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GCF and serum levels of ANG2 (angiogenic peptide) in chronic periodontitis with and without type II diabetes mellitus- a randomized controlled clinical trial

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Introduction

Angiogenesis is defined as the formation of new blood vessels from the endothelium of pre-existing vessels.[1] It is a complex process that involves endothelial cell (EC) division, selective degradation of vascular basement membranes and of surrounding extracellular matrix, and EC migration.[2] This process has a significant role in the etiology, progression, and repair of inflamed tissues .[3, 4] Angiogenesis is an essential process in the development of chronic inflammatory diseases [5] and contributes to the degree of the inflammation as a result of the ability of new blood vessels to transport proinflammatory cells to the lesion and supply oxygen and nutrients to the inflamed tissues.[6]

Periodontitis is a chronic inflammatory disease of richly vascularised supporting tissues of the teeth. It has active as well as quiescent period during its course. The number of blood vessels increases during the progression of periodontal disease, which has been suggested to either enhance the severity of inflammation [7] or promote healing of the inflamed site.[8] Although, in both, inflammation and healing, angiogenesis is a prominent feature, and then also there is limited information about role of angiogenesis in periodontal lesions.[7, 9-16]

Diabetes is a metabolic disease that, due to disturbances in insulin production, leads to abnormal fat, sugar, and protein metabolism and resultant hyperglycemia that can ultimately induce diverse multiple systems pathologies.[17] Periodontitis is one of the most common oral problems observed in diabetes mellitus (DM), and many studies suggest the relationship between periodontitis and DM.[18-23] Infact periodontitis has been referred as the sixth complication of diabetes mellitus (DM).[24] Diabetic subjects with periodontal infection have a greater risk of worsening glycemic control over time compared to diabetic subjects without periodontitis.[25]

Diabetic patients have a 2-3-fold higher risk of developing severe periodontitis and progressive periodontal disease [22] and mechanisms such as vascular changes, neutrophil dysfunction, altered collagen synthesis, and genetic disposition may play a role in this increased risk. [23]

Because of the gingival microangiopathy, oxygen supply and diffusion removal of metabolic end products, leukocyte migration and diffusion factors are impaired in diabetic patients, consequently leading to tissue repair and regeneration inability.[26] Our understanding has been increased in recent years regarding the molecules associated in the pathogenesis of diabetic microvasculopathy.

Ang2, an angiogenic peptide, activates endothelial cells and increases vascular inflammation. It functions as an autocrine mediator of the endothelium and is stored predominantly in endothelial cells.[27] Ang2 is a ligand of the tyrosine kinase receptor, Tie-2, and antagonises the Ang1 induced Tie-2 receptor autophosphorylation responsible for the maintenance of endothelial cell quiescence.[28] This results in endothelial cells being sensitized to the effects of pro-inflammatory cytokines and Vascular Endothelial Growth Factor (VEGF), resulting in a loss of endothelial cell quiescence and an increase in vascular activation and inflammation. Growing evidence suggests an involvement of Ang-2 and its receptor Tie-2 in the pathophysiology of different vascular and inflammatory diseases such as arteriosclerosis [29], hypertension[30], idiopathic pulmonary arterial hypertension[31], chronic kidney disease[32], and rheumatoid arthritis.[33]

Plasma Ang2 (but not Ang1), like VEGF levels, are selectively elevated in patients with diabetes and are associated with indexes of endothelial damage/dysfunction.[34, 35] Ang2 plays a critical role in diabetic retinopathy as it is found to be upregulated in diabetic retina in rats[36] and humans.[37] In an immunohistochemistry study, Yuan K et al. [13] reported that positive detection rate of Ang2 were significantly higher in periodontitis and pyogenic granuloma subjects than healthy subjects.

