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High-Sensitivity C-Reactive Protein and Irisin: Comparison of Serum and Saliva Levels in MI Patients

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Abstract

Objective: Cardiovascular diseases are the main cause of mortality worldwide. Despite the importance of early detection and advancements in the field of medicine, one third of the Myocardial Infarction (MI) cases remain undiagnosed. Biomarkers are a reflection of the physiologic function and pathologic changes in the body. Saliva is a refined plasma and given the benefits of its collection and storage, it can replace blood to facilitate and accelerate the diagnosis process. We studied the correlation between serum and saliva hs-CRP and Irisin concentration in MI patients.

Methods: In this case-control study 46 cases, (24 acute MI, 22 controls), the serum and saliva samples were collected and studied for hs-CRP and irisin concentration.

Results: Saliva irisin was significantly higher (P=0.021) and hs-CRP level was significantly lower in the serum and saliva of the control group (P<0.001). Aging resulted in an insignificant decrease in serum but a significant increase in the saliva (P<0.001, P=0.001) of the control group. With an increase in the serum irisin and hs-CRP concentration, a slight decrease in irisin and a significant increase in hs-CRP (P<0.001) was observed in the saliva.

Conclusion: The serum and saliva hs-CRP concentration had a correlation with MI. Unlike irisin, the concentration of hs-CRP in serum significantly correlated with its concentration in saliva. Since a decrease in saliva irisin concentration can also be a reliable criterion for predicting MI, it seems that both methods can be used in AMI diagnosis.

Keywords: Myocardial infarction; Biomarkers; Saliva; Serum; Irisin; C-reactive protein

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Introduction

Cardiovascular diseases are the most common cause of mortality in many nations; one third of such patients die and the other two third never experience full recovery [1]. Accordingly, Acute Myocardial Infarction (AMI) is the leading cause of death and disability in all age groups in both developed and developing countries [2]. Despite the advancements made in diagnostic procedures and screening stages, many such patients remain undiagnosed or have a delayed diagnosis [3].

Clinical evaluation, Electrocardiogram (ECG) and biomarkers are the keystones for the diagnosis, risk stratification and treatment plan of suspected Non-ST-Elevated MI (NSTEMI) patients. High-sensitivity cardiac troponin measurement is another essential test in such patients [4]. However, in emergent cases biochemical markers and cardiac enzymes are preferred over ECG [5].

Biomarkers are biomolecules found in body fluids or tissues and are a sign of a normal or abnormal disease process. Many of such molecules enter the blood through active or inactive transportation and extracellular ultra-filtration [6]. Therefore, as a biological marker, they can be a good reflection of the physiological function and pathological changes of the body [7].

On the other hand, the saliva is an important source of blood and urine biomarkers. Recent proteomic studies have reported that over 1,000 proteins and 19,000 unique peptide sequences are present in the saliva [8]. Therefore, saliva has been recently introduced as an important mediator in

the diagnosis and evaluation of certain systemic diseases [6].

Given that atherosclerosis has been defined as an inflammatory disorder and the underlying cause of AMI, studying the inflammatory markers in such patients is of great value [9]. Although Troponin I (TnI) is the gold standard in AMI diagnosis, many studies have suggested the measurement of other biomarkers with high sensitivity and specificity as diagnostic and prognostic factors in such patients [10].

In this respect, C-Reactive Protein (CRP) is one of the systemic indices of the acute phase of inflammation [1] which is produced in the initial phases of tissue injury or inflammation onset with the stimulation of cytokines and its level increases during the acute phase reactions; its concentration rises to 10 times during 6 h and reaches its peak value by 48 h [11]. Moreover, it is regarded as a cardiovascular risk factor as its high concentrations have a higher risk of mortality, MI and require emergent revascularization. Measurement of its high serum level is done by the conventional CRP technique while its lower concentrations are measured by high-sensitivity-CRP (hs-CRP) [12]. However, hs-CRP level measurement has not yet been widely recommended in the guidelines [13]. As it is the most reliable blood biomarker in MI and atherosclerotic patients, it is a beneficial method for risk stratification of healthy controls and identification of high-risk patients for CVD. Therefore, hs-CRP measurement in body fluids is of great importance in this respect [14].

