Aspirin Resistance in Different Doses by Bleeding Time and Urinary 11-dehydro-thromboxane B2

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Abstract

The aim of the present study was the evaluation bleeding time (BT) in comparison to Urinary 11-dehydro thromboxane B2 (TXB2) regarding different ASA frequent dosages used in Borujerd city. This is a double blind randomized clinical trial on 370 subjects aged 35 years and older, referred to clinical offices in Borujerd. All ischemic heart disease’s patients were randomly assigned to 4 ASA dose groups (80 mg, 81 mg, 100 mg and 325 mg) and one group-matched control group without any IHD. BT was measured by Ivy method; TXB2 was measured in a urine sample, both at least 5 days after ASA consumption. Probale AR was indicated if TXB2 was normal or higher than normal higher limit values, or BT was normal or lower than normal higher values. (IRCT201202026958N3) Probale AR was present in 37.6% and 64% resistance by BT and TXB2, respectively. All 4 treated groups had higher TXB2 levels than the control group/normal values (p>0.05). Also, urinary TXB2 level correlated positively with BT. Given the simplicity and low costs of its performance it might be of some potential use in developing countries. However, due to IVY method limitations it cannot be perceived as a tool to assess such specific aspects of platlat function or aspirin resistance.
Introduction

Aspirin, also known as acetylsalicylic acid (ASA) is one of the most used drugs worldwide. It was discovered in 1890s as an anti-inflammatory drug; however, its anti-platelet activity was not known till the 1960s. Nowadays, ASA is used as an antipyretic, anti-inflammation and anti-platelet agent (1, 2). ASA is absorbed by the upper-GI in less than an hour and interrupts the platelet activity by inhibiting the production of thromboxane (Specially TXA2), which under normal circumstances binds platelet molecules together to create a patch over damaged walls of blood vessels (3, 4). Low dose ASA (80 mg) has been effective on lowering the risk of myocardial infarction (MI) and cerebral vascular attacks (CVA) and furthermore near 25% reduction in mortality in patients with other cardiac risk factors; however, different doses of ASA are used for different patients (5-7). Aspirin resistance (AR) is a newly introduced term. The Laboratory definition of AR is failing in inhibition of TXA2 or failing in reduction of platelet aggregation; which results in increase of TXA2 metabolites such as TXB2 in plasma and urine. Additionally, Clinical AR is defined as failing in prevention of ischemic events in the patients (8-10). As ASA consumption can increase Bleeding Time (BT), this test can be used for assessing its clinical effect regarding the BT individual or population baseline (9, 10). However, both test are not quite accurate and should be used with caution. There are several ways to evaluation of AR. A method that has been used extensively is measurement of the urinary TXA2 metabolite 11-dehydro-TXB2 using both radioimmunoassay (RIA) and enzyme immunoassay (EIA) (11). Another automated, point-of-care platelet function assay, Platelet Function Analyzer 100 (PFA-100), has also been introduced; most of them are expensive and hard to interpret (8-11). There are several studies which have compared these tests to find the best method to assess AR; however, the results were controversial (8-11). Regarding different doses of ASA used in this field and several complications which can occur due to AR, finding a regional-tested, cost-benefit and easy-to-use method seems to be inevitable. This study was aimed to measure BT and Urinary 11-dehydro-TXB2 regarding different ASA frequent dosages and calculate probable AR population in Borujerd, Lorestan; a city in western Iran.

Materials & Methods

Experimental Design

This is a double blind, placebo controlled, parallel designed randomized clinical trial on 370 subjects, age 35 years and older, referred to clinical offices in Borujerd city in Lorestan province, western Iran. We calculated the sample size by Altman Nomogram (12). All patients were new referrals of definite diagnosis of Ischemic heart disease, without any history of aspirin and any other non-aspirin NSAIDs taking and indicated for ASA medication. The in-charge physicians, researchers and the patients were all blind to the type of therapy. The patients received a code by Random Allocation Software (Isfahan University of Medical Sciences, Iran) at the drug registry in the medical central office. The patients' medical history was recorded according to a designed checklist. Patients with past medical history of congenital or acquired coagulation disorders, renal failure, blood cell dyscrasia, medications affecting blood cells, platelets less than 150000, simultaneous use of other anti-platelets and anticoagulants such warfarin or heparin in the recent month and previous history of hypersensitivity to ASA were omitted from our samples.

