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Gingival Gene Expression of Endocan as Biomarker of Periodontitis and Diabetes Mellitus – An Invivo Comparative Analysis

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KEYWORDS

Periodontitis, Type II Diabetes mellitus, Endocan mRNA, Periodontal inflamed surface area

ABSTRACT:

Periodontal disease is an inflammatory disease that is initiated by microbial infection, subsequently progresses via an aberrant host response, and primarily contributes to periodontal tissue destruction. Studies have revealed possible link between periodontitis and different systemic diseases. Periodontitis and diabetes mellitus are chronic inflammatory diseases with an established bi-directional relationship. It has been well documented that Endocan mRNA levels reported to modulate inflammatory dependent pathways between periodontal disease and systemic diseases.The aim of the present study is to evaluate and compare the level of Endocan mRNA expression in gingival tissues of periodontally healthy subjects and periodontitis patients with and without type II diabetes mellitus and also to correlate the inflammatory burden (PISA score) with Endocan mRNA expression.

INTRODUCTION

Periodontitis, a quotidian immunoinflammatory pathological entity is no more limited to the extent of a mere bacterial disease based on currently evolving knowledge regarding it's chronicity. At present, dysbiotic interference in dental plaque biofilm has been strongly stringed with the resultant upshots of periodontal disease , principally the periodontal attachment and bone loss. [1] Moreover, the disease consecution is very well attributed to the complex interactions between the host and

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periodontopathic pathogens owing to the release of cluster of cytokines, chemokines and lipid derivatives. [2]

Periodontal disease is acknowledged as a potent mainspring of several systemic comorbidities involving cardiovascular diseases, diabetes, pneumonia. [3,4,5] The two way interaction between diabetes mellitus and periodontal disease is credited to the cumulative inflammatory impact on each other. [6] There are evidences highlighting the intimately linked periodontal status and glycemic metabolics with a consistent nature. [7,8] Hyperglycemic state altered neutrophil function is regarded as an exceptional culprit for dysregulated inflammatory reponse which enhances level of proinflammatory cytokines TNF alpha, IL-6, and leptin. [9] Periodontitis mediated backtrack interaction also exist due to inflammation obliged glycemic state resulting in insulin resistance. [10]

Evolving periodontal research has revealed amazing mechanism linking periodontal pathologic entity and host immune response which has shed light on futuristic periodontitis diagnosis based on biomarkers. Endothelial-specific molecule-1 (Endocan) is a novel inflammatory biomarker which reflects dysfunction of endothelium. [11] It is secreted by endothelial cells when stimulated by pro-inflammatory factors, such as vascular endothelial growth factor (VEGF) and cytokines (interleukin-1, tumor necrosis factor-alpha) . [12] In recent times, there has been studies highlighting association of Endocan expression with periodontal and glycemic status. [13] From that perspective, this invivo study is the first of its kind to evaluate Endocan expression in gingival tissue of periodontally healthy subjects and periodontitis patients with and without type II Diabetes Mellitus. The study has attempted to explore association of Endocan expression on inflammatory burden in periodontitis and Diabetes Mellitus.

MATERIALS AND METHODS

Study Population and Clinical Examination

This was an analytical comparative exploration in 36 voluntary participants from the outpatient department of Periodontology and Oral Implantology within age group of 30-60 years including both sexes and were categorized into 3 groups based on periodontal health status and glycemic levels as Group I: Periodontally Healthy Subjects, Group II: Periodontitis patients without Type II Diabetes Mellitus and Group III: Periodontitis patients with controlled Type II Diabetes Mellitus. The participants were recruited based on World workshop 2017 Classification of Periodontal and Periimplant diseases and Conditions,European Federation of Periodontology (EFP) and the American Academy of Periodontology (AAP) classification and diagnosis of Diabetes(ADA); standards of medical care - 2020. [14,15] The protocol was summarized and approved by the institutional ethics committee (PMS/IEC/2020-21/19 and a written approval was obtained before commencement of the procedures from all the participants. Clinical examination included, Full mouth plaque score (FMPS) [16], Full mouth bleeding score (FMBS) [17], Periodontal Inflamed Surface Area (PISA) Score [18] ,and full mouth periodontal status evaluation on four sites per tooth including probing pocket depth (PPD), clinical attachment loss (CAL), recession/enlargement, mobility, bleeding on probing and furcation involvement and the readings recorded. Dentition status were recorded using WHO proforma.

