

TNF- α and IL-8 in Acute Stroke and the Modulation of these Cytokines by Antiplatelet Agents

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Abstract: Stroke is associated with elevation of several proinflammatory cytokines such as tumor necrosis factor alpha (TNF- α) and interleukin (IL)-8 that are correlated with central nervous system (CNS) injury. Anti-platelet therapies are important agents in stroke management. The role of antiplatelets as anti-inflammatory agents is not known in acute stroke patients. Furthermore, their effect on induction of potential cytokines as TNF- α and IL-8 in those patients is still not clear. Thus, we herein examined the induction of TNF- α and IL-8 in acute stroke patients and examined the effects of the antiplatelets drugs aspirin, clopidogrel and dipyridamole, and piracetam in their induction. Cytokines were detected intracellularly in leukocytes from patients who had first acute ischemic stroke and from matched controls by immunocytochemistry. The results showed significant increase of spontaneously produced TNF- α and IL-8 in patients compared to control. This induction was significantly inhibited differently by each drug and dual drug agents. The data of this work suggest that antiplatelets agents may have a role in inhibition of stroke mediated proinflammatory cytokine effects, which may initiate a new aspect of the role of antiplatelets in the treatment of acute ischemic stroke.

Key Words: Brain, ischemia, cytokine, inflammation, aspirin, clopidogrel, dipyridamole, piracetam.

INTRODUCTION

Stroke occurs when blood circulation of the brain is interrupted by a blockage in a blood vessel supplying the brain. About 700,000 people experience have a new or recurrent stroke in United States (US) every year, of which 500,000 of them are first attacks. Direct and indirect cost of stroke in US in 2004 was estimated at \$53.6 billion (American Heart Association, 2004). A growing number of recent investigations have established a critical role for leukocytes in propagating tissue damage after ischemia and reperfusion in stroke (Montaner *et al.*, 2003, Grau *et al.*, 2001). Data from recent animal and human studies demonstrated that inflammatory events occurring at the blood-endothelium interface of the cerebral capillaries underlie the resultant ischemic tissue damage. For example, inflammatory interactions that occur at the blood-endothelium interface, involving cytokines, adhesion molecules, chemokines and leukocytes, were found to be critical to the pathogenesis of tissue damage in cerebral infarction (Huang *et al.*, 2006). However, inflammatory mediators may have dual roles, with the above mentioned detrimental effects but beneficial effects in long-term repair and recovery, leading to complications in their application as novel therapies (Lucas *et al.*, 2006).

Cytokines such as TNF- α , IL-6, IL-1 and IL-8 have been associated with CNS injury and ischemic injury (Kostulas *et al.*, 1999). In experimental models of stroke, increased expression of the cytokines TNF- α and IL-8-like cytokines

within the ischemic lesion were reported. Several therapies aimed at preventing this inflammatory response have demonstrated neuroprotective efficacy and significant reduction of the lesion volume that was associated with a marked functional improvement (Beech *et al.*, 2001).

Antiplatelet drugs are important therapies in prevention of acute myocardial ischemia and acute stroke (Bednar *et al.*, 1999). By using antiplatelet therapies, vascular morbidity and mortality were reduced by 27% among patients with evidence of cardiovascular disease and with high risk for recurrent illness. In addition, these therapies reduced non-fatal myocardial infarction by about 30% and non-fatal stroke by about 25% (Patrono *et al.*, 2004). Platelet stimulation and activation are known not only as prerequisite of clot formation but are increasingly recognized as important contributors to inflammation and vascular injury and the role of antiplatelet drugs in stroke prevention is well established. Antiplatelet drugs have also antiinflammatory effects as for example the role of aspirin, as antiplatelet agent and an irreversible COX-1 inhibitor, is well elucidated and recognized (Kulbertus 2004). Also, clopidogrel was demonstrated to improve endothelial nitric oxide bioavailability and diminish biomarkers of oxidant stress and inflammation in patients with symptomatic coronary artery disease, suggesting that beyond inhibition of platelet aggregation, adenosine phosphate receptor blockade may also have promising vasoprotective effects (Heitzer *et al.*, 2006). Dipyridamole, a drug that simultaneously decreases platelet function exhibited selective antiinflammatory properties, which was suggested to contribute to its actions in the prevention of stroke (Weyrich *et al.*, 2005).

