

ORIGINAL RESEARCH

PINE BARK EXTRACT REDUCES PLATELET AGGREGATION

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The effects of long-term consumption of the bioflavonoid mixture, French maritime pine bark extract (Pycnogenol®), were assessed on aggregation of platelets from cigarette smokers and nonsmokers. Previously we showed that a single dose of Pycnogenol® reduced platelet aggregation in cigarette smokers in a dose-response fashion. Cigarette smoking increased platelet reactivity aggregation when measured 2 h after smoking the first cigarette of the day. Blood was collected immediately before and 5 min after smoking three cigarettes each. Smoking increased platelet aggregation (1.17 ± 0.04). However 200 mg Pycnogenol®/day, taken 3 h prior to first cigarette for the day for 2 months, significantly ($p < .0023$) reduced smoke-induced platelet aggregation (0.98 ± 0.05) to the level of nonsmokers. In a group of 19 nonsmokers, platelet aggregation was measured during in vitro stimulation by platelet aggregation factor (PAF) after 4 or 8 weeks of 200 mg/day of Pycnogenol® consumption. Platelet aggregation was significant when induced in vitro by PAF. However, Pycnogenol® consumption did not change platelet aggregation, suggesting that Pycnogenol®'s regulation of aggregation is by another mechanism. Thromboxane A2 (TxA2) is increased in smokers by release from platelets and rapidly becomes thromboxane B2 (TxB2). Smoking increased TxB2, which was prevented by Pycnogenol®, lowering TxB2 levels to those of nonsmokers. However, Pycnogenol® had no effect on the lower levels of TxB2 in nonsmokers. These observations suggest that Pycnogenol® supplementation reduces a risk factor for cardiovascular diseases, that is, platelet aggregation in smokers. The bioflavonoids in Pycnogenol® reduced platelet aggregation stimulated by tobacco smoke. (Int Med 1999;2:73-77) © 2000 Elsevier Science Inc.

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Tobacco smoking is the leading preventable cause of death in the United States. Cigarette smoking [1,2] as well as aging result in platelet aggregability [3-8], promoting vascular disease [9]. Smoking even one cigarette significantly increases platelet aggregation [7]. After smoking, plasma epinephrine increased more than twofold as did thrombin-induced platelet aggregation [8]. Cigarette smoking also stimulated formation of the proaggregatory thromboxane A2 (TxA2), while significantly decreasing anti-aggregatory prostacyclin [10,11]. Although treatment with aspirin prevented smoking-induced platelet aggregation [8,11], it was less effective in diabetics [12] and patients with stable coronary disease [7], while frequently in-

creasing bleeding time. Platelet activation occurred after ischemic stroke [13-15].

Procyanidins, found in a variety of edible plants, inhibit thromboxane B2 (TxB2) and platelet aggregation in vitro. Polyphenolic compounds from *Lonicera japonica* have anti-aggregatory effects [16]. Although procyanidins in red wine may provide some cardiac protection, the adverse cardiovascular consequences of high alcohol intake in many individuals encouraged us to search for other sources of procyanidins. The polyphenolic compounds, procyanidins and phenolic acids, in Pycnogenol® [16,17] inhibited epinephrine-induced platelet aggregation in vitro [17].

Recently we found that a single dose of Pycnogenol® prevented smoke-induced platelet aggregation [4]. Therefore, smokers were used in the present study to determine the long-term effects on platelet aggregation of Pycnogenol®. We investigated whether changes in platelet aggregation in smokers could be produced by long-term Pycnogenol® supplementation.

Cigarette smoking also facilitates platelet formation of proaggregatory TxA2, which has a vasoconstrictive effect.

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Cigarette smoking is the only established risk factor for coronary vasospasm (variant angina). An urinary metabolite of Tx_{A2}, 11-dehydro-TxB₂, is higher in patients with peripheral arterial disease. TxB₂ is a stable hydrolyzed product of unstable Tx_{A2}. It is derived from prostaglandin, which is synthesized from arachidonic acid through the cyclooxygenase pathway. TxB₂ is a major product following platelet aggregation induced by a variety of agents such as thrombin and collagen. We report here that Pycnogenol® reduced smoke-enhanced TxB formation in vivo.