Gingival Tissue Collection

Gingival tissue samples (2mm wide×2mm high×1-3mm thick) of healthy subjects were obtained through, crown lengthening procedure and gingival tissue samples of periodontitis patients with and without Type 2 Diabetes Mellitus and healthy subjects were taken during flap surgery. Samples were stabilized in 5 volumes of RNA later (Invitrogen) to minimize the need to immediately process the tissue samples or to freeze samples in liquid nitrogen for later processing .It was stored at -200 C until it was transported for evaluating Endocan expression in ice bag.

Isolation of Total RNA (trizol method)

Total RNA was isolated using the total RNA isolation kit according to the manufacture instruction (Invitrogen – Product code10296010). The tissue samples were washed with sterile PBS and 1ml of trizol reagent was added to the 100mg tissue sample and homogenized until it formed a fine paste. The contents were then transferred to a fresh sterile Eppendorf tube. 200 µl of chloroform was added and shaking was done vigorously for 15

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seconds and incubated for 2-3minutes at room temperature, followed by centrifugation at 14000 rpm for 15 minutes at 4 ◦C. The aqueous layer was collected and 500 µl of100% isopropanol was added. It was incubated for 10 minutes at room temperature and then centrifuged at 14000 rpm for 15 minutes at 4 ◦C. Supernatant was discarded and pellet thus obtained was washed with 200 µl of 75% of ethanol (Merck). It was then centrifuged at 14000 rpm for 5 minutes at 4 ◦C in a cooling centrifuge (RemiCM12). The RNA pellet was dried and suspended in TE buffer.

Gene Expression Analysis by RT-qPCR

Total RNA was extracted using TRI Reagent (Sigma). The purity and the concentration of total RNA was determined. Template complementary DNA was synthesized using the cDNA preparation kit (Thermoscientific, Product code AB1453A, Verso cDNA Synthesis kit). Real-Time qRT-PCR analysis was carried out using SYBR Green Master Mix (Applied Biosystem, Life technologies).

Statistical Analysis

Categorical and quantitative variables were expressed as frequency (percentage) and mean ± SD respectively. One way ANOVA test and Bonferroni test adjusted for multiple comparisons (post hoc test) was used to compare quantitative parameters among the groups. Chi-square test was used to find association between categorical variables. Spearman correlation was used to find out the relationship of Endocan and PISA score. For all statistical interpretations, $p<0.05$ was considered the threshold for statistical significance. Statistical analysis were performed by using a statistical software package SPSS, version 22.

RESULTS

Graph 1 shows comparison of age between Group I(Healthy), Group II (Periodontitis without type II Diabetes Mellitus) and Group III (Periodontitis with type II Diabetes Mellitus) using ANOVA. Mean age of the healthy group was 36.3 ± 4.7 years, Periodontitis without type 2 diabetes mellitus was 39 ± 6.2 years, Periodontitis with type 2 diabetes mellitus was 50.3 ± 6.6 years. ANOVA test was used, there was a statistically significant difference of mean age between three groups ($p <0.01$).

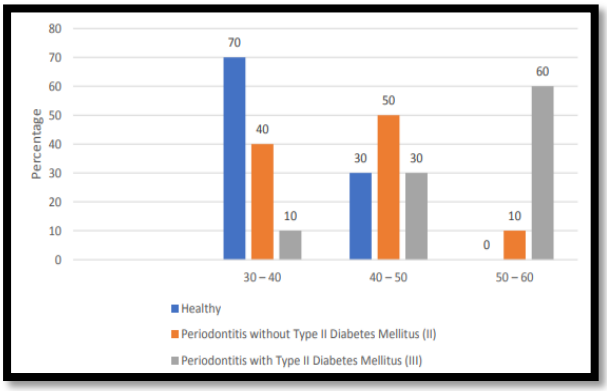

Figure 1:Comparison of age between Group I(Healthy), Group II (Periodontitis without type II Diabetes Mellitus) and Group III (Periodontitis with type II Diabetes Mellitus)

The difference among the mean and standard deviation of Endocan mRNA levels in 3 groups were calculated by ANOVA (Analysis of variance) (Table 1). Mean of Endocan mRNA in Group I was 1, Group II was 1.13±0.13, Group III was 1.63±0.40. The analysis of variance showed that difference in mean Endocan mRNA levels among all groups were statistically significant.

