposition of plaque which might be an early host defense mechanism against bacterial proliferation in plaque [68]. NO is known to potentiate matrix degradation which includes suppression of proteoglycan and collagen synthesis[120, 121] and upregulation of metalloproteinases activity. [120,122]

Elevated NO production in chronic periodontitis is a reflection of an immune-activated state on which inflammatory cytokines and other mediators are upregulated. Cytokine and bacterial toxin –induced NO upregulates IL-1, TNF-α and IL-8 production by neutrophils a possible mechanism contributing to the escalation of inflammation in periodontal disease. [120] NO has been shown to directly activate both constitutive and inducible forms of cycloxygenase (COX) enzyme, leading to increase in PGE_2 production and enhances bone resorption in periodontal disease. [76,123] It was also reported that Th1 type cytokines increase the expression of NOS and prime the

membrane bound NADPH oxidase of neutrophils and monocytes of mice leading to an activated state, which upon second stimulus releases upto six fold increased levels of ROS than do unprimed phagocytes and enhances tissue destruction. [120] In our study , lower value of serum NO levels were observed in group III when compared to group II at baseline, which was statistically significant ($p<0.05$). The possible underlying mechanism for the significant decrease in serum nitric oxide levels in patients with chronic periodontitis and diabetes mellitus in our study could be due to impaired circulation observed in diabetic patients which limits the availability of NOS and NO[124]. It has been reported that glycosylation of haemoglobin impairs the NO vasodilator function of RBC. Glycosylated haemoglobin binds NO in the form of nitrosothiols very tightly so that any NO that is formed cannot be easily released from RBC and aggravates the consequences of endothelial dysfunction in diabetes mellitus[124]. Moreover, there is evidence that AGEs can have an inhibitory action on NO. Several mechanisms by which AGEs reduce or block NO activity have been proposed. AGEs reduce the half-life of endothelial NO synthase (eNOS) mRNA through an increased rate of mRNA degradation and reduced eNOS activity. Another mechanism proposes that AGEs impair NO production via the binding of CML (N^{ε}-(carboxymethyl) lysine- a stable AGE compound) residues to endothelial AGE receptors, causing a reduction in phosphorylation of serine residues in eNOS, resulting in deactivation of the enzyme.[125]

Studies have shown that hyperglycemia- -induced oxidative stress increases assymetrical dimethyl arginine (ADMA) , an endogenous inhibitor of NO synthesis, in type 2 diabetes mellitus. Oxidative stress can reduce the bioavailability of NO and activation of the polyol pathway, which increases the use of nicotinamide adenine dinucleotide phosphate can reduce the biosynthesis of NO.[57,126] It has also been

reported that the kidney dysfunction associated with diabetes mellitus may prevent the elimination of major NOS inhibitor, asymmetrical dimethyl arginine (ADMA) thereby limiting the production of NO in type 2 diabetes mellitus patients.[124]

Our study was an interventional study in which all the clinical parameters and serum nitric oxide levels were re-estimated four weeks after initial periodontal therapy in all the three groups. Results showed a reduction in all the clinical parameters -OHI-S, GI,PPD,CAL- in all the three groups. However the reduction in OHI-S and GI values were statistically significant ($p<o.o5$). This is in agreement with previous studies[127,128] in which initial periodontal therapy was associated with improved periodontal health in type 2 diabetes mellitus patients.

The serum nitric oxide levels in all the three groups after initial periodontal therapy also showed a decline from its baseline values . We observed a statistically significant reduction in group II when compared to group III after initial periodontal therapy. However there is no evidence to support the influence of initial periodontal therapy in lowering the serum levels of NO in chronic periodontitis with or without type 2 diabetes mellitus.

Currently an alternative approach to reduce periodontal inflammation using NOS inhibitors has been proposed. Selective inhibitors of iNOS -mercaptoethylguanidine, guanidoethyldisulfide, aminoguanidine- are shown to reduce peroxynitrite formation as well as inhibiting prostaglandin production via inhibition of COX.[37, 62,129] It is therefore logical that modulation of this biological messenger might be useful for the treatment of chronic inflammatory diseases such as periodontal disease and diabetes mellitus.

There were several drawbacks in this study. 1) Age group included in the study ranged from 20-70 years. 2) Male to female ratio was not matched 3) Sample size selected for the study was small. 4) The initial periodontal therapy was limited to subgingival scaling alone and no root planing was performed.