Furthermore, an increase in serum irisin level, a peptide hormone obtained from the Fibronectin Type III Domain Containing Protein 5 (FNDC5) [15], has been reported to be associated with the progression of Major Adverse Cardiovascular Events (MACE) in those with CVD [16]. Irisin is highly expressed in the myocardium and its serum concentration originates from the cardiac muscle [17]. However, and in spite of the promising results in animal studies, the role of serum irisin as a predictive factor for mortality in Acute Heart Failure (AHF) patients in not known [15], therefore, the evaluation of this marker in human studies is essential [16].

Regarding the importance of biomarkers in diagnostic processes, special focus has been given to the saliva in recent studies as it is a refined plasma containing thousands of biomolecules derived from the local capillary bed. Different saliva biomarkers including IL-6, matrix metalloproteinase-9, Soluble Intercellular Adhesion Molecule (sICAM-1), soluble ligand form CD40 (sCD40-L), hs-CRP, cTnI, MPO and TNF-α have been studied as MI diagnostic factors [18]. Some saliva biomarkers have up to 90% to 100% sensitivity in this respect [7].

The saliva as a diagnostic environment has many advantages regarding its collection, storage and control [18]. Its sampling is non-invasive, simple, rapid, painless, low cost and low-stress and the duration required for markers measurement is similar to serum. Therefore, it can be a suitable alternative for blood sampling in the emergent diagnosis of AMI [11]. Nevertheless, very few studies have investigated the association between serum and saliva biomarkers in cardiac diseases [6,16]. Herein we aimed at studying the association between serum and saliva hs-CRP and irisin and AMI incidence so that by the earlier diagnosis and prompt treatment a better quality of life could be anticipated in such patients.

Materials and Methods

In this case-control study 50 cases, 25 AMI patients and 25 controls were studied. The research protocol was approved by the Ethics Committee of Mashhad University of Medical Sciences and an informed consent was obtained from each participant prior to study entrance. The AMI patients were selected from the patients visiting the Emergency Unit of Javad-al-Aemeh Cardiology Hospital, Mashhad, Iran whom had experienced ischemia and chest pain during the past 48 h and in whom the diagnosis of AMI was made by two experienced cardiologists and confirmed by paraclinical study including ECG and troponin test [4,15]. All patients over 18 years of age, with a BMI between 19 Kg/cm^2 to 25 Kg/cm^2 who consented to participate were enrolled. Patients with the following criteria were excluded from the study: Obesity [19], history of smoking or alcohol consumption, breast feeding and pregnancy [20], under treatment with thrombolytic drugs or antianginal therapy, consuming antibiotics or steroid drugs, inflammatory or immunologic diseases, malignancy and its related treatments [19], active infection, history of CABG [21], systemic, chronic or metabolic diseases, trauma, neuromuscular diseases or consuming drugs which affect the cardiac or skeletal muscles [17], psychotic and neurodegenerative diseases and other uncontrolled systemic diseases [22]. The control groups were selected from the healthy individuals accompanying the cases. Demographic data of all studied cases was initially recorded and then blood and saliva samples were taken from both groups for studying certain biomarkers. The blood sample was taken at fasting in the sitting position and from the radial or femoral blood vessel with a 5cc syringe and 21-gauge needle [23]. In order to collect the saliva sample, the unstimulated whole saliva method was used under the supervision of an oral medicine specialist. They were required to stop eating, drinking, brushing teeth and smoking for 2 h prior to the saliva sample collection. The sample was taken after a full mouthwash in the sitting position [24]. The collected samples were then promptly delivered to the biochemistry lab of the Medical Faculty and stored at -80°C until analysis. The serum and saliva irisin and hs-CRP levels were measured based on the kit instructions and by the Enzyme-Linked Immunosorbent Assay (ELISA) method as follows [23]:

