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Research Article

Prevalence of Aspirin Resistance among Jordanian Patients with Cardiovascular Disease

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ABSTRACT

Introduction: Despite the wide range of aspirin indications, there is a considerable amount of patients do not respond to aspirin, who also are referred to as aspirin non-responders or aspirin resistant patients. Aims and objectives: This study was carried out to prospectively evaluate the prevalence of aspirin resistance in Jordanian patients with cardiovascular disease and further clarify the clinical predictors of aspirin resistance. Materials and methods: Biochemical aspirin response was assessed based on the measurements of urinary11-dehydro thromboxane B_2 (11-dhTxB_2) levels using FDA approved diagnostic kit. Patients taking aspirin (75-325mg) for at least 7 days were prospectively enrolled from all stable cardiac patients presenting at Jordan University hospital outpatient clinics. Results: Eighty six (86) patients were enrolled in this study. Another twenty four healthy individuals were enrolled to function as a control group. The mean urinary levels of 11- dhTxB_2/creatinine were significantly lower almost 3- times in patients in the primary and secondary aspirin prevention group compared with the control group (1567.58 vs. 4236.19 pg/mg, p-value <0.005). Thirty-one patients were found to be aspirin resistant with a prevalence of 36%. Conclusion: Our findings of aspirin resistance are particularly important given the large number of patients using this medication for prevention of atherothrombotic events. These results indicate that aspirin resistance should be diagnosed so that individuals with no response to aspirin can receive an alternative or an additional antiplatelet therapy.

Keywords: Aspirin resistance, dehydrothromboxane B_2, cardiovascular disease, Jordan.

INTRODUCTION

Cardiovascular diseases (CVD) are responsible for the majority of deaths in the developed countries[1,2]. Aspirin is recommended by different agencies for primary and secondary prevention of different cardiovascular diseases[3,4]. It exerts its effect by the inhibition of cyclooxygenase enzyme which is responsible for the conversion of arachidonic acid to prostaglandins (PG) and thromboxane A_2 (TxA_2)[3,4].

Despite the wide range of aspirin indications, there is a considerable amount of patients do not respond to aspirin, who also are referred to as aspirin non-responders.

The term Aspirin resistance is used to describe different events including: i) the failure of aspirin to protect against thrombotic complications; ii) the inability of aspirin to result in prolonged bleeding time; iii) the failure of aspirin to inhibit TxA_2 production and iv) the inability of aspirin to prevent platelet function *in vitro*[5,6]. Aspirin resistance is defined as laboratory and clinical resistance[7]. Laboratory aspirin resistance can be defined as the failure of aspirin to inhibit the production of platelet TxA_2 or inhibit tests of platelet function that depend on platelet thromboxane production. However, clinical aspirin resistance is the failure of aspirin to protect the patient from an ischemic event despite regular intake of appropriate doses[7].

Different methods have been employed for the detection of aspirin resistance but none of them is considered a specific and sensitive test to be routinely recommended as a screening method to detect aspirin resistance in clinical practice[8]. On the other hand, urinary levels of 11-dehydrothromboxane B_2 (11-d $HTxB_2$) have been proved to coorrelate with cardiovascular mortlity in high risk patients treated with low dose aspirin[9]. and are directly dependent on aspirin's target COX-1[8,9]. 11-d HTxB2 levels also reflect *in vivo* thromboxane production[8] and are measured by a non-invasive method which is normalized with standard controls[10].

This study was carried out to prospectively evaluate the prevalence of aspirin resistance in Jordanian patients with cardiovascular disease using urinary 11-d$HTxB_2$ level measurement and further clarify the clinical predictors of aspirin resistance.

MATERIALS AND METHODS

Patients and controls

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Table 1: Demographic and clinical characteristics of patients (N=86).

