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ORIGINAL ARTICLE

CRP and IL-6 levels of Gingival Crevicular Fluid in stroke survivors: A pilot study

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ABSTRACT

Introduction: The aim of this study was to evaluate the levels of C-reactive protein (CRP) and interleukin-6 (IL-6) in the gingival crevicular fluid (GCF) among stroke survivors in relation to the oral hygiene status and stroke characteristics. **Methods:** A multi-centre cross-sectional study was conducted among hospitalised stroke survivors. Socio-demographic data were collected. Oral clinical assessment (dental plaque scores) and functional dependency levels scores were carried out. Gingival Crevicular Fluids were collected, using absorbent papers and analysed using ELISA kit. Descriptive statistic and correlation analyses were performed using proportion and Spearmen correlation coefficient test. **Results:** A total of 53 patients were recruited from five public hospitals. There was a significant correlation between CRP and IL-6 levels of GCF (P=0.021, r=0.21). A significant correlation was also observed between CRP levels in GCF with dental plaque scores and functional dependency levels. There was no statistically significant correlation found between IL-6 levels in GCF with dental plaque scores and functional dependency levels. Dental plaque scores were associated with high levels of CRP in GCF (P=0.014), and swallowing problem was associated with high levels of IL-6 in GCF (P=0.002). **Conclusions:** IL-6 levels in GCF were correlated with the levels of CRP in GCF. High CRP and IL-6 levels in GCF were significantly associated with dental plaque scores and swallowing condition (presence of dysphagia), respectively. Thus, this pilot study suggests that CRP level in the oral cavity respond to the oral health conditions and may not be a predictor factor of stroke outcomes. Further studies are warranted to compare the level of inflammatory biomarkers from the oral cavity and serum in relation to the stroke conditions and outcomes.

KEYWORDS: C-reactive protein, interleukin-6, oral health, stroke

INTRODUCTION

Inflammatory biomarkers may reflect the inflammatory status of a disease and their profiles vary across diseases. A review identified that the production of inflammatory biomarkers which included the changes of acute-phase protein responses is different, based on conditions such as infection, trauma, surgery, tissue infarction, inflammation, exercise and psychological stress [1]. Among the cytokines, interleukin-6 (IL-6) is the most common and one of the main stimulators for the production of acute-phase proteins [2]. The increased level of inflammatory biomarkers has been associated with the risk of acute and chronic diseases. Systematic reviews on inflammatory cytokines including IL-6 and C-reactive protein (CRP) have been shown to associate with spontaneous preterm birth and chronic obstructive pulmonary disease (COPD) [3].

High level of CRP is associated with the risk of coronary heart disease, ischaemic stroke and vascular mortality, and death from several cancers and lung diseases. Among the reported major cardiovascular events include myocardial infarction (MI), fatal and non-fatal stroke, coronary revascularization, and all-cause mortality. CRP has also been reported as a predictor of mortality in critically ill patients [4] and ischemic stroke [5], as well as a bacterial infection marker among critically immunosuppressed patients [6].

High level of IL-6 is associated with the magnitude of surgical stress, severity of sepsis and illness, as well as a prognosis biomarker for several diseases such as colorectal cancer [7] and ischaemic stroke [8]. IL-6 level is also associated with a high risk of cardiovascular diseases [9], cerebrovascular disease [10], diabetes mellitus [11] and rheumatoid disease [12]. Increased level of IL-6 is found to associate with larger infarct volume, low prognosis of neurological deficits and mortality [13]. However, the evidence is insufficient due to limited studies.