Hs-CRP

The serum and saliva samples were centrifuged for 10 min at 3500 rpm and 4°C. Then a 500 µl of each sample was placed in the sample tray of the Mindray biochemistry auto analyzer device (Model Bs-800, manufactured by China). The No. 1 and 2 solutions of the kit were added to the sample tray as follows: 100cc of No. 1 solution was added to the samples and blanch (10cc distilled water). After mixing it was incubated at 37°C for 5 min. then 100 µl of the No. 2 solution was added and after mixture it was incubated at 37°C. The initial light absorption of the calibrator and samples was also measured. The second light absorption was measured after 90s.

The changes in light absorption of different calibrators was then entered into a logarithmic table and the samples and control concentration was determined based on the achieved curve.

Irisin

Serum and saliva samples were centrifuged for 10 min at 3500 rpm and 4°C and the standard samples were prepared as follows:

The standard solution No. 5 with a final concentration of 32 ngr/ml (100 µl of the main standard solution with 64 ng/ml concentration plus 100µl of the standard diluting solution).

Solution No. 4 with an end concentration of 16 ng/ml (100 µl of No. 5 solution plus 100 µl of the main diluting solution).

Table 1: Comparison of serum and saliva irisin and hs-CRP between the two studied groups.

Variable	Group	Mean*	SD&	Lowest*	Highest*	Median	T-test result
Serum Irisin	Study	8.9	1.03	5.8	10.6	8.8	T=1.29
	Control	9.28	1	7.6	11.3	9.4	P=0.205
Saliva irisin	Study	6.49	0.84	5.3	8.3	6.5	T=2.40
	Control	7.14	0.99	4.6	9	6.9	P=0.021
Serum hs-CRP	Study	8.65	1	7.2	10.9	8.55	T=34.79
	Control	1.05	0.24	0.7	1.6	1	P<0.001
Saliva hs-CRP	Study	2.99	0.84	1.7	4.9	2.95	Z=5.83
	Control	0.58	0.13	0.4	0.8	0.55	P<0.001

*ng/ml & SD: Standard Deviation

Solution No. 3 with an end concentration of 8 ng/ml (100 µl of No. 4 solution plus 100 µl of the main diluting solution).

Solution No. 2 with an end concentration of 4 ng/ml (100 µl of No. 3 solution plus 100 µl of the main diluting solution).

Solution No. 1 with an end concentration of 2 ng/ml (100 µl of No. 2 solution plus 100 µl of the main diluting solution).

Standard solution No. 0 only contained 200 ml of the main diluting solution.

50 µl of the above-mentioned standard solutions along with 50 µl of saliva and serum samples (40 µl of saliva and serum solution plus 10 µl of antibody) was transferred to the plate wells. 50 µl of the Streptavidin-HRP solution was also added to all wells. In order to mix the content, the plate was placed on a shaker and reserved in an incubator for 50 min at 37°C. To prepare the wash solution, the concentration of the washing liquid was diluted with distilled water up to 30 times. The plate was removed from the incubator and carefully emptied, while by shaking the plate complete liquid discharge was ensured.

Each well was filled with the washing solution and emptied after 30s; this was repeated 4 times. In the next step 50 µl of the A chromogenic substrate solution and then 50 µl of B chromogenic substrate solution was added. After mixing the material the plate was placed in the incubator for 10 min at 37°C with no light exposure. It was then removed from the incubator and 50 µl was added to each well in order to cease the reaction. The blue color of the plate changed to yellow immediately after adding the ceasing solution. The irisin serum and saliva level were measured by the ELISA reader at 450 nm wave length [16]. Eventually the data were entered into the SPSS software and analyzed by Pearson's correlation test.

Results

In total 46 patients were studied, 27 males and 19 females with the mean age of 50.43 ± 16.84 yrs (range: 23 to 82 years). The study and control group included 24 and 22 cases, respectively. The mean serum level of irisin showed no meaningful difference between the two groups (P=0.205); however, the saliva irisin level was significantly lower in the cases compared to controls (P=0.021). The mean level of hs-CRP in both the serum and saliva had significantly increased in the cases compared to controls (P<0.001) (Table 1).