In view of the anti-inflammatory role of the antiplatelet drugs and the suggested role of pro-inflammatory cytokines

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as TNF- α and IL-8 in stroke development, we investigated the effects of these antiplatelet agents in the production of the cytokines TNF- α and IL-8.

METHODS

Subjects

Patients presented the first acute ischemic stroke (n=14) who didn't use any antiplatelet drug were chosen from those presented to the emergency department at the Bahrain Defense Forces Hospital. Matched control group with similar risk factors who were not under any antiplatelet drugs (n=14) was used. All subjects were asked to fill a consent form and sign it, indicating their acceptance to participate in the study.

Cell Culture

Patient and control venous blood samples (10 ml) were collected into EDTA tubes. Leukocytes were isolated by overlaying carefully the diluted blood on Ficoll and centrifuged at 3200 rpm for 20 minutes (min.) at room temperature (RT), followed by discarding the plasma layer and collecting the buffy coat layer. Cells were then washed for 3 times with 1X PBS buffer (Phosphate Buffer Saline, Ph 7.2), then maintained in culture medium RPMI 1640 (ICN, Biomedicals, Inc., Costa Mesa CA, US) supplemented with 5% fetal bovine serum, 1M HEPES buffer, 50 μ mole Mercaptoethanol, 2ml of 200 mM L-glutamine, and 5IU/5 μ g penicillin streptomycin (Gibco, Gaithersburg, MD, US). The cells were then transferred to adhesion slides (BioRad Lab, Munich, Germany).

Intracellular Detection of Cytokines

Cell cultures from patients and controls were either incubated with aspirin (0.03 mg/ml), clopidogrel (0.015 mg/ml), dipyridamole (0.015 mg/ml), dual drug of three agents or piracetam (0.96 mg/ml) or no drug. As positive control, some cultures were stimulated with LPS (5pg/ml). Cells were then incubated for overnight at 37°C in 5% CO₂ followed by fixation with methanol for 5min and then incubated with avidin-biotin blocking kit (Vector Laboratories, Burlingame, CA, US) to block endogenous biotin or biotin-binding proteins. Cells were permeabilized by 0.1% saponin dissolved in PBS to allow the intracellular access of the cytokine-specific antibody. A total of 50 μ l cytokine specific monoclonal antibody (mAb) (mouse anti-human IL-8 and TNF- α R&D Systems, Oxon, United Kingdom) diluted in PBS-saponin to a final concentration of 5 μ l/ml was added and allowed to incubate for overnight at 4°C followed by several washes in PBS. Non-specific staining by the second-step biotinylated goat antibody caused by Fc-interactions was prevented by a subsequent incubation with 1% goat serum (Dako, Glostrup, Denmark) dissolved in PBS-saponin for 30 min. at RT. The biotin-conjugated secondary antibodies were then added. The cells were incubated with an avidin-biotin horse-radish peroxidase complex (Vectastain, Vector Laboratories, Burlingame, CA, US) for 30 min. in the dark at RT. A color reaction was developed by 3-diaminobenzidine tetra hydrochloride (DAB) (Vector Laboratories, Burlingame, CA, US) and stopped after 5-10 min. by washes in PBS. The cells were counterstained with hematoxylin and the slides were left to dry before mounting in buffered glycerol.

The immunocytochemically stained cells were examined in a Leica RXM microscope (Leica, Wetzlar, Germany) equipped with a 3CDD color camera (Sony, Tokyo, Japan). Enumeration of cytokine producing cells was performed manually at X 100 objective. The frequency of cytokine expressing cells was assessed by examination of at least 10,000 cells.

Statistic Analysis

Analysis of variance (ANOVA) was used for multiple comparisons. Mann-Whitney's test was used to calculate level of significance (*=p<0.01, **=p>0.001, ***=p>0.0001).

