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Correlation between increased urinary serotonin levels and coronary artery disease in cigarette smoking patients

Necat YILMAZ[1], Yakup MERMERDAŞ[2], Esin EREN[3], Ayşenur YEĞİN[1], Emine NAMIDURU[2]

Aim: Platelets secrete serotonin (5-hydroxytryptamine), which has several effects on the vascular wall and promotes thrombogenesis, mitogenesis, and the proliferation of smooth muscle cells. We therefore measured excreted levels of 5-hydroxytryptamine (5-HT) concentrations in urine as a means of assessing vascular lesions in patients with coronary artery disease (CAD).

Materials and methods: Subjects who underwent coronary angiography were classified into 2 groups according to their diagnosis. The CAD group consisted of 33 patients with coronary artery stenosis, whereas the healthy control group consisted of 30 subjects. An isocratic high-performance liquid chromatographic (HPLC) system with an electrochemical detector was used for the HPLC analysis of serotonin in urine.

Results: The mean urine 5-HT concentration was significantly ($P < 0.01$) higher in CAD patients than in the healthy control patients. Interestingly, the excretion of the urinary 5-HT levels in CAD patients were positively associated with cigarette smoking ($r = 0.411$, $P < 0.01$), serum hs-CRP levels ($r = 0.198$, $P < 0.05$), and age ($r = 0.132$, $P < 0.05$).

Conclusion: The increase in the amount of serotonin in urine in correlation with cigarette smoking, age, and hs-CRP may be important for future treatment strategies.

Key words: Smoking, serotonin, 5-HT, urine, coronary, platelet

Sigara içen koroner arter hastaları ve artmış idrar serotonin seviyeleri arasındaki ilişkinin korelasyonu

Amaç: Trombositler serotonin salgılar (5-hydroxytryptamine); bu salgının damar duvarı üzerinde çeşitli etkileri vardır ve düz kas hücreleri üzerinde mitogenezi, trombogenezi artırıcıdır. Bu sebeble biz koroner arter hastalarındaki (KAH) vasküler lezyonları değerlendirmek için serotonin'in (5-HT) idrardaki atılım seviyelerini ölçtük.

Yöntem ve gereç: Koroner anjiografi yapılan kişiler teşhislerine göre iki gruba ayrıldı. KAH grubunda koroner arter stenozu olan 33 hasta vardı. Sağlıklı kontrol grubunda 30 kişi vardı. İzokratik yüksek basınç sıvı kromatoğrafisi (HPLC) ve elektrokimyasal dedektör idrardaki serotonin ölçümünde kullanıldı.

Bulgular: Sağlıklı kontrollere oranla KAH hasta grubunda idrardaki 5-HT konsantrasyonu anlamlı miktarda yüksekti ($P < 0,01$). İlginç olarak, KAH hasta grubunda idrara atılan 5-HT miktarı ile sigara içilmesi ($r = 0,411$, $P < 0,01$), serum hs-CRP ($r = 0,198$, $P < 0,05$) ve yaş ($r = 0,132$, $P < 0,05$) arasında pozitif korelasyon gözlendi.

Sonuç: İdrara atılan serotonin miktarındaki artışın sigara yaş ve hs-CRP ile korelasyonu gelecekte kullanılacak tedavi stratejileri için önemli olabilir.

Anahtar sözcükler: Sigara içmek, serotonin, 5-HT, idrar, koroner, trombosit

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[1] Central Laboratory, Educational and Research Hospital, Antalya - TURKEY
[2] Department of Clinical Biochemistry, School of Medicine, University of Gaziantep, Gaziantep - TURKEY
[3] Public Health Laboratory, Antalya - TURKEY
Correspondence: *Necat YILMAZ, Central Laboratory, Educational and Research Hospital, Antalya - TURKEY*
E-mail: necatyilmaz@hotmail.com

Introduction

Serotonin, chemically known as 5-hydroxytryptamine (5-HT), is a biogenic monoamine with a molecular weight of 176 Da. 5-HT, a decarboxylated derivative of tryptophan, is synthesized in the enterochromaffin cells and released into the blood stream to be incorporated into platelets. At the site of endothelial lesions, platelets aggregate and secrete 5-HT that presents several vascular actions involved in thrombosis and atherogenesis (1). In fact, 5-HT may induce vasoconstriction in the presence of an endothelial injury, the aggregation of platelets, and the mitogenesis of arterial smooth muscle cells and endothelial cells. The serotonin is released at the site where platelets are activated, in situations such as atherosclerotic vascular lesions (2,3).