The volunteer subjects were divided into five groups, consisting of 4 case groups and one age and sex and lifestyle and hookah smoking matched control group of placebo (enrolled from the patients' next of kin who did not have any CVD). The Four case groups were: group 1 (ASA 80 mg per day - Parsdaru Inc., Tehran, Iran); group 2 (ASA 81 mg per day - Kirkland Signature Low Dose Aspirin, LNK international inc., NY, USA); group 3 (ASA 100 mg per day - Parsdaru Inc., Tehran, Iran); and group 4 (ASA 325 mg per day - Parsdaru Inc., Tehran, Iran). The drug manufacturer provided us plain tablets- including the placebo- in the same shape, color and size of the ASA 81 mg tablets. The participants BT were tested using Ivy method. Since this technique is painful, we performed it once for each participant after using ASA for at least five simultaneous days. In this
research, we defined probable AR as no statistical increment of BT more than upper limit BT of control group (13). All patients were aware of the study and signed a written consent regarding the trial. We kept all information confidential and presented the results as anonymous. The ethical board of Lorestan University of Medical Sciences approved the study with the ethical code 200/61140 and the research deputy granted the study without any third party support.

**Determination of bleeding time**

Ivy method is a standard and less invasive method (compared to other method such as Duke) to assess BT (14). A standard-sized incision around 10 mm long and 1 mm deep is made by lancet or a specific device. The time from when the incision is made until all bleeding has stopped is measured and is called bleeding time (BT). An absorbent is used to draw off the blood in time equal time blocks, mostly every 20-30 seconds. The test is finished when bleeding has stopped completely. In our study, we have carried out all BT assessment in a single laboratory (in a stable humidity and temperature during the study) and by a single operator after 2 h from ASA used and the time was measured by a chronometer.

**Determination of 11-dehydro-Thrombozane B2**

TXB2 is produced from the breakdown of thromboxane A2 (11). It is released by activated platelets and urine levels of 11-dehydro-TXB2 can be used to monitor the response to aspirin therapy. In this study, Urinary TXB2 concentrations were measured using a polyclonal antibody enzyme immunoassay kit (11-dehydro-thromboxane B2 EIA Kit, Cayman Chemical, MI, USA). We perform an overnight collection, where the excretion rate of the metabolite and its values are less variable. Urinary dTxB2 concentrations were normalized for urinary creatinine concentrations. This kit could measure the concentrations in the range of 101000 pg/mL with a specificity approaching 100%. Subjects presenting TXB2 levels ≥294 mg/pg creatinine were considered as AR, based on the maximum normal of the EIA kit. We also took blood sample to count white cells and platelets; and measure hematocrite, PTT, PT and concentrations of hemoglobin. Serum urea and creatinine, uric acid and fasting blood sugar (FBS) concentration were measured by biochemical analyzer using commercial kits (Olympus AU-600, Tokyo, Japan).

**Statistical analysis**

All values are expressed as means. The data were compared between groups by statistical test such as independent samples t-test, one-way ANOVA and bonferroni post-hoc test. A P value of < 0.05 was considered statistically significant.

**Results**

**Demographic data**

At the end of the study, 370 patients were enrolled with 179/191 male to female ratio. Two hundred-fifty five patients (69%) lived in the rural areas of the Province and 115 (31%) were suburb residents. No significant difference was seen in gender ratio (p=0.678). In their medical history, 170 (46.3%) patients suffered from hypertension; 124 received medication. Furthermore, 67 (18.4%) subjects had history of under treatment type 2 diabetes (13 with life style modification, 47 with oral agents and 7 with Insulin). 51 patients had BMI >25 kg/m². Forty (11%) smoked cigarettes, 30 (8.2%) used opium and 9 used hookah in their life-course. No significant difference was seen in gender ratio (p>0.05). In Electrocardiographic study, 6 patients had cardiac arrhythmia at the time of diagnosis (MAT, heart blocks and junction rhythm) and 5 patients presented PVC. The average ejection fraction was 51.50±8.63%. 59 out of 76 available Angiographies shown abnormality and 116 out of 135 available Stress exercise tests were not in acceptable range. Other Demographic and laboratory data are stated in Table I.