Summary & Conclusion

Periodontal disease and diabetes mellitus are strongly interrelated and have common pathobiology. Inflammatory events during periodontal disease may play a important role on development of diabetes and insulin resistance probably facilitates the progress of periodontal disease.

Indeed virtually every cell and many immunological parameters are modulated by nitric oxide. It has been shown that nitric oxide can have pro-inflammatory or anti-inflammatory actions during infections. For these reasons nitric oxide has been described as 'double edged sword mediator'. And this phenomenon is often referred to as nitric oxide paradox. Hence serum NO levels can be utilized as a good indicator of the inflammatory status of the periodontium and evaluating these levels in the serum by Spectrophotometric method using Griess reaction is a reliable and faster method to estimate the level of inflammation in the periodontal tissues.

However further studies need to be conducted as to whether the administration of some chemical inhibitors of NOS or antioxidants in addition to mechanical therapy can be of some help to modulate the host response in inflammatory periodontal diseases.

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APPENDICES

SREE MOOKAMBIKA INSTITUTE OF MEDICAL SCIENCES
(V.P.M Hospital Complex, Padanilam)
KULASEKHARAM, Kanyakumari District Tamilnadu – 629161

Institute Human Ethics Committee

No. SMIMS/IHEC/2012/B1 **Date:** 10th November 2012

Certificate

This is to certify that the research protocol No. **SMIMS/IHEC/2012/B1/13**, entitled **"A Comparative Study on Serum Levels of Nitric Oxide Before and After Initial Periodontal Therapy in Healthy and Type 2 Diabetes Mellitus Patients with Chronic Periodontitis"** submitted by Dr. Gayathri S, Post Graduate, Dept. of Periodontics, has been approved by the Institute Human Ethics Committee by expedited review on 10th of November 2012.

10/11/12

(Dr. T. Ashok Kumar)
CHAIRPERSON
Institute Human Ethics Committee
SMIMS, Kulasekharam

SREE MOOKAMBIKA INSTITUTE OF DENTAL SCIENCES,
KULASEKHARAM – 629161

Part-I

PATIENT INFORMATION SHEET

Study title: **"A comparative study on serum levels of nitric oxide before and after initial periodontal therapy in healthy and type 2 diabetes mellitus patients with chronic periodontitis"**

You are being invited to take part in the above mentioned study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

1) What is the purpose of the study?

- To assess the influence of nitric oxide in serum of healthy and type 2 diabetes mellitus patients with chronic periodontitis.
- To find out the effect of initial periodontal therapy on the serum levels of nitric oxide in healthy and type 2 diabetes mellitus patients with chronic periodontitis.

2) Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive.

3) What will happen to me if I take part?

The investigator will ask you few questions and will evaluate your oral hygiene status and periodontal health status. 5ml of venous blood will be

collected from the antecubital fossa by venipuncture before and 4 weeks after initial periodontal therapy for the assay.

4) What are the risks of taking part?
   There are no risks in taking part this study.

5) Will my taking part in this study be kept confidential?
   All information collected from you will be kept confidential. No personal details will be revealed to anyone.

Contacts for Further Information

Investigator

Dr.Gayathri.S

Mob: 08903247177

Mail Id: gkathu@gmail.com

Guide

Dr. Elizabeth Koshi , MDS

Mob: 09447154335

Mail Id: elizabethkohi_dr@yahoo.com

Co- guide

Dr. Arun Sadasivan , MDS

Mob: 09847246961

Mail Id: sadasivan_arun@hotmail.com

Thank you for taking part in this study.

ஸ்ரீ மூகாம்பிகா பல் மருத்துவக் கல்லூரி

குலசேகரம் - 629 161

பகுதி I

நோயாளிகளின் தகவல் அறிக்கை

ஆய்வின் தலைப்பு

ஆரோக்கியமான மற்றும் நீரழிவு நோயாளிகளின் ஈறு நோய்களின் முதற்கட்ட ஈறு நோய் சிகிச்சைக்கு முன்பும் பின்பும் ரத்தத்தில் உள்ள Nitric Oxide எனப்படும் சுரப்பிகளின் அளவை தெரிந்து கொள்ளுதல்.

உங்களையும் இந்த ஆய்வில் கலந்து கொள்ளுமாறு அழைக்கிறோம். இதில் பங்கேற்பதற்கு முன் நீங்கள் இந்த ஆய்வின் நோக்கம் மற்றும் செயல்பாடு பற்றி அறிந்து கொள்ளுங்கள். கீழே கொடுத்துள்ளவற்றை கவனமாக படிக்கவும், சந்தேகங்களோ விளக்கமோ தேவையொன்றால் எங்களை அணுகவும். அதற்கு பின் உங்கள் பங்களிப்பை உறுதிப்படுத்தவும்.

