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Activation of platelets exposed to shear stress in the presence of smoke extracts of low-nicotine and zero-nicotine cigarettes: The protective effect of nicotine

Jaimohan Ramachandran, David Rubenstein, Danny Bluestein, Jolyon Jesty

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Mainstream and sidestream smoke extracts of both high-tar and low-tar cigarettes have been shown to increase platelet activation directly and to sensitize them to further activation by exposure to mechanical stimuli such as shear stress. However, nicotine has an inhibitory effect on platelet activation, opposite to that of whole cigarette smoke extracts. To distinguish between the nicotine-dependent and non-nicotine-dependent effects of smoke, platelets were exposed to mainstream and sidestream smoke extracts of low-nicotine and zero-nicotine cigarettes in vitro under flow conditions comparable with the normal circulation, and their activation state was measured using a modified prothrombinase-based assay. Both low-nicotine and zero-nicotine extracts caused increased platelet activation upon exposure to shear stress, and they caused significantly greater activation than extracts from ordinary high-tar, high-nicotine cigarettes. That nicotine was crucial was confirmed by adding 50 nM nicotine (comparable with the nicotine level in smokers' plasma) to zero-nicotine smoke extracts and demonstrating a reduction in the shear-dependent rate of platelet activation of more than 75%.

Introduction

Cigarette smoke is a primary risk factor for cardiovascular disease, including the development of atherosclerosis and, in particular, coronary heart disease and peripheral vascular disease (Kritz & Sinzinger, 1996). Enhanced function of the hemostatic system, in which platelets play a major role, is a significant underlying mechanism in cardiovascular disease and its progression. The problems associated with tobacco smoke are not confined to smokers but also apply to nonsmokers exposed to environmental, or secondhand, smoke, which is derived mostly from the smoldering end of the cigarette. Like mainstream smoke, secondhand smoke adversely affects platelet function, causing high states of activation, increased response to physiological and mechanical agonists, and high thrombogenic potential (Glantz & Parmley, 1991; Rubenstein, Jesty, & Bluestein, 2004).

The general role of platelets in cardiovascular disease associated with smoking is reasonably well established, but the role of nicotine in smoking-related cardiovascular disease has not been demonstrated (Benowitz & Gourlay, 1997). It has sometimes been assumed tacitly that the various deleterious effects of tobacco smoke are related mainly to nicotine, but in the case of platelets, no evidence supports this assumption, and the effects of nicotine on platelets remain unclear. The key focus of the present study was to discriminate between the nicotine-dependent and non-nicotine-dependent effects of tobacco smoke on platelets.

Enhanced thrombin generation is an important factor in the pathogenesis of cardiovascular diseases. Platelet-dependent thrombin generation is elevated in smokers, even after abstaining from smoking for several hours (Hioki et al., 2001). Nicotine, however, which is the major psychoactive constituent of cigarette smoke, does not appear to be directly

Jaimohan Ramachandran, B.Tech., David Rubenstein, and Danny Bluestein, Ph.D., Department of Biomedical Engineering; Jolyon Jesty, Ph.D., Division of Hematology, Stony Brook University Health Sciences Center, Stony Brook, NY.

Correspondence: Jolyon Jesty, Ph.D., Division of Hematology, Stony Brook University Health Sciences Center, Stony Brook, NY 11794-8151, USA. Tel.: +1 (631)-444-2059; Fax: +1 (631)-444-7530; E-mail: jolyon.jesty@sunysb.edu

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responsible for these changes (Blann, Steele, & McCollum, 1997; Ludviksdottir, Blondal, Franzon, Gudmundsson, & Sawe, 1999). Nicotine replacement therapies, such as nicotine gum and transdermal patches, deliver doses of nicotine comparable with those obtained through cigarette smoke but do not entail the inhalation of smoke. These therapies are reported not to influence platelets (Benowitz, Fitzgerald, Wilson, & Zhang, 1993; Wennmalm et al., 1991). Rubenstein et al. (2004) demonstrated that small concentrations of pure nicotine, in the absence of smoke, desensitize platelets to activation, under both static and dynamic flow conditions.

In the present study, we measured the thrombogenic properties of platelets exposed to mainstream and sidestream smoke extracts from low-nicotine and zero-nicotine cigarettes under flow conditions that approximately mimicked normal vascular blood flow, and we determined the effect of adding nicotine to the zero-nicotine smoke extracts.