**ASA resistance**

All patients underwent BT testing and urinary TXB2 measurements. Mean BT was 4.83±1.42 minutes in the case groups and 2.79±0.78 minutes in the control group. Mean TXB2 was 338.1±106.3 mg/pg creatinine
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and 236.4±101.2 mg/pg creatinine in the control group. Table 2 demonstrates these data regarding cardiac risk factors; as seen, so significant differences was found. On base of the pilot study and case control group, the mean and maximum normal values of BT in Borujerd normal population are 2.79 and 4.25 minutes, respectively. Furthermore, the maximum normal value for Urinary TXB2 regarding the kit manuals was set on 294 mg/pg creatinine. Table 3 illustrates AR calculated by BT and TXB2. As shown, significant difference was seen between normal values and all 4 groups receiving ASA; however, there were so significant difference among groups. Furthermore, urinary TXB2 levels were significantly higher than normal values in all 4 groups (p<0.05) and between the first group (80 mg) with other testing groups (p<0.05).

Table I: Demographic data regarding all 4 testing groups; significant difference was seen among platelet counts and Partial thrombin time. (*: p values resulted from one-way ANOVA test). (ASA 80 mg group, ASA 81 mg group, ASA 100 mg group and ASA 325 mg group; significance results from bonferoni post-hoc test).

<table>
<thead>
<tr>
<th></th>
<th>Total N=70</th>
<th>Controls n=72</th>
<th>80 mg n=76</th>
<th>81 mg n=71</th>
<th>100 mg n=71</th>
<th>325 mg n=71</th>
<th>P-value*</th>
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<tr>
<td>Age (years)</td>
<td>60.6±11.5</td>
<td>56.2±5.2</td>
<td>62.0±9.9</td>
<td>58.6±12.0</td>
<td>60.9±11.7</td>
<td>61.6±13.0</td>
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</tr>
<tr>
<td>PLT (x10⁶)</td>
<td>290.7±80.9</td>
<td>293.0±67.1</td>
<td>263±78.0*</td>
<td>302±74.0*</td>
<td>288±63.4</td>
<td>326±109*</td>
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<tr>
<td>WBC (x10⁹)</td>
<td>6.1±1.6</td>
<td>6.2±1.1</td>
<td>6.09±1.83</td>
<td>5.97±1.51</td>
<td>6.22±1.71</td>
<td>6.38±1.74</td>
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<tr>
<td>Hb (mg/dl)</td>
<td>13.3±1.5</td>
<td>13.1±0.9</td>
<td>13.1±1.50</td>
<td>13.6±1.32</td>
<td>13.6±1.76</td>
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<tr>
<td>PTT (s)</td>
<td>39.3±6.7</td>
<td>24.8±3.3</td>
<td>41.2±6.19</td>
<td>38.2±6.07</td>
<td>39.0±6.3</td>
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<td>PT (s)</td>
<td>12.6±1.1</td>
<td>12.1±0.8</td>
<td>12.8±0.74</td>
<td>12.3±0.87</td>
<td>12.6±1.15</td>
<td>12.7±1.85</td>
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<tr>
<td>Cr (mg/dl)</td>
<td>1.06±0.25</td>
<td>1.06±0.30</td>
<td>1.08±0.30</td>
<td>1.06±0.25</td>
<td>0.99±0.19</td>
<td>1.12±0.23</td>
<td>0.074</td>
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<tr>
<td>BUN (mg/dl)</td>
<td>15.9±6.0</td>
<td>15.8±5.5</td>
<td>16.5±8.0</td>
<td>15.7±6.0</td>
<td>14.9±4.1</td>
<td>16.7±4.7</td>
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<tr>
<td>Uric Acid (mg/dl)</td>
<td>5.7±1.8</td>
<td>5.77±2.03</td>
<td>5.89±1.89</td>
<td>5.77±2.03</td>
<td>5.67±1.64</td>
<td>5.62±1.79</td>
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<tr>
<td>FBS (mg/dl)</td>
<td>106.1±54.7</td>
<td>100.1±54.2</td>
<td>101±51.2</td>
<td>103±43.5</td>
<td>107±58.6</td>
<td>118±71.8</td>
<td>0.516</td>
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Table II: BT and urinary TXB2 regarding cardiac risk factors in our enrolled subjects (* p values resulted from student’s t test).