1. இந்த ஆய்வின் நோக்கம் என்ன?

- ஆரோக்கியமான மற்றும் நீரழிவு நோயாளிகளில் ஈறு நோய் உள்ளவர்களின் ரத்தத்திலுள்ள Nitric Oxide சுரப்பியின் பங்களிப்பு.
- இந்த சுரப்பிகளின் அளவில் முதற்கட்ட ஈறு சிகிட்சையால் ஏற்படும் தாக்கம்.

2. நான் இதில் கலந்து கொள்ள வேண்டுமா?

அதை நீங்கள் தான் முடிவு செய்ய வேண்டும். நீங்கள் கலந்து கொள்வதென்றால் இந்த தகவல் அறிக்கை கொடுக்கப்படும். உறுதிப்படிவத்தில் கையொப்பும் பெறப்படும். கலந்து கொள்ளும் பட்சத்தில் இதிலிருந்து விலகும் உரிமையும் உள்ளது. இது சிகிச்சையை பாதிக்காது.

3. ஆய்வில் கலந்து கொண்டால் எனக்கு ஏற்படுபவை?

மருத்துவர் சில வினாக்கள் கேட்டு உங்கள் ஈறுகளின் ஆரோக்கிய நிலையை கண்டறிவார். முதற்கட்ட ஈறுசிகிச்சைக்கு முன்பும் 4 வாரங்களுக்கு பின்பும் ரத்தம் சோதனைக்கு எடுக்கப்படும்.

4. ஆய்வில் கலந்து கொள்வதால் இடையூறுகள் என்ன?

எந்த இடையூறும் இல்லை.

5. என் பங்களிப்பு ரகசியமானதா?

ஆம் எல்லா தகவல்களும் ரகசியமாக வைக்கப்படும். வேறொரு நபருக்கு தெரிவிக்கப்படாது.

மேலும் விபரங்களுக்கு தொடர்பு கொள்க :

ஆய்வாளர்

மரு. காயத்ரி.எஸ்

கைபேசி : 08903247177

மின்னஞ்சல் : gkathu@gmail.com

வழிகாட்டி

மரு. எலிசபெத் கோஸி MDS

கைபேசி : 09447154335

மின்னஞ்சல் : elizabethkoshi_dr@yahoo.com

துணைவழிகாட்டி

மரு. அருண் சதாசிவன் MDS

கைபேசி எண் : 09847246921

மின்னஞ்சல் : sadasivan_arun@hotmail.com

இந்த ஆய்வில் கலந்து கொண்டதற்கு நன்றி.

குறிப்பு : பயனாளிக்கு இந்த தகவல் குறியீட்டுடன் கையொப்பமிட்ட உறுதிப்படிவத்தின் நகலும் கொடுக்கப்படும்.

രോഗികളുടെ അറിവിലേക്കായി

ആരോഗ്യമുള്ളതും പ്രമേഹമുള്ളതുമായ മോണരോഗികളിൽ മോണ ചികിത്സയ്ക്ക് മുൻപും പിൻപും ഉള്ള നൈട്രിക് ഓക്സൈഡ് എന്ന പദാർത്ഥത്തിന്റെ രക്തത്തിലെ അളവിലുണ്ടാകുന്ന വ്യതിയാനത്തിന്റെ ഒരു താരതമ്യ പഠനം

നിങ്ങളെ മേൽ പറഞ്ഞ പഠനത്തിലേക്ക് സ്വാഗതം ചെയ്യുന്നു. ഈ പഠനത്തിൽ പങ്കെടുക്കുന്നതിന് മുൻപ് താങ്കൾ ഇതിന്റെ പ്രാധാന്യത്തെപ്പറ്റി അറിഞ്ഞിരിക്കേണ്ടതുണ്ട്. ഈ പഠനത്തെപറ്റി എന്തെങ്കിലും സംശയമുണ്ടെങ്കിൽ ചോദിച്ചു മനസ്സിലാക്കാനുള്ള എല്ലാ സ്വാതന്ത്ര്യവും താങ്കൾക്കുണ്ട്. ഇതിൽ പങ്കെടുക്കാനും, പങ്കെടുക്കാതിരിക്കുവാനുമുള്ള പൂർണ്ണ അവകാശവും താങ്കൾക്കുണ്ട്.

1. ഈ പഠനത്തിന്റെ ഉദ്ദേശ്യം എന്ത് ?

