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Assessment of platelet aspirin responsiveness in Turkish Population with PFA-100, serum thromboxane B_2, soluble CD40 ligand and soluble P-Selectin]

[Türk Populasyonunda aspirin cevapsızlığının PFA-100, serum tromboksan B_2, çözünür CD40 Ligand ve çözünür P-Selektin ile değerlendirilmesi]

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ABSTRACT

Aim: Recurrent thromboembolic vascular events have been reported in 5.5 to 60% of the population despite aspirin therapy. We aimed to determine the frequency of aspirin non-responsiveness in our population with "Platelet Function Analyzer-100 Closure Time" (PFA-100 CT) and serum tromboxane B_2, soluble CD40 Ligand and soluble P-selectin levels.

Material and Methods: Patients (n=118) taking 80-300 mg/day aspirin were enrolled into the study. PFA-100 of 66 healthy people was used to predict cut-off value in our population. PFA-100 test results and serum TXB_2, sCD40L and sP-selectine levels of the patients were compared with each other. A TXB_2 level of ≥80 ng/ml was accepted as an indicator of aspirin non-responsiveness.

Results: According to PFA-100, 40% of the patients revealed aspirin non-responsiveness which was found 8% with serum TXB2 levels. Patients with aspirin non-responsiveness according to the PFA-100 showed significantly higher serum TBX_2, sCD40L and sP-selectin ($p<0.001$, p,=0.018, p=0.037 respectively).

Conclusions: PFA-100 results showed higher frequency of aspirin non-responsiveness in our population, however biochemical tests of the platelet function indicated the possibility of lower ratios. Therefore the prevelance of aspirin non-responsiveness may not be so frequent in our population as established before and PFA-100 test results need confirmation with the serum TXB2 level.

Key Words: Aspirin resistance; PFA-100; thromboxane B_2; ($TXB2_)$; CD40 ligand, sP-selectin
Conflict of interest: Authors have no conflict of interest.

ÖZET

Amaç: Aspirin tedavisi alan hastalarda tekrarlayan tromboembolik olayların sıklığının toplumda %5.5-60 olduğu bildirilmiştir. Bu çalışmada, toplumumuzdaki aspirin cevapsızlığı sıklığının "*Platelet Function Analyzer-100 (PFA-100)"* kapanma süresi (CT), serum tromboksan B_2 (TXB_2), sCD40 ligand ve sP-selektin düzeyleri ile taranması amaçlanmıştır.

Materyal ve metod: Günlük 80-300 mg aspirin kullanmakta olan hastalar (n=118) çalışmaya alındı. Sağlıklı 66 bireyin PFA-100 CT'si kullanılarak toplumumuz için "cut-off" değeri belirlendi. Hastaların PFA-100 test sonuçları, serum TXB_2, sCD40L ve sP-selektin düzeyleri ölçülerek birbirleriyle karşılaştırıldı. Bu esnada TXB_2 düzeyinin ≥80 ng/ml olması aspirin cevapsızlığının bir göstergesi olarak kabul edildi.

Bulgular: PFA-100 testine göre hastaların %40'nda, serum TXB_2 sonuçlarına göre ise hastaların %8'inde aspirin cevapsızlığı bulundu. PFA-100 testine göre cevapsız hastalarda serum TXB2, sCD40L ve sP-selektin düzeyleri aspirine duyarlı olan kişilere göre daha yüksek bulundu ($p<0.001$, p=0.018, p=0.037).

Sonuç: Bundan önce bulunan sonuçlara benzer şekilde toplumumuzda PFA-100 testi ile aspirin cevapsızlığı oranının oldukça yüksek çıktığı, diğer laboratuvar belirteçlerinin sonuçlarına göre bu oranın daha düşük olabileceği anlaşılmaktadır. PFA-100 testi pozitif bireylerde sonucun serum TXB2 düzeyi ile doğrulanması önerilir.

Anahtar kelimeler: Aspirin direnci; tromboksan B_2 ($TXB2_)$; PFA-100; CD40 ligand, sP-selektin
Çıkar Çatışması: Yazarların çıkar çatışması yoktur.

Introduction

Aspirin (acetyl salicylic acid) is a trusted drug in the prevention of atherothrombotic clinical events. It irreversibly inhibits platelets by acetylating cyclooxygenase-1 (COX-1) enzyme, thereby interfering the synthesis of cyclic prostanoids: thromboxane A_2 (TXA_2), prostacyclins and other prostanoids. The decrease in the synthesis of prostaglandins and TXA_2 results with antithrombotic action [1,2]. Inhibition of COX-1 is rapid, saturable at low doses and lasts for the platelet's lifespan (8-10 days). The platelets are anucleated and they can not generate new COX.