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>No</th>
<th>P*</th>
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<tr>
<td>Smoking BT</td>
<td>4.8±1.4</td>
<td>4.8±1.3</td>
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<tr>
<td>TXB2</td>
<td>341.5±102.7</td>
<td>335.3±109.6</td>
<td>0.491</td>
</tr>
<tr>
<td>Opium BT</td>
<td>4.3±0.9</td>
<td>4.8±1.4</td>
<td>0.095</td>
</tr>
<tr>
<td>TXB2</td>
<td>365.5±105.7</td>
<td>335.8±106.7</td>
<td>0.257</td>
</tr>
<tr>
<td>Diabetes BT</td>
<td>4.8±1.4</td>
<td>4.7±1.4</td>
<td>0.517</td>
</tr>
<tr>
<td>TXB2</td>
<td>353.5±124.9</td>
<td>334.9±102.1</td>
<td>0.317</td>
</tr>
<tr>
<td>Obesity BT</td>
<td>4.7±1.4</td>
<td>5.2±1.1</td>
<td>0.092</td>
</tr>
<tr>
<td>TXB2</td>
<td>338.0±104.1</td>
<td>338.9±120.2</td>
<td>0.963</td>
</tr>
</tbody>
</table>

BT: bleeding time (minutes), TXB2: urinary 11-dehydro-thromboxane B2 (mg/pg creatinine).

Fig. 1: Correlation between bleeding time and urinary 11-dehydro-thromboxane B2 treated groups with aspirin. (r=0.114, p=0.045).
Correlation of urinary 11-dehydro thromboxane B2 with bleeding time

The level of urinary 11-dehydro thromboxane B2 correlated positively with bleeding time treated groups with aspirin. \((r=0.114, p=0.045, \text{Fig. 1})\).

Discussion

All individuals with or prone to IHD take ASA which can reduce the risk of cardiac ischemia to near 22% in cases with atherosclerosis (15, 16); furthermore, it can lower the infarct size in case of MI (17). However, the dose used in different patients varies among many factors and patients respond differently to ASA (8-11). This study was designed to evaluate the response to different types of ASA. Previous studies demonstrated that near 45% of patients do not respond to ASA as expected. Researchers have stated different theories to explain this fact; however, they have not yet come to a single conclusion (18, 19). This study have compared the 2 most easily-performed and low price methods.

In our study using BT, we assume that 37% of included individuals were AR; however, 64% of our patients were considered AR regarding urinary TXB2. Bochmann have studies AR in 2 different studies had demonstrated 42% and 54% AR by using BT (20, 21). The results of other studies have a wide range results (0.5% to 70%) which can be explained by variation of ethnicity, ASA dosage and the measuring AR method used (22-25). In Iran, Asadian et al reported that AR is present in 16% of patients (by using PFA-100) (26) and Sadeghi et al have showed that this rate is near 75% (using TXB2 levels) (27). In our study, all 4 test groups had higher BT than the control group and furthermore, there were no significant difference among the test groups. These results represent that all 4 doses can increase BT nearly by the same rate and increasing the ASA dosage does not give any additional protection; which rephrasing: conventional 80 mg ASA does not have any difference with American 81mg ASA in increasing BT (and therefore, preventive effect for IHD). Kojouri et al have also come up with the same results (28); however, Grubel et al have demonstrated that 81 mg ASA has less resistance than 162 mg and 325 mg conventional ASAs (29). Therefore, lower doses of ASA are good enough for giving protection against IHD.

