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Secondhand tobacco smoke impairs neurogenic and endothelium-dependent relaxation of rabbit corpus cavernosum smooth muscle: improvement with chronic oral administration of L-arginine

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The first goal of this study was to examine the effect of secondhand smoking on neurogenic, endothelium- and cGMP-dependent relaxant responses of rabbit corpus cavernosum smooth muscle. Our second goal was to determine whether such an effect can be prevented by oral administration of L-arginine. Male New Zealand rabbits were divided into control, chronic passive cigarette smoking and L-arginine treatment groups. Relaxant or contractile responses in isolated corpus cavernosum smooth muscle strips were determined by using *in vitro* muscle technique. There was no significant difference in the relaxant response of the strips to papaverine, sodium nitroprusside and contractile response to KCl among the groups. Relaxant responses to acetylcholine and electrical field stimulation and contractile response to phenylephrine were significantly decreased in the strips of the smoking group than that of the control group. The impaired relaxations of strips were markedly improved by treatment of L-arginine, but the contractile responses to phenylephrine were not affected. These data indicate that secondhand smoking may impair both neurogenic and endothelium-dependent relaxation of corpus cavernosum smooth muscle, and may contribute to the etiology of impotence. Chronic dietary supplementation with L-arginine offsets the impairment of neurogenic and endothelial relaxation. Therefore, we suggest that secondhand smoking exposure to cigarette produces selective impairment of neurogenic and endothelium-dependent relaxation of corpus cavernosum smooth muscle via a mechanism related to the decreased production and/or availability of nitric oxide.
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Introduction

Nitric oxide (NO) is strongly associated with physiology of penis, based on mounting basic science and clinical evidence.[1,2] NO is synthesized from its precursor, L-arginine, by the enzyme NOS from two sources, nonadrenergic noncholinergic nerves and the sinusoidal endothelium. NO diffuses into the adjacent smooth muscle cells and activates soluble guanylate cyclase to increase the intracellular cGMP concentration. The cGMP appears to be the major intracellular effector of the smooth muscle cell relaxation.[3–5]

Epidemiological evidence shows that chronic smoking is a major risk factor in the development of erectile dysfunction independent of smoking-related chronic illness.[6–8] It is reported that cigarette smoking was associated with reduced endothelium-dependent vasodilator,[9–12] NO generation and eNOS activity in the presence of increased eNOS protein expression. Also, chronic dietary supplementation with NO precursor L-arginine prevents smoking-induced endothelial dysfunction.[12–14] In this background, we investigated the effects of secondhand smoking on neurogenic, endothelium- and cGMP-dependent relaxant responses of corpus cavernosum smooth muscle in rabbits. We also examined whether treatment with L-arginine prevents the development of functional impairment of NO/

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cGMP-mediated corpus cavernosum smooth muscle relaxation.

Materials and methods

Experiments were performed on mature male albino rabbits obtained from Experimental Medical Research Unit (DETAB, Kocaeli University, Kocaeli, Turkey) (2.5–3 kg). The albino rabbit was chosen as the model based on the close similarities that have been reported in the reactivity *in vitro* of human and rabbit corpus cavernosum. The rabbits were randomly assigned to control ($n=9$), cigarette smoking ($n=9$), and L-arginine treatment ($n=9$) groups.

Treatment schedule

The experimental protocol was essentially the same as described previously.[13] Briefly, for the secondhand smoking procedure, rabbits were housed in a secondhand smoking exposure cage that measured $1.92 \times 1.92 \times 0.97\,m^3$ ($3.58\,m^3$). Rabbits were exposed to sidestream smoke from cigarettes (four cigarettes every 15 min for 6 h per day, 5 days per week) for 10 weeks. At the end of the 6-h exposure period, rabbits were housed in separate cages. Rabbits randomized to the L-arginine treatment group received L-arginine in their drinking water when they exposed to smoke for 10 weeks (1 mg/ml solution of L-arginine *ad libitum*). Control animals were also placed in the cage, but they inspired fresh air without cigarette smoke.

Urine cotinine and nicotine metabolite levels were measured at the end of the 10-week secondhand smoking (SHS) exposure and were determined by radioimmunoassay (RIA).