RESULTS

Anti-platelet drugs are central agents in the management of stroke, but their role as anti-inflammatory agents is not known in acute stroke patients. Accordingly, this study examined the induction of TNF- α and IL-8 as potential pro-inflammatory cytokines in acute stroke patients and determined the effects of the antiplatelet drugs aspirin, clopidogrel and dipyridamole, and piracetam in their induction. Fourteen patients (9 males and 5 females) at an age range of 31-72 years (mean = 55.93) with the first attack of acute ischemic stroke were used in the study and compared to 14 matched control subjects (9 males and 5 females) at an age range of 30-73 years (mean = 54.29). Patients group had 25.71% occurrence of hypertension and 57.14% of diabetes compared to 25.87% hypertension and 55.5% diabetes in controls group. Hyperlipidemia occurred in 64.28% of patients group (1.77 mmol/l Triglyceride, 4.94 mmol/l Cholesterol, 3.61 mmol/l LDL, 1.39 mmol/l HDL) compared to 62.41% hyperlipidemia in controls group (1.62 mmol/l Triglyceride, 4.87 mmol/l Cholesterol, 3.18 mmol/l LDL, 1.22 mmol/l HDL). Mean of fasting blood glucose was 7.35 mmol/l in patients group and 7.36 in controls group. White Blood Cell (WBC) and platelet counts in patients group were 6.72x10³ and 250.68 x10⁶ respectively compared to 6.48x10³ and 250.68 x10⁶ respectively in controls group (all characteristics of patients and controls were shown in Table 1).

Illustration of intracellular the cytokine TNF- α and IL-8 protein production as detected by immunohistochemistry was shown in Fig. (1). Leukocytes from patients with first attack of ischemic stroke cultured and stained by intracellular method for cytokine detection as described in the Methods. Only patients who presented with first acute ischemic stroke (n=14) and who didn't use any antiplatelet drug were examined. Patients with hemorrhagic stroke or those who started any antiinflammatory treatment were excluded. Matched subjects with similar risk factors who were not under any antiplatelet or antiinflammatory drugs (n=14) were used as control.

Spontaneous induction of TNF- α was significantly increased in patients compared to controls (p<0.001) (Fig. 2A and B). Aspirin, clopidogrel and dipyridamole had a significant inhibitory effect on TNF- α production (p<0.001, p<0.01) respectively (Fig. 2A). Aspirin and clopidogrel combination significantly reduced TNF- α production (p<0.001). Also, aspirin dipyridamole combination significantly reduced TNF- α production (p<0.001) (Fig. 2B). However, the most potent inhibition on TNF- α was demonstrated by the

Table 1. Characteristics of Patients and Controls

Characteristics	Patients (n=14)	Controls (n=14)
Age (years, range)	55.93 (31-72)	54.29 (30-73)
Gender (n, %)	5 Female (35.71%); 9 Male (64.29%)	5 Female (35.71%); 9 Male (64.29%)
Hypertension (%)	25.71	25.87
Diabetes (%)	57.14	55.5
Hyperlipidemia (%)	64.28	62.41
Glucose (3.4-6.1mmol/l)	7.35	7.36
Triglyceride (0.11-2.15mmol/l)	1.77	1.62
Cholesterol (3.88-6.47mmol/l)	4.94	4.78
LDL-chol. (<3.4mmol/l)	3.61	3.18
HDL-chol. (F= 0.91-1.168 mmol/l); (M= 0.91-2.07mmol/l)	1.39	1.22
WBC (4.4-11 ×10 ³ /μl)	6.72	6.48
Platelets count (150-450 ×10 ⁶ /μl)	250.68	245.59
% age of Ischemic stroke	100	0