Table 1: Comparison of Expression fold change of Endocan mRNA in Group I(Healthy), Group II (Periodontitis without type II Diabetes Mellitus) and Group III (Periodontitis with type II Diabetes Mellitus) using ANOVA

Expression fold change of Endocan mRNA	N	Mean	Standard Deviation	p value
Group I	12	1	0.00	< 0.001
Group II	12	1.13	0.13	< 0.001

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Group III	12	1.63	0.40	< 0.001
p-value based on Analysis of Variance (ANOVA) * = Statistically Significant (p< 0.05); N- Sample size				

The difference among the mean and standard deviation of PISA score in 3 groups were calculated by ANOVA (Table 2). Mean and standard deviation of PISA score in Group I was 442.1657 +575.0092, Group II was 1126.5959 + 161.7863, Group III was 1549.1192 + 215.6988. The analysis of variance showed that difference in mean PISA score among all groups were statistically significant.

Table 2: Comparison of PISA Score in Group I(Healthy), Group II (Periodontitis without type II diabetes mellitus) and Group III (Periodontitis with type II diabetes mellitus) using ANOVA

PISA Score	N	Mean	Standard Deviation	p value
Group I	12	442.1657	575.0092	< 0.001
Group II	12	1126.5959	161.7863	< 0.001
Group III	12	1549.1192	215.6988	< 0.001
p-value based on ANOVA (Analysis of Variance) Test * = Statistically Significant (p < 0.05)				

Pair-wise comparison of expression fold change of Endocan mRNA was done by post-hoc analysis using Bonferroni Test adjusted for multiple comparisons (Table 3). There was no statistically significant difference between group I and II. There was statistically significant difference between group I and III and II And III.

Table 3: Inter-group Pair-wise Comparison of Expression Fold Change of Endocan mRNA in Group I(Healthy), Group II (Periodontitis without type II diabetes mellitus) and Group III (Periodontitis with type II diabetes mellitus)

Inter-group Pair-wise Comparison of Expression Fold Change of Endocan mRNA	Mean Difference	p value
Group I and Group II	0.13	0.708
Group I and Group III	0.63	< 0.001*
Group II and Group III	0.49	< 0.001*

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p-value based on post-hoc analysis using Bonferroni Test after adjusted for multiple comparisons * = Statistically Significant (p < 0.05)

Pair-wise comparison of PISA score was done by post-hoc analysis using Bonferroni Test adjusted for multiple comparisons (Table 4). There is statistically significant difference between group I, II and III were reported.

Table 4 : Inter-group Pair-wise Comparison of PISA score in Group I(Healthy), Group II (Periodontitis without type II Diabetes Mellitus) and Group III (Periodontitis with type II Diabetes Mellitus).

Inter-group Pair-wise Comparison of PISA score	Mean Difference	p value
Group I and Group II	684.4302	0.001*
Group I and Group III	-1106.9534	< 0.001*
Group II and Group III	422.5232	0.047*
p-value based on post-hoc analysis using Bonferroni Test after adjusted for multiple comparisons * = Statistically Significant (p < 0.05)		

Correlation analysis was performed using Spearman correlation coefficient test. In Group I, no correlation was obtained between Endocan and PISA Score (Figure 2) ; and a positive moderate correlation which was not statistically significant was obtained between Endocan and PISA score in group II (ρ=0.307 , p=0.987) (Figure 3); and positive strong correlation which was not statistically significant was obtained between Endocan and PISA score in group III (ρ=-0.485, p=0.199) (Figure 4).

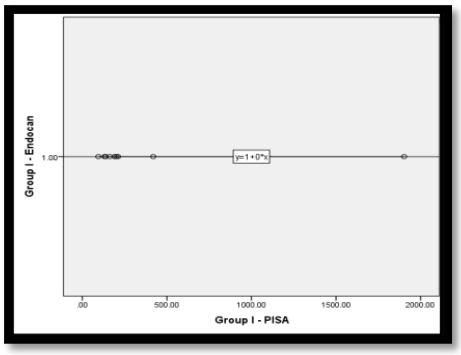

Figure 2: Scatter plot for correlation of Endocan with PISA score in Group I(Healthy)