With regards to stroke, there is limited number of studies pertaining to inflammatory biomarkers, specifically to IL-6 and CRP. Inflammation is the main features of stroke pathology. Pro-inflammatory biomarkers have been associated with the risk and prognosis of stroke [14], and prediction of functional outcome after stroke [15]. Hence, they have the potential in future, to facilitate stroke therapy [16]. The inflammatory biomarkers such as CRP, TNF-α and IL-6 are higher compared to those without stroke [16]. A review reported that the increased levels of specific pro-inflammatory biomarkers, in particular the CRP and IL-6 in response to wider inflammatory conditions, may increase the risk of stroke [14]. Thus, the presence of these inflammatory biomarkers in chronic inflammation may be indicators for risk of stroke. Further studies need to be carried out to reduce the inflammation and therefore decrease the risk of stroke. Another review of 24 studies also reported that IL-6 levels are associated with poor outcome after stroke [15]. Furthermore, CRP, IL-6, fibrinogen and white blood cells were found to have strong association with poor outcome following stroke [17].

The increase in IL-6 levels in serum was significantly correlated with the level of CRP, and the magnitude of the response was suggested to relate to the infarct volume and degree of disability [18]. A high baseline CRP concentration was a predictor to risk of future myocardial infarction and stroke, compared to those who did not develop the diseases [19]. Studies also showed that, reducing the level of CRP using anti-inflammatory medicine significantly decreased the risk of first stroke [20] and myocardial infarction [19]. Inflammatory biomarkers have been suggested to be useful and beneficial diagnostic tools in predicting stroke occurrence, as well as to improve clinical management and outcomes following stroke [21].

IL-6 and CRP are also associated with systemic and local inflammatory conditions such as with increased risk of cardiovascular diseases, and those affected by periodontal diseases. CRP is also associated with other oral health conditions such as, denture-related mucosal infection and an increase in oral microbial counts [22]. Inflammatory biomarkers can also be detected through saliva and gingival crevicular fluid (GCF) [23]. The presence of dental plaque around the gingival area may lead to the release of inflammatory biomarkers at the gingival crevicular area, as a result of interactions between the pathogens in the dental plaque with the host immune response [24]. Salivary and gingival crevicular fluid (GCF) interleukins that are present during the inflammation stage can be diagnostic markers of the degree of inflammation [25]. Hence, whether or not they are related to the systemic conditions is still questionable.

The evidence of detection of IL-6 and CRP from GCF is still lacking in stroke patients, either with relations to periodontal diseases or chronic systematic diseases. Moreover, a systematic review revealed that limited number of studies have explored the association of IL-6 and CRP with oral health conditions in stroke survivors [26]. Thus, this pilot study aimed to evaluate the levels of CRP and IL-6 in the GCF among stroke survivors in relation to the oral health status and stroke characteristics.

METHODS

Population Sampling

A total of 86 hospitalised stroke patients were approached from five public hospitals in Klang Valley, Malaysia. The study was registered with the National Medical Research Registry of Malaysia, [NMRR-13-1664-17247(IIR)] and was conducted from June 2015 to August 2016. Ethical approval was obtained from the Medical Research and Ethics Committee (MREC) of National Institutes of Health Malaysia in accordance with the World Medical Association Declaration of Helsinki. Permissions to conduct the study were obtained from the directors and heads of rehabilitation department of the hospitals. The reporting of the study followed the STROBE (Strengthening the Reporting of

Observational studies in Epidemiology) statement for dissemination of the findings.

The inclusion criteria were: the stroke patients who were under the management of rehabilitation physicians team, functional dependency level >70, medically stable, and were not edentulous (on average minimum of 20 teeth present). Patients who were on antibiotics or antimicrobial agents were not recruited into the study. Patients who agreed to participate in the study were enrolled into this trial. Information sheets about the study were given to the patients or caregivers prior to commencement of the study. Written informed consent was obtained from all participants who agreed to participate in the study.

Collection of Samples and Data

Gingival crevicular fluid (GCF) samples were obtained using 'PerioPaper' collection strips (Oroflow Inc., Smithtown, New York, USA). It is the absorbent type of paper strips [27] and is one of the commonest methods because of its simpler procedures [28]. The GCF was collected using "Brill technique", which is also known as intra-sulcular method. The PerioPaper strip was carefully inserted into the gingival sulcus until mild resistance was achieved, where it was left in situ for 30 seconds [29]. In this study, the sample sites were either the mesiobuccal or distobuccal sites of teeth. The sites were identified, isolated and dried using sterile cotton pellet/rolls [30]. The site should appear healthy with no pocket depth or sign of inflammation. PerioPaper strips contaminated with blood and saliva were discarded, while the uncontaminated ones were kept in the sterile Eppendorf tubes. The Eppendorf tubes were labelled and kept in a sealed plastic bag. The samples were placed in an icebox before it was transported back to the same lab. The Eppendorf tubes were kept in a freezer at -80ºC until required for analysis [31].