The pathophysiological mechanisms by which platelets contribute to the atherosclerotic process are not fully understood. Low doses of 5-HT dilate the arteries through stimulation of 5-HT1 receptors, but high doses elicit vasocontraction by way of stimulation of the 5-HT2 receptors (4). Experimental animal studies have demonstrated that platelets are activated and aggregate at the sites of coronary artery stenosis and endothelial injury (5). Activated platelets release serotonin in substantial quantities, causing vasoconstriction and the recurrent aggregation of platelets with cyclic flow reductions (1). Serotonin also acts as a growth factor, stimulating mitogenesis and the migration of arterial smooth muscle cells. Furthermore, serotonin has shown it promotes the proliferation of vascular endothelial cells, and direct endothelial injury in cell cultures suggests the release of lactate dehydrogenase and preloaded (14C)-adenine when exposed to serotonin (2). Thus, serotonin clearly has important vascular actions, and may be involved in atherogenesis as well (3).

Moreover, studies indicate that nicotine increases serotonin release in the brain, while nicotine withdrawal has the opposite effect. Some of the behavioral effects of nicotine, including its reinforcing efficacy, result, in part, from the activation of mesolimbic dopamine neurons. Serotonergic neurons modulate the functioning of dopamine neurons in a complex fashion. Much of this complexity arises from the fact that serotonin (5-HT) exerts its effects through multiple receptor subtypes, some of which even act in apparent functional opposition to one another (6,7).

In this study, we assessed the urinary excretion of serotonin as a clinical marker of coronary artery disease in cigarette smoking and non-smoking subjects with stable symptoms admitted to elective coronary angiography for chest pain.

Materials and methods

Subjects

All patients referred to the Department of Cardiology, University of Gaziantep, between March 2007 and May 2007, whose clinical data were available were included in our study. The CAD group consisted of 33 patients (7 females and 26 males; mean age 60.54 ± 2.16 years) with stenosis of the coronary arteries. The healthy control group consisted of 30 subjects (8 females and 22 males, mean age 58.95 ± 2.2 years). Each angiogram was evaluated jointly by at least 2 cardiologists. Coronary artery lesions were magnified, traced, and measured with calipers to determine the percentage of diameter narrowing the artery. All coronary angiographies were performed in the same center. Coronary artery disease was classified into groups according to the maximum coronary stenosis at the angiography: 0%-19% (no detectable CAD), 20%-49% (mild disease), 50%-69% (moderate disease), and 70%-100% (severe disease). Another classification of the severity of the disease was assessed by counting the number of diseased vessels (0 to 3). All subjects were questioned for established cardiovascular risk factors including diabetes, smoking, renal disease, and hypertension.

Obesity was defined as a body mass index (BMI) greater than 27.8 kg/m^2 as proposed by the National Institutes of Health Consensus Statement. Diabetes mellitus was considered present in patients with a known history of diabetes and patients with a fasting blood glucose ≥ 126 mg/dL (7.0 mmol/L) according to the American Diabetes Association criteria. The study was approved by the local Ethics Committee, and the individuals participating in the study gave their informed consent.

The healthy subjects (n = 30) also had coronary angiograms, and they underwent a comprehensive physical examination by a physician, completed the World Health Organization standard Rose questionnaire on chest pain, and answered other questions about their past medical history. All of them had normal electrocardiograms according to Minnesota Coding Criteria.