Moreover, with an increase in age the serum irisin concentration decreased in both groups with no meaningful correlation (r= -0.348, P=0.096 and r= -0.157, P=0.484). In the study group an insignificant increase in the saliva irisin level by age was observed (r=0.192, P=0.369); however, with a rise in serum irisin level, its concentration slightly decreased in the saliva (r= -0.048, P=0.823).

In the control group an insignificant decrease in saliva irisin by age was observed (r= -0.239, P=0.258). However, with a rise in serum irisin level, its saliva concentration also increased, yet the correlation was insignificant (r=0.157, P=0.486).

Regarding hs-CRP level, an insignificant increase in its level by age was observed in both saliva and serum in the study group (r=0.154, P=0.471 and r=0.127, P=0.554). This increase was significant both in serum (r=0.765, P<0.001) and saliva (r=0.656, P=0.001) of the control group. In both groups with an increase in the serum hs-CRP concentration, its saliva level increased significantly (P<0.001) (Table 2).

Discussion

Very few human studies have been performed in this respect whereas in this study the changes in serum and saliva irisin and hs-CRP concentration were studied for the first time in AMI patients, yet the main limitations were the small study sample and the interventional effect of the treatment type. Despite the vast advancements to date, AMI still results in considerable mortality, therefore its early diagnosis is of great importance. Very few human studies have been performed on the role of saliva biomarkers in AMI diagnosis. In this study we aimed at investigating the relationship between two serum and saliva biomarkers, irisin and hs-CRP, in AMI patients.

Although cardiac tissue is the main site for irisin production [16], and a decrease in serum irisin level is a predictor of CVD severity, yet the etiology of irisin reduction following MI has not been fully understood [21].

In the present study serum irisin concentration in the control group was significantly higher than the cases whereas the saliva concentration was significantly higher than the cases. It seems that our obtained results are in accordance to that of a previous animal study reporting that irisin concentration gradually decreases in MI cases. In Sarioglu et al. [25] study a significant increase in the irisin level of cardiac tissue of 21 rats following lab-induced MI was reported by immunohistochemistry study (P=0.01). Moreover, in Anastasilaks et al. [26] study it was shown that MI or CVD patients had a lower irisin level compared to controls, which is consistent with our findings. Similar results were reported in Aydin et al. [16] study performed on 11 AMI patients and 14 controls (P<0.05). They also confirmed a reduction in serum/saliva irisin in MI patients compared to controls by another immunohistochemistry study [27].

In a study on 75 males aged 30 to 70 years with AMI, the irisin level was significantly less in those with atherosclerosis and MI in comparison to controls. Although this study was limited to a certain

Table 2: The association between serum and saliva Irisin and hs-CRP level and age.

Group (Test)			Serum Irisin	Saliva Irisin	Age Irisin	Serum hs-CRP	Saliva hs-CRP	Age hs-CRP
Study group	Serum	Pearson's correlation test	1	-0.048	-0.348*	1	0.895	0.154*
		PV @		0.823	0.096		<0.001	0.471
	Saliva	Pearson's correlation test	-0.048	1	0.192*	0.895	1	0.127*
		PV	0.823		0.369	<0.001		0.554
	Age	Spearman's correlation test	-0.348*	0.192*	1	0.154*	0.127*	1
		PV	0.096	0.369		0.471	0.554	
Control group	Serum	Pearson's correlation test	1	0.157	-0.157	1	0.753*	0.765
		PV		0.486	0.484		<0.001	<0.001
	Saliva	Pearson's correlation test	0.157	1	-0.239	0.753*	1	0.656*
		PV	0.486		0.285	<0.001		0.001
	Age	Pearson's correlation test	-0.157	-0.239	1	0.765	0.656*	1
		PV	0.484	0.285		<0.001	0.001	

@ PV: P Value

age and sex group, yet in accordance to our findings it showed that irisin is an indicator for CVD and it seems that the drop in irisin level in MI patients may be a protective mechanism for the heart. Due to the effect of irisin in thermogenesis increase, when it decreases tissue damage due to reduced energy may be more common [23]. Moreover, studies have shown that the injured cardiac cells during MI require more energy for myocytes regeneration, therefore the production and release of irisin is restricted [28] and the main generative tissues due to the loss of ATP produce more heat through the uncoupling effect [29].