Biomarkers IL-6 and CRP were identified using Enzyme-linked Immunosorbent Assay (ELISA) (R&D systems™, USA). The frozen samples were thawed and diluted with phosphate buffered saline before preparing for the ELISA test. Manufactured instruction for ELISA test was followed including Quantikine® ELISA for human CRP and Quantikine® HS ELISA High Sensitivity for IL-6.

Dental plaque score was assessed following the methods and criteria of the Silness and Löe plaque index [32]. The plaque index (PI) records level of dental plaque at six sites per tooth. The dental plaque score was assessed by one calibrated investigator (NAM). The Kappa values of the dental plaque scores obtained was 0.75. Presence and type of dental prosthesis were recorded. Functional dependency level was assessed using the Modified Barthel Index (MBI) score [33].

Data Analysis

The collected data were analysed using the IBM SPSS Statistics 23 software. Descriptive statistic was performed for each categorical variable. Analyses of logistic regression and univariate regression were performed to determine factors associated with the level of CRP and IL-6 in GCF; socio-demographic background, oral hygiene (plaque index scores), functional and cognitive status, and stroke characteristic (such as type of stroke). The correlation between CRP and IL-6, and that with dental plaque percentage and functional dependency level were performed using Spearman correlation analyses. The level of significance for all tests was set at 0.05.

RESULTS

A total of 53 patients were recruited for the study. The profile of participants is presented in Table 1. Approximately half of the patients were age between 40 to 59 years old. Majority of them (62.3%) were male and two third were of Malay ethnicity. High percentage (98.1%) reported to brush their teeth at least once daily. Approximately, 86.8% was their first-ever stroke and of ischemic type. More than one third (37.7%) had swallowing problem (dysphagia). Almost two third (71.7%) were in the severe or total functional dependency level (measured by MBI scores, with MBI scores less than 50).

Table 1 Participant's profiles

N=53	n	Percentage (%)
Socio-demographic		
Gender		
Male	33	62.3%
Female	20	37.7%
Age		
20 – 39 years	7	13.2%
40 – 59 years	27	50.9%
60 ++ years	19	35.8%
Ethnic group		
Malay	39	73.6%

Chinese	6	11.3%
Indian	8	15.1%
Oral health		
Visit dentist		
Once / more a year	3	5.7%
When have problem	50	94.3%
Brushing frequency		
Twice a day	52	98.1%
Once a day	1	1.9%
Denture		
No denture	43	81.1%
Partial	10	18.9%
PI Score		
Mean (SD)	1.78	(SD) 0.481
DMFT		
Mean (SD)	13.51	(SD) 8.201
Stroke risk factor		
Alcohol intake		
No	46	86.8%
Yes	7	13.2%
Smoking		
No	38	71.7%
Yes	15	28.3%
Stroke features		
Stroke incidence		
Recurrent	7	13.2%
First stroke	46	86.8%
Stroke type		
Haemorrhagic	7	13.2%
Ischemic	46	86.8%
Hemiparesis side		
Left side	35	66.0%
Right side	18	34.0%
Dominant hand		
Left hand	7	13.2%
Right hand	46	86.8%
Swallowing		
Safe	33	62.3%
Unsafe	20	37.7%
Comorbidities		
Hypertension	45	84.9%
Diabetes mellitus	27	50.9%
Dyslipidaemia	5	9.4%
Asthma	2	3.8%
Heart disease	1	1.9%
Other / Unknown	1	1.9%
Functional & cognitive status		
MBI_0mth		
Total dependence	15	28.3%
Severe dependence	23	43.4%
Moderate dependence	15	28.3%
MMSE_0mth		
Severe cognitive impairment	19	35.8%
Mild cognitive impairment	12	22.6%
No cognitive impairment	22	41.5%