Organ chamber experiments

After 10 weeks of exposure, rabbits were anesthetized with ketamine (25 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.). Rabbit penises were surgically removed at the level of the crural attachments to the pubo-ischial bones, and the corpus spongiosum and urethra were excised. The corpus cavernosum tissue was immediately placed in Krebs-bicarbonate solution composed of (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11. The tunica albuginea was cleared of overlying tissue and opened. The proximal half of the corporal body was dissected free from the tunica and harvested en block. Each corporal body was cut transversely to obtain two longitudinal strips and strips of corpus cavernosum tissue were mounted in organ chambers for isometric tension measurement. The organ chambers contained Krebs-bicarbonate solution. The solution was gassed with 95% O_2 and 5% CO_2 during the study. The temperature was maintained at 37°C by a thermoregulated water circuit. Each strip was connected to a force transducer (FDT 10 A Commat Iletisim) for the measurement of isometric force, which was continuously displaced and recorded on an online computer via a four-channel transducer data acquisition system (TDA 94, Commat Iletisim) using a software (Polywin 95 ver 1.0, Commat Iletisim) that also had the capacity to analyze the data. After mounting, each strip was allowed to equilibrate with a basal tension of 1.5 g for 1 h, and during this time Krebs-bicarbonate solution was replaced every 15 min with fresh solution. At the end of the equilibration, strips were depolarized with 124 mM KCl in Krebs-bicarbonate solution and allowed to equilibrate for 30 min. This procedure increases and stabilizes subsequent contractile responses to phenylephrine and decreases spontaneous contractions. After equilibration, the contractile responses to phenylephrine (10^{-8}–10^{-4} M) were obtained cumulatively. After addition of each dose, we waited until a plateau response was obtained before adding the next one. Following completion of phenylephrine dose–response curve, tissues were washed for a further 30 min, precontracted with a submaximal concentration of phenylephrine (10^{-6}–3×10^{-5} M). After the contraction reached a plateau, concentration–response relationships for acetylcholine (10^{-8}–10^{-4} M), sodium nitroprusside (10^{-10}–10^{-4} M) or papaverine (10^{-4} M) were obtained by adding one of those agents to the bath in a cumulative manner.

Electrical stimulation (EFS) was provided by a stimulator (ST 95PT, Commat Iletisim) and applied via two platinum wire electrodes set vertically within the opposite organ bath sides of the suspended tissue. Prior to EFS, tissue was treated with guanethidine (3×10^{-6} M) (adrenergic nerve blocker) and atropin (10^{-6} M) (muscarinic receptor blocker) for 30 min. Strips were precontracted with phenylephrine at 10^{-6}–3×10^{-5} M; electric stimulation was then performed. Square-wave pulses of 10 V, 0.5 ms duration in 10-s trains with varying frequency (1, 2, 4, 8, 16 and 32 Hz) were applied at 5-min intervals. The strips were allowed to return to baseline precontractile tension between the tests at each frequency.

Analysis of data

The results are expressed as the mean ± s.e. of different experiments. Contractile responses to phenylephrine were calculated as percentage of the maximal contraction caused by KCl (124 mM). Relaxant effects of agonists were expressed as a percentage of the precontraction to phenylephrine. In

order to evaluate the effects of agonists, maximum responses (E_{max}) and pD_2 values (apparent agonist affinity constants; $-\log ED_{50}$) were calculated. Agonist pD_2 value was calculated from each agonist dose–response curve by linear portion of the curve and taken as a measure of the sensitivity of the tissues to each agonist.

Statistical comparison between groups was performed using ANOVA *post hoc* Tukey test. Results were considered to be significantly different at a *P*-value of <0.05.

Drugs

The following drugs were used: phenylephrine hydrochloride (Sigma Chemical, St Louis, MO, USA), atropine sulfate (Sigma Chemical, St Louis, MO, USA), guanethidine sulfate (Sigma Chemical, St Louis, MO, USA), acetylcholine chloride (Sigma Chemical, St Louis, MO, USA), papaverine hydrochloride (Sigma Chemical, St Louis, MO, USA) and sodium nitroprusside (Nipruss, Adeca). All drugs were dissolved in distilled water and were freshly prepared on the day of the experiments.

Ethical approval was granted by the Kocaeli University Ethics Committee (Kocaeli, Turkey, AEK-341/6).

Results

The urine nicotine metabolite concentrations of all groups at the end of 10 weeks are shown in Table 1. It was significantly increased in the secondhand smoking group and L-arginine treatment group compared with the control group ($P<0.05$). The contractions elicited by KCl (124 mM) were similar in three groups (Table 2).