Patient Characteristic	(Frequency) % or mean ± SD
Age of patients (mean ± SD)	58.8 ± 10.7
Min	28
Max	83
Gender (females)	(30) 34.9%
Primary vs. secondary prevention of CVD	(37) 43 % vs. (49) 57%
Diabetes	(43) 50%
Hypertension	(81) 94.2%
Congestive Heart Failure (CHF)	(8) 9.3%
Atrial fibrillation (A Fib)	(4) 4.7%
Peripheral arterial disease (PAD)	(3) 3.5%
Dyslipidemia	(71) 82.6%
Coronary artery disease (CAD)	(52) 60.5%
History of myocardial infarction (MI)	(11) 12.8%
History of stroke	(5) 5.8%
Aspirin dose (mg/ day)	
75	(2) 2.3%
81	(1) 1.2%
100	(61) 70.9 %
150	(1) 1.2%
162.5	(6) 7.0%
325	(15) 17.4%
Smokers	(11) 12.8%

Table 2: Drug therapy regimen of patients (N = 86).

Drug category	Frequency (N) %
B blockers	(57) 66.3%
Statins	(53) 61.6 %
ACE inhibitors*	(33) 38.34 %
ARBs **	(20) 23.3%
Thiazide diuretics	(18) 20.9%
Loop diuretics	(18) 20.9%
Clopidogrel	(23) 26.7%
Metformin	(23) 26.7%
Sulfonylureas	(16) 18.6%
Warfarin	(4) 4.7%

*ACE: angiotensin converting enzyme

**ARB: angiotensin receptor blocker

Patients were prospectively enrolled from all stable cardiac patients presenting to Jordan University (JUH) hospital outpatient clinics.

All patients who were ≥ 21 years old and who had taken 75-325 mg of aspirin for at least 7 days were eligible for enrollment. Exclusion criteria included: ingestion of other non-steroidal anti-inflammatory drugs (NSAIDs), myocardial infarction or ischemic stroke within the period < 6 weeks. The study was reviewed and approved by the Institutional Review Board of JUH. All participants gave informed written consent before enrollment. The patient group was compared to a control group of healthy individuals who are not taking aspirin.

Materials

Reagents and pharmaceutical products

Stabilized antibody (goat) coated microwells with frame, sample diluent, reference solution (11- dhTxB_2 in buffer), alkaline phosphatase-tracer solution (purified 11- dHTxB_2 conjugated to alkaline- phosphatase), antibody solution (purified anti 11- dhTxB_2 antibody) stabilized para-nitrophenylphosphate (pNPP) substrate, stopping solution ethylenediaminetetra acetic acid (0.1M EDTA), tris buffered saline TBS/Tween wash concentrate. All reagents were purchased from Corgenix Inc (USA).

Instruments and apparatus

Biotek Microplate reader (USA), Centrifuge from Hettich (Germany), Spectrophotometer (Spectroscan80 D (Germany)), plate shaker HeidolphUnimax (Germany).

Methods

In this study, we used a method to measure the concentration of urinary 11- dhTxB_2 which function as a substitute for the measurement of TxA_2. TxA_2 has extremely short half-life and is rapidly cleared from the bloodstream. Furthermore, arachidonic acid release from biological membranes (and thus synthesis of TxA_2) is likely to be phasic rather than continuous (FitzGerald et.al, 1983) Urine samples were analyzed to measure the level of 11- dhTxB_2 using a special ELISA kit called Aspirin Works® Test Kit from Corgenix Inc. (USA). It measures urinary 11- dhTxB_2 and is performed as a competitive ELISA.