Method

Smoke extracts

Three cigarette brands were used to prepare smoke extracts (tar and nicotine contents refer to mainstream smoke contents as determined by industry-standard smoking-machine protocols): (a) High nicotine (high tar)—Marlboro 100's (16 mg tar, 1.2 mg nicotine), Philip Morris, Richmond, Virginia; (b) low nicotine (medium tar)—Quest 1 (10 mg tar, .6 mg nicotine), Vector Tobacco, Timberlake, North Carolina; and (c) zero nicotine (medium tar)—Quest 3 (10 mg tar, $\leq$.05 mg nicotine), Vector Tobacco. None of these cigarettes has filter bypass ventilation holes of the type found in light cigarettes (Rubenstein et al., 2004).

Aqueous smoke extraction was carried out using a modification of Su, Han, Giraldo, De Li, and Block's (1998) method. In the mainstream extraction, all smoke passes through the cigarette and the filter; whereas in the sidestream extraction, smoke is collected only from the smoldering end of the cigarette, and none passes through the filter (Rubenstein et al., 2004). In addition to these extracts, one mainstream extract was prepared from zero-nicotine cigarettes from which the filters had been removed. A standard extract of each type was prepared by extracting the smoke from two cigarettes into 100 ml of Hepes-buffered saline pH 7.4 (depth $\sim$4.5 cm). In the case of the cigarettes with filters removed, mounting in the smoking apparatus prevented complete burning, and instead four half-cigarettes were burned to make an equivalent extract. To minimize the effects of variations in combustion and extraction, each standard extract was pooled from four separate extractions. Extracts were stored in 30-ml aliquots at -20°C.

(−)-Nicotine hydrogen tartrate (>98% by thin-layer chromatography) was obtained from Sigma-Aldrich and prepared as a stock solution of 1 mM in water. It was stored at -20°C and used at a final concentration of 50 nM.

Platelets

After we obtained informed consent from participants (approved by the Stony Brook University institutional review board), blood was drawn by venipuncture from healthy adult volunteers who had never smoked or who had quit smoking more than a year previously, and who had abstained from aspirin or ibuprofen for at least 2 weeks. Gel-filtered platelets were prepared as described by Jesty, Yin, Perrotta, and Bluestein (2003) and were used within 4 hr of gel filtration.

Measurement of platelet activation state

Platelet activation state (PAS) of timed samples was measured using a prothrombinase-based assay that measures acetylated thrombin generation from acetylated prothrombin by factor X-a (Jesty & Bluestein, 1999; Jesty et al., 2003). Activated platelets contributed two essential cofactors in this reaction: Negative phospholipids and factor Va. Hence, the rate of thrombin generation reflects the activation state and thrombogenic potential of platelets. To account for initial PAS variability in individual platelet preparations, which vary significantly in base activation state and maximum attainable activity, PAS data values from individual experiments were normalized to full activation of the same platelets with 5 μM calcium ionophore A23187 (Jesty & Bluestein, 1999; Jesty et al., 2003). Hence, PAS data are dimensionless with a maximum value of 1. PAS values reported by this assay have been shown to correlate with those reported by measurement of annexin V binding by flow cytometry (Jesty et al., 2003).

Circulation system

Calcium ions (final concentration, 3 mM) and smoke extracts (final concentration, 1% of the standard extracts—see above) were added to platelets (100,000/μl) 15 min before each circulation experiment. The platelets were then subjected to intermittent shear stress in a flow loop containing a 1-m section of PTFE .86-mm capillary tubing, as described previously (Jesty et al., 2003). Conditions were such that the shear stress in the capillary section was 12 dyne/cm^2, and the time of exposure to shear was 25% of the overall 21-min circulation time. Experiments were carried out at $37 \pm 2^{\circ}$C, and samples were taken every 3 min for PAS assay.

Statistical analysis

The final result of each individual experiment is given as a platelet activation rate (PAR, min^{-1}), which is the rate of increase in normalized PAS value per unit time. PAR values for each individual experiment were determined by linear regression. Because platelets are subject to substantial daily variation in baseline PAS and susceptibility to shear, and because all platelets slowly activate between preparation and use, experimental comparisons were designed rigorously to minimize the effects of conditions. Statistical comparison of PAR values was restricted to paired datasets collected together on the same days and with the same platelet preparations. To account for the slowly rising base activation state over the course of a day, palindromic arrangements were used, as follows: Daily sets of pairwise comparisons between two conditions, e.g., A and B (Figures 1 and 2), were done in the order ABBABA in the first day's series, followed by the order BAABAB in the next daily series. For triple comparisons of conditions, e.g., A, B, and C (Figure 3), the daily order of experiments was ABCCBA throughout.

