SHORT COMMUNICATION

Antioxidants Aleviate Nicotine-Induced Platelet Aggregation in Cerebral Arterioles of Mice *in Vivo*

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Summary

Experimental data on the effect of nicotine on cerebral microvessel thrombosis is lacking. Therefore, this study was carried out to elucidate the effects of nicotine on platelet aggregation in cerebral (pial) microcirculation of the mouse, and the possible protective effect of vitamins C and E. Male TO mice were divided into six groups, and injected i.p. with saline as a control, nicotine (1 mg/kg), vitamin C alone (100 mg/kg), vitamin E alone (100 mg/kg), nicotine plus vitamin C or nicotine plus vitamin E, all for one week before the experiment. After one week, platelet aggregation in cerebral microvessels of these groups of mice were studied *in vivo*. The appearance of the first platelet aggregation and total blood flow stop in arterioles and venules were timed in seconds. In the animals treated with nicotine, venules did not show any alteration in the platelet aggregation time in comparison to the control animals. However, in arterioles platelet aggregation time was significantly accelerated (p<0.001) in nicotine-treated animals as compared to controls. Both vitamins C and E prevented the shortening of arteriolar platelet aggregation time significantly (p<0.001) when applied with nicotine but not alone. It can be concluded that nicotine enhances the susceptibility to thrombosis in the cerebral arterioles *in vivo* and that vitamins C and E have alleviating effect on nicotine-induced thrombotic events in mice pial microvessels.

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Tobacco smoke, a health hazard most widely associated with cancer, is also a significant independent risk factor for cardiovascular disease and a leading cause of structural and functional alterations of the cardiovascular system (Bernhard *et al.* 2003, Rahman and Laher 2007, Shireen *et al.* 2008).

Most clinical and experimental investigations on the pathophysiology of cigarette smoking have studied the effects of smoke as a whole, while a few studies focused on specific components of cigarette smoke, e.g. nicotine (Rubenstein *et al.* 2004). Nicotine exposure *via* cigarette smoking has been implicated in the pathogenesis of cardiovascular disorders like atherosclerosis and hypertension (Lindenblatt *et al.* 2007). Nicotine causes vasoconstriction and increased blood pressure (Lindenblatt *et al.* 2007). A drop in blood-flow velocity in humans, endothelial damage in rats, and inhibition of platelet aggregation in rabbit blood, have all been shown experimentally (Lindenblatt *et al.* 2007). Nicotine has been reported to upregulate the expression of various proteins such as basic fibroblast growth factor, tumor necrosis factor-α and plasminogen activator inhibitor-1 (Lau *et al.* 2006). In addition, nicotine induces mononuclear leukocyte adhesion and expression of adhesion molecules such as vascular cell adhesion molecule-1 and intracellular adhesion molecule in endothelial cells (Albaugh *et al.* 2004).

Circulating platelets do not normally adhere to the endothelial cells of the vascular wall or aggregate, except when activated or when the endothelium is damaged. Delivery of blood to various organs and proper tissue perfusion are significant for normal tissue function.

Development of thrombi in the circulatory system would result in reduced blood delivery and consequently, in tissue hypoxia/ischemia. Smoking is linked to thromboembolic events (Hioki *et al.* 2001), but a relationship between nicotine exposure and cerebral microvessel thrombosis has not been established.

Oxidative stress has been shown to be the major cause of atherosclerosis and smoking is considered to induce oxidative stress. Oxidative stress arises from an imbalance between the production of free radicals and physiological antioxidant capability. The supplementation with vitamin C and E has been reported to provide health benefits against cardiovascular diseases through its antioxidant activity, and the inhibition of platelet aggregation (Niki, 2004, Singh and Jialal 2006, Sesso *et al.* 2008, Violi *et al.* 2010).

Therefore, the aim of this study was to examine the influence of nicotine exposure on the *in vivo* platelet aggregation in cerebral, pial, microcirculation of mice, and to assess the possible protective effects of vitamin C and vitamin E.

All animal treatment procedures met both the NIH and Institutional Animal Ethics Committee guidelines. Adult male mice, TO strain, weighing 30-35 g from a colony maintained in our animal house facility were used. Animals were group-housed in a temperature maintained (22 ± 2 °C) and a light/dark cycle (12/12 h) environment. They had access to standard pelleted diet and water *ad libitum*. Except for their intended treatment all animals were handled in the same manner.

Nicotine (Sigma) aqueous solution (40 %) in a dose of 1 mg/kg body weight was dissolved in normal saline and injected intraperitoneally (i.p) for one week every day. Normal saline (vehicle) was injected in the same manner to the control. Concurrently with the nicotine or saline treatment, animals were injected (i.p.) with antioxidants: vitamin C (100 mg/kg b.w./day), or vitamin E (100 mg/kg b.w./day) for the same week.