All the subjects identified themselves as either current smokers (N = 21), or current non-smokers (N = 12), and were compared by chi-square for CAD. In addition to this, they were all monitored for somatic illnesses throughout the investigation period and were excluded if symptoms of any infectious or systemic illness were present (i.e. acute or chronic liver disease, cancer, renal disorder, rheumatic disease, etc). Patients diagnosed with acute coronary syndrome 6 months prior to the study were excluded. Additional exclusion criteria included alcohol intake, and the use of some types of drugs such as antidepressants, anticonvulsants, estrogen, lipid-lowering therapy, and other medications that might affect serotonin metabolism. The subjects were also asked to avoid these foods containing large amounts of serotonin for 2 days prior to and during specimen collection: pineapples, eggplants, avocados, bananas, redcurrants, kiwis, melons, mirabelle, gooseberries, tomatoes, walnuts, and plums.

Blood samples

Fasting blood samples were collected by a standard venipuncture technique between 08.30 and 11.30. The blood was taken into K3 EDTA vacutainer tubes for hemogram and into plain tubes for serum samples. Serum and plasma were separated after the centrifugation of the blood at +4 °C, 1500 × *g* for 10 min and stored at –70 °C until analysis.

Urine collection

Urine samples for the determination of serotonin and creatine excretion were obtained from a 24-h urine specimen, including the early morning portion. After their division into aliquots, the urine samples were also frozen at –70 °C before assay. Urinary serotonin concentrations were expressed relative to the urinary creatinine levels.

Analytical methods

We measured urinary 5-HT in the samples using an HPLC method (8) involving a column-switching system, including a pretreatment reverse-phase column (internal diameter of the column 4.6 mm, and 35 mm long), an analytical reverse-phase column (internal diameter of the column 4.6 mm, and 120 mm long) and a post-column reaction. 5-HT in 500 µL of sample was bound in the pretreatment column with an eluent containing glycine, sodium perchlorate, and disodium EDTA. We transferred the 5-HT bound in the pretreatment column to the analytical column and separated it with an eluent containing sodium acetate, ammonium nitrate, acetonitrile, and disodium EDTA. We used a post-column reaction, employing benzylamine for the derived fluorescent reagent, to detect 5-HT in the HPLC method.

For the HPLC analysis of serotonin in urine and plasma, an isocratic HPLC system with electrochemical detector is suitable (Figure). Accessories for electrochemical detectors were obtained from Chromsystems® (urine serotonin normal range: 28.4-125 µmol/day, intra-assay: CV < 2%, inter-assay: CV < 3%, linearity: up to 1000 µg/L, limit of quantification: 5 µg/L, run time: approximately 8 min, injection volume: 20 µL, potential: approximately +400 to +500 mV, internal standard solution of N-methylserotonin).

Routine laboratory measurements

Routine laboratory investigations were carried out by using a Roche® autoanalyzer with Roche reagents (Roche®, Hitachi®, Mannheim/Germany) for serum creatine, urinary creatine, serum alanine

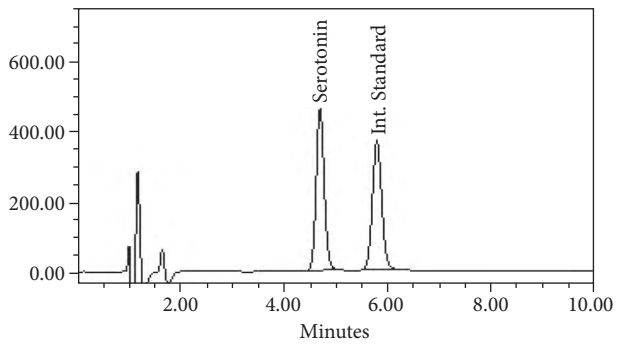

Figure. Typical chromatogram of urine 5-HT.

transaminase (ALT), aspartate transaminase (AST), triglyceride (TG), total cholesterol (TC), HDL-cholesterol (HDL-C), uric acid (UA), urea, CK-MB and platelet (Plt), Hemoglobin (Hb), and WBC concentrations. Serum hs-CRP levels were determined by using the Immulite® analyzer with DPC reagents (Los Angeles, CA, USA).