For each pairwise comparison, a total of six determinations of PAR was made for each condition, generating a mean PAR and standard deviation for each. These pairs were then analyzed by Student's paired *t*-test.

Results

Mainstream smoke extracts

A major purpose of the present study was to determine whether low-nicotine and zero-nicotine cigarette smoke extracts render platelets more susceptible to activation than extracts from ordinary (high-nicotine) cigarettes. Platelets were circulated for 21 min in the presence of smoke extracts at an intermittent shear stress of 12 dyne/cm^2, which roughly approximates normal shear exposure in the vasculature. Figure 1 shows the effect of exposing platelets to extracts of zero-nicotine, low-nicotine, and high-nicotine cigarettes. The results of pairwise comparisons (Table 1) demonstrate that platelet activation is inversely related to nicotine content;

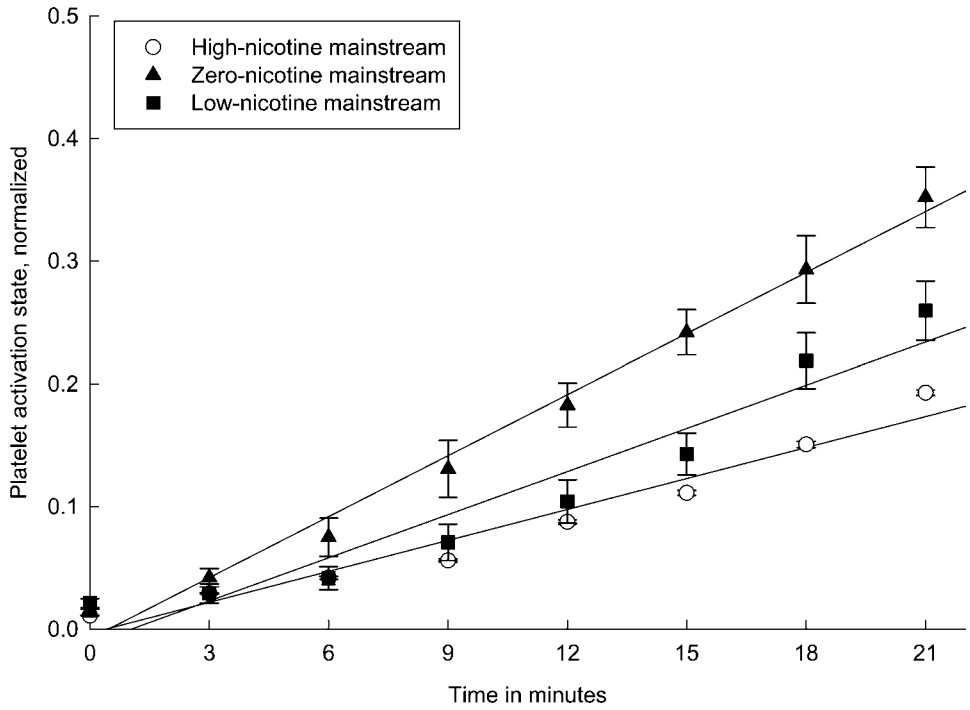

Figure 1. Platelet activation under intermittent shear stress of 12 dyne/cm^2 in the presence of mainstream smoke extracts of zero-nicotine, low-nicotine, and high-nicotine cigarettes. Platelets were circulated at 37°C for 21 min through a capillary loop. Samples were removed every 3 min for the determination of platelet activation state (PAS). Plots of PAS vs. time for each circulation run were fitted by linear regression to obtain a platelet activation rate (PAR). The data shown represent the sum of three separate pairwise experimental setups, organized as described in the Methods section. The pairs are as follows: (A) zero-nicotine ($n=6$) vs. high-nicotine ($n=6$), (B) zero-nicotine ($n=6$) vs. low-nicotine ($n=6$), and (C) low-nicotine ($n=6$) vs. high-nicotine ($n=6$). Statistical comparisons on the collected PAR values from each pair (6 vs. 6) were carried using Student's paired *t*-test (Table 1). Each condition appears in two pairwise comparisons, and for visual brevity, the points and lines shown represent the sum of all data (12 PAR values for each condition), even though each pairwise analysis only used the strictly paired data.