After the treatments, mice were anesthetized with urethane (25 %, 0.1 ml/10 g b.w., i.p) and trachea was intubated. Cerebral microvessels were exposed after performing craniotomy on the anesthetized mice on the left side of the head, with the aid of a microdrill and dura was carefully removed. The body temperature was stabilized at 37 °C with the help of an infrared lamp and monitored with a rectal thermoprobe (Physitemp Model RET-3). The preparation was kept moist with artificial cerebrospinal fluid (ACSF). Composition of the ACSF used was (mM): NaCl 124, KCl 5, NaH_2PO_4 3, $CaCl$ 2.5,

$MgSO_4$, 2.4, $NaHCO_3$ 23, and glucose 10; pH 7.3. Preparation showing any level of trauma to the vessel or brain tissue was discarded.

The closed circuit set up used consisted of a fluorescence microscope (Olympus, BH-2), a video camera (JVC, TK-890E), a VHS-VCR (JVC, BR-S600E) and a television monitor (JVC, TM-1500PS). All microscopic observations utilized a fiber-optic light source and were made using a 4 x objective lens and a 10 x eye piece. Total magnification of the observed field, from the microscope stage to the television monitor was x250.

Sodium fluorescein (2 %, 0.1 ml/10 g b.w.) was injected *via* the tail vein. It was allowed to circulate in the body for 30 seconds before mercury light was switched on. The combination of mercury light exposure and fluorescein dye produces a free radical which injures the endothelium of the microvessel lumen. This in turn, causes platelets to adhere at the sites of endothelial damage and then aggregate. The fluorescence of aggregates was readily visible as they adhered to the damaged endothelium (Fig. 1). Platelet aggregates grow in size until complete vascular occlusion (Fig. 1). The time when the first observable aggregate appeared (time to first aggregate) and until full vascular occlusion (time to flow stop) in both venules and arterioles (Fig. 1) were measured in seconds. The time of aggregate growth was calculated as the difference between the time to flow stop and the time to first aggregate. Diameters before insult and after full vascular occlusion were measured in control and treated mice from video recordings and changes were calculated. All events were video-taped for analysis and measurement of microvessel diameter changes.

All data were analyzed with GraphPad Prism Version 4.01 for Windows software (Graphpad Software Inc., San Diego, CA). Data are expressed as means ± S.D. Comparisons between groups were performed using the Mann-Whitney U test. A *p* value <0.05 was considered to indicate statistical significance.

The effects of nicotine treatments on the time required for platelet aggregation and for full occlusion in pial microvessels are summarized in the Table 1. Our results show that in the animals treated with nicotine (1 mg/kg) one week before the experiment, venules did not show any alteration in the platelet aggregation timings in comparison to the control animals. But in arterioles platelet aggregation time was significantly accelerated (p<0.001) in nicotine treated animals. In both arterioles and venules, vascular diameter changes

throughout the photochemical insult among both groups were within a very narrow range and no statistically significant differences were present (Table 1). Data on platelet aggregation revealed significant shorter times (P<0.001) for the appearance of the first aggregate in pial arterioles of the nicotine-treated group, thus indicating an increased susceptibility to thrombosis in nicotine-exposed animals. In contrast to arterioles, measured platelet aggregation parameters in venules were not significantly different in control and nicotine-exposed animals.

When vitamins C and E were injected alone, the time for the first observed platelet aggregation and for full occlusion in pial microvessels did not significantly change. However, when both vitamins were injected concurrently with nicotine, they reduced significantly (p<0.001) the time required to initiate the thromboembolic platelet aggregation and the time for full occlusion in pial microvessels as compared with nicotine-treated mice (Table 1).

The present study revealed that nicotine treatment accelerated the time required for both the appearance of the first aggregate and the time for full occlusion in pial arterioles. Although both vitamins C and E when injected alone decreased the timing of the pial arteriols thrombotic events not significantly, but their concurrent injection along with nicotine indeed had a significant effect (p<0.001) when compared to nicotine treatment alone (Table 1).

The nicotine dose and the duration of the treatment was selected according to the excellent review (Matta and Elberger 2007) that explains the approaches which have been developed, strain-dependent differences, modes of nicotine administration, each resulting in elevated blood nicotine concentration.