Urine samples of both patients and controls were brought to room temperature and centrifuged at 4000xg for 4 minutes. A hundred microliter of both patients' and controls' urine were added to 400 µL of sample diluent in Eppendorf tubes and were vortexed (1:5 sample dilution). One hundred microliters (100 µL) of all dilutions (patients' urine samples, controls' urine samples, and reference solution dilution) were pipetted into well of the microplate provided with the kit. Duplicate well determination was performed for all of the above dilutions. Fifty microliters (50 µL) of AP-tracer solution were added to each of the reference solution, patient sample and control wells. Fifty microliters (50 µL) of antibody solution were added to each well of the reference, patient and control samples. The plate was covered with an adhesive cover and then incubated for 2 hours at room temperature on a rotary shaker at 300 rpm. Incubation allows the endogenous 11-dhTxB_2 present in the samples to compete with the purified AP-conjugated 11- dhTxB_2 for binding to the mouse monoclonal anti-11-dhTxB_2 antibody. The monoclonal antibody then binds to the poly-clonal anti-mouse antibody coated on the micro-titer plate. The complex formed on the plate is composed of monoclonal antibody and endogenous or AP-conjugated 11- dhTxB_2.

After removal of unbound complexes by washing, the bound AP-conjugated 11- dhTxB_2 is assayed by the addition of 200 µL pNPP chromogenic substrate. The plate was covered and then incubated for another 30 minutes at room temperature with rotary shaking. Color develops in the wells at intensity inversely proportional to the sample urine concentration of 11- dhTxB_2 and is read at 405nm. After incubation was completed, 100 µL of the stopping solution were added to each well to stop the enzyme reaction. Results (pg/ml) were calculated against a

Table 3: Patients' characteristics for aspirin-resistant and aspirin-sensitive patients.

Variable	Aspirin-resistant patients (n=31)	Aspirin-sensitive patients (n=55)	*p*-value
Age	60.7 ±11.1	57.7 ± 10.5	0.217
Gender (female)	(10) 32.3%	(20) 36.4%	0.701
Hypertension	(30) 96.8%	(51) 92.7%	0.650
Diabetes	(16) 51.6 %	(27) 49.1%	0.822
CAD	(15) 48.4%	(37) 67.3%	0.085
CHF	(3) 9.7%	(5) 9.1%	0.928
History of MI	(5) 16.1%	(6) 10.9%	0.486
History of stroke	(4) 12.9%	(1) 1.8%	0.055
Use for secondary prevention	(15) 48.4%	(34) 61.8%	0.227
Cigarette smokers	(5) 16.2%	(6) 10.9%	0.486
Dyslipidemia	(23) 74.2%	(48) 87.3%	0.125

reference curve prepared from the reference solution provided in the kit (Six different concentrations of the reference solution (11- $dhTxB_2$ in buffer) were freshly prepared by serial dilution with the following concentrations 5000, 2500, 1250, 625, 312.5 and 156.25 pg/ml). Final results were reported as the ratio of the amount of 11- $dhTxB_2$ (pg) to the amount of creatinine (mg) in the urine sample to normalize results for urine concentration. Urinary creatinine concentrations were determined using the kinetic method. *Statistical analysis*

Data were analyzed using SPSS© software (version 16; SPSS, Inc, Chicago, IL).

For all statistical analysis, *p*-values of 0.05 or less were considered statistically significant. Continuous variables are presented as mean ± SD and categorical variables are presented as frequencies and percentages. Unpaired sample t test and nonparametric Mann–Whitney test were used to compare between continuous variables. Chi-square or Fisher exact test were used to compare between categorical variables.

RESULTS

Demographic and clinical characteristics of patients

Eighty six (86) patients from Jordan University hospital clinics were enrolled in this study. Thirty five percent were females, 50% were diabetics, 12.8% were smokers and 57% were taking aspirin for secondary prevention of CVD. The mean age of patients was 58.8 ± 10.7 years. The most commonly prescribed dose of aspirin was 100 mg daily (70.9 % of patients). Twenty four healthy individuals were enrolled to function as a control group (mean age was 34 ± 9.5 years, 58 % females). The control subjects were not age-matched with the patients due to the high number of older people who are already on prophylactic long-term aspirin therapy.

