ANALYSIS OF DIFFERENCES OF SERUM THROMBOXANE B_2 LEVEL AFTER TAKING ACETOSAL IN ACUTE THROMBOTIC STROKE WITH DIABETES MELLITUS AND NON-DIABETES MELLITUS

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ABSTRAK

Disfungsi endotel dan kerusakan vaskular merupakan awal dari pembentukan trombus pada stroke trombotik. Hal ini akan memicu aktivasi platelet yang ditandai dengan sintesis Tromboksan A_2 sebagai agonis pada proses agregasi platelet. Asetosal (ASA) 100 mg yang umum diberikan pada pasien stroke trombotik bekerja dengan menghambat sintesis TxA_2 sehingga mampu mencegah pembentukan trombus. diabetes mellitus (DM) sebagai salah satu faktor risiko stroke trombotik menunjukkan peningkatan sintesis Tromboksan A_2 (TxA_2). Belum diketahui apakah ASA 100 mg cukup untuk menghambat sintesis TxA_2 pada pasien DM. Tujuan penelitian ini adalah menganalisis perbedaan kadar serum TxA_2, yang diukur melalui kadar serum TxB_2 sebagai metabolit stabil TxA_2, pasca pemberian ASA 100 mg pada pasien stroke trombotik dengan DM dan non-DM. Penelitian dengan desain prospektif observasional dilakukan di Departemen Ilmu Penyakit Saraf, RSUD Dr. Soetomo, Surabaya. Sebanyak 27 pasien stroke trombotik terbagi menjadi 15 pasien DM dan 12 non-DM diukur kadar serum TxB_2 sebelum dan setelah 5-7 hari pemberian ASA 100 mg. Rerata kadar serum TxB_2 sebelum dan sesudah ASA 100 mg pada pasien DM adalah 16,43 ± 16,08 ng/mL dan 2,93 ± 1,83 ng/mL dan 27,36 ± 21,04 ng/mL dan 5,36 ± 4,06 ng/mL pada non-DM. Sedangkan rerata penurunan kadar serum TxB_2 pada DM dan non-DM adalah 13,49 ± 15,9 ng/mL dan 22,00 ± 21,65 ng/mL. Terdapat perbedaan bermakna kadar serum TxB_2 pasca pemberian ASA 100 mg 5-7 hari pada kelompok DM dan non-DM, namun penurunan kadar serum TxB_2 pasca pemberian ASA 100 mg antar kedua kelompok tidak berbeda bermakna. ***(FMI 2018;54:53-58)***

Kata kunci*: Stroke trombotik; asetosal; tromboksan A_2; tromboksan B_2; diabetes mellitus*

ABSTRACT

Endothelial dysfunction and vascular injuries are the early processes in thrombogenesis leading to thrombotic stroke. These processes trigger platelet activation characterized by synthesis of Thromboxane A_2, potent agonist in platelet aggregation. Acetosal (ASA) 100 mg usually given to thrombotic stroke patients exerts its pharmacological effect by inhibition of TxA_2 synthesis, thus could prevent thrombus formation. Diabetes mellitus (DM) as risk factor of thrombotic stroke exhibits an increase in TxA_2 synthesis. It is not known whether ASA 100 mg could inhibit TxA_2 adequately in diabetic patients. This study aimed to analyze the differences of serum TxA_2 level, which was measured by serum TxB_2 level as stabile metabolite of TxA_2, after taking ASA 100 mg in diabetic and non-diabetic thrombotic stroke patients. This prospective observational study was held in Neurology Department of Dr. Soetomo Hospital, Surabaya. Total 27 patients, consisted of 15 patients with DM and 12 patients with non-DM were enrolled. Serum TxB_2 was measured before and after 5-7 days 100 mg ASA 100 administration. Mean value of serum TxB_2 level before and after taking ASA was 16.43 ± 16.08 ng/mL and 2.93 ± 1.83 ng/mL in diabetic and 27.36 ± 21.04 ng/mL and 5.36 ± 4.06 ng/mL in non-diabetic group. Mean reduction of serum TxB_2 level in diabetic and non-diabetic group was 13.49 ± 15.9 ng/mL and 22.00 ± 21.65 ng/mL. There were significant differences in serum TxB_2 level after taking ASA 100 mg in diabetic and non-diabetic group but the mean reduction of serum TxB_2 level were not significantly different. ***(FMI 2018;54:53-58)***