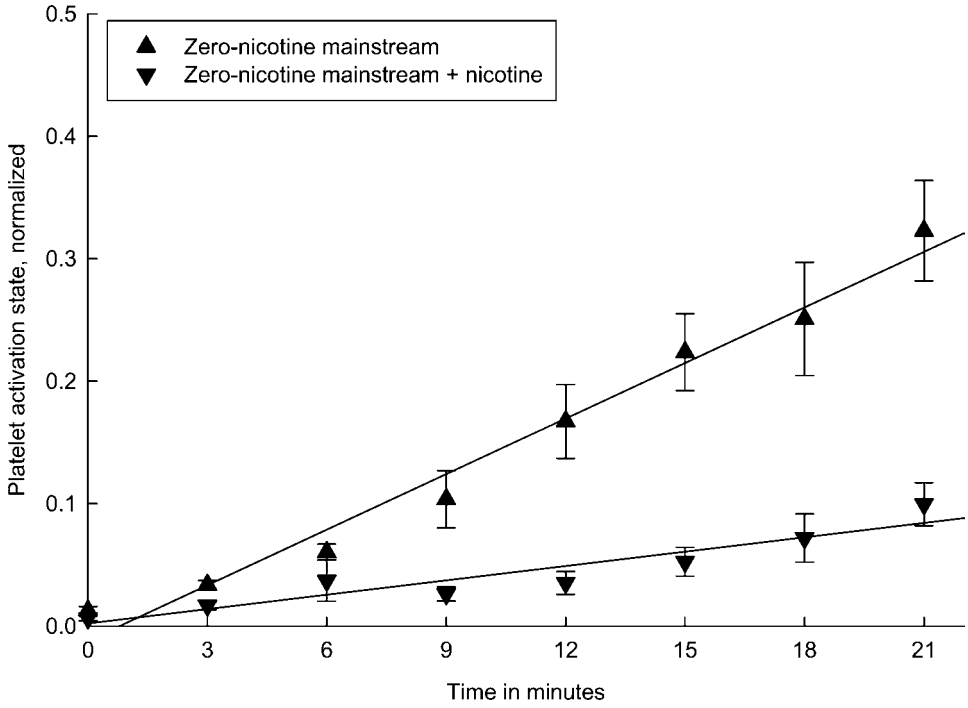

Figure 2. Effect on platelet activation of adding nicotine to the zero-nicotine mainstream smoke extract. Platelets were circulated, as described for Figure 1, in the presence of (A) zero-nicotine extract ($n=6$) and (B) zero-nicotine extract + 50 nM nicotine ($n=6$); and platelet activation rates (PAR) were determined, using a palindromic organization (see Methods section). Statistical comparison of the collected PAR values for the two conditions was carried out using Student's paired *t*-test ($p<.01$; Table 1).