PATIENTS AND METHODS

Subjects

Approval of the Institutional Review Board of the University of Arizona was obtained for these studies. Volunteers gave written consent to participate and were free to withdraw at any time. All smokers, four men and four women, smoked at least 15 cigarettes (Marlboro Red) per day for more than 5 years. Exclusion criteria were pregnancy, or regular intake of anticoagulants, antioxidant vitamin supplements, analgesics, or nonsteroidal anti-rheumatics. Volunteer smokers were screened for those with more than 10% increased platelet aggregation upon smoking. They received 200 mg/day of Pycnogenol® for 8 weeks. A group of 16 nonsmokers received 200 mg of Pycnogenol® supplements daily for 8 weeks. The subjects were asked to ingest the Pycnogenol® supplements 3 h before blood collection. No adverse effects were reported. Smoking subjects reported feeling more energetic with none of the common morning pulmonary congestion when taking Pycnogenol®. The nonsmokers did not report any clinical changes due to supplementation.

Supplementation

Smoking-volunteers received a single dose of 200 mg Pycnogenol® (Pycnogenol tablets, Henkel, Chicago, IL) per day. Nonsmokers volunteers received 2 mg/kg body weight with 200 mL of tap water. Both groups were supplemented for 60 days. In the week prior to supplementation, platelet aggregation was determined on two separate days. Thereafter, each smoker consumed 200 mg Pycnogenol®/day while each nonsmoker took 2 mg/kg body weight of Pycnogenol®. Smokers were instructed not to smoke at least 8–10 h before blood sampling. Collection of heparinized blood occurred between 5:00 and 7:00 A.M. with breakfast 1 h later. Juice, jam, and fruits were excluded to reduce bioflavonoids. Then, 3 h after supplementation, blood samples were taken. Smokers smoked 3 cigarettes within 30 min and immediately after smoking, blood samples were taken again. Nonsmokers ingested Pycnogenol® pills before 10:00 A.M. each day with consumption of juice, jam, and fruits excluded the day of sampling.

Platelet Reactivity Index (PRI) in Smokers

Platelet aggregation in smokers was measured by the method of Grottemeyer [18]). This method yields a PRI as described in detail elsewhere [4]. This assay quantifies the ratio of the number of nonaggregated platelets and the total number of platelets measured immediately. The mean PRI of 120 healthy volunteers was 0.98 ± 0.09 [19] over a period of 260 days. The index was significantly higher in persons with transitory ischemic attack [13]. Blood in smokers was collected from the antecubital vein using an R-21 butterfly in 3-mL syringes with the first 200 μ L being discarded. The blood was collected immediately before and 5 min after smoking three cigarettes. Then 300 μ L blood were collected directly into two syringes containing either 1 mL ethylene diamine tetraacetic acid (EDTA) buffer (11.4 mmol Na EDTA in 1/15 mmol NaK hydrogenphosphate, pH 7.4) or EDTA-formaldehyde buffer (EDTA buffer containing 1% formaldehyde). Erythrocytes were counted in both samples. Both samples were centrifuged for 20 min at $52 \times g$. Supernatants were collected and platelets were counted by means of a platelet counter [13]. PRI was calculated [4,18,20] by the following formula:

$$\text{PRI} = \frac{\text{Platelets in EDTA} \times \text{Erythrocyte number in EDTA-formaldehyde}}{\text{Platelets in EDTA-formaldehyde} \times \text{Erythrocyte number in EDTA}}$$

Platelet Aggregation Activity Induced In Vivo in Cells from Nonsmokers

Blood was collected from the antecubital vein using a R-21 butterfly in 3-mL syringes with the first 2 mL being discarded. Then 3 mL blood were collected directly into two syringes containing either 9 μ L heparin (3.0-unit heparin/mL blood). Clotting time was measured automatically by Medtronic (HMS Instruments, Hemo Status, MN) via Platelet function-PAF Test Cartridges. Platelet activating factor (PAF) concentrations of 0, 1.25, 6.25, 12.5, and 150 nM were used to test platelet aggregation time (clotting time) in nonsmokers (Table 1).

Tx Assay

TxA₂ is produced by platelets, fibroblasts, and microphages. Quantitation of Tx formation can be made by determining the level of the more stable TxB₂. After blood was drawn and centrifuged, serum was separated. Then 0.2 mL methanol was added to 1 mL serum and vortexed. After centrifugation for 10 min at 1,200 rpm serum was applied into a preconditioned C18 Sep-Pak® column (Waters® Corporation) with a flow rate of 1 mL/min. Then the column was washed with 15% methanol in water and then petroleum ether. Finally TxB₂ was eluted by methyl formate, which was evaporated with a stream of nitrogen gas. After dissolving the residue with 1 mL of diluted extraction buffer, 50- μ L samples were assayed in triplicate using a Neogen TxB₂ enzyme-linked immunosorbent assay (ELISA) kit.