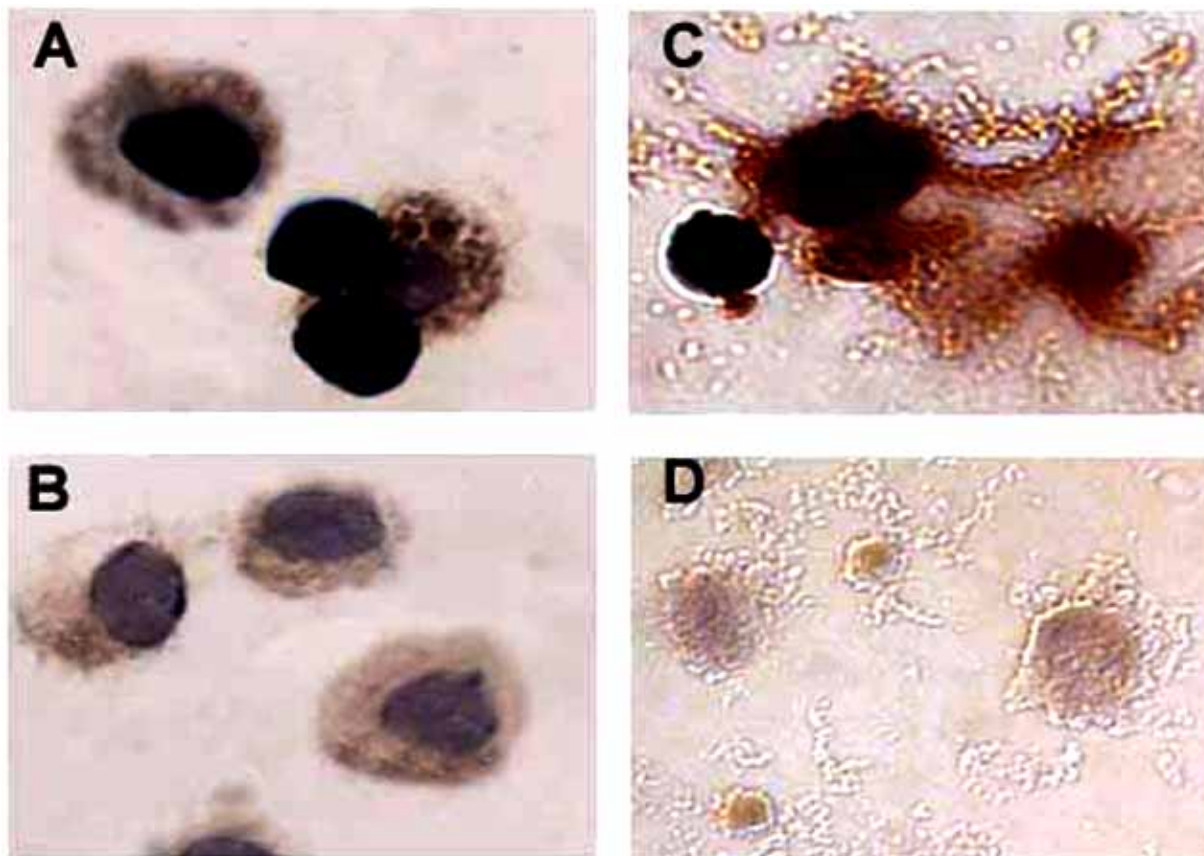


Fig. (1). Illustration of tumor necrosis factor alpha (TNF-α) and Interleukin (IL)-8 producing cells detected by immunohistochemistry.

TNF-α and IL-8 protein as detected by immunohistochemistry. Leukocytes from patients with first attack of ischemic stroke cultured and stained by intracellular method for cytokine detection as described in the Methods. Note the very high production of TNF-α in (A) and IL-8 in (B) compared with the low production in the control (C) and (D) respectively.