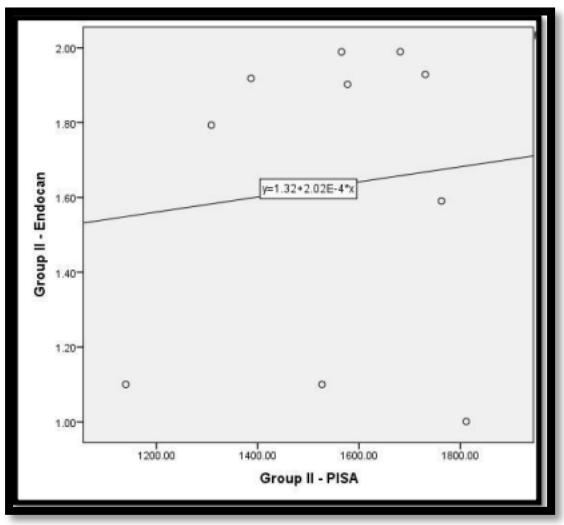

Figure 3:Scatter plot for correlation of Endocan with PISA score in Group II (Periodontitis without type II Diabetes Mellitus)

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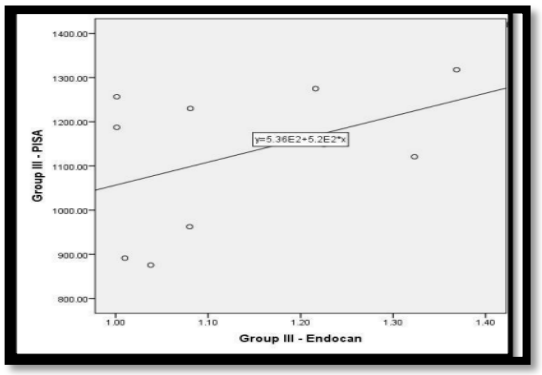

Figure 4:Scatter plot for correlation of Endocan with PISA score in Group III (Periodontitis with type II Diabetes Mellitus)

DISCUSSION

The present study demonstrated that Group III individuals have significantly higher levels of Endocan mRNA (1.63 + 0.40, $p<0.001$) as compared to Group II (1.13+ 0.13, $p<0.001$) and Group I (1.00, $p<0.001$) which was statistically significant. On correlation of Endocan mRNA with PISA score there was no correlation in group I and there exist a positive moderate correlation ($\rho=0.307$, $p=0.987$) in group II and positive strong correlation ($\rho=-0.485$, $p=0.199$) in group III. There is 2-fold rise in expression fold change of Endocan mRNA. Our study is in agreement with the other studies which have demonstrated independently an increased Endocan mRNA expression in chronic inflammatory diseases such as type II diabetes mellitus and periodontal disease. [19]

The dysfunctional endothelium is an early pathologic change occurring before detectable morphologic changes in the blood vessel wall is thought to be an independent predictor of the risk and prognosis of inflammatory diseases. [20] It has been reported that the expression of Endocan in endothelial cells can be upregulated in response to inflammatory triggers, such as lipopolysaccharide and cytokines leading to the activation of an inflammatory cascades and cause endothelial dysfunction. [21] Lee et al. demonstrated that Endocan is related to the upregulation of cellular adhesion molecules(CAMs). [22] These CAMs mediate the firm adhesion of leukocytes to the endothelium and subsequent transmigration to the inflammatory sites and contribute to the adhesion of activated lymphocytes and monocytes to ECs in acute and chronic inflammatory tissues . Endocan upregulated CAMs causes endothelial cytoskeletal rearrangement leading to cellular contraction, which also alters cellular permeability via interacting MAPK signaling pathways and NF-kB.. Thus, endocan activates components that provide the necessary substrate for recruitment, adhesion, and transmigration of leukocytes across an activated endothelium. Thus, it can influence recruitment and activation of immune cells to inflammatory sites leading to overexpression of inflammatory cytokines ,growth factors and reactive oxygen species leading to tissue destruction . In addition, accumulation of AGEs resulting from long-standing hyperglycemia may stimulate endocan secretion from endothelial cells and exaggerates further inflammation. [23] Increased expression of Endocan mRNA in group III could be attributed to cumulative inflammatory burden caused by Diabetes Mellitus and periodontal disease .

CONCLUSION

In line with the aforementioned findings, the results of our study showed there exist a direct relationship between greater inflammatory burden as measured by PISA score and Endocan mRNA expression in chronic inflammatory diseases. The limitation of the present study was the small sample size which was insufficient to make significant correlations with the inflammatory burden of periodontal disease and type II Diabetes Mellitus. Further research with elaborate study design with larger sample sizes are needed to validate the use of Endocan as an inflammatory biomarker to reinforce the concept of bidirectional relationship between periodontal disease and type II Diabetes Mellitus.

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