Keywords*: Thrombotic stroke; acetosal; thromboxane A_2; thromboxane B_2; diabetes mellitus*

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INTRODUCTION

Ischemic stroke is defined as rapidly developing signs of focal (or global) neurological dysfunction lasting for >24 hours (unless interrupted by surgery or death), caused by cerebral infarction attributable to vascular ischemic based on pathological, clinical and imaging evident (Thorvaldsen 1997, Smith et al 2013). The incidence and prevalence of stroke tend to increase over the year especially in low to middle income country. According to data from Indonesian Health Ministry in 2013, the prevalence of stroke in Indonesia was 12.1 per 1000 (Balitbangkes 2013). There are several factors that can increase the risk for stroke. It can be divided into non-modifiable risk factors such age, sex, gender and genetics and modifiable risk factors such hypertension, dyslipidemia, diabetes mellitus (DM) and smoking (Biller et al 2012).

The most common form of ischemic stroke is thrombotic stroke. It occurs when a blood clot inhibits blood flow in the lumen of cerebrovascular, leading to the decrease of cerebral blood flow (Smith et al 2013). As cerebral blood flow decline to less than 20ml/100 g/min and is not rapidly restored, the brain becomes infarcted and thus manifested as neurological deficit (Smith et al 2013, Langhorne 2014). Endothelial dysfunction and vascular injury as the main cause of thrombus formation induce the exposure of subendothelial matrix, consisting of collagen to blood component, such as platelet (Golan et al 2012). In addition, injured endothelial cell expresses von Willebrand factors. Altogether, these processes cause platelet adhesion to the injured blood vessel via its receptor (GPVI and GPIb) and thus become activated and release its granule containing Adenosine Diphosphate (ADP) (DeLoughery 2015). Stimulation by ADP activates platelet membrane Phospholipase A_2 (PLA_2). PLA_2 cleaves membrane phospholipid and liberated Arachidonic Acid (AA). Via Cyclooxygenase (COX) and Thromboxane A_2 (TxA_2) Synthase enzyme, AA will subsequently be converted to Prostaglandin H2 and TxA_2. Furthermore, TxA_2 and ADP activates nearby platelets in circulation thus increases the platelet adherence and enlarges the size of aggregating platelet (Furie & Furie 2008). Altogether with fibrin formed through coagulation cascade, aggregating platelet forms a stable hemostatic plug called thrombus (Widmaier et al 2014). Synthesized TxA_2 is rapidly being hydrolyzed to its stable metabolite, Thromboxane B_2 (TxB_2). Due to its instable characteristic in circulation, TxA_2 cannot be measured directly. Hence, it could be measured through its stable metabolite level (Hankey & Eikelboom 2006).

Acetosal (ASA), a non-selective COX-inhibitor, is a common antiplatelet used in thrombotic stroke. American Heart Association/American Stroke Association (AHA/ASA) recommends ASA at low dose (50-325 mg daily) as a secondary prevention of stroke (Kernan et al 2014). It prevents a thrombus formation by covalently acetylating a serine residue near the active site of COX enzyme in platelet, thus inhibits TxA_2 synthesis. Once inhibited, unnucleated platelet cannot express COX enzyme. Therefore, the inhibition of ASA is irreversible and last in platelet lifetime (7-10 days) (Golan et al 2012). Once daily low dose ASA suppresses the TxA_2 formation by at least 95% within 5 days (Hankey & Eikleboom 2006).