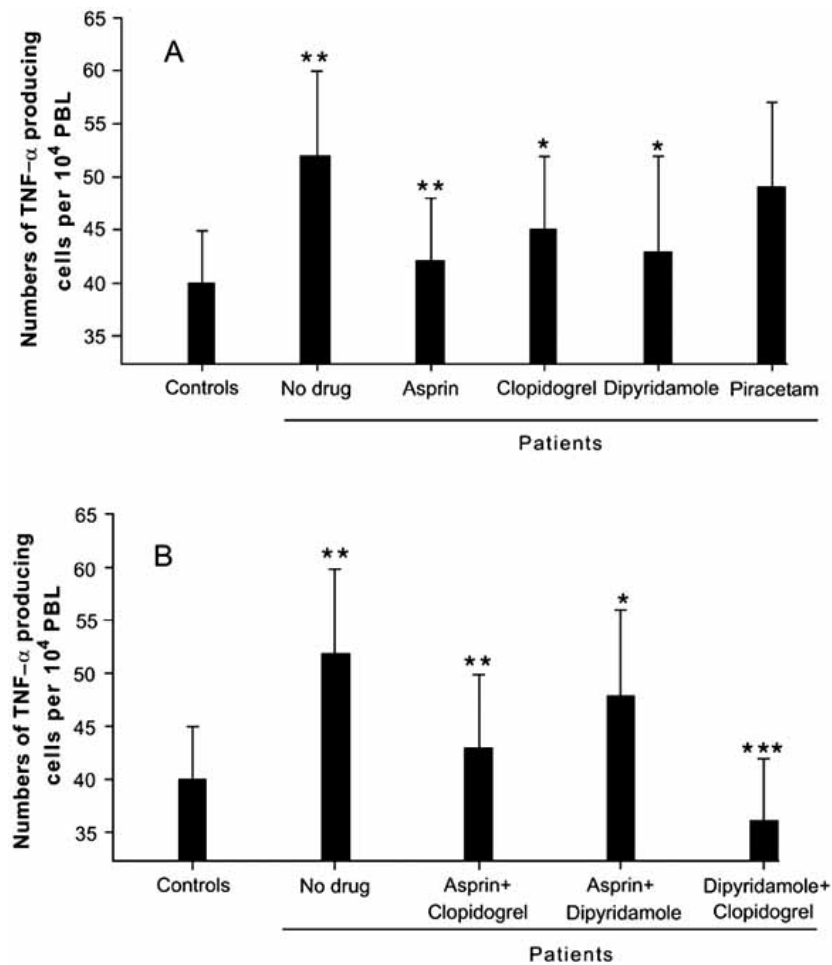


Fig. (2). TNF- α producing cells.

Fig. (2A) illustrates constitutive tumor necrosis factor alpha (TNF- α) expressing cells per 10⁴ leukocytes and the effects of antiplatelets and piracetam on the spontaneous TNF- α production. Fig. (2B) demonstrates the effects different drug combinations on the spontaneous TNF- α production as measured by immunohistochemistry. Leukocytes suspensions were prepared from patients with first attack of acute stroke and from matched controls and stained as described in the Methods. Mean and standard deviations are shown. Stars denote statistical differences between ischemic stroke patients (number=14) and matched controls (number=14) (*= $p < 0.01$, **= $p > 0.001$, ***= $p > 0.0001$).

dipyridamole clopidogrel combination ($p < 0.0001$) (Fig. 2B). On the other hand, Piracetam did not show significant effects on TNF- α production.

In case of IL-8, the spontaneous induction of this cytokine was also significantly increased in patients compared to controls ($p < 0.001$) (Fig. 3A and B), which was significantly inhibited by the action of the antiplatelet drugs aspirin, clopidogrel and dipyridamole ($p < 0.001$, $p < 0.01$) respectively (Fig. 3A). However, aspirin and clopidogrel combination did not influence the induction of IL-8. This was also the case when aspirin dipyridamole combination was used. As for TNF- α , the most potent inhibition of IL-8 was demonstrated by the dipyridamole clopidogrel combination ($p < 0.0001$) (Fig. 3B) and that Piracetam did not show significant effects on the induction of IL-8.

DISCUSSION

This study demonstrated a significant increase of spontaneous TNF- α and IL-8 induction in patients with first attack of acute ischemic stroke compared to matched control group

and inhibition of this activity by antiplatelet agents. TNF- α has been associated with stroke in various roles such as initiation and propagation of inflammatory process (Feuerstein *et al.*, 2002), cell apoptosis in ischemic injury (Akasaka *et al.*, 2006) and CNS reperfusion injury (Kostulas *et al.*, 1999). Systemic increase of IL-8 was noted in ischemic stroke patients (Alberts *et al.*, 2002). IL-8 has also been associated with atherogenesis, reperfusion injury and plaque destabilization in stroke patients (Grau *et al.*, 2001). When anti-IL-8 antibodies were used, both cerebral edema and ischemic damage were declined (Alberts *et al.*, 2002).

Antiplatelet therapies are an important strategy in stroke management. With regards to efficiency and safety issues, there is growing evidence that a combination of antiplatelet therapies may have an important benefit of vascular event prevention when they use different mechanisms of platelets inhibition. However, combination of therapies is now recommended for patients with high risk to atherosclerotic events (Zhao *et al.*, 2001). Zhao and his colleagues (Zhao *et al.*, 2001) tested effect of combination of triple antiplatelet