Several studies have shown that chronic daily administration of low-dose aspirin (40-60 mg) is sufficient to supress TXA_2 synthesis as higher doses of aspirin (500 mg) [3-6]. However, in a substantial portion of patients, aspirin does not inhibit platelet function and thromboembolic events may occur. This situation in which aspirin can not reduce production of TXA_2 in platelets and thereby platelet activation/aggregation persists in vivo and in vitro, is defined as clinical aspirin resistance or aspirin non-responsiveness [7,8]. Aspirin resistance has been determined in 5.5-60% of the population by different assays in patients with various vascular diseases [9-11]. The reason of the aspirin non-responsiveness is likely a combination of clinical, biological and genetic properties affecting platelet function. Measurements of platelet aggregation, platelet activation, glycoprotein expression and bleeding time have all confirmed variability in patients' antithrombotic responses to aspirin therapy.

Several different platelet function tests are in use to diagnose aspirin resistance or non-responsiveness such as Optical Platelet Aggregometry (OPA), Platelet Function Analyzer-100 (PFA-100), VerifyNow system (VN, recently known RPFA or Ultegra), serum TXA_2, serum TXB_2, urinary TXB_2, serum sP-selectine and sCD40 ligand. Many studies have been reported to have the advantages and disadvantages of the non-spesific tests including small sample size, different dose regimens and non-adherence [12,13].

All these reasons directed us to show the platelet activation by measuring the serum concentrations of thromboxane B2 (TXB2), sP-selectine and sCD40 ligand to confirm and compare the results of PFA-100 system. Measurement of TXA_2 production in terms of the serum TXB_2 level was used to assess biological effect of aspirin [14]. P- selectine, an adhesion molecule and a constitutent of the platelet α-granule membrane, is released from activated platelets [15]. sCD40 is also other protein expressed by platelet activation, 95% of sCD40L in blood is known to be associated with the platelets [16].

Many mechanisms were proposed to explain aspirin resistance or non-responsiveness (non-compliance, inadequate aspirin dosage, drug interactions, genetic polymorphsims, up-regulations of non-platelet pathways and increased platelet turnover) [17,18].

In this study, we aimed to determine aspirin non-responsiveness in our population according to the results of the platelet function analyzer (PFA-100), serum TXB_2, sP-selectine and sCD40 ligand and compare these different platelet function tests in patients on regular aspirin therapy.

Material and Methods

One hundred-eighteen patients (70 women and 48 men) taking 80-300 mg/day aspirin at least for the last four weeks were enrolled into the study. Patients were screened during their routine follow-up visits at our outpatient clinic. Patients taking ticlopidine, dipyridamole, non-steroidal anti-inflammatory drugs (NSAIDs) and any other drug containing acetylsalicylic acid or NSAID, patients with any infectious disease and with personal/family history of bleeding disorder, hematocrit (Htc) <25%, platelet count <125 $x10^3/mm^3$ or >450$x10^3/mm^3$ and serum creatinine level >2.5 mg/dl were excluded.

Platelet function was determined by the PFA-100 (Platelet Function Analyzer, Siemens, Marburg, Germany) in a fasting sample 24 h after the last dose of aspirin. Citrated blood (0.129 mol/l trisodium citrate,1:10) was used and the analyses were performed within 2 h after blood collection. The PFA-100 measures the time (closure time) required for platelets to plug an aperture in the membrane of the test cartridge, simulating an injured vessel. The membrane is coated with collagen and an agonist (ADP or epinephrine). The collagen/epinephrine (CEPI) cartridge is the primary cartridge for the detection of aspirin effect on plateletes. The reference range of CEPI cartridge is 84-160 s based on values 127 healthy subjects [19]. Maximum closure time is 300 s and any value greater than 300 s is accepted as nonclosure. PFA-100 CT of 66 healthy people were used to predict cut-off value for aspirin non-responsiveness in our population. The normal range for the PFA-100 was calculated as "mean±2SD (standart deviation)" of the healthy volunteers. Then 95th percentile of the baseline PFA-100 CT values were used as cut-off value for our population which was <174 s. Precision testing revealed an inter-assay CV of < 10% for collagen/ADP cartridges and a <5% for the collagen/epinephrine cartridges. Previously, Fontana et al. defined aspirin non-responsiveness by PFA-100 (Dade Behring, Marburg, Germany) as collagen and epinephrine CT <186 s, and serum TxB_2 ≥80 ng/ml [14]. During aspirin treatment, PFA-100 CT <174 s together with serum TxB2 <80 ng/ml was accepted as aspirin pseudo-resistance.