The cumulative addition of phenylephrine (10^{-8}–10^{-4} M) produced concentration-dependent contractions of the corpus cavernosum strips and the contractility was significantly lower in secondhand smoking and L-arginine treatment groups ($P<0.05$). The concentration–response curve for phenylephrine was shifted to the right, with the significantly lower E_{max} values in both secondhand smoking and L-arginine treatment groups than in controls ($P<0.05$), but there was no change in the corresponding pD_2 values (Figure 1; Tables 2 and 3).

In corpus cavernosum strips precontracted with phenylephrine at submaximal concentration, endothelium-dependent relaxation to acetylcholine was significantly decreased in the secondhand smoking group compared with the control and L-arginine treatment group ($P<0.05$). The concentration–response curve for acetylcholine was shifted to the right with significantly lower E_{max} in the secondhand smoking group than in controls ($P<0.05$), but there was no change in the corresponding pD_2 values (Figure 2; Tables 2 and 3). The impairment of relaxation in the L-arginine treatment group was returned to controls. There was no significant difference in pD_2 and E_{max} values between the control and L-arginine treatment groups (Figure 2; Tables 2 and 3).

In corpus cavernosum strips precontracted with phenylephrine, sodium nitroprusside (10^{-10}–10^{-4} M) caused concentration-dependent relaxation (Figure 3), but there were no significant

Table 1 The urine levels of nicotine metabolites (ng/ml)

Control	n	*SHS*	n	*L-arginine*	n
176.84±34.06	5	2727.74±609.52*	9	3429.03±921.47*	4

*$P<0.05$, statistically different from control rabbits.

Table 2 E_{max} (% of 10^{-6} M phenylephrine) values for acetylcholine, sodium nitroprusside and EFS, E_{max} values (% of 124 mM KCl) for phenylephrine and E_{max} values (mg) for 124 mM KCl in corpus cavernosum strips obtained from SHS, L-arginine treatment and control rabbits

	Control	n	*SHS*	n	*L-arginine*	n
Phenylephrine	155.9±20	6	97.5±6.7*	8	103.9±11.9*	6
Acetylcholine	72.0±3.6	9	50.4±9.4*#	6	85.7±6.0	7
Sodium nitroprusside	97.3±2	6	98.0±1.1	7	98.4±1.7	7
EFS	64.8±10.9	6	44.9±7.4*#	6	75.5±6.7	8
KCl	2430.3±471	9	2658.6±381	8	2238.3±394	7

*$P<0.05$, statistically different from the response of strips from control rabbits.
#$P<0.05$, statistically different from the response of strips from L-arginine groups.

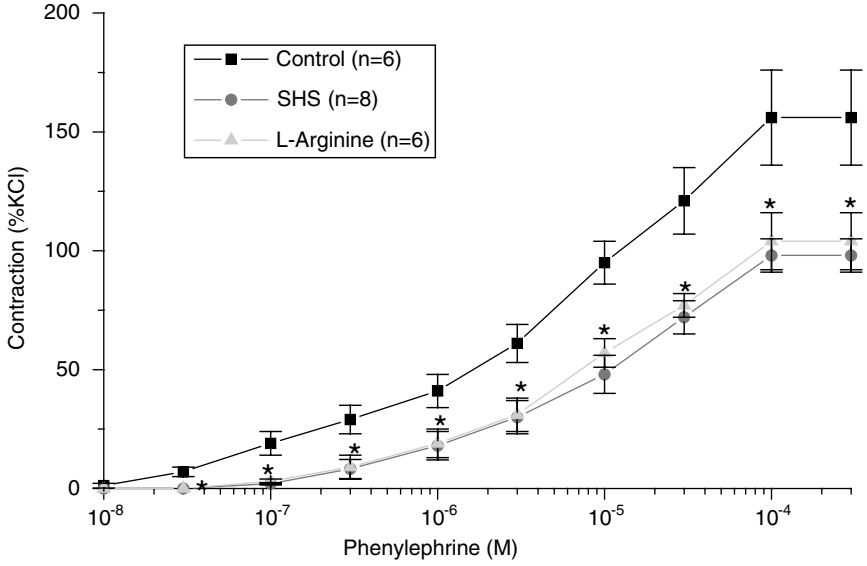


Figure 1 Phenylephrine concentration–response curves in isolated rabbit corpus cavernosum strips. Each point is expressed as a percentage of the contraction induced by 124 mM KCl and is given as the mean ± standard error of the mean (s.e.m.). Number of rabbits in each group is shown in parentheses.