The results of this study complement the data from previous studies in animals and humans. For example, reported data indicate that nicotine and cotinine exert direct effects on endothelial cells and smooth muscle cells, shifting them toward a pro-thrombotic state *via* induction of tissue factor expression (Massberg *et al.* 2002). These effects on cells of the vessel wall might explain, at least in part, the deleterious cardiovascular consequences of smoking. Platelet-dependent thrombin level is enhanced in smokers, even when not smoking, when compared with non-smokers and increases immediately after smoking. Increases in nicotine and cotinine levels caused by smoking induced a prothrombotic state in smokers *via* increased platelet-dependent thrombogenesis (Hioki *et al.* 2001).
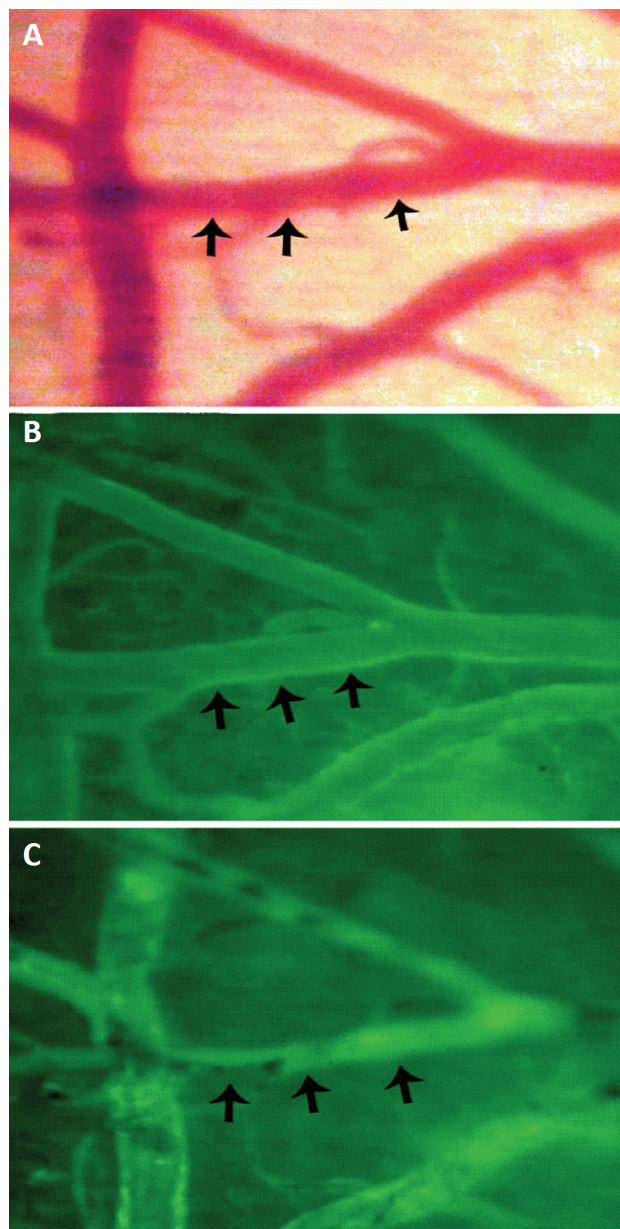
Fig. 1. Representative pictures of a field containing cerebral pial microvessels in control group before **(A)** and immediately after i.v. injection of fluoroscein **(B)**, showing clearly the microvessels. Arrows indicate pial arteriole. As consequence of the photochemical reaction between fluorescein and continues illumination with mercury light, platelets start to aggregate and thrombus forms and grows in size until it causes a complete vascular occlusion **(C)**. The differences in the occlusion times between the groups are indicated in Table 1.

Our data show that one week of antioxidants treatments revealed that both vitamins C (100 mg/kg) and E (100 mg/kg) caused a significant reduction in platelet aggregation compared with control (Table 1, *P*<0.001). The doses of vitamin C and E used in the current study are comparable to the those reported by others in rats and mice (Komeima *et al.* 2006, Ming *et al.* 2006, Ocak *et al.* 2007). However, with respect to human studies, it has

Table 1. Platelet aggregation timings and diameter changes in control vs nicotine and antioxidants treated animals.