Table 1. The effect of 200 mg Pycnogenol consumption on *in vitro* clotting time in nonsmokers

PAF level	n	Age (years) (mean ± SE)	Clotting time (s)		p
			Baseline (mean ± SE)	9th week (mean ± SE)	
None	8	32.1 ± 1.42	687.44 ± 48.03	618.55 ± 54.5	.2
	8	65.28 ± 1.62	503.29 ± 24.07	541 ± 37	.12
125 nM	8	32.1 ± 1.42	614.77 ± 48.61	471.66 ± 36.01	.015***
	8	65.28 ± 1.62	315 ± 21.31	304.44 ± 22.49	.41
150 nM	8	32.1 ± 1.42	315.33 ± 21.35	293.33 ± 20.14	.5
	8	65.28 ± 1.62	249.86 ± 8.13	289.166 ± 15.07	.03***

PAF, platelet aggregation factor.

*** Significantly different from baseline value prior to supplementation.

Statistics

The data were analyzed using two-way analysis of variance (ANOVA) Student's *t*-test and verified using the Wilcoxon rank-sum (Mann–Whitney) test. Data expressed as means ± SEM with significance considered when $p < .01$.

RESULTS

Platelet Aggregation

The PRI of smokers was 1.17 ± 0.04 after smoking (Fig. 1). This was higher than previously reported values of 0.98 ± 0.05 for nonsmoking adults [21]. Smoking significantly increased ($p < .05$) the PRI. Intake of single dose of 200 mg of Pycnogenol®/day for 8 weeks prevented this enhancement of platelet reactivity completely after smoking (Fig. 1). In a group of heavy smokers, 200 mg Pycnogenol® significantly ($p < .008$) reduced platelet reactivity from 1.17 ± 0.04 to 1.01 ± 0.04 (Fig. 1).

Platelet reactivity during PAF activation *in vitro* was assessed in nonsmoker volunteers who ingested 2 mg/kg-body weight of Pycnogenol®/day for 8 weeks. PAF increased platelet aggregation *in vitro* dramatically using cells from nonsmokers. PAF is one of several mechanisms the

body uses to aggregate platelets. After Pycnogenol® supplementation there was a nonsignificant trend toward less aggregation, which was significant for young smokers at 125 nM PAF (Table 1).

TxB2 Levels

Cigarette smoking significantly increased serum TxB2 concentration in smokers ($p = .0026$). When these smokers were supplemented with Pycnogenol® for 8 weeks, serum TxB2 levels were reduced to the level of nonsmokers (Fig. 2). Although in nonsmokers Pycnogenol® supplementation resulted in lower serum TxB2 concentrations, the level was not statistically significant from that associated with nonsupplementation (0.30 ± 0.01 vs. 0.31 ± 0.03 , respectively).

DISCUSSION

Enhanced formation of lipid hydroperoxides after smoking is responsible for the acute and marked platelet hyperactivity [21]. Therefore, supplementation with the antioxidant Pycnogenol® should provide protection from such adverse effects of smoking [5]. Among smokers, supplementation with Pycnogenol® for 8 weeks substantially reduced their

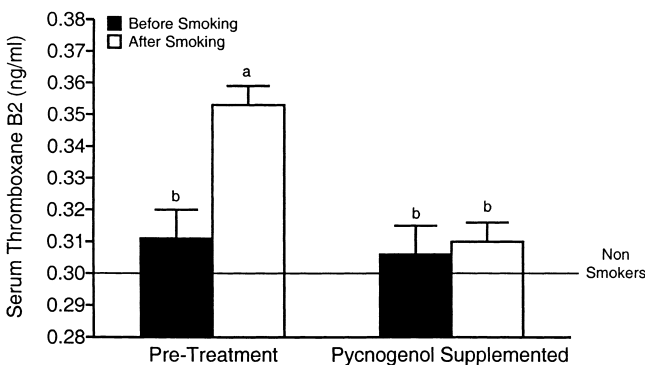


Figure 1. Dose–response effects of Pycnogenol® on platelet reactivity index in smokers ($n = 19$). Significant p values as marked above each bar are compared with respective baselines. The values are means ± SE. ^a Significantly different ($p < .05$) from those groups with letter “b” over them.