Some inflammatory biomarkers such as cytokines, chemokines and bone-related factors have also been found to play a very important role in the pathogenesis of both chronic periodontitis (CP) and type 2 DM.[38, 39] Studies of the links between periodontal disease and cardiovascular disease have indicated that C-reactive protein (CRP) is one the inflammatory biomarkers involved.[40] CRP is an acute-phase reactant synthesized by the liver in response to the inflammatory cytokines IL-6, IL-1, and tumor necrosis factor-alpha (TNF-α). The level of circulating CRP is a marker of systemic inflammation, and is associated with periodontal disease.[41] Recent studies support a significant association between Ang2 and inflammation via CRP.[42-44]

With the increasing number of diabetics in an aging population, determination of Ang2 levels in periodontitis patients with DM and its association with inflammatory biomarker CRP may be beneficial in establishing appropriate health/oral care.

Till now, the levels of Ang2 and hs-CRP in gingival crevicular fluid (GCF) and serum in subjects with type 2 DM and chronic periodontitis (CP) and CP without type 2 DM have not been explored. Thus, considering the aforementioned findings the present study was designed to evaluate and correlate the levels of Ang2 and hs-CRP, in GCF and serum with periodontal clinical parameters in CP subjects with and without type2 DM.

Materials & Methods

This cross-sectional study of 4 months duration was conducted from July 2017 until October 2017 and involved 44 (22 males, 22 females) age- and gender-balanced subjects divided into three groups. The subjects were selected from among patients referred to the Department of Dentistry, ESIC PGIMSR, ESIC Medical College and Hospital & ODC (EZ), Joka, Kolkata, India. Ethical clearance was approved by Institutional Ethics Committee and Review Board, ESIC Medical College, Joka, Kolkata All the subjects, who agreed to participate, voluntarily signed a written informed consent.

Inclusion criteria

The inclusion criteria for the study subjects were an age of 25-45 years, presence of at least 20 natural teeth with a diagnosis of CP based on clinical parameters such as probing depth (PD), clinical attachment level (CAL)[45] and gingival index (GI)[46], body mass index (BMI) of 18.5-22.9 kg/m2 and a waist circumference of < 90 cm (men) or < 80 cm (women) (WHO)[47], diabetic patients had well-controlled type 2 diabetes, classified according to the 2011 criteria of the American Diabetic Association (ADA) and the level of glycated haemoglobin.[48]

Exclusion criteria

The exclusion criteria for the study subjects were consumption of tobacco in any form, consumption of alcohol, periodontal therapy within the 6 months preceding the study , presence of any other systemic disease capable of affecting the course of periodontal disease, or those who had any course of medication affecting periodontal status,.

Grouping criteria

Group 1 (healthy) comprised 10 subjects with clinically healthy periodontium, GI = 0 (absence of clinical inflammation), PD ≤ 3 mm, and CAL = 0, with no evidence of bone loss on radiographs. Group 2 (CP patients without type 2 DM) comprised 17 subjects who had signs of clinical inflammation, GI > 1, more than 30% of sites showing PD ≥ 5 mm, and CAL ≥ 3 mm, and HbA1c ≤ 7% with radiographic evidence of bone loss. Group 3 (patients with type 2 DM and CP) comprised 17 subjects who had signs of clinical inflammation, GI > 1, more than 30% of sites showing PD ≥ 5 mm, and CAL ≥ 3 mm, with radiographic evidence of bone loss. Only subjects with well controlled (HbA1c ≤ 7%) type 2 DM were selected based on the ADA criteria for diagnosis of diabetes.[48]

Clinical evaluation of subjects

Group allocations and sample site selection were performed by the chief coordinator (ARP). An examiner (SPS) performed the clinical evaluation and determined the clinical parameters including PD, CAL, and GI using a University of North Carolina-15 (UNC-15) periodontal probe (Hu-friedy, Chicago, IL, USA). The same examiner (SPS) also performed the radiographic evaluations and collected the GCF samples.