Serum total cholesterol, LDL-cholesterol, urea, creatinine levels were measured by Roche modular system (Mannheim, Germany). Hemoglobin (Hb), Htc, platelet count (PLT), mean platelet volume (MPV) were determined by Coulter HMX analyzer (Miami, U.S.A). Serum sP-selectine, sCD40 ligand (Biosource, California, U.S.A) and TXB_2 concentrations (Cayman Chem., Miami, U.S.A.) were measured by ELISA. This study was appro-

ved by the local ethical committee of Istanbul University Istanbul School of Medicine and written consents were obtained from all volunteers those enrolled into the study.

Continuous variables were given as mean±SD. Categorical variables were compared using chi-square test. Mann-Whitney U test was used to compare the means and correlation analysis was done by using Pearson test. A p value of <0.05 was considered statistically significant. Statistical analyses were performed with SPSS 15.0 (SPSS Inc, Chicago, Illinois, U.S.A).

Results

Baseline characteristics of the study population are shown in Table 1. The cut-off value for PFA-100 CT was accepted as 174 sec depending on the results of our healthy population. According to PFA-100, 47 of 118 patients (40%) were aspirin non-responders, males showed increased aspirin non-responsiveness when compared to females (46% vs 36%) and 41% of the elderly patients (≥ 65 years) revealed non-responsiveness. No significant difference was found between aspirin responders and non-repsonders when age, sex, history of diabetes mellitus, blood HbA1c, Hb concentration, platelet count and MPV levels, serum creatinine, ALT, AST, TSH, LDL, HDL and triglyceride levels were taken into consideration. Bleeding time was shorter in patients with aspirin non-responsiveness (117±67 vs 160±174 s, p=0.01).

Results of the platelet function tests were summarized in Table 2. Patients with aspirin non-responsiveness according to the PFA-100 showed higher serum TXB_2, sCD40L and sP-selectin levels (TXB_2: 45.02±28.40 vs 13.86±12.15 ng/ml, p <0.001; sCD40L: 0.88±0.72 vs 0.66±0.64 ng/ml, p=0.018; sP-selectin: 35±20 vs 30±31 ng/ml, p= 0.037). Ten patients had serum TXB_2 levels ≥80 ng/ml (8%) and only 6/47 patients with PFA-100 CT <174 sec had serum TXB_2≥80 ng/ml (Table 3).

Table 1. Baseline characteristics of the study population

	PFA-100 results		P value
	Aspirin non-responders (n=47)	Aspirin responders (n=71)	
Age (mean ± SD)	56±13	58±12	NS
Women / Men	25/22	45/26	NS
Diabetes mellitus	10	16	NS
Hb (g/dl)	13.3±1.6	13.5±1.4	NS
Platelet count (10^3 /mm^3)	269±66	275±84	NS
Mean platelet volume ,fl)	8.7±0.9	8.4±1.0	NS
Bleeding time (second)	117±97	160±174	0.01
Creatinine (mg/dl)	0.9±0.2	0.9±0.2	NS
Total cholesterol (mg/dl)	202±48	194±39	NS
Triglycerides (mg/dl)	161±116	139±62	NS
ALT (U/l)	30.4±44.1	22.8±10.6	NS
AST (U/l)	21.2±9.3	21.0±8.6	NS
TSH (IU/l)	2.0±1.9	2.1±1.3	NS
HbA1c (%)	7.1±1.8	6.9±1.7	NS

Hb; hemoglobin, AST; aspartate aminotransferase, ALT; alanine aminotransferase, TSH; thyroid stimulating hormone, NS; not significant.

Table 2. Cyclic prostanoid levels of the patients in aspirin responder and non-responder groups by the PFA-100

	PFA-100 results		P Value
	Aspirin non-responder (n=47)	Aspirin responder (n=71)	
TXB_2 (ng/mL)	45,02±28,40	13,86±12,15	<0.001
sCD40L (ng/mL)	0,88±0,72	0,66±0,64	0,018
sP-selectin (ng/mL)	35±20	30±31	0,037

Table 3. Aspirin responsiveness of the patients by PFA-100 and serum TXB_2

PFA-100	TXB_2 ≥80 ng/ml	TXB_2 <80 ng/ml	Total
Closure time <174 s	6	41	47 (40%)
Closure time ≥174 s	4	67	71 (60%)
Total	10 (8%)	108 (92%)	118

According to data after exclusion of diabetic patients, 37 patients (40%) had PFA-100 < 174 sec which were aspirin non-responders and 55 patients had normal PFA-100 results. Bleeding time was shorter in patients with aspirin non-responsiveness (117±97 vs 160±174 sec, p=0.013). Aspirin non-responders according to the PFA-100 showed higher serum TXB_2, sCD40L and sP-selectin levels, however difference in sP-selectin levels did not reach statistical significance (TXB_2: 49.96±89.95 vs 13.91±32.75 ng/ml, p =0.041; sCD40L: 0.88±0.72 vs 0.66±0.64 ng/ml, p=0.04, sP-selectin: 35±21 vs 31±31 ng/ml, p= 0.4). Eight patients had serum TXB_2 levels ≥80 ng/ml (8.6 %) and only 5/37 patients with PFA-100 CT <174 sec had serum TXB_2≥80 ng/ml. Aspirin pseudo non-responsiveness was 37.8 %.