If the amount of irisin in MI is not reduced, due to the release of energy in the form of heat the cardiomyocytes and other cells are at higher risk of higher irisin concentrations. Therefore, it is assumed that if ATP is required in local tissues, it reduces the production of irisin at these sites [28]. Irisin synthesis and release appears to depend on the energy requirements of the tissues and it may act as a hormone for controlling energy. Therefore, the lower expression of irisin may have a positive role in the pathogenesis of acute cardiac events [16]. Changes in the mitochondrial functions can affect the pathogenesis and cardiovascular events following MI. Studies have shown that irisin can prevent cellular proliferation and increased oxygen consumption in cardiomyoblasts through mitochondrial regulation [30-32]. Irisin is not a passive byproduct of cardiomyocytes injury, but an energy-mediated secreted molecule. Therefore, the myocardium may release less irisin in cases of reduced myocardial oxygenation and blood circulation to suppress the cardiac metabolic needs and compensate for low energy availability [26]. Unlike other studies Hsieh et al. [30] study showed that with increased irisin concentration, the risk of cardiovascular diseases increases; further studies are still required to confirm this finding.

Given that Rahim et al. [11] described saliva proteins as highly beneficial in MI diagnosis, the comparison of blood and saliva markers is of great importance. In the present study the serum irisin concentration was insignificantly higher among the controls, whereas its changes in serum did not significantly differ from that of saliva, in accordance to Aydin et al. [16] study. This may be due to the saliva constitution and its secretion as evidence suggests that certain peptides are formed in the saliva which have higher concentrations than serum [33]. Moreover, salivary glands can themselves secrete irisin [16]. Therefore, it seems that saliva irisin concentration for MI diagnosis and prediction is appropriate due to its high accuracy and non-invasive nature.

Similar to Li et al. [33], Aydin et al. [16] and Efe TH studies [19], our findings showed that serum irisin concentration decreases by age [34]. However, Rmanuele et al. [35] reported that irisin level is significantly lower in young people with MI. Huh et al. [36] demonstrated a positive correlation between irisin concentration and age. The inverse correlation between age and irisin concentration in our study can be justified by the fact that in Rana et al. [37] study, the circulated irisin level had a positive association with the telomere length and a shorter telomere length was associated with age and the MI risk.

Hs-CRP as a systemic inflammatory marker is a predictor of cardiovascular diseases risk [12,14]. In the current study the serum and saliva hs-CRP level was significantly lower in the control group. A rise in serum/saliva hs-CRP was reported in Ebersole et al. [2] study. Lie et al. [12] also reported a rise in this marker 3 days following MI; therefore, hs-CRP can be regarded as a specific marker in the diagnosis of left ventricular disorders. Due to the significance of hs-CRP in MI diagnosis, a significant relationship was achieved between its serum and saliva concentration in our study. Ouellet et al. [38] also confirmed a moderate to strong association between saliva and serum hs-CRP.

Given the advantages of saliva (its content and simple sampling) in comparison to serum, it seems that saliva biomarkers such as irisin and hs-CRP could be regarded as desirable markers in the diagnosis and prediction of AMI. Considering that different post MI treatments (primary PCI, thrombolytics or antianginal therapy) can affect the irisin concentration, performing future studies with a larger study sample and taking into account the various treatment options and other saliva biomarkers is highly recommended.

The serum and saliva hs-CRP concentration had a direct correlation with AMI incidence. In contrast to irisin, the serum and saliva hs-CRP concentration also significantly correlated. Therefore, both methods can be used in the move toward a better assessment of the MI risk, prognosis, as well as diagnosis.

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