As seen in the results, TXB2 levels were higher than the upper limit in all 4 test groups. Eskandarian et al have demonstrated a 49% AR by using TXB2 (30). Other studies have also demonstrated a range of 14-50% AR (8-11, 21-25). However, all of these studies have shown that there is a positive correlation between ASA dose and TXB2 levels. TXB2 is one of the end metabolites of arachidonic acid in cyclogenase-2 pathway along with other thromboxanes. This pathway is not limited to the platelets and other blood cells such as monocytes, macrophages and endothelial cells even without TXA2 mediation (11, 21, 26, 31). As some of the mentioned studies have stated, using TXB2 for measuring AR is outdated and should not be used for clinical purposes.

This study also assessed the relation between AR and cardiac risk factors; which demonstrated no significant relation in none of the BT and TXB2 levels. Salama et al have also stated the same results (32). There are studies which sporadically shown some
difference among these risk factors; however, these results are redundant to our study due to different type of diseases and adjuvant medication and also different ethnicity and genetic pool (33-36). As far as we know, there are no explanations for these relations, if there are any, indeed.

The most important point in this study is the imbalance of AR rate using BT (as a direct clinical test) and TXB2 (as a para-clinic laboratory test). Comparing to the pilot study (12) and the results of other published data, Using BT is more practical and nearer to fact than any other para-clinic method; if a hospital can provide an optimum setting (considering temperature, humidity, sunlight and experienced operator). In this study, Ivy method for calculating BT was performed in a single setting by a single operator, but the TXB2 levels was measured by the kit sample. Given the simplicity and low costs of its performance it might be of some potential use in developing countries (for approximate estimation of the presence of some major hemostatic disorders). However, due to Ivy method limitations it cannot be perceived as a tool to assess such specific aspects of platelet function as COX activity (35, 36). Furthermore, Based on our r value (0.114) correlation between bleeding time and is urinary 11-dehydrothromboxane B2 weak, so it can’t be used as a predictor of COX-1 inhibition.

ASA is an effective inhibitor of platelet TxA2 production, nevertheless is often considered as overall relatively weak platelet inhibitor because of its limited effects on aggregation in the presence of high concentrations of such agonists as adenosine diphosphate (ADP) or collagen. This may account for some of the variability of the response to these stimulants, which activate platelets through both COX-dependent and COX-independent pathway (37). Moreover, urinary dTxB2 is a global index of TxA2 synthesis, which may originate from other blood elements such as erythrocytes and monocytes and from renal biosynthesis. Accordingly, high levels of urinary dTxB2 despite daily aspirin therapy may be a reflection of a larger non-platelet production, unaffected by cardioprotective aspirin doses, as opposed to increased platelet activity as it has been previously suggested (38).

Regarding our results, AR in our population had not have a significant difference with AR rate in other parts of Iran or in the world; however, our study had the limitation of using gold standards of AR such as LTA. And furthermore, as ASA was used by the patients themselves, these are some concerns regarding the full use of ASA by all patients. However, all of them have complied that they have “token their drug”. It is recommended that AR should be measured in other parts of Iran and the countries which do not have any documented report of their AR rate by specific (not sensitive) methods.

Conclusion

This randomized clinical trial, was conducted to calculate the aspirin resistance rate in different doses of aspirin in patients with ischemic heart disease by two methods; bleeding time and urinary thromboxane B2. This study showed that level of urinary 11-dehydro thromboxane B2 was correlated weekly with bleeding time. Regarding the difficulties in using thromboxane B2 (such as non-logical higher rate of resistance and dose dependent increase), it seems that calculating BT by Ivy method in an optimum situation (when operated by a stable laboratory settings) is a more reasonable method as a clinical easy way to evaluate AR in developing countries, if we have the normal values of the general population. However, this information has huge controversies and should be used with caution.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.
References