Immunological Findings

The mean levels of IL-6 in GCF were 0.95 pg/mL (σ 1.63) and the levels CRP in GCF were 2.62mg/mL (σ 6.15 mg/mL). The median of GCF IL-6 levels were 0.17 (IQR: 0.00, 0.95), and GCF CRP levels were 0.61 (0.25, 1.55). Approximately half of the participants had IL-6 level ≥ 0.17 pg/mL, and CRP level ≥ 0.61 pg/mL.

Figure 1: IL-6 and CRP levels in GCF of participants.

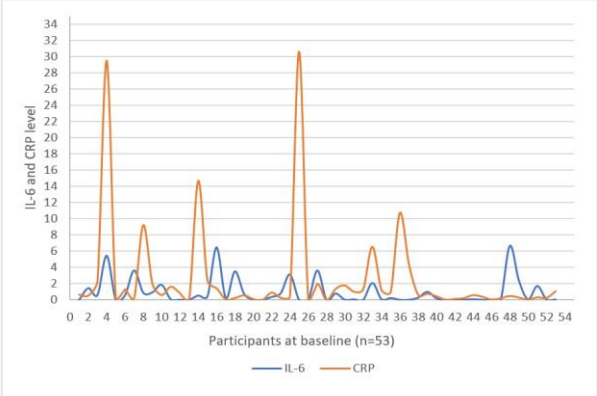

Figure 1 illustrates the IL-6 and CRP levels in GCF of each participant. There was a significant correlation between IL-6 and CRP levels in GCF among the patients, r=0.21 (P=0.021).

Figure 2: CRP levels in GCF with dental plaque percentage and MBI scores

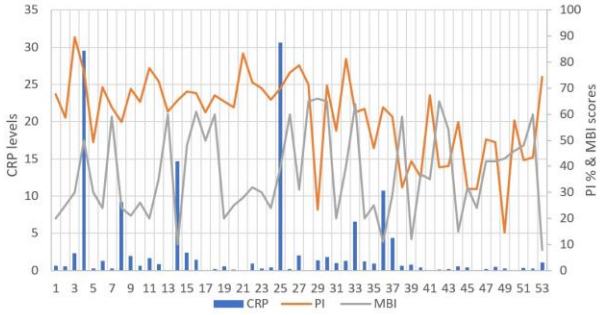

Figure 2 illustrates the levels of CRP in GCF (n=53), dental plaque percentage and MBI scores. A significant correlation was observed between CRP levels in GCF and dental plaque percentage (r=0.28, P<0.05), as well as MBI scores (r=-0.28, P<0.05).

Figure 3: IL-6 levels in GCF with dental plaque percentage and MBI scores

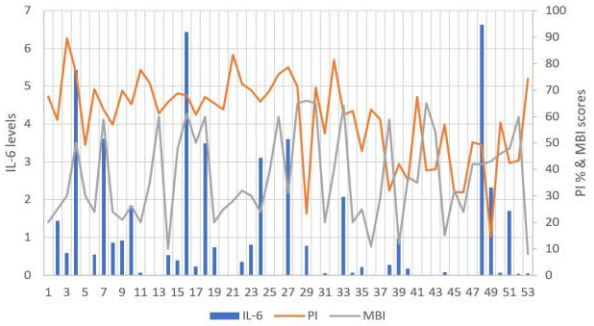

Figure 3 illustrates the levels of IL-6 in GCF (n=53), dental plaque percentage and MBI scores. No significant correlation was observed between IL-6 levels in GCF and dental plaque percentage (P>0.05) and MBI scores (P>0.05).

Regression Analyses Findings

Table 2 presents factors associated with CRP and IL-6 in GCF. Unadjusted and adjusted logistic regression analyses indicated that age and dental plaque were significantly associated with high GCF CRP levels (≥ 0.61 pg/mL). Both adjusted and unadjusted logistic regression analyses indicated that dental plaque scores

were significantly associated with high GCF CRP levels (≥ 0.61 pg/mL) [adjusted (OR 6.35, 95%CI 1.45, 27.81, P<0.05), unadjusted (OR 4.34, 95%CI 1.15, 16.33, P<0.05)].