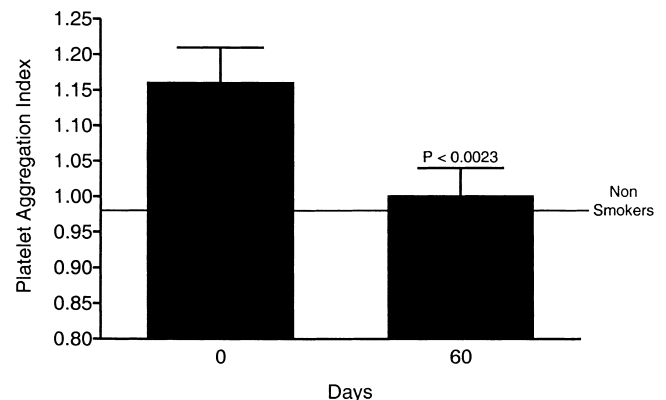


Figure 2. Effects of 200 mg Pycnogenol® on TxB2 levels in serum from smokers ($n = 19$). Significant p values are compared with the baseline. The values are means ± SE.

otherwise excessive platelet aggregation. Previously we reported that the platelet reactivity dropped significantly ($p < .005$) to 0.97 ± 0.03 , 2 h after smoking and taking a single 200-mg of Pycnogenol® when compared with untreated smokers [4]. Moreover, the reduced platelet aggregation remained below the values of smokers without Pycnogenol® supplementation for several days after the single dose [4]. The Pycnogenol® anti-aggregatory effects was diminished gradually after the 10th day with platelet reactivity index of 1.02 ± 0.09 ($p < .11$) in the same subjects [4].

PAF and TxA2 are two different mediators stimulating platelet aggregation upon release into the body. The prostaglandin product, TxA2, is increased by aging or smoking, resulting in an increase of platelet aggregation and the incidence of cardiovascular diseases. Smoking significantly increased serum TxB2 levels. Pycnogenol® prevented the effect of smoking and maintained TxB2 levels, similar to nonsmokers. This finding supports the effects of Pycnogenol® in reducing tobacco smoke-enhanced platelet aggregation. It also suggests that regulation of Tx is a mechanism of action of Pycnogenol®. Supplementation with Pycnogenol® tended to decrease serum TxB2 concentration in nonsmokers, a change that was not statistically significant.

The standardized blood sampling procedure and fixation of platelet aggregation immediately after blood sampling resulted in a PRI that reflected actual platelet aggregation. The PRI was almost 1.0 in nonsmoking volunteers [20–23], showing no enhanced aggregation. In our study, prior to smoking that morning, smokers' platelet aggregation was significantly greater than 1.0. This result shows some aggregation likely due to residual effects of long-term smoking, which was enhanced further by smoking that day. We found that Pycnogenol®'s effects were present more than 6 days after intake of a single 200-mg dose [4,5]. Platelet reactivity remained below baseline for over 10 days after a single dose of 200-mg Pycnogenol®. Although 100- or 150-mg doses of Pycnogenol® also significantly normalized PRI, their effects did not persist for as long as with the larger dose, the 200-mg intake [4].

Several possible mechanisms may explain the anti-aggregation activity in smokers. Procyanidins, like Pycnogenol®, block the smoking-induced formation of TxA2 [13,19], by stimulating production of nitric oxide in the endothelium [16]. Thus, inhibition of platelet aggregation by nitric oxide [24] could be part of the mechanism of Pycnogenol®'s preventive effects. In a dose-dependent manner, Pycnogenol® inhibited epinephrine-induced platelet aggregation in vitro [17]. As smoking doubles plasma epinephrine concentration [25], prevention of epinephrine-induced platelet aggregation by Pycnogenol® may also contribute the in vivo platelet activity. The broad antioxidative properties of Pycnogenol® should also benefit in microcirculation by reducing lipid peroxides [4], which promote formation of atherosclerotic plaques. Pycnogenol® exerts other positive effects including vasoprotection by strengthening capillary walls

and resistance [26]. Pycnogenol® also reduced edema formation in inpatients. Our results further support the conclusions of a recent review [5], which found that Pycnogenol® use strengthened the blood vessels and circulation. Pycnogenol® may be useful in other conditions that commonly promote heart disease such as stress, age, and hypertension. We recently found that Pycnogenol® supplementation among older, nonsmoking adults with mild hypertension dramatically reduced TxB2 (personal communication).

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