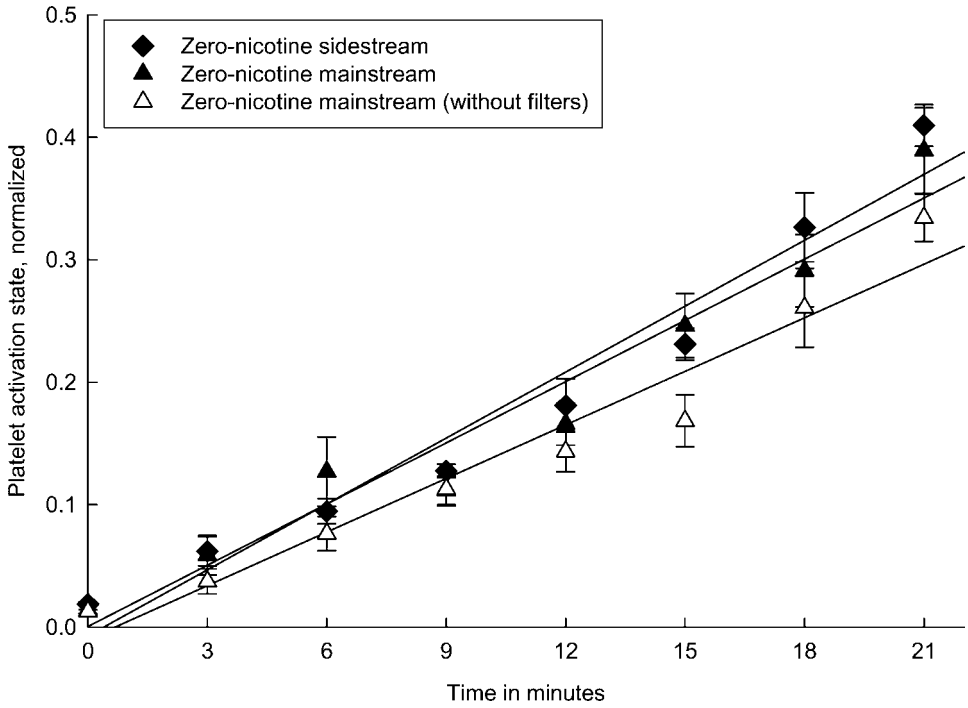

Figure 3. The effect of the zero-nicotine cigarette filter on platelet activation rate (PAR), and comparison with sidestream extract. Platelets were circulated, as described for Figure 1, under three extract conditions: (A) zero-nicotine mainstream, (B) zero-nicotine mainstream without cigarette filter, and (C) zero-nicotine sidestream. The relevant comparisons here are A vs. B and A vs. C. All three conditions were tested in each experimental set, in balanced order (see Methods section). Three such experimental sets provided six PAR estimates for each condition. Statistical pairwise comparisons were done using Student's paired *t*-test ($p>.2$ for both A vs. B and A vs. C; Table 1).

Table 1. Mean platelet activation rates (PARs) under intermittent shear stress of 12 dyne/cm^2 in the presence of smoke extracts.

Extract	High-nicotine mainstream	Low-nicotine mainstream	Zero-nicotine mainstream	Zero-nicotine sidestream	Zero-nicotine mainstream + nicotine	Zero-nicotine mainstream without filters
PAR (min^{-1})	.0084 ($n=12$)	.0117 ($n=12$)	.0159 ($n=24$)	.0179 ($n=6$)	.0039 ($n=6$)	.0146 ($n=6$)
Zero-nicotine mainstream	$p=.005$	$p=.2$	—	$p>.2$	$p=.007$	$p>.2$
Low-nicotine mainstream	$p=.09$	—				

Probabilities (p) for the null hypothesis that activation rates were identical were determined from the individual experimental pair comparisons ($n=6+6$), as described in the Methods section. The platelet activation rates (PARs) are the combined means of PAR values for each extract condition in all its experimental pairings (Figures 1–3). Thus, for extract conditions that were measured in more than one experimental pairing, total $n>6$.

mainstream extracts of zero-nicotine cigarettes render platelets more susceptible to activation than do extracts from low-nicotine or high-nicotine cigarettes.

Nicotine. To confirm the direct inhibitory effect of nicotine in flow-induced platelet activation, we added 50 nM nicotine (the approximate plasma concentration in smokers immediately after smoking one light cigarette) to the mainstream smoke extract of zero-nicotine cigarettes. The effect was a highly significant reduction in the rate of platelet activation, of more than 75% ($p<.01$; Figure 2 and Table 1). At this nicotine concentration, the rate of platelet activation was significantly lower than with the smoke extract of high-nicotine cigarettes. The cause for this finding is unknown but likely reflects the aqueous extraction procedure producing fairly dilute extracts, when compared with the acute concentrations of smoke components that may be found in smokers' plasma.

Sidestream smoke extracts

Sidestream smoke, which issues directly from the tip of a burning cigarette, is by far the major constituent of secondhand smoke. Whereas the effects of mainstream smoke are relevant for smokers, the effects of secondhand smoke are critical for nonsmokers. Sidestream extracts were prepared from zero-nicotine cigarettes and examined in the same way as mainstream extracts under conditions of intermittent shear. The results demonstrate that for the cigarettes used here, which did not incorporate filter bypass ventilation holes of the type found in light cigarettes, sidestream and mainstream extracts were equally potent in their sensitization of platelets to shear stress (Figure 3).

We noted in preparing the extracts that the two Quest cigarette brands, in addition to being manufactured with zero-nicotine or low-nicotine tobacco, also incorporate carbon in their filters. Although the equivalence of mainstream and sidestream extracts suggested that these filters had no effect on the platelet-sensitizing potency of the smoke, we decided to address the question directly by preparing smoke extracts from zero-nicotine cigarettes from which the filters were removed. The rates of platelet activation in the presence of this extract were slightly lower than those seen with the regular mainstream and sidestream smoke extracts of these cigarettes, but the differences were not statistically significant (Figure 3 and Table 1). Although the filters of the zero-nicotine cigarettes might reduce slightly the levels of putative inhibitors of platelet activation (perhaps traces of nicotine still present in these cigarettes), the effect is not large.