Table 2 Factors associated with the CRP and IL-6 levels in GCF: finding from the logistic regression analyses

	Unadjusted			Adjusted#		
N=53	**OR**	**95% CI**	**P-value**	**OR**	**95% CI**	**P-value**
CRP						
Age	1.06	1.01, 1.12	0.015 *			
PI at 0mth	4.34	1.15, 16.33	0.030 *	6.35	1.45, 27.81	0.014 *
Swallowing	2.52	0.80, 7.95	0.115	3.86	1.00, 15.04	0.051
IL-6						
Denture	0.34	0.08, 1.49	0.152	0.04	0.01, 0.40	0.006 **
Comorbidities	2.33	0.77, 7.00	0.133	0.07	0.01, 0.46	0.005 **
Hemiparesis	3.90	1.14, 13.39	0.031 *			
Swallowing	3.59	1.10, 11.73	0.034 *	19.47	2.88, 131.5	0.002 **

*P-value < 0.05, **P-value < 0.01

Unadjusted and adjusted logistic regression analyses indicated that having denture, number of comorbidities, hemiparesis and swallowing were significantly associated with IL-6 levels (≥ 0.71 pg/mL). In adjusted analyses, having a denture was associated with less likelihood to have high level of IL-6 (≥ 0.71 pg/mL), compared with not having denture (OR 0.04, 95%CI 0.01, 0.40, P<0.01). Having multiple comorbidities was associated with less likelihood to have high level of IL-6 (≥ 0.71 pg/mL), compared with not having multiple comorbidities (OR 0.07, 95%CI 0.01, 0.46, P<0.01). Swallowing problems was a key factor associated with high likelihood to have high level of IL-6 (≥ 0.71 pg/mL), compared with not having swallowing problems (OR 19.47, 95%CI 2.88, 131.0, P<0.01).

DISCUSSION

The concentrations of biomarkers varied widely among the samples, and this might be related to variation in participant's conditions during the study or due to the diverse effect of stroke [34]. This is consistent with findings of previous study in the variation of the biomarkers levels among the participants [35]. This study revealed significant correlation between IL-6 and CRP. This is consistent with the role of IL-6 in stimulating C-reactive protein synthesis [36, 37]. CRP is mainly synthesized by the hepatocytes. However, there are other cells that synthesize CRP such as respiratory tract, coronary artery and kidney [35].

Interestingly, with regards to oral cavity, a study reported that gingival epithelia is also capable to produce CRP [35]. Thus, this might suggest that improvement in stroke conditions with probability of decreasing in the CRP serum levels, might not be significantly related with the level of CRP in GCF. The reason could be because of the ability of gingival epithelial to produce only CRP, with response to the local conditions and was not influenced by the systemic conditions. Of note, serum CRP levels were found to be elevated in persons with periodontal disease, who are seemingly healthy but are at high risk of cardiovascular disease [38]. This was in agreement with regards to the correlation between the CRP levels in GCF and the functional dependency level (MBI scores) of the participants, which explained that high scores in functional dependency level among stroke survivors was negatively associated with the level of CRP in GCF in this cohort. This study has revealed the correlation between the level of CRP and the dental plaque scores and functional dependency levels scores [39]. However, no statistically significant correlation was found in this study with regards to the level of IL-6 and the dental plaque scores and functional dependency levels scores. Although a study on induced gingivitis among healthy participants reported that the IL-6 levels in GCF increased during the induction period (abstain from all oral hygiene procedures) [40].

In regression analyses, oral health factor; having a removable partial denture was significantly associated with low level of IL-6 in GCF, controlling for other factors (adjusted regression models). However, a study on teeth supporting fixed prosthesis has shown that the tooth of fixed prosthesis in the oral cavity was associated with increased level of IL-6 in GCF [41]. Hence, direct comparison cannot be made because this study sample was not necessarily collected from the abutment tooth, as reported in the fixed prosthesis study.