Discussion

The prevalance of aspirin non-responsiveness or resistance was reported 5.5 to 60% according to the method used to measure platelet functions. In our study, 40% of the patients were aspirin non-responders by the PFA-100 test, while only 8% of the patients had serum TXB2 levels ≥80 ng/ml. Incoherence between different platelet function assays limits the reliability of these tests and may cause unrealistic increases in aspirin non-responsiveness.

Although optical platelet aggregometry (OPA) is accepted as a "gold standart" in the assessment of in vitro platelet function, PFA-100 and RPFA are simple and rapid point of care tests that are used in studies. In vitro platelet function tests (e.g. turbidimetric aggregometry, PFA-100 and VerifyNow) underestimate thromboxane production from non-platelet sources that will result in in vivo platelet activation [20]. Therefore it may be difficult to assess aspirin responsiveness with these tests when they are used alone. PFA-100 test was proposed as an ideal and rather physiological platelet function test because of using whole blood instead of platelet rich plasma and having a high shear system. Analyzers with high shear system have superiority because cyclooxygenase inhibitors (e.g. aspirin) and purinergic receptor inhibitors (e.g. clopidogrel) are being used primarily for arterial disorders which are characterized by high shear rate [21,22]. However, there are many preanalytical factors such as platelet function, platelet count, red blood cells and plasma von Willebrand factor are known to affect the PFA-100 results. High prevalance of short PFA-100 CT has been associated with high vWF in aspirin-treated patients [23].

Gum et al. reported poor correlation between OPA and PFA-100 in detection of aspirin resistance, and in their recent work, long-term outcomes (death/ myocardial infarction/ stroke) were not found to be related with aspirin resistance determined by PFA-100 [24,25].

In our study, serum TXB_2 levels were significantly reduced in aspirin-sensitive patients compared to aspirin-nonresponder group (according to PFA-100 test) as is true for sCD40 ligand and sP-selectin. However, only few patients had increased serum TXB_2 levels despite aspirin treatment. Fontana et al. proposed serum TXB_2 as a reliable test in the evaluation of aspirin resistance [14].

Many factors may increase the risk of aspirin non-responsiveness. Transient expression of COX-2 in newly formed platelets during increased platelet turnover such as infection, inflammation and following major surgery can lead to an increased proportion of non-aspirinated sources of TXA2 during 24-hours period [26,27]. Extraplatelet sources of TXA_2 (e.g. monocyte/macrophage COX-2) cause aspirin insensitive TXA_2 in acute coronary syndrome [28]. However, Pamukcu et al did not show any significant differences in major risk factors of coronary artery disease, number of involved coronary vessels, serum lipid levels, and blood counts between aspirin-resistant and aspirin–sensitive subjects [11].

In our study, we could not reveal any differences in platelet count, hemoglobin, cholesterol, triglyceride and HbA1c% levels. The other reason of reduced efficiency in aspirin treatment was reported in the patients with diabetes mellitus [29]. High blood glucose causes in glycation of platelet proteins, resulting them less accessible to acetylation, potentially predisposing to treatment failure [30]. But in the present study, no differences were existed in HbA1c % levels and the history of diabetes between two groups. Furthermore, similar data was achieved after diabetic patients were excluded from the study population.

Concomitant intake of NSAIDs (e.g. ibuprofen and naproxen) has been reported to prevent access of aspirin to the COX-1 substrate-binding site causing impaired supression of platelet COX-1. This effect has not been shown with COX-2 selective rofecoxib or diclofenac [31]. Poor compliance, drug interactions especially those reduce enteral absorption of aspirin such as proton pump inhibitors [32-34], long-term aspirin treatment (tachyphylaxis) [35] and genetic polymorphisms involving COX-1 and COX-2 have been reported as the reasons for reduced response to aspirin treatment [36,37].

Our results suggest that aspirin non-responsiveness is not so frequent in our population as reported previously by the point of care test PFA-100. The confirmation of the results with TXB2 level in the patients which are determined aspirin non-responsiveness by the PFA-100 CT will reduce the risk of future thrombotic complications and improve their long-term outcome. Further studies are needed to establish the current ratio of true aspirin non-responsiveness in our population.

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