ആരോഗ്യമുള്ളതും പ്രമേഹമുള്ളതുമായ മോണരോഗങ്ങളിൽ നൈട്രിക്ക് ഓക്സൈഡ് എന്ന പദാർത്ഥത്തിനുള്ള സ്വാധീനം മനസ്സിലാക്കുന്നതിനും മോണചികിത്സ മുലം നൈട്രിക്ക് ഓക്സൈഡിന്റെ അളവിലുണ്ടാകുന്ന വ്യതിയാനം കണ്ടുപിടിക്കുന്നതിനുമായാണ് ഈ പഠനം നടത്തുന്നത്.

2. എന്നെ എന്തുകൊണ്ട് ഈ പഠനത്തിൽ ഉൾപ്പെടുത്തുന്നു ?

ഈ പഠനത്തിലേക്കായി 30 വീതം ആരോഗ്യമുള്ളതും പ്രമേഹമുള്ളതുമായ മോണരോഗികളേയും 30 പൂർണ്ണ ആരോഗ്യമുള്ളവരേയും ആവശ്യമുണ്ട്. നിങ്ങളിൽ ഈ പഠനത്തിനു വേണ്ട എല്ലാ മാനദണ്ഡങ്ങളും ഉണ്ടെന്നതിനാലാണ് ഈ പഠനത്തിൽ ഉൾപ്പെടുത്തുന്നത്.

3. ഞാനിതിൽ പങ്കെടുക്കണമോ ?

ഈ പഠനത്തിൽ പങ്കെടുക്കണമോ വേണ്ടയോ എന്നു തീരുമാനിക്കുവാനുള്ള പൂർണ്ണ അവകാശം നിങ്ങൾക്കുണ്ട്. നിങ്ങൾ ഇതിൽ പങ്കെടുക്കുവാൻ തീരുമാനിക്കുകയാണെങ്കിൽ ഒരു സമ്മത പത്രത്തിൽ ഒപ്പിട്ടു നൽകേണ്ടതുണ്ട്. ഈ പഠനത്തിൽ നിന്നും ഏതു സമയത്തും പിൻവാങ്ങാനുള്ള സ്വാതന്ത്ര്യവും നിങ്ങൾക്കുണ്ട്. ഇതു നിങ്ങളുടെ മറ്റു ചികിത്സകളെ യാതൊരുവിധത്തിലും ബാധിക്കുന്നതായിരിക്കില്ല എന്നുള്ളത് പ്രത്യേകം പറയേണ്ടതുണ്ട്.

4. ഞാൻ ഈ പഠനത്തിൽ പങ്കെടുത്താൽ എന്തു സംഭവിക്കാം ?

പരിശോധകൻ നിങ്ങളോട് ചില ചോദ്യങ്ങൾ ചോദിക്കുകയും ദന്തശുചിത്വവും മോണയുടെ ആരോഗ്യവും പരിശോധിക്കുന്നതുമാണ്. മോണ ചികിത്സയ്ക്ക് മുൻപും 4 ആഴ്ചകൾക്ക് ശേഷവും 4 ml വീതം രക്തം ശേഖരിക്കുന്നതുമായിരിക്കും.

5. ഈ പഠനത്തിൽ പങ്കെടുക്കുന്നതുകൊണ്ട് എന്തെങ്കിലും അപകട സാധ്യതഉണ്ടോ ?

ഇതിൽ പങ്കെടുക്കുന്നതുകൊണ്ട് യാതൊരുവിധത്തിലുമുള്ള അപകടസാദ്ധ്യതയും ഇല്ല.

6. ഞാൻ ഈ പഠനത്തിൽ പങ്കെടുക്കുന്നുവെന്നുള്ള വിവരം രഹസ്യമാക്കി വെയ്ക്കുമോ ?

നിങ്ങളിൽ നിന്നും ശേഖരിക്കുന്ന എല്ലാ വിവരങ്ങളും രഹസ്യമാക്കി വെയ്ക്കുന്നതായിരിക്കും. നിങ്ങളെ പറ്റിയുള്ള വിവരങ്ങൾ ആരോടും വെളിപ്പെടുത്തുന്നതായിരിക്കില്ല.

കൂടുതൽ വിവരങ്ങൾക്കായി താഴെ പറയുന്നവരെ നിങ്ങൾക്ക് ബന്ധപ്പെടാവുന്നതാണ്.