Statistical analysis

The data were evaluated for all variables (mean ± SD). The differences between the groups were tested with a non-paired t-test for continuous variables, and the categorical variables were examined by Pearson's χ^2 test for exact values. The difference in the current smoking frequency between cases and controls was assessed by Fisher's exact test. Statistical analysis was considered significant at P values of 0.05.

Results

The results of our statistical data are given in Table 1. The table shows the summary statistics obtained for the patients with CAD and for apparently healthy subjects. There was no significant difference between the groups in BMI, waist/hip ratio and age. The mean urinary 5-HT level in the control group was 28.6 ± 6.9 μmol/mmol of creatinine, whereas it was 55.1 ± 14 μmol/mmol of creatinine in the CAD group ($P < 0.01$). The correlation between urinary serotonin excretion and CAD was strengthened when the heavy smoker (> 40/day) patients (n = 12) or serum hs-CRP levels were included in the analysis ($r = 0.410$, $P < 0.039$ and $r = 0.199$, $P < 0.05$ respectively). Urinary serotonin was neither related to the number of diseased vessels, degree of occlusion, nor to the other routine measurement results ($P > 0.05$).

Table 1. Summary statistics of subjects groups (mean ± SD).

	Healthy (n = 30)	CAD (n = 33)
Female (%)	40%	35%
Age (year)	58.95 ± 2.2	60.54 ± 2.16
Smoking (%)*	16%	63%
Hypertension (n)	0	14
Diabetes (n)	0	11
BMI (kg/m2)	24.8 ± 1.74	26.2 ± 1.96
Waist/Hip ratio (cm/cm)	0.89	0.95
Urine 5-HT (μmol/ mmol of creatinine) **	28.6 ± 6.9	55.1 ± 14
Serum creatinine (mg/dL)	1.09 ± 0.32	0.95 ± 0.39
Maximum coronary stenosis	Not detectable	Mild: 14% Moderate: 42% Severe: 44%
hs-CRP (mg/dL) **	0.47 ± 0.61	1.54 ± 0.87
WBC (103/μL)	7219 ± 1768	8426 ± 2527
TC (mg/dL)	164 ± 42	158 ± 42
TG (mg/dL)	170 ± 37	155 ± 25
HDL-C (mg/dL) **	43 ± 11.12	31 ± 7.93
UA (mg/dL) *	5.1 ± 1	6.4 ± 1
ALT (U/L) *	21 ± 2.3	37 ± 5.8
AST (U/L) *	20 ± 2.5	34 ± 2.3
CK-MB (U/L) *	11 ± 2.2	44 ± 13
Urea (mg/dL)	35 ± 9.78	38 ± 17.8
Plt (103/μL)	252 ± 76.5	249 ± 64.3
Hb (g/dL)	13.3 ± 1.6	14.2 ± 1.1
Htc(%)	40.1 ± 4	41 ± 3

* Healthy versus CAD, $P < 0.05$, **Healthy versus CAD, $P < 0.01$

Table 2. Urinary 5-HT and significant correlation coefficients of biomarkers of coronary artery disease (Pearson's coefficients).

Variable	5-HT (urinary serotonin)
Number of cigarettes smoked daily	r = 0.410, P < 0.001
hs-CRP	r = 0.199, P < 0.05
Age	r = 0.132, P < 0.05

Discussion

This study has shown that urinary 5-HT concentration is considerably higher in CAD patients than in healthy control subjects, and it has a strong association with smoking in CAD patients. In urine, serotonin excretion was previously evaluated by HPLC methods with fluorometric or amperometric detection, and by enzyme immunoassay (4). Normal values obtained with our method and expressed in nmol/24 h or in nmol/mmol of creatinine agreed with those previously described (4). Our sophisticated chromatographic conditions allowed us to separate and to quantify 5-HT in urine more definitively.

