Comparison of the Salivary Levels of Homocysteine and C-Reactive Protein in Type 1 Diabetic Patients and Healthy Individuals

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Abstract

Background: Diabetes mellitus type 1 (DM-1) is associated with pancreatic beta-cell destruction, inflammatory processes, and cardiovascular disorders. C-reactive protein (CRP) and homocysteine are considered as inflammatory processes and cardiovascular disorder indicators that can be used for monitoring patients with DM-1. The present study aimed to compare the salivary levels of homocysteine and CRP of DM-1 patients with those of healthy people.

Methods: In this case-control study, 82 patients participated, including 41 DM-1 patients (case group) and 41 healthy people (control group). The case and control groups were matched in terms of age, gender, and body mass index, and 5 mL of the saliva was collected from each participant. Then, the salivary levels of CRP and homocysteine were measured for each patient. Finally, several parameters were recorded for diabetic patients, including fasting blood glucose (FBS), 2-hour postprandial glucose (2hpp), and glycosylated hemoglobin (HbA1c), as well as the duration of the disease and the type and amount of insulin injections. Eventually, data were analyzed by SPSS software using descriptive statistics, independent t-test, and Pearson correlation coefficient.

Results: The salivary CRP and homocysteine concentration had no significant difference between patients and controls (P>0.05). There was no significant correlation between the salivary level of homocysteine and CRP and FBS, 2hpp, HbA1c, albuminuria, duration of disease, type and amount of insulin injection (P<0.05).

Conclusions: According to the results of the current study, the measurement of the salivary levels of CRP and homocysteine could not be helpful for monitoring patients with DM-1.

Background

If the immune system makes antibodies against Langerhans islet cells, the destruction of especially beta cells results in insulin deficiency, which may lead to diabetes mellitus type 1 (DM-1) (1). The results of a systematic review study show that the prevalence of undiagnosed diabetes and prediabetes is high and increasing. Therefore, the notion of universal health coverage is a priority, namely, integrating primary, secondary, and tertiary health levels, as well as employing the available action plans (2). DM has many micro- and macro-vascular complications and retinopathy is the most common micro-vascular one (3). Diabetes increases the risk of cardiovascular diseases. Patients with diabetes are 2-3 times more likely to develop atherosclerotic diseases and have a 2-4 times increase in the cardiovascular-related mortality rate compared to healthy individuals (4).

Homocysteine is a sulfur-containing amino acid, produced during the metabolism of methionine which is an essential amino acid (5). The plasma concentration of homocysteine is associated with the incidence of vascular disease, atherothrombosis, and cardiovascular-related mortality in the general population. Insulin plays a key role in regulating the methionine-homocysteine metabolism.
in humans, and insulin deficiencies in patients with DM-1 can change homocysteine levels (6,7).

C-reactive protein (CRP) is a systemic biomarker released in the acute phase of inflammation. It is produced by the liver and secreted in the presence of cytokines including tumor necrosis factor (TNF) and interleukin-1 (IL-1) in localized or systemic inflammations such as the periodontal one (8). The upper level of CRP can be considered as an indicator for the progression of DM-1 and is a useful biomarker for predicting cardiovascular-related events and deaths (9,10).

These two biomarkers are also present in the human saliva although few studies have addressed their concentration in the saliva (8,11,12) and its relationship with the complications of diabetes. Most studies have examined these two factors in the blood and serum (4,5,7,9) while testing them in the saliva can suggest it as an affordable and available method for determining the prognosis and severity of diabetes and even monitoring the response to treatment (11).

In this regard, the present study sought to compare the salivary levels of homocysteine and CRP of MD-1 patients with those of healthy people.

**Materials and Methods**

In the current case-control study, 41 patients with DM-1 referring to Hamadan Endocrinopathy Clinic were enrolled as the case group and 41 healthy individuals were included as the control group. The number of participants in the present study was calculated according to (9) and considering α at the level of 0.05.

All subjects in the control group were matched in terms of age and gender with patients in the case group. Those in the control group were evaluated for having DM-1 by an endocrinologist in Hamadan Endocrinopathy Clinic. The participants of the control group included those having a fasting blood glucose (FBS) level of 110 mg/dL or lower and no DM-1 suggesting signs or symptoms.

The inclusion criteria were FBS greater than 126 mg/dL, hemoglobin A1c (HbA1c) greater than 6.5%, and 2-hour postprandial glucose (2hpp) greater than 200 mg/dL measured for 2 times, along with the presence of the symptoms and signs of diabetes.

On the other hand, the exclusion criteria included taking medicine with probable effects on homocysteine levels (medications that interfere with folate and B12), drugs affecting CRP (i.e., angiotensin–converting renin inhibitors), aspirin, and non-steroidal anti-inflammatory drugs (NSAIDs). The other criteria were a periodontologist’s confirmed periodontitis, any diseases affecting salivary glands or salivary secretions, the presence of oral lesions, and factors such as smoking, alcohol drinking, and bleeding (13).