DM as one of the major modifiable risk factor can increase the risk of stroke 2 to 4-folds than non-diabetic patients (Sacco et al 1997). DM which characterized by hyperglycemia, hyperinsulinemia and insulin resistance, can affect the endothelial cell (EC) and platelet function (Ferroni et al 2004). The increase of oxidative stress (ROS), Protein Kinase C (PKC) activation and Receptor for advanced glycation end products (RAGE) activation are thought to be the underlying mechanisms of diabetic vascular complication (Ferroni et al 2004, Powers 2012). These mechanisms can induce the activation of EC and platelet which causes the switch toward a prothrombotic pro-inflammatory condition. High blood glucose also impairs the calcium (Ca2+) homeostasis in platelet, leading to increase level of cytosolic Ca2+ in platelet and eventually increase the platelet activation (Natarajan et al 2008, Hess & Grant 2011). It is stated that DM exhibits a platelet hyper-activation and hyper-aggregation (Ferreiro & Angiolilo 2011). Some established studies support this statement. Different methods, such as AA-induced Light Transmittance Aggre-gometry (LTA) (Pulcinelli, et al 2009), Verify-Now (DiChiara et al 2007), measurement of urinary 11-Dehydro-TxB_2 (DiChiara et al 2007, Lopez et al 2014), Platelet Function Analyzer (PFA-100) (DiChiara et al 2007) and Mean Platelet Volume (Kodiatte et al 2012), showed that a diabetic platelet tends to have a higher activity or aggregation compared with non-diabetic. However, none of this study can evaluate specifically the inhibitory effect of ASA on platelet (Cattaneo 2007).

MATERIALS AND METHODS

Patients came between August and October 2016 to the Neurology Department of Dr. Soetomo Hospital who diagnosed as having thrombotic stroke and met the inclusion and exclusion criteria. The inclusion criteria were: 1) male and female at least 17 years of age in time of admission, 2) was diagnosed with diabetes mellitus by clinician, 3) was about to be given with 100 mg ASA for at least 5 days during hospitalization 4) signed the informed consent and information for consent. The exclusion criteria were: 1) was about to be given another antiplatelet agent, 2) was diagnosed with altered liver, renal and hemostatic function, 3) had taken Non-Steroidal Anti-Inflammatory Drugs (NSAID), including ASA, steroid or any other drugs, affecting platelet aggregation by at least 2 weeks before admission. Patients were dropped out under the following criteria: 1) ASA discontinued due to allergic or adverse event reaction 2) died or discharged earlier, 3) experience hemorrhagic transformation, 4) experi-ence an altered liver, renal and or hemostatic function, and 5) was given with NSAID (except ASA), steroid or any other drugs which affected platelet aggregation concomitantly.

Ethical clearance for this study was obtained from the Ethics Committee for Clinical Research at Dr. Soetomo Hospital No. 446/Panke. KKE/ VI/ 2016. Patients who met the inclusion and exclusion criteria and agreed to

participate in this study were provided an informed consent and information for consent written and orally.

Twenty-seven patients (DM=15; Non-DM=12) were given with the same enteric-coated ASA 100 mg once daily every morning at 8 a.m. Two mL blood venous sample of all patients was obtained at 6 a.m before ASA 100 administration at day-1 of admission (data pre serum TxB_2 level). During hospitalization, all patients were given therapy as needed by clinician. Data on post serum TxB_2 level was obtained at day 5 (or up to 7) days of ASA 100 mg administration at 6 a.m. The blood venous were collected in Serum Separator Tube (SST) 3 mL and were allowed to clot for 1 hour at room temperature before centrifugation for 15 minutes at 1000 x g. Serum obtained then kept and stored in -80ºC prior to assay. Serum TxB_2 level was measured using ELISA Commercial Kit (R&D Systems Inc., Minneapolis, USA) in the Laboratory of Clinical Pathology, Central Diagnostics, Dr. Soetomo Hospital, Surabaya.

Data of serum TxB_2 level obtained were analyzed using statistical software program Statistical Package for the Social Science (SPSS) 20.0 version. Kolmogorov-Smirnoff test was used to assess the normality of the variables distribution. Chi-Square test was performed to assess the distribution of demographic data between groups. Paired t-test was used to assess the difference of data pre- and post-treatment in both group. Independent t-test were performed on all baseline characteristic and to assess the difference between groups. $P<0.05$ was considered as significant for all statistical tests.