	Control (saline)	Nicotine (1 mg/ml)	Vitamin C alone	Vitamin E alone	Nicotine + Vitamin C	Nicotine + Vitamin E
Weight of the animals (g)	33.7±1.1	32.6±1.9	31.6±1.5	32.5±1.6	31.9±1.5	32.5±2.4
Venules						
Time to first aggregate (s)	28.1±2.0	25.4±1.6	29.4±2.7	27.4±2.2	28.2±1.5	26.9±2.1
Lower 95 % CI	26.7	24.3	27.5	25.8	27.1	25.4
Upper 95 % CI	29.5	26.5	31.3	29.0	29.3	28.4
Time to flow stop	167.1±31.7	151.3±19.9	174.0±27.6	172.7±28.5	159.6±20.1	155.7±24.8
Lower 95 % CI	144.4	137.0	154.2	152.3	145.2	138.0
Upper 95 % CI	189.8	165.6	193.8	193.1	174.0	173.4
Arterioles						
Time to first aggregate (s)	94.3±12.9	54.7±9.9*	94.0±10.8	91.4±8.1	71.7±11.1*	70.5±12.0*
Lower 95 % CI	85.1	47.6	86.3	85.6	63.8	62.0
Upper 95 % CI	103.5	61.8	101.7	97.2	79.7	79.1
Time to flow stop	201.1±23.8	137.6±9.9	196.1±14.2	200.2±20.1	155.1±21.3*	154.8±25.1*
Lower 95 % CI	184.0	130.5	185.9	185.8	139.9	136.8
Upper 95 % CI	218.0	144.7	206.3	214.6	170.3	172.8
Venules diameter (μm)						
Initial diameter	42.6±3.5	41.0±4.4	39.8±3.3	41.1±5.0	44.1±2.4	43.2±2.4
Lower 95 % CI	40.1	37.9	37.4	37.6	42.4	41.2
Upper 95 % CI	45.1	44.1	42.2	44.6	45.8	44.9
Diameter after the aggregation	51.2±7.5	51.9±11.9	48.9±5.3	52.7±7.5	53.6±4.7	52.5±4.4
Lower 95 % CI	45.8	43.4	45.1	47.4	50.3	49.3
Upper 95 % CI	56.6	60.4	52.7	58.0	56.9	55.7
Arterioles diameter (μm)						
Initial diameter	41.1±4.3	37.8±2.0	39.4±5.2	39.9±3.8	41.5±3.0	41.7±2.8
Lower 95 % CI	38.0	36.4	35.7	37.2	39.3	39.7
Upper 95 % CI	44.2	39.2.1	43.1	42.6	43.7	43.7
Diameter after the aggregation	50.3±9.9	48.8±8.6	49.9±7.4	49.7±6.5	50.1±4.7	51.4±5.5
Lower 95 % CI	43.2	42.7	44.6	45.1	46.8	47.4
Upper 95 % CI	57.4	55.0	55.2	54.4	53.5	55.4

* $P < 0.001$, number of animals n=10, values are means ± standard deviation.

been reported that doses of vitamin C are generally tolerated up to a level of 2000 mg/day (Standing Committee on the Scientific Evaluation of Dietary Reference Intakes 2000, Sesso *et al.* 2008). A dose of 400 IU/ day (~268 mg/day) vitamin E has been used in large-scale clinical trial for cancer prevention with cardiovascular disease as secondary endpoints (Lippman *et al.* 2005). Thus, compared to human studies, the doses of vitamins E and C in our study were relatively high.

Vitamin E is the principal lipid-soluble antioxidant in human plasma, and some studies indicate that it may provide cardiovascular protection (Violi *et al.* 2010). Oxidative stress has been considered to play a major role in the development of atherosclerosis.

Consistent with this idea, numerous studies have shown an inverse association between atherosclerosis and antioxidant intake (Golestani *et al.* 2006, Guney *et al.* 2007, Demiralay *et al.* 2008, Chiu *et al.* 2009). The development of atherosclerosis and myocardial ischemia-reperfusion injury; both of these phenomena are associated with platelet activation (Ulker *et al.* 2003). It has been reported that tissue-type plasminogen activator enhanced platelet adhesion, and co-administration of vitamin E further decreased platelet adhesion in a model of thrombosis (Hioki *et al.* 2001, Cirillo *et al.* 2006). Moreover, an inverse relationship between the intake of vitamin C and risk of an acute myocardial infarction was found in humans (Antoniades *et al.* 2003, Chiu *et al.* 2009). Non-toxic doses of vitamin E in treated rats can upregulate SOD activity, but cumulative effect of the same doses can lead to the attenuation of SOD activity and hence antioxidant defense (Golestani *et al.* 2006). Moreover, vitamin E treatments significantly reduced the rate of nicotine-induced hippocampal neural cell apoptosis (Demiralay *et al.* 2008).

In animal models of endothelial dysfunction, vitamin E improved the activity of endothelium-derived nitric oxide, in part due to the inhibition of protein kinase C (PKC) stimulation (Freedman and Keaney Jr. 2001). This activity of vitamin E was examined in platelets, and vitamin E inhibited platelet aggregation in part through a mechanism that involves PKC (Freedman and Keaney Jr. 2001).

It can be concluded that one week exposure to nicotine causes prothrombotic events in the cerebral arterioles, and that the antioxidant vitamins C and E alleviate these effects. Further experiments are needed to investigate the mechanism underlying the observed effects.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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