Site selection and GCF collection

GCF samples were collected from two selected test sites. In Groups 2 and 3, the sites showing the greatest CAL and signs of inflammation, along with radiographic confirmation of bone loss assessed by intraoral periapical radiographs taken by the paralleling technique, were selected for sampling. One of the two sites selected per subject was used for Ang2 and the other for hs-CRP

analysis. In the healthy group, to standardize site selection and obtain an adequate fluid volume, sampling was predetermined to be from the mesio-buccal region of the maxillary right first molar, in the absence of which the left first molar was sampled. First, to avoid contamination of the paper strips, the selected site was cleaned, isolated and air-dried using sterile cotton rolls, and the supragingival plaque was removed gently using a Gracey curette (Universal Gracey curette #4R/4L, Hu-friedy, Chicago, IL, USA). The paper strips (Periopaper, Ora Flow Inc., Amityville, NY, USA) were placed gently at the entrance of the gingival sulcus/crevice until light resistance was felt [49], taking care to avoid mechanical injury, and left in place for 60 seconds. The absorbed GCF volume of each strip was determined by electronic impedance (Periotron 8000, ProFlow Inc.). Samples that were suspected to be contaminated with blood and saliva were excluded. After collection of the gingival fluid, the two periopaper strips per site that had been used to absorb GCF from each subject were pooled and then immediately transferred to microcentrifuge tubes (premarked with the biomarker name) containing 400 μL of phosphate-buffered saline and stored frozen at -70°C for subsequent analysis. Periodontal treatment (scaling and root planing) was performed for CP subjects at the same appointment after collection of GCF by the operator (SSM).

Blood collection

Two milliliters of blood was collected using a 20-gauge needle with a 2-mL syringe from the antecubital fossa by venipuncture and immediately transferred to the laboratory. The blood sample was allowed to clot at room temperature, and after 1 h serum was separated from blood by centrifuging at 3,000 × g for 5 min. The serum was immediately transferred to a plastic vial and stored at -70°C until the time of assay.

Ang2 analysis

The samples were assayed for Ang2 using an enzymelinked immunosorbent assay (ELISA) kit in accordance with the manufacturer's instructions. The GCF sample tubes were first homogenized for 30 seconds and centrifuged for 5 minutes at 1,500 × g to yield an eluate. The eluate was then used as a sample for ELISA estimation of Ang2. Each sample was assayed using a commercially available ELISA kit (human Ang2, RayBiotech, Inc, USA) in accordance with the manufacturer's instructions. Color development was monitored using a microplate reader until an optimum optical density was reached, then a stop solution was added and the optical density was read at 450 nm. The total Ang2 was determined in picograms (pg), and the calculation of the concentration in each sample was performed by dividing the amount of Ang2 by the volume of the sample (pg/mL).

hs-CRP analysis

The samples for CRP were measured immunoturbimetrically. The microcentrifuge tubes containing the periopaper strips and plastic vials containing serum were transferred to the laboratory for immunoturbedimetric analysis. Serum was used undiluted. The measurement range for CRP was 0-220 mg/L.

Statistical Analysis:

Analysis of variance (ANOVA) was carried out for a comparison of GCF and serum Ang2 and hs-CRP levels between the groups. Power calculations were performed before the study was initiated and the sample size was selected based on the previous study.[9] Based on the power of the study and the confidence interval of 95% ($p<0.05$) three groups were made.

Using Pearson's correlation coefficient, the relationships between Ang2 and hs-CRP concentrations and the clinical parameters were analyzed using a software program

(SPSS Inc. version 10.5, Chicago, IL, USA). Differences at $p < 0.05$ were considered statistically significant. The intra-group correlation of serum and GCF concentrations of Ang2 and hs-CRP was also performed using Pearson's correlation coffecient. The mean intra-examiner standard deviation of differences in repeated PD measurements and CAL measurements obtained using single passes of measurements with a UNC- 15 probe (correlation coefficients between duplicate measurements; $r = 0.95$).

Results

Table 1 shows the data (mean ± SD) for the study population. The mean Ang2 and hs-CRP concentrations in both serum and GCF were highest in Group 3, followed by Group 2, and were lowest in Group 1. To determine the equality of means between the three groups, ANOVA was carried out (Table 2). Significant differences in the serum and GCF levels of Ang2 and hs-CRP were found between the three groups. The Pearson correlation coefficient test was applied to evaluate the correlation and statistically significant correlation exist between the serum level of Ang2 and the serum level of hs-CRP, and also the GCF values between the both. Table 3 shows the correlation coefficients and p values. The serum and GCF levels of Ang2 and GCF levels of hs-CRP were found to be significantly correlated ($P < 0.05$) with all the clinical parameters in Group 2 and Group 3. The serum concentration of hs-CRP was significantly correlated with GI in Group 2 and PD and CAL in Group 2 and Group 3. The correlations between the GCF and serum levels of the two biomarkers and clinical parameters are presented in Table 4.