The data (i.e., age, gender, and body mass index) of those meeting the inclusion criteria were completed based on their medical records and entered into the questionnaire. The values of FBS, 2hpp (blood sugar (BS)), HbA1c, albuminuria, duration of the disease, and the type and amount of insulin injections were recorded in the questionnaire, and then saliva samples were collected as well (14).

The unstimulated saliva sample was taken between 8 and 11 AM and all study participants were asked not to eat, drink, and brush their teeth 90 minutes before sample collection. Then, patients spat for 5 minutes into a calibrated Falcon tube (ISOLAB, Wertheim, Germany) in a sitting position while slightly bending forward. The saliva samples were stored immediately on the ice at 4°C and sent to the laboratory in 20 minutes, where they were kept at -20°C.

Before testing the levels of homocysteine and CRP biomarkers, all samples were unfrozen at 4°C and centrifuged for 15 minutes at 4°C at 1500 g. The saliva floating on the surface was transferred to fresh tubes (14). Next, the CRP level was measured in μg/L by the MININEPH TM human CRP kit (The Binding Site Company, Birmingham, the UK). A salivary level of homocysteine was measured in μmol/L using the radioimmunoassay method by IMMULITE Kit (SIEMENS, Munich, Germany). Then, SPSS 16 was used for the statistical analysis of the collected data. First, descriptive statistics were tested for all variables. For quantitative variables, the normal distribution was tested by the Shapiro-Wilk method. In addition, an independent t test was used to compare the mean of quantitative variables between case and control groups. Finally, the relationship between the studied variables was evaluated using the Pearson correlation coefficient. The significance level was considered less than 0.05 for all tests.

**Results**

The demographic data of the participants are provided in Table 1. According to the results, the case and control groups had no statistically significant difference in terms of age (\(P = 0.14\)), gender distribution (\(P = 0.001\)), and body mass index (\(P = 0.33\)).

In general, 17 out 41 patients used regular/NovoRapid-Lantus, and the mean repetition rate of injection was 4.2 per day. Biochemical parameters including HbA1c, 2hpp, and FBS are presented in Table 2.

The mean level of salivary homocysteine in the control and case groups was 1.41 ± 0.27 and 1.41 ± 0.24 μmol/L, respectively, which was not statistically different between the two experimental groups (\(P = 0.82\)).

The mean level of salivary CRP was 0.67 ± 0.06 and 0.81±0.09 in the control and case groups, respectively. The results of the independent t-test showed no significant difference between the two groups regarding the mean salivary CRP level (\(P = 0.97\)).

Based on the results (Table 3), there was no statistically significant relationship between the salivary levels of CRP and homocysteine with 2hpp, FBS, HbA1c, the frequency of insulin injections, type of insulin, and the duration of disease.
increases the risk of cardiovascular disorders (8). The results of our study showed that the CRP level increased in patients compared with the control group although this increase was not statistically significant. Contrary to our findings, in a study on biochemical analysis and periodontal health status in patients with DM-1 and DM-2, Leka’a and Ibrahim found that CRP levels were significantly higher in DM patients compared to the control group and higher in patients suffering from DM-1 and DM-2 (15). In a similar study by Zaciragic et al, it was also demonstrated that CRP levels significantly increased in the serum samples of patients with DM-1 (16). The mechanism of the relationship between the increased levels of CRP in patients with DM represents that hyperglycemia in diabetic patients leads to the formation of advanced endogenous glycation (AGE) products. Subsequently, these products exacerbate oxidative stress, activate macrophages, and the expression of IL-6 and IL-1 and the TNF, which all induce CRP synthesis. In addition, the activation of the inherent immune system can increase the CRP level in patients with DM-1. Additionally, fat-derived cytokines can be another source of CRP in this group of patients. It seems that the discrepancy between the results of our study and those of the above-mentioned studies is due to the difference in the sample size, as well as the inclusion and exclusion criteria. In our study, patients were excluded if they smoked, used NSAIDs, and had periodontal disorders, which have not been considered in the above-mentioned studies. Furthermore, fluctuations in salivary CRP levels with the serum or plasma levels can be another reason for such a difference. Further, one of the reasons for the independence of the salivary level of CRP from that of blood is the independent production of CRP. Although the liver produces CRP, mRNA expressing CRP in salivary glands, producing this factor independent of the liver. Thus, the change in CRP synthesis in the liver is not expected to be comparable to the change in CRP synthesis in the salivary glands.