RESULTS

From August to October 2016, 27 enrolled patients who met all the inclusion and exclusion criteria were divided into 15 diabetics and 12 non-diabetics. There was no significant difference in demographic data between two groups (Table 1). Sixty-percent subjects in diabetic group were men, while it was 66.7% in non-diabetic group. The men:women ratio between both groups was not significantly different (p=0.722). Patients who participated in this study were at least 40 years olds. In both groups, most of the patients were in between 40-60 years old. The distribution of ages in both groups could be seen in Table 1. Performed Chi-square showed there were no significant difference in the distribution of ages in both groups. Beside sex and age, other risk factors such as hypertension (HT), dyslipidemia, smoking and history of stroke were observed in all subjects. The most prevalence risk factor in this study was HT. Around 87% in diabetics and 83% in non-diabetic group had HT. In diabetic group, none of the subject had stroke prior to admission while in non-diabetic group there were 7 subjects who had. Nevertheless, the distribution of other risk factors was not significantly difference between two groups ($p>0.05$).

The serum level of TxB_2 in both groups was in broad ranges (Fig. 3). The serum TxB_2 level pre ASA 100 in diabetic and non-diabetic groups were in a range between 0.56 ng/mL – 55.77 ng/mL and 4.24 ng/mL – 60.31 ng/mL while the serum TxB_2 level post ASA 100 mg were about 0.90 ng/mL – 6.58 ng/mL and 0.95 ng/mL – 16.23 ng/mL, respectively. The mean value of serum TxB_2 level pre ASA in diabetic (16.43 ± 16.08 ng/mL) was not significantly difference compared to that in non-diabetic group (27.63 ± 21.04 ng/mL). After taking ASA 100 mg for 5-7 days, the mean value of serum TxB_2 declined significantly both in diabetic and non-diabetic group to 2.93 ± 1.83 ng/mL (p= 0.006) and 5.36 ± 4.06 ng/mL (p= 0.005). The difference level of serum TxB_2 in both groups were also statistically significant (p=0.048). Meanwhile, the mean reduction of serum TxB_2 level in diabetic (13.49 ± 15.98 ng/mL) and non-diabetic groups (22.00 ± 21.65 ng/mL) showed no significant difference (p= 0.251) (Table 2).

DISCUSSIONS

TxA_2, a prostaglandin derivative, is an agonist in platelet aggregation and plays a pivotal role in thrombus formation (Golan et al 2012). It has a short half-life in circulation and being rapidly hydrolyzed to its stable metabolite, Thromboxane B_2 (TxB_2). Due to its short half-life and unstable characteristic, it is impossible to measure TxA_2 directly. It can only be measured through TxB_2 which can be detected in serum or urine as urinary 11-dehydro-Thromboxane B_2 (11-dehydro-TxB_2). It is estimated that 30% of TxB_2 measured in urine are from extra-platelet source (Hankey & Eikelboom 2006).

This was the first study which compared and analyzed the difference of serum TxB_2 level between DM and non-DM thrombotic stroke patients. It is stated that man has a higher risk than woman in experiencing stroke (Biller et al 2012). The older the patient, the higher the risk of being attacked by stroke. As it is getting older, the physiological function of endothelial cell is also declining. It is also stated that there was a correlation between age and platelet function (Jones, 2015). Despite all the suggestions, it is still unclear whether sex and age contribute to the formation of TxB_2. HT, dyslipidemia and smoking are affecting on platelet activity by different mechanism. Endothelial dysfunction occurred in HT can increase the platelet activity and the expression of Tissue Factor (TF), a substance which plays a role in coagulation cascade (Lip et al 2003, Gkaliagkousi et al 2013). In dyslipidemia, the oxidized low

density lipoprotein (LDL-Ox) activates platelet, increases the formation of TF and is associated with increased platelet biogenesis (Wang & Tall 2016). Platelet in actively smoking patients also tends to turn-over more rapidly and aggregates easier than in those who are not (Fuster et al 1981, Takajo et al 2001).