Discussion

PAS

The modified prothrombinase-based assay has been used successfully in previous studies to determine the thrombogenic state of platelets without discriminating between the activation related to whole platelets and microparticles, which may be released upon activation or mechanical damage. PAS is therefore a global measure. The results of Rubenstein et al. (2004) suggest that microparticle generation is not a major confounding parameter, given that platelet loss in this circulation system is fairly small (approximately 11%).

Mainstream smoke extracts

We hypothesized that reduced-nicotine cigarettes might enhance thrombogenic risk by rendering platelets more susceptible to activation, compared with ordinary high-nicotine cigarettes. We tested this hypothesis by using the mainstream smoke extracts of zero-nicotine and low-nicotine cigarettes (Figures 1 and 2, Table 1). These were both Quest cigarettes, and they had identical tar ratings. Smoke extracts prepared from these cigarettes were more potent in sensitizing platelets to flow-induced activation than were extracts from even high-tar, high-nicotine cigarettes (we used the most common such cigarette, Marlboro red pack). Quest cigarettes are manufactured from genetically modified tobacco, in which the

gene of an essential enzyme in nicotine biosynthesis, quinolinate phosphoribosyl transferase, is knocked out (Fogarty, 2003).

Although the experimental results are clear, they do not prove that the low nicotine content is the direct cause. However, there is no reason a priori to suspect that genetically modified tobacco is significantly different in other respects from ordinary tobacco, and this is borne out partly by the fact that the tar content of smoke from these cigarettes (10 mg tar in a standard machine-smoking protocol) is not a great deal less than that from ordinary high-tar cigarettes (approximately 15 mg). Nonetheless, the possibility that components other than nicotine are different in these cigarettes cannot be discounted. For example, higher-than-normal levels of a platelet-activating component in the genetically modified tobacco would produce the same results that we observed.

That possibility notwithstanding, the major platelet-desensitizing effect of nicotine remains clear (Figure 2). The addition of pure nicotine, at a level in the range of its plasma concentration after a smoker smokes just one light cigarette, reduced the rate of platelet activation by more than 75%. Notably, the PAR with added nicotine was less than that observed with the ordinary high-nicotine extract. In this regard, it is relevant that our aqueous extraction procedure involved only brief exposure of the smoke to the extraction buffer and was therefore likely quite inefficient compared with the extraction efficiency of a smoker's lungs.

Because low-nicotine cigarettes are a new product, no information exists about whether smokers might compensate for the lower nicotine content by increasing puff frequency and puff volume, as they do with the more common light cigarettes (U.S. Department of Health and Human Services, 2001). Should such cigarettes become popular, the question is an important one. In the present study, we used the same extraction procedure for the three cigarette types, the concentrations of smoke extracts were held constant, and zero-nicotine and low-nicotine cigarettes were still more thrombogenic to platelets. If smokers were to compensate for the nicotine reduction by increasing their smoke intake, the increased thrombogenic effects would be expected to be even greater. Although the increases in platelet thrombogenicity observed in vitro have not been proven to bear direct relation or relevance to the situation when people smoke or are exposed to secondhand smoke, the possibility should be kept in mind.

Sidestream smoke extracts

Secondhand smoke is mainly sidestream smoke from the smoldering end of a cigarette. Exposure to secondhand smoke remains a major worldwide health risk, despite major headway made in various cities, states, and countries. As expected for cigarettes that incorporate no filter bypass (Rubenstein et al., 2004), the platelet-activating properties of Quest variable-nicotine cigarettes did not differ significantly between mainstream and sidestream smoke at the same dose; thus, the highly potent nature of the zero-nicotine extract was essentially the same for both mainstream and sidestream extracts.

Summary

We assessed the thrombogenic activation of platelets subjected to mainstream and sidestream smoke extracts of cigarettes with varying nicotine content under flow conditions approximating those in the vasculature. Smoke extracts from low-nicotine and zero-nicotine cigarettes caused substantially greater activation of platelets than extracts from ordinary high-tar, high-nicotine cigarettes. In support of the conclusion that this is a direct result of nicotine reduction, the addition of a low concentration of nicotine to zero-nicotine smoke extracts was shown to substantially reduce the platelet-activating potential of the extracts. Although the in vitro experiments described here do not constitute definite proof, we conclude that even though low-nicotine and zero-nicotine cigarettes may be useful in weaning smokers from cigarettes, they also might cause a significantly increased risk of thrombotic disease in smokers and others exposed to the secondhand smoke from such cigarettes.

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