Stroke features (having hemiparesis and swallowing problems) were significant factors associated with a high level of IL-6 in GCF after

controlling for other confounding factors. Having multiple comorbidities was another factor associated with low level of IL-6 in GCF in adjusted (multi-variate) regression analyses. Approximately half of the participants had more than one comorbid at baseline, and more than one type of medication. The common types of medications prescribed were antiplatelet drugs such as aspirin, anti-cholesterol drug such as simvastatin, and anti-hypertensive such as amlodipine. These medications were associated with reduction of circulating IL-6 [42]. A study showed reduction of GCF inflammatory biomarkers levels in chronic periodontitis patients of statin users and it was due to the anti-inflammatory properties of statin medication [43]. However, no statistically significant association was found in this study with regards to the level of IL-6 and CRP with medications taken by the participants.

Right hemiparesis was found to be associated with high level of IL-6 in GCF at baseline in unadjusted (univariate) regression analyses. This concurs with finding that stroke severity associated with left-hemiparesis lesion, which indicate greater impact if the lesions were on the left side of the brain [44]. Having swallowing problems was also a key factor associated with a high level of IL-6 in GCF among stroke survivors. IL-6 is one of the pro-inflammatory biomarkers which raised locally in inflammatory conditions. The presence of a nasogastric tube might cause a reaction to local tissues and increased the likelihood to have a high level of IL-6 in GCF. Complications such as swelling of the larynx and sinusitis caused by a nasogastric tube have been reported [45]. In addition, dysphagia is a consequences of systemic inflammatory conditions, which may play a role in the raise of IL-6 levels. Thus, this may effect the circulating IL-6 level.

With regards to CRP levels in GCF, finding from regression analyses, controlling for other characteristics; socio-demographic variables such as age were associated with a high level of CRP in GCF at baseline in unadjusted regression analyses. A review on inflammatory biomarkers among aging population reported that CRP levels increase with age [46]. Oral health factor such as dental plaque levels at baseline was a key factor associated with high level of CRP in GCF at baseline in unadjusted and adjusted regression analyses. Increase in dental plaque scores was found to be a factor associated with a high CRP level in GCF. This was in agreement with previous study that high dental plaque scores and periodontal diseases were associated with a high level of CRP [47]. Again, swallowing emerged as factor associated with high level of CRP in GCF but was not statistically significant (borderline significant level in adjusted regression analyses).

The sampling procedures of inflammatory biomarkers from GCF was considered sensitive and difficult technique as compared with obtaining the inflammatory biomarkers from plasma [48]. The samples were difficult to be obtained from inflamed gingiva, dry mouths or uncooperative patients. In addition, patients position and conditions during the sampling play a crucial role with regards to the sample collected and obtained. More than half of the participants (68.9%) were on antiplatelet drugs such as aspirin, a high percentage (83.7%) of them were on anti-cholesterol drug such as simvastatin, and about half (58.1%) of them were on anti-hypertensive or beta-blocker. Thus, it has been shown to associate with low IL-6 and CRP levels [49]. The laboratory techniques with regards to the detection of inflammatory biomarkers, might also play a role in contributing to the outcomes of this study. In addition, the cytokines concentrations varied widely among samples due to diverse in participant's conditions. Thus implications might have been caused on the amount of GCF retrieved from the subjects, and outcomes of the study. Furthermore, this pilot study has no control group. Therefore, the result cannot be generalized and a direct comparison cannot be made in the level of inflammatory biomarkers. Thus, it is highly recommended to perform a comparison study to determine the exact association of the variables.

CONCLUSION

To conclude, the study showed significant association between CRP levels in GCF and dental plaque scores, and swallowing problems with the levels of IL-6 in GCF. Therefore, a further study is warranted to explore the relationship between the level of inflammatory biomarkers in the oral fluid with oral health condition and stroke outcomes among stroke survivors.

Conflict of Interest

Authors declare none.

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