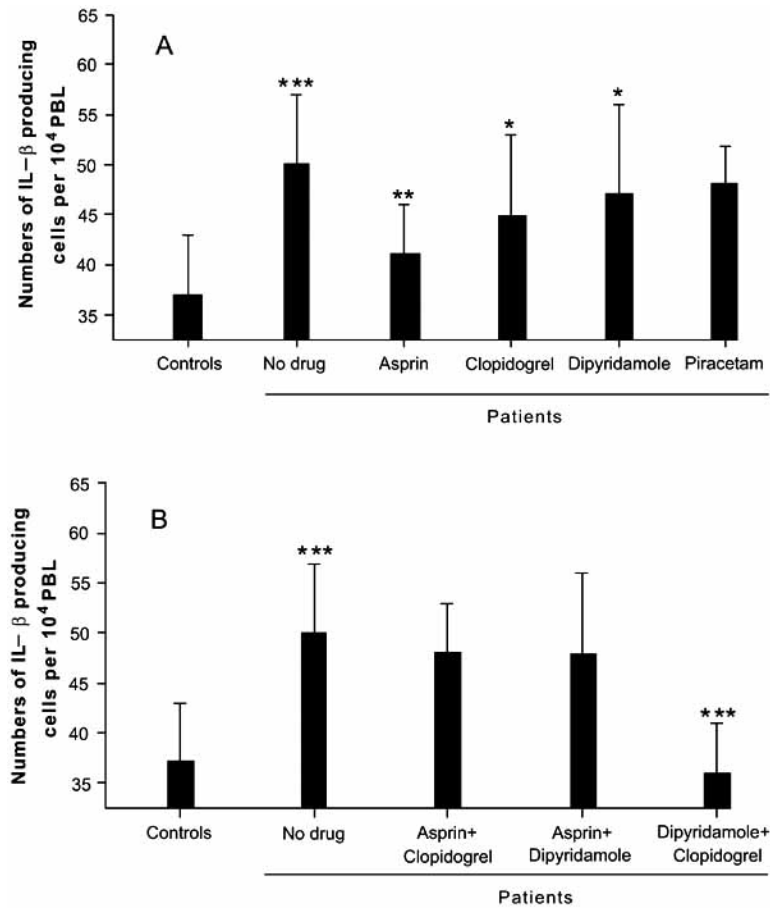


Fig. (3). IL-8 producing cells.

Fig. (3A) illustrates constitutive interleukin (IL)-8 expressing cells per 10⁴ leukocytes and the effects of antiplatelets and piracetam on the spontaneous IL-8 production. Fig. (3B) demonstrates the effects different drug combinations on the spontaneous IL-8 production as measured by immunohistochemistry. Leukocytes suspensions were prepared from patients with first attack of acute stroke and from matched controls and stained as described in the Methods. Mean and standard deviations are shown. Stars denote statistical differences between ischemic stroke patients (number=14) and matched controls (number=14) (*=p<0.01, **=p>0.001, ***=p>0.0001).

agents on platelets and leukocytes activity. They found that the triple combination of aspirin, dipyridamole and AR-C69931 inhibited monocytes and neutrophils activation; and platelet-monocyte and platelet-neutrophil conjugate formation. Consequently, using triple antiplatelet drug together, which work through different mechanisms, might be a new strategy in vascular disease prevention (Gorelick *et al.*, 1999). This study is the first one investigated effects of single or dual antiplatelet therapy (aspirin, clopidogrel and dipyridamole) and piracetam drug on TNF- α and IL-8 productions. Interestingly, we found that these antiplatelet drugs had inhibitory effect on TNF- α and IL-8 productions.

In the 1950s, aspirin was recognized as an agent, which reduces the incidence of myocardial infarctions (Goldstein *et al.*, 2001). Aspirin is one of common efficacy preventive agent for vascular events, at least in part of its antiinflammatory effects (Yang *et al.*, 2004). In this experiment, aspirin was the best agent in reducing of TNF- α and IL-8 releases compared to other drugs. It is possible that these reductions were consequence of antiinflammatory mediator's effect of aspirin (Yang *et al.*, 2004). From that, a new dimension of