Homocysteine is a sulfuric amino acid that does not exist in the diet and can be synthesized in the body from methionine. This amino acid is prone to auto-oxidation and exacerbates the oxidative damage of vascular cells. It is suggested that the increased concentration of homocysteine is one of the factors contributing to the incidence of cardiovascular disorders in diabetic patients (5). Insulin plays an essential role in the regulation of methionine-homocysteine metabolism in humans,
and the amount of homocysteine may change due to insulin deficiency in patients with DM-1. However, the mechanism for regulating homocysteine metabolism in humans remains largely unknown (6). Based on the results of our study, no significant difference was found between the control and case groups regarding the salivary level of homocysteine. However, the results of various studies regarding homocysteine levels in patients with DM are somewhat controversial, indicating no difference, decrease, or increase in homocysteine levels between patients with DM-1 and controls (17-19). One of the reasons for the inconsistency of the results of the studies is neglecting factors affecting homocysteine levels, including the nutritional status, folate, and vitamin B12 of participants of the study. In some studies, the high-performance liquid chromatography method was used to evaluate the homocysteine level and the results indicated that homocysteine levels in the saliva and blood are directly related to one another, and therefore, saliva can be used as a non-invasive alternative for assessing homocysteine levels. Therefore, a highly sensitive method should be used for evaluating the effect of homocysteine fluctuations in blood samples on the saliva sample, and it seems that the lack of using a high-sensitive method caused a contradiction in the results of our study with those of some other studies (20,21).

In the study of Zaciragic et al, there was a significant relationship between the serum CRP level and HbA1c. This can be justified by HbA1c in uncontrolled glycemic conditions (high blood sugar or FBS in diabetic patients), as well as the increased level of AGE products, both of which increase the levels of inflammatory factors such as CRP and homocysteine. Accordingly, a significant relationship is expected between HbA1c, 2hpp, and FBS levels and the serum levels of inflammatory factors such as CRP and homocysteine (16). Heydari-Zarnagh et al (22) also found that high levels of uncontrolled blood glucose increased HbA1c levels in patients with DM, and the oxidative stress caused by hyperglycemia damaged the vascular wall and released homocysteine. Thus, there is a significant and direct correlation between the levels of glucose, HbA1c, and homocysteine in the blood while the result of our study and some other studies revealed a significant correlation between the CRP level and serum HbA1c. The difference in the results of this study with those of previous studies may be due to the difference in the sample size. On the other hand, the salivary level of the factors is lower than that of the serum, and therefore, the sensitivity of the measurement method can affect the study outcome. Moreover, given that CRP is produced by the salivary glands in addition to the liver and there is no evidence on the association between the liver and the CRP production of salivary glands, the results on the relationship between serum CRP and glycemic condition parameters cannot be expected to resemble the relationship between salivary CRP and these parameters.

In the present study, the relationship between salivary CRP and homocysteine was also examined with the duration of DM-1. Inflammatory reactions and tissue damage in DM patients are related to the duration of DM and the CRP level increases in inflammatory reactions and tissue damage (23). Accordingly, the statistically direct and significant association between the duration of DM and the CRP level is not out of expectation. However, considering the difference in the source and amount of CRP in the saliva, the generalization of the association to the saliva is incorrect. The results of the study by Huang et al showed that homocysteine levels in patients with DM are related to the duration of the disease so that higher homocysteine levels were observed in patients with a disease duration of more than 10 years (24). Additionally, homocysteine is one of the inflammatory factors associated with vascular injury thus the level of vascular damage is expected to increase by an increase in the duration of DM. Therefore, the homocysteine level will increase with the increased duration of DM. However, low concentrations of homocysteine and CRP in the saliva sample and the lack of using a high sensitivity method for detecting low salivary levels of homocysteine and CRP can be the reasons for the difference between our study results and those of other studies.

In this study, no relationship was observed between salivary homocysteine and CRP with the type of insulin regimen and the frequency of insulin administration. Although no study was found on the relationship between salivary homocysteine and CRP and the frequency and type of insulin intake in patients with DM-1, Khatana et al examined the relationship between insulin dose and serum CRP levels in obese and normal-weighted patients with DM-2 and reported a direct correlation between the CRP level and insulin consumption in normal-weighted individuals (25).

Conclusions
Based on the results of the current study, the salivary levels of homocysteine and CRP were approximately the same in patients with DM-1 and healthy individuals. Therefore, these two factors cannot be measured for checking the status of DM. The results of this study showed that salivary homocysteine and CRP have a direct and significant relationship with each other although salivary homocysteine and CRP levels cannot predict the level of blood glucose and the duration of DM-1. Eventually, the frequency and type of the insulin regimen seem to have no effect on the salivary level of homocysteine and CRP.

Authors’ Contribution
MJ, HA and ZR developed the concept and design of the study. MF and MH Performed laboratory steps. AS performed statistical analyzes. MF was primarily responsible for writing the manuscript. All authors have read and approved the final.

Ethical Statement
The study was approved by the Ethics Committee of Hamadan University of Medical Sciences (IR.UMSHA.Rec.1394.393).
Conflict of Interest Disclosures
The authors declare that they have no conflict of interests.

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