Discussion

Periodontal diseases are a complex group of diseases characterized by inflammation and the subsequent destruction of the tooth-supporting tissues. Angiogenesis is a prominent feature of inflammation and healing and although aberrant angiogenesis is associated with lesion formation in chronic periodontitis but its role in promoting the progression or healing of periodontal lesions and the mediators that contribute to angiogenesis or therapeutic agents that control the action of the mediators have not been well described.[9, 12]

The role of DM in various periodontal diseases has been extensively investigated, and an impact of periodontal inflammation on diabetic balance has also been indicated in a study by Katz J et al.[18] It is reported that both severity and progression of periodontal disease has been aggravated by DM [22], and especially poor metabolic control of DM has often been associated with the severity of periodontitis.[26]

The structural changes characterizing diabetic microangiopathy, which may be referred to as abnormal growth and impaired regeneration, strongly suggest a role for a number of aberrantly expressed growth factors, possibly acting in combination, in the development of these complications.[54]

Ang2, an angiogenic peptide, activates endothelial cells and increases vascular inflammation. It functions as an autocrine mediator of the endothelium and is stored predominantly in endothelial cells.[27] It has been reported that Ang2 regulates vascular remodelling and endothelial responsiveness to pro-inflammatory cytokines and has a crucial role in the induction of inflammation .[50] In addition, recent in vitro and in vivo studies have demonstrated that Ang-2 acts as a chemoattractant for pro-angiogenic Tie2- expressing monocyte/macrophages.[51, 52]

Ang2 plays a critical role in diabetic retinopathy[36, 37] and its plasma levels selectively elevated in patients with diabetes and are associated with indexes of endothelial damage/dysfunction.[34, 35]

We decided to investigate the role of Ang2 in the pathogenesis of CP with and without DM by comparing

and correlating its levels with a marker of inflammation (in this case, hs-CRP) that has been clearly proven to play a role in inflammation in various systemic diseases. The level of circulating CRP is a marker of systemic inflammation, and is associated with periodontal disease.[41] Recent studies support a significant association between Ang2 and inflammation via CRP.[42-44]

It was anticipated that such a comparison and correlation would further validate the role of the new molecule (in this case, Ang2) being tested.

This cross-sectional study attempted to evaluate the GCF and serum levels of Ang2 and hs-CRP in CP patients with and without type 2 DM. The results clearly indicated increasing GCF and serum levels of the Ang2 and hs-CRP in patients with CP and those with type 2 DM with CP, relative to healthy controls.

The observed increase in hs-CRP, an acute-phase reactant protein and one of the most important markers of inflammation from Group 1 to Group 2, was in accord with a previous study by Pradeep et al.[53] in which CRP levels in GCF and serum were measured using ELISA, and also with a study by Noack et al.[55]

The increase in values from health to CP and further in type 2 DM with CP is similar to a previous study but the levels are higher than those found in that study.[56] The higher GCF and serum values in the CP group could have been due to higher mean values of periodontal parameters recorded in this series of subjects. The higher mean serum values in patients with type 2 DM and CP could have been due to the fact that the corresponding group in that study had coronary artery disease with CP and were receiving statin therapy for the same and statins lower CRP.[55-56] The highest serum levels of hs-CRP in CP with type 2 DM could be attributed to the presence of type 2 DM in which hs-CRP levels were found to be elevated in a previous study[41]

The increase in the GCF levels of Ang2 from Group 1 to Group 2, corresponds to a previous study by Yuan K et al. in which the GCF levels of Ang2 were found to be highly elevated in patients with CP as compared to those with chronic gingivitis and healthy individuals.[13] The GCF concentration was the lowest in periodontaly healthy individuals, followed by those with CP, and was highest in patients with CP and type 2 DM. This could be related to the fact that Ang2 is clearly implicated in periodontal disease severity.[13] The higher production of Ang2 in the serum of CP patients as compared to healthy subjects could have resulted from spillover of the increased GCF Ang2 levels from diseased periodontal tissues, leading to a concomitant increase in serum Ang2. The further increase in the levels of Ang2 in serum and GCF of CP patients with type 2 DM is in accordance with the studies by Lim HS et al. [34, 35] and Cai J et al.[37] The present study showed that the GCF and serum levels of Ang2 and hs-CRP were significantly correlated with CP ($p < 0.05$) and this data supports the fact that significant association exist between Ang2 and inflammation via CRP.[42-44]