aspirin uses may be created not only in stroke management but also in all vascular event diseases. However, aspirin had less inhibitory effect when combined with other antiplatelet drugs. It may have an antagonism to them. Aspirin (325 mg/day) reduced the incidence of stroke, systemic embolism by 32% and the incidence of myocardial infarction and sudden death by 34% (Goldstein *et al.*, 2001). Unfortunately, aspirin has been associated with several side effects such as gastric ulcers, gastrointestinal bleeding and other bleeding episodes (including hemorrhagic stroke). Low aspirin dose (75mg - 150mg daily), which induces maximum platelet inactivation (Patrono *et al.*, 2004), and enteric-coated or highly buffered aspirin may reduce aspirin gastric toxicity (Goldstein *et al.*, 2001). The U.S. Food and Drug Administration (FDA) recommends using 50mg to 325 mg of aspirin daily for the prevention of ischemic stroke, transient ischemic attack (TIA) and recurrent myocardial infarction (Goldstein *et al.*, 2001).

Clopidogrel was approved by FDA in 1997 as drug for secondary prevention of heart attacks and stroke (Lalouschek *et al.*, 2001). Compared with aspirin, we found that clopi-

dogrel generally had less inhibitory effect on our target cytokines. It has been found that clopidogrel was slightly more effective than aspirin at preventing vascular events (Patrono *et al.*, 2004). However it is recommended for patients who are intolerant of aspirin, have recurrent ischemic events, and have additional vascular diseases such as coronary artery disease (CAD) and a known cause of vascular event (Warlow 2002). Recommended clopidogrel dose is 75mg daily (Patrono *et al.*, 2004).

Further, the present study revealed that aspirin and clopidogrel combination had more inhibitory effect on only production of TNF- α than clopidogrel alone but not more than aspirin alone. Compared with aspirin, clopidogrel plus aspirin group in the CURE study showed 20% relative risk reduction and 2.1% absolute risk reduction in the primary outcome of nonfatal myocardial infarction, stroke, and vascular death. Furthermore, 14% relative risk reduction in stroke was also documented (Zhao *et al.*, 2001). This combination might be more suitable for the first few months after unstable angina (De Schryver *et al.*, 2003). Also, it may enhance anti-thrombotic activities (Zhao *et al.*, 2001). In general, this combination is not preferred because aspirin alone works perfectly even in absence of clopidogrel (De Schryver *et al.*, 2003).

This study demonstrated that aspirin and dipyridamole combination showed higher inhibition only in stimulated TNF- α and IL-8 productions compared with dipyridamole alone. As results of 25 trials, this combination has not been clearly indicated as reducing agent of serious vascular events (Patrono *et al.*, 2004). This combination (ASA 25 mg BID plus extended-release dipyridamole 200 mg BID) had a relative reduction in stroke risk by 23% compared with aspirin alone (Zhao *et al.*, 2001). Compared with placebo, it has been found that aspirin plus slow-release dipyridamole reduced risk of fatal and non fatal stroke by 37%. Consequently, aspirin and dipyridamole combination is better than aspirin alone in secondary stroke prevention (De Schryver *et al.*, 2003). It is usually classified as the first line of stroke management for secondary prevention especially if patients have an unknown cause of vascular event (Warlow 2002). In 1999, FDA approved this combination as drug called (Aggrenox) to reduce risk of stroke for patients who had previous stroke or TIA (Lalouschek *et al.*, 2001).

We found that the combination of dipyridamole and clopidogrel had significant inhibitory effect on both cytokines. Generally, it inhibited our target cytokines better than clopidogrel alone. Unfortunately, there is no data concern with using of this combination in the clinics. Piracetam did not show inhibitory activity of spontaneous induction of IL-8 or TNF- α . However, neuroprotective and antithrombotic properties of piracetam may have a role in reducing the disability of acute stroke patient (Hankey and Eikelboom 2003), in addition to the demonstrated importance of piracetam in increasing of compromised regional cerebral blood flow and in improving of motor function, aphasia and level of consciousness (De Schryver 2003).

The observation that the agents examined can alter platelet function may be completely independent from the mechanisms that alter cytokines. This was considered in view of action of effects of certain drugs on inflammatory

mediators by a mechanism that differs from their known mechanism of action (Ji *et al.*, 2006, Harada *et al.*, 2006, Park *et al.*, 2006, Kast RE 2006). In conclusion, TNF- α and IL-8 and may be involve in stroke pathogenesis and that antiplatelet agents may have an important role in inhibition of stroke mediates cytokines. This aspect may initiate a new dimension of using antiplatelet agents as cytokines inhibitors.

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