Sex, age, HT, dyslipidemia and smoking habit are known to affect the platelet and endothelial cell. Thus, it is anticipated that these factors could also affect the serum TxB_2 level. Therefore, to minimize the effect of those risk factors on the difference of measured serum TxB_2, the demographic data of all subjects in both group must be homogeneous. In Table 1, it can be seen that all the demographic data of subjects are not significantly different between two groups, except the history of prior stroke. There were 7 non-diabetic patients had stroke before admission. These patients still included in this study as they had not visited neurologist and had not taken ASA by at least 2 weeks before admission, thus, it was assumed that these patient's platelet were not suppressed.

Diabetes mellitus (DM) as one of modifiable risk factor of thrombotic stroke alters the metabolic and functional properties of multiple cells, including endothelium and platelet. It is suggested that metabolic changes in DM increase platelet turnover and TxA_2 synthesis (Hankey & Eikelboom 2006). The high recurrence rate of stroke in DM was considered due to ASA resistance. ASA resistance was defined as the inability of ASA to inhibit the COX enzyme, thus the inhibition of TxA_2 synthesis was inevitable (Zehnder et al 2016). By that definition, it is logical to assess the pharmacological effect of low-dose ASA by serum TxB_2 level.

As seen on Table 2, the mean value of serum TxB_2 level before taking ASA 100 mg in diabetic group is lower than that in non-diabetic group although not significant ($p>0.05$). After ASA has been administered for 5-7 days, serum TxB_2 levels in both groups were measured. The results showed that serum TxB_2 level in diabetic and non-diabetic groups declined significantly from 16.43 ± 16.08 ng/mL to 2.93 ± 1.83 and 27.36 ± 21.04 to 5.36 ± 4.06 ng/mL respectively ($p<0.05$). It means that ASA 100 mg administered for 5-7 days exhibited its pharmacological effect in both groups. Despite all the suggested theories that DM exhibits higher thrombo-xane synthesis (Hess & Grant 2010), this study showed that ASA 100 mg could suppress the formation of thromboxane by platelet.

Table 1. Patients' demographic

Characteristic	Diabetic Group (N=15) n (%)	Non-Diabetic Group (N=12) n (%)	Sig. level (*P*)
Sex			
Man	9 (60%)	8 (66.7%)	0.772
Woman	6 (40%)	4 (33.3%)	
Ages			
40-50 y.o	5 (33.3%)	4 (33.3%)	0.825
51-60 y.o	5 (33.3%)	4 (33.3%)	
61-70 y.o	4 (26.7%)	2 (16.7%)	
<70 y.o	1 (6.7%)	2 (16.7%)	
Other RF			
HT	13 (86.7%)	10 (83.3%)	0.809
Dyslipidemia	5 (33.3%)	3 (25%)	0.637
Smoking	4 (26.7%)	3 (25%)	0.922
Prior stroke	0 (0%)	7 (58.3%)	n/a

Table 2. Serum TxB_2 in diabetic and non-diabetic stroke patients before and after ASA 100 mg

	DM (mean ± SD)	Non-DM (mean ± SD)	Sig. level (*P*)*
Serum TxB_2 pre (ng/mL)	16.43 ± 16.08	27.36 ± 21.04	0.138
Serum TxB_2 post (ng/mL)	2.93 ± 1.83**	5.36 ± 4.06***	0.048
Reduction of Serum TxB_2	13.49 ± 15.98	22.00 ± 21.65	0.251

Note:
*: test were performed using independent t-test to assess the difference between two groups
**: test were performed using paired t-test to assess the difference of serum TxB2 pre and post within diabetic group (p= 0.006)
***: test were performed using paired t-test to assess the difference of serum TxB2 pre and post within non-diabetic group (p= 0.005)

Compared to non-diabetic group, the serum TxB_2 post level in diabetic patients had significantly higher mean value ($p>0.05$). This finding was in contrary with previous study that diabetic patient had higher platelet activity compared to non-diabetic patients. Although diabetic patients had lower mean value of serum TxB_2 post level than non-diabetic group, it cannot be concluded that this group had higher mean reduction of serum TxB_2. Mean reduction of serum TxB_2 in diabetic group (13.49 ± 15.98 ng/mL) was less than that in non-diabetic group (22.00 ± 21.65 ng/mL). Although the difference of mean reduction in both groups was not significant ($p>0.05$), ASA exerted its pharmacological effect in non-diabetic group higher.