Table (1) summarizes the demographic and clinical characteristics of patients. The most commonly prescribed drugs were beta blockers, statins, angiotensin converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARBs), clopidogrel, and metformin as shown in Table (2)

The mean urinary levels of 11- $dhTxB_2$/creatinine were significantly lower almost 3- times in patients in the primary and secondary aspirin prevention group compared with the control group of healthy individuals who were not taking aspirin (1567.58 vs. 4236.19 pg/mg, *p*-value <0.005). Thirty-one patients, who were taking aspirin, were found to be aspirin resistant (11- $dhTxB_2$/creatinine>1500 as specified by the test kit) with a prevalence of 36%. Aspirin resistance was associated with no significant differences with regard to age, gender, diabetes, hypertension, coronary artery disease (CAD), smoking, aspirin dose and the aim of aspirin use (primary vs. secondary prevention). Results are summarized in table (3).There were no significant differences between the aspirin-resistant and aspirin-sensitive group when comparing different drugs including; statins, ACE inhibitors, beta blockers, and ARBs as summarized in Table (4).

DISCUSSION

Among patients with cardiovascular diseases, our study showed that 36% are aspirin resistant. Considering the standard guideline use of aspirin and its wide indications to decrease atheroembolic events by its antiplatelet action, this prevalence is particularly of great potential clinical importance. Prevention of atheroembolic events with aspirin treatment may not be achieved in all patients, such as those in this study who did not attain aspirin's antiplatelet effect. Further research concerning clinical predictors of aspirin resistance is needed. Although findings from our study indicate that aspirin resistance is not significantly associated with age or gender, larger numbers are needed to make definitive statements about demographics that are associated with aspirin resistance.

In other populations, the prevalence of aspirin resistance is higly variable (5-60%), this can be attributed to the method used for detection and the population studied[10]. In a cohort of American cardiac patients, aspirin resistance was found in 5-9% of patients using the methods of platelet aggregation and platelet function analyzer (PFA 100). Aspirin semi-responders accounted for an additional of

Table 4: Drug therapy in aspirin-resistant and aspirin-sensitive patients.

Drug category	Aspirin resistant (n=31)	Aspirin sensitive (n=55)	*p*-value
B blockers	(20) 64.5%	(37) 67.3%	0.795
Statins	(16) 51.6%	(37) 67.3%	0.152
ARBs	(8) 25.8%	(12) 21.8%	0.674
ACE inhibitors	(10) 32.3%	(23) 41.8%	0.389
Thiazide diuretics	(9) 29.0 %	(9) 16.4 %	0.166
Loop diuretics	(7) 22.6 %	(11) 20.0 %	0.778
Clopidogrel	(5) 16.1%	(18) 32.7%	0.095
Sulfony urea	(5) 16.1 %	(11) 20.0%	0.658
Metformin	(6) 19.4 %	(17) 30.9%	0.245
Warfarin	(1) 3.2%	(3) 5.5%	1.00

23% of patients[11]. The total number of aspirin non-responders in this cohort (28-34%) is not far from that in our study (36%).

Observations in this study did not show any significant differences among patients at different levels of CVD risk. Although most of the frequently discussed possible risk factors for aspirin resistance are found in patients enrolled in this study, their real clinical relevance remains unclear. Diabetes is considered as one of the possible risk factors of aspirin resistance [11-12]. In our study, we couldn't not prove a significant difference in the biochemically measured aspirin efficacy between diabetics and nondiabetics and this was consistent with the findings of other studies[11,14]. This diversity can be explained again in terms of the different methods used to evaluate aspirin resistance in these studies, as well as to different populations studied for example, Mehta *et al*, included both type 1 and type 2 diabetic patients[12] , while others included only type 2 diabetics[13]. Another important limitation to the studies that assess the rate of aspirin resistance in diabetes is the assessment of platelet function and reactivity using methods that are independent of TxA_2 release. These tests do not specifically measure how effectively aspirin has inhibited its target which is COX-1 enzyme[15]. When other methods for evaluating aspirin resistance were used, mainly those dependent on thromboxane release (e.g. serum TxB_2 levels or other surrogate markers of arachidonic acid-induced platelet aggregation); the prevalence of aspirin resistance was very low[15].