5-HT affects the peripheral circulation in 2 opposing ways: it constricts the arteries and augments platelet aggregation by way of 5-HT_2 receptors, but it dilates arteries and inhibits platelet aggregation by way of 5-HT_1 receptors. 5-HT_1 receptors are predominantly distributed on the surfaces of vascular endothelial cells, which are damaged by many mechanisms, particularly by shear stress to the vascular wall and by oxidized low-density lipoprotein in patients with risk factors for atherosclerosis (9,10). The vasodilatation is mediated by NO released on stimulation of 5-HT_{1B} receptors on the vascular endothelial cells. Therefore, when 5-HT is applied to the diseased arteries, vasoconstriction instead of vasodilatation occurs, in vitro and in vivo. The 5-HT released locally from the activated platelets would have increased NO release, which would have dilated the arteries to decrease the shear stress to the vascular wall and suppressed the platelet activation to restore the perturbation in the normal vessels. However, when the endothelial cells are damaged, NO release may be deficient, enabling 5-HT to reach the vascular smooth-muscle cells because of deficient barrier function of the endothelial cells (11,12). Stimulation of 5-HT_2 receptors on the smooth-muscle cells constricts the arteries, and shear stress can be augmented by the narrowed arteries. Consequently, athermanous lesions advance (13-16).

Recent evidence points to the role of a polymorphism in the promoter region of the serotonin transporter gene in predisposing patients to depression after stressful life events. Other work has investigated the possibility that this polymorphism is common to both altered serotonergic function and cardiovascular reactivity to stress. A study of monozygotic and dizygotic twins has provided further evidence for a genetic risk factor common to both depression and heart disease (6).

As an inductor of platelet aggregation and vascular contraction, 5-HT has been implicated in coronary artery disease. This is also supported by the observation that inhibitors of 5-HT re-uptake protect against MI. In addition, 5-HT stimulates the hyperplasia of artery smooth muscle, thus contributing to the endothelial dysfunction that characterizes the pathogenesis of cardiovascular disease (7). Atherosclerotic lesions are advanced in patients with CAD and because these lesions can be confirmed with the use of coronary angiography. We performed coronary angiography in all subjects enrolled in this study**.** Since it is obvious that atherosclerotic vascular lesions advance with aging, the increased plasma 5-HT level may have been a result of the activation of the circulating platelets releasing 5-HT into the circulation (17). Due to limited information, it is hard to explain the exact reason why the 5-HT increased significantly in the urine. Serotonin is released from nerve terminals and distributed at the intestinal chromaffin cells, which are taken up into the platelets. Most likely, the increased 5-HT content in the urine might mean that the platelets had been activated or that uptake into the platelets had been impaired (8,18).

Some of the effects of 5-HT, such as vascular contraction and platelet aggregation, can lead to thrombus formation and have been implicated in the pathogenesis of myocardial infarction (MI). Moreover, smoking is a well-documented risk factor for cardiovascular disease, and an increased 5-HT receptor density among smokers has been described (7). This fact might explain our results about the correlation between higher urinary 5-HT levels in smoking patients.

Emerging evidence suggests that alterations in immune functioning and inflammation may contribute to the development and clinical manifestations of CAD. The body's inflammatory response to chronic hypercholesterolemia and hypertension may contribute to atherosclerosis as damage to the arterial lining occurs over time. The increase in hs-CRP levels in our patients with CAD (Table 1) might be related to this explanation, and such a correlation has been shown by other researchers. For example, Ridker et al. showed that patients who were initially disease free but who developed peripheral arterial disease over 5 years differed from the controls by having higher levels of C-reactive protein after controlling for other risk factors (6).

Several limitations of the study should be pointed out. First of all, the study sample was relatively small. Secondly, we have not genotyped a *5*-HTTLPR. Furthermore, the smoking status has not been verified with the measurement of exhaled CO concentration of smokers to avoid the classification error attributed to self-report.

Clinical studies are necessary for further conclusions. From these findings, we propose that urinary serotonin may be useful as a marker for CAD. We have confirmed in this study that urinary 5-HT concentrations increase in patients with coronary artery disease.

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