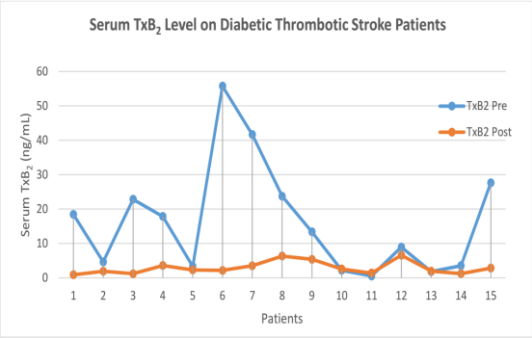

Fig. 1. Graphic of serum TxB2 in diabetic Group before and after ASA Administration.

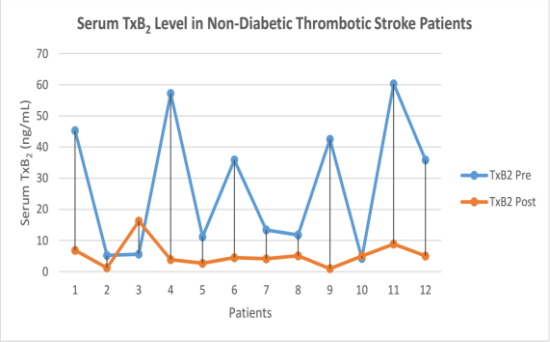

Fig. 2. Graphic of serum TxB_2 in non-diabetic group before and after ASA administration.

Figs. 1 and 2 show that there are 5 patients (patients no. 10, 11 and 13 in DM; patients no. 3 and 10 in non-DM) who experience anomaly. After ASA 100 mg administration, these patients had increased serum TxB_2 instead. All of them had slightly increased (± 0.56 ng/mL) serum TxB_2 level after taking ASA 100 mg except patient no.3 in non-diabetic group (Mr.P). The serum TxB_2 level of Mr.P increased up to 10.63 ng/mL (from 5.60 ng/mL before to 16.23 ng/mL after ASA 100 mg for 5 days). His clinical conditions were predicted contributed to this anomaly.

Unlike any other patient who experienced anomaly, One patient was diagnosed with hyper-fibrinogenemia and psoriasis. Hyper-fibrinogenemia is a condition in which the level of fibrinogen is increasing and it reaches up to 575 mg/dL during hospitalization. There was a study suggested that fibrinogen-induced platelet aggregation was associated with the increase of TxA_2 synthesis. Besides, psoriasis as an inflammation disease could increase the formation of TxA_2 via COX-2 pathway from up-regulated proinflammatory cells, such as monocyte and macrophage (Hankey & Eikleboom, 2006, Langhorne 2014).

It is still unclear the causal of increasing serum TxB_2 in 4 other patients. There were no other clinical conditions, drugs interaction and non-compliance issues found. It is predicted that these 4 patients experiencing ASA resistance. Some suggested hypothesis that ASA resistance is due to genetic polymorphism, the used of enteric-coated ASA, drugs interaction (NSAID, PPI), TxA_2 extra-platelet source and increased platelet turnover in several condition, such as post-surgery, infection and inflammation (Hankey & Eikleboom 2006, Zehnder et al 2016).

CONCLUSION

There was a significant difference of serum TxB_2 level after ASA 100 mg administration, although the reduction of serum TxB_2 level was not significantly different between diabetic and non-diabetic thrombotic stroke patients.

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