Previous studies have demonstrated that smoking[16], congestive heart failure[17] and history of stroke[18] are associated with aspirin resistance. Although this was not the case in our study, small sample size may be the reason for this disparity.

Different doses of aspirin inhibit serum TxB_2 levels and urinary excretion of thromboxane metabolite in a dose dependent manner (99 % vs. 95 % average inhibition of serum thromboxane by 160 and 80 mg/day respectively and 77% and 61 % average inhibitions of urinary excretion of thromboxane metabolites by 160 and 80 mg/day respectively). This suggests that in some instances, such difference would translate into a greater clinical benefit with higher aspirin dosage[19]. These small differences between different doses of aspirin were not translated into real differences in clinical practice as was the case in our study. There was no significant difference in the levels of 11-dhTxB_2 between patients using different doses of aspirin.

Many different drugs (statins and ACE inhibitors), by their effect on oxidative stress, nitric oxide metabolism, inflammation, and coagulation, can represent the ideal candidates to increase antiplatelet effectiveness of aspirin in diabetic patients. Our results did not prove that statins and ACE inhibitors are associated with lower prevalence of aspirin resistance among diabetic patients (*p*-value for statins was 0.594 and for ACE inhibitors 0.189).

The most relevant factors for aspirin treatment failure are patient non-compliance and interaction with ibuprofen. The need for long term daily treatment of aspirin predisposes to non-compliance, which may result in high risk of atherothrombotic events. The role of aspirin non-compliance in the prevention of aspirin resistance is well documented. Several studies showed that when patient compliance was enhanced and ensured, the frequency of aspirin resistance decreased dramatically[20,21]. All patients enrolled in this study were compliant to aspirin as assessed in the data collection sheet.

The clinical relevance of interactions of aspirin with ibuprofen was also proved in clinical trials. The concomitant treatment with ibuprofen may lead to the failure of aspirin protection, as ibuprofen binds to the same target place of COX-1, thus preventing aspirin from its antiplatelet effect [22, 23]. For this reason, patients using NSAIDs were excluded from this study.

One option to overcome aspirin resistance is to provide a newer antiplatelet agent like clopidogrel. The effect of clopidogrel on aspirin resistance may be well characterized by the addition of clopidogrel to already known aspirin resistant patients and evaluating their status after a certain follow up period[9].

There are some limitations to this study. The number of patients of this study was small to draw epidemiologic conclusions. Nevertheless, it suggests the need for studying larger cohorts to make an accurate estimate of the frequency of aspirin resistance and factors affecting it. This modest-sized, heterogeneous group of patients makes

it difficult to assess the effect of different CVDs, concurrent use of other medications, and demographic background on aspirin resistance. Examination of various well-characterized populations is necessary to fully elucidate the epidemiology of aspirin resistance. However, data shown here reflect what is seen in an actual large community practice.

One more limitation in this study is related to thromboxane metabolism. Although the majority of endogenous TxA_2 comes from activated platelets, there are some alternative sources of thromboxane synthesis via COX-2 pathway that is not affected by aspirin. These include young platelets, monocytes, macrophages and endothelial cells especially in pathological conditions of inflammation and high-risk CVD, therefore results of this test may be inflated and, thus, should be reported cautiously as a basis for therapeutic decision making.

For now, every effort should be done to improve patients' compliance and to prevent clinically relevant interactions of aspirin with ibuprofen. The elimination of these two factors may provide better efficacy of the antithrombotic effect of aspirin.

Some researchers argue that aspirin resistance may not be absolute over time and measurement of aspirin resistance should be performed more than once as a single measure may overestimate its prevalence[14]. Thus, measurement of aspirin resistance prospectively should be recommended.

CONCLUSION

Our findings of aspirin resistance are particularly important given the large number of patients using this medication for prevention of cardio-embolic events. These results indicate that aspirin resistance should be diagnosed so that individuals with no response can receive alternative or additional antiplatelet therapy. Moreover, mechanistic work to fully elucidate the biology of aspirin resistance is clearly warranted.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest to disclose.

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