To our knowledge this is the first study to have evaluated and correlated Ang2 and hs-CRPin CP patients with and without type 2 DM. One limitation of this crosssectional study was the small sample size evaluated. Further long-term longitudinal studies with larger sample sizes should be undertaken to validate these results. Based on the present findings, it can be proposed that Ang2 and hs-CRP play roles in the pathogenesis of periodontal disease. The highest levels of these two mediators in CP patients with type 2 DM may indicate an active inflammatory process, both locally in periodontal tissues, and also systemically.[57]

Conclusion

The present study showed that the Ang2 and hs-CRP concentration in GCF increases proportionally with the

progression of periodontal disease, i.e., gingival inflammation and CAL and in DM.

Thus, within the limits of the present study, the role of Ang2 as a biochemical marker of periodontal disease and its progression could be proposed. However, long-term prospective studies involving a larger sample size need to be carried out to confirm the above findings. In addition, chair-side diagnostic tests and Ang2-specific therapeutic strategies could be developed to arrest the periodontitis-associated alveolar bone destruction.

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Legend Tables

Table 1: Descriptive data for study population (Mean ± SD)

Study Group	Group 1 (n=10)	Group 2 (n=17)	Group 3 (n=17)
Age (in years)	39.40±3.86	39.59±4.53	41.24±4.15
Sex (M/F)	4/6	10/7	8/9
GI	0	2.18±0.38	2.32±0.39
PD(mm)	1.80±0.63	6.12±0.78	7.12±1.05
CAL(mm)	0	6.82±1.07	7.88±1.27
Serum Ang2(pg/ml)	1650.00±218.02	3354.12±286.82	4622.35±342.90
GCF Ang2(pg/ml)	600.00±164.18	861.76±74.01	965.88±121.86
Serum hs-CRP(mg/l)	2.32±0.58	4.15±0.52	5.56±1.03
GCF hs-CRP(mg/l)	0.56±0.25	0.78±0.18	0.86±0.29

Table 2: Results of ANOVA comparing the mean serum and GCF concentrations of Ang2 and hs-CRP among the three groups

Study Groups	Ang2				hs-CRP			
	Serum		GCF		Serum		GCF	
	F value	p-value	F value	p-value	F value	p-value	F value	p-value
Group1 Group2 Group3	316.21	<0.0001*	30.80	<0.0001*	55.87	<0.0001*	4.97	0.012*

*Significant at $p < 0.05$

Table 3: Correlations of serum and GCF concentrations of Ang2 and hs-CRP in each group using Spearman's rank correlation coefficient test

Study Groups	Serum		GCF	
	Correlation coefficient	p-value	Correlation coefficient	p-value
Group1	0.989	<0.0001*	0.795	0.006*
Group2	0.911	<0.0001*	0.659	0.004*
Group3	0.697	0.002*	0.944	0.004*

*Significant at $p < 0.05$

Table 4: Relationship of Ang2 and hs-CRP levels to clinical parameters

	Parameters	Group1	Group2	Group3
Ang2				
Serum	GI	-	<0.0001*	0.008*
	PD	0.006*	<0.0001*	<0.0001*
	CAL	-	0.001*	0.001*
GCF	GI	-	0.006*	0.013*
	PD	0.029*	0.044*	<0.0001*
	CAL	-	<0.0001*	0.002*
hs-CRP				
Serum	GI	-	0.001*	0.068
	PD	0.009*	<0.0001*	0.012*
	CAL	-	0.011*	0.032*
GCF	GI	-	0.002*	0.002*
	PD	0.007*	0.001*	<0.0001*
	CAL	-	<0.0001*	<0.0001*

*Significant at $p < 0.05$