Part-II

PATIENT CONSENT FORM

I hereby declare that I will comply with all the treatment procedures needed for "A comparative study on serum levels of nitric oxide before and after initial periodontal therapy in healthy and type 2 diabetes patients with chronic periodontitis", for which blood samples will be drawn before and four weeks after periodontal therapy. Doctor has explained to me all the procedures. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to participate as a participant in this study and understand that I have the right to withdraw from the study at any time without in any way affecting my dental care.

Doctor's Name Patient's Name

(Signature) (Signature)

உறுதிமொழிப்படிவம்

இதன்படி நான் உறுதிபட கூறுவது என்னவென்றால் ஆரோக்கியமான மற்றும் நீரிழிவு நோயாளிகளின் முதற்கட்ட ஈறு நோய் சிகிச்சைக்கு முன்பும், பின்பும் ரத்தத்திலுள்ள நைட்ரிக் ஆக்ஸைடு (Nitric oxide) எனப்படும் சுரப்பிகளின் அளவை தெரிந்து கொள்ளும் ஆய்விற்குதேவையான அனைத்து சிகிட்சை முறைகளையும் நான் மனப்பூர்வமாக ஏற்கிறேன். இந்த ஆய்விற்கான 4 வாரத்தில் ஈறு சிகிட்சையின் முன்னும் பின்னுமாக இரத்த பரிசோதனை செய்யப்படும். மருத்துவர் செய்முறை விளக்கமளித்து எனது சந்தேகங்களுக்கும் பதிலளித்தார். எனது சிகிட்சை முறையில் எந்த பாதிப்பும் ஏற்படாமல் தேவைப்பட்டால் ஆய்விலிருந்து விலகிக்கொள்ளும் உரிமை உண்டு என்றும் அறிந்து கொண்டு நான் மனபூர்வமாக இந்த ஆய்வில் பங்கேற்கிறேன்.

மருத்துவர் பெயர்

கையொப்பம்

சிகிட்சை பெறுபவர் பெயர்

கையொப்பம்

സമ്മതപത്രം

'ആരോഗ്യമുള്ളതും പ്രമേഹമുള്ളതുമായ മോണരോഗികളിൽ മോണ ചികിത്സയ്ക്കു മുൻപും പിൻപും ഉള്ള നൈട്രിക് ഓക്സൈഡ് എന്ന പദാർത്ഥത്തിന്റെ രക്തത്തിലെ അളവിലുണ്ടാകുന്ന വ്യതിയാനത്തിന്റെ ഒരു താരതമ്യ പഠനം' എന്ന പഠനത്തിനുവേണ്ടിയുള്ള എല്ലാ ചികിത്സാവിധിക്രമങ്ങളും യഥാവിധി അനുവർത്തിക്കുമെന്ന് ഞാൻ ഉറപ്പു നൽകുന്നു. മേൽ പറഞ്ഞ പഠനാവശ്യത്തിനായി എന്റെ രക്തത്തിന്റെ സാമ്പിൾ നൽകുന്നതിനായി പൂർണ്ണ മനസ്സോടെ സഹകരിക്കുമെന്ന് അറിയിച്ചുകൊള്ളുന്നു. ഈ പഠനത്തിൽ നിന്നും ഏതു സമയത്തും എന്റെ ദന്ത ചികിത്സയെ ബാധിക്കാത്ത തരത്തിൽ പിൻവാങ്ങാനുള്ള അവകാശമുണ്ടെന്നും ഞാൻ മനസ്സിലാക്കുന്നു.

ഡോക്ടറുടെ പേര്

ഒപ്പ്

രോഗിയുടെ പേര്

ഒപ്പ്

A comparative study on the serum levels of nitric oxide before and after initial periodontal therapy in healthy and type 2 diabetes mellitus patients with chronic periodontitis

Patient record form (Pre- treatment)

GROUP I / GROUP II/ GROUP III

Serial No: Date:

Name: Age: Sex: M / F

Address: Occupation:

Medical history:

Drug history:

CLINICAL PARAMETERS

I. Oral Hygiene Index – Simplified (Greene and Vermilion-1964)

Debris Index-S

16	**11**	**26**
46	**31**	**36**

Calculus Index-S

16	**11**	**26**
46	**31**	**36**

Score:

Interpretation:

Good		Fair		Poor	

II. Gingival Index (Loe and Silness-1963)

16			**12**			**24**		
44			**32**			**36**		

Score:

Interpretation:

Mild		Moderate		Severe	

BLOOD INVESTIGATION

Random blood sugar (RBS) / Glycated haemoglobin (HbA_1c) level:

NO level:

PERIODONTAL STATUS

MAXILLA

	D.B B M.B	D.B B M.B	D.B B M.B	D.B B M.B	D.B B M.B	D.B B M.B	D.B B M.B	D.B B M.B	M.B B D.B	M.B B D.B	M.B B D.B	M.B B D.B	M.B B D.B	M.B B D.B	M.B B D.B	M.B B D.B
	D.P P M.P	D.P P M.P	D.P P M.P	D.P P M.P	D.P P M.P	D.P P M.P	D.P P M.P	D.P P M.P	M.P P D.P	M.P P D.P	M.P P D.P	M.P P D.P	M.P P D.P	M.P P D.P	M.P P D.P	M.P P D.P
Recession	18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28
Enlargement	18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28
PPD	18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28
CAL	18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28

PERIODONTAL STATUS

MANDIBLE

	D.B B M.B			D.B B M.B			D.B B M.B			D.B B M.B			D.B B M.B			D.B B M.B			D.B B M.B			D.B B M.B			M.B B D.B			M.B B D.B			M.B B D.B			M.B B D.B			M.B B D.B			M.B B D.B			M.B B D.B		
	D.L L M.L			D.L L M.L			D.L L M.L			D.L L M.L			D.L L M.L			D.L L M.L			D.L L M.L			D.L L M.L			D.L L M.L			D.L L M.L			D.L L M.L			D.L L M.L			D.L L M.L			D.L L M.L			D.L L M.L		
Recession	48			47			46			45			44			43			42			41			31			32			33			34			35			36			37			38		
Enlargement	48			47			46			45			44			43			42			41			31			32			33			34			35			36			37			38		
PPD	48			47			46			45			44			43			42			41			31			32			33			34			35			36			37			38		
CAL	48			47			46			45			44			43			42			41			31			32			33			34			35			36			37			38		

A comparative study on the serum levels of nitric oxide before and after initial periodontal therapy in healthy and type 2 diabetes mellitus patients with chronic periodontitis

Patient record form (Post- treatment)

GROUP I / GROUP II/ GROUP III

Date:

Name: Age: Sex: M / F

CLINICAL PARAMETERS

I. Oral Hygiene Index – Simplified (Greene and Vermilion-1964)

Debris Index-S

16	**11**	**26**
46	**31**	**36**

Calculus Index-S

16	**11**	**26**
46	**31**	**36**

Score:

Interpretation:

Good		Fair		Poor	

II. Gingival Index (Loe and Silness-1963)

16			**12**			**24**		
44			**32**			**36**		

Score:

Interpretation:

Mild		Moderate		Severe	

BLOOD INVESTIGATION

NO level:

PERIODONTAL STATUS

MAXILLA

	D.B B M.B	D.B B M.B	D.B B M.B	D.B B M.B	D.B B M.B	D.B B M.B	D.B B M.B	D.B B M.B	M.B B D.B	M.B B D.B	M.B B D.B	M.B B D.B	M.B B D.B	M.B B D.B	M.B B D.B	M.B B D.B
	D.P P M.P	D.P P M.P	D.P P M.P	D.P P M.P	D.P P M.P	D.P P M.P	D.P P M.P	D.P P M.P	M.P P D.P	M.P P D.P	M.P P D.P	M.P P D.P	M.P P D.P	M.P P D.P	M.P P D.P	M.P P D.P
Recession	18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28
Enlargement	18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28
PPD	18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28
CAL	18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28

PERIODONTAL STATUS

MANDIBLE

	D.B B M.B			D.B B M.B			D.B B M.B			D.B B M.B			D.B B M.B			D.B B M.B			D.B B M.B			D.B B M.B			M.B B D.B			M.B B D.B			M.B B D.B			M.B B D.B			M.B B D.B			M.B B D.B			M.B B D.B		
	D.L L M.L			D.L L M.L			D.L L M.L			D.L L M.L			D.L L M.L			D.L L M.L			D.L L M.L			D.L L M.L			D.L L M.L			D.L L M.L			D.L L M.L			D.L L M.L			D.L L M.L			D.L L M.L			D.L L M.L		
Recession	48			47			46			45			44			43			42			41			31			32			33			34			35			36			37			38		
Enlargement	48			47			46			45			44			43			42			41			31			32			33			34			35			36			37			38		
PPD	48			47			46			45			44			43			42			41			31			32			33			34			35			36			37			38		
CAL	48			47			46			45			44			43			42			41			31			32			33			34			35			36			37			38		