Table 3 pD_2 values for phenylephrine, acetylcholine and sodium nitroprusside in corpus cavernosum strips obtained from SHS, L-arginine and control rabbits

	Control	n	*SHS*	n	*L-arginine*	n
Phenylephrine	5.24 ± 0.06	6	5.14 ± 0.15	8	5.10 ± 0.11	6
Acetylcholine	5.78 ± 0.21	9	5.94 ± 0.17	6	5.92 ± 0.14	7
Sodium nitroprusside	5.72 ± 0.20	6	5.55 ± 0.43	7	5.59 ± 0.71	7

Values are arithmetic means ± s.e., n = the number of rabbits.

differences in E_{max} and pD_2 among the groups (Tables 2 and 3).

In precontracted strips, electric stimulation (1–32 Hz) evoked frequency-dependent relaxations. In strips from secondhand smoking group, the relaxant EFS effects remained unaltered at lower freqency (1–8 Hz) but at higher frequencies, EFS responses decreased significantly ($P<0.05$, Figure 4). In strips from L-arginine treatment group, the relaxant effects of EFS were similar to those in the control (Figure 4).

Discussion

This study demonstrates that secondhand smoking impaired both neurogenic and endothelium-dependent relaxant or phenylephrine-induced contractile responses in the isolated rabbit corpus cavernosum smooth muscle, whereas the response to sodium nitroprusside, an NO donor, and to KCl, a non-receptor-mediated constrictor, is not affected. The impaired relaxations, but not contractions of corpus cavernosum smooth muscle, markedly improved by L-arginine treatment.

Endothelium-dependent relaxation to acetylcholine and electrical field stimulation-induced neurogenic relaxation of cavernosal tissue are mediated largely by the NO/cGMP pathway.[5] NO is widely accepted to have an important role in the relaxation of corporal smooth muscle and vasculature. It has been well documented that impairment of neurogenic and/or endothelium-dependent relaxation may result in erectile dysfunction.[15–18] Therefore, this study focused on endothelium-dependent and neurogenic relaxation, but we also showed that secondhand smoking can inhibit corpus cavernosum smooth muscle reactions to contractile agents. At present, the mechanism underlying reduced contractile responsiveness of corpus cavernosum smooth muscle after their exposure to secondhand smoking is not known, but the proposed mechanisms include modified receptor-coupled transduction mechanisms controlling corpus cavernosum smooth muscle contraction and enhanced synthesis

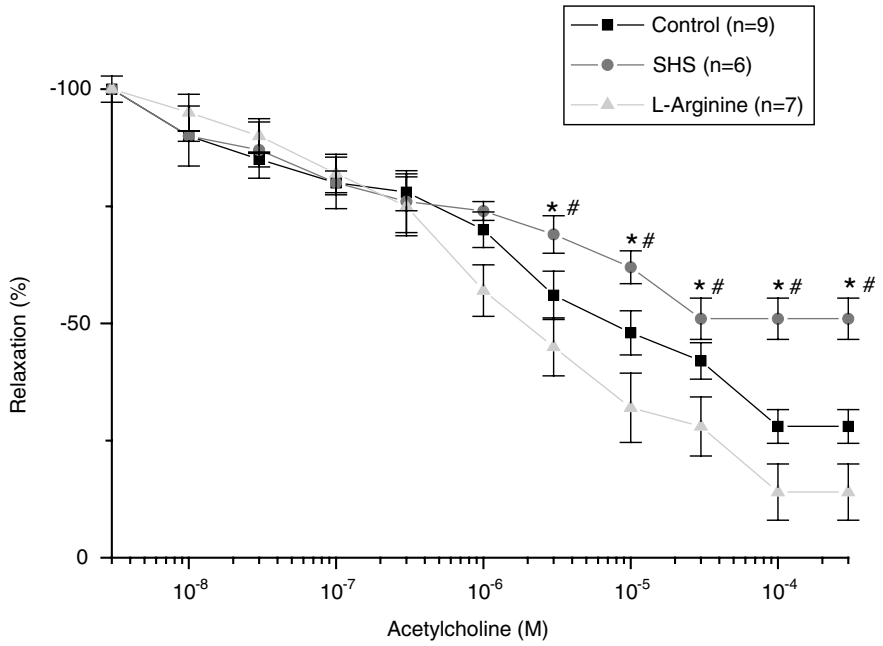


Figure 2 Acetylcholine concentration–response curves in isolated rabbit corpus cavernosum strips precontracted with phenylephrine 10^{-6}M. Each point is expressed as a percentage of the contraction induced by phenylephrine, and is given as the mean ± standard error of the mean (s.e.m.). Number of rabbits in each group is shown in parentheses. $*P<0.05$ statistically different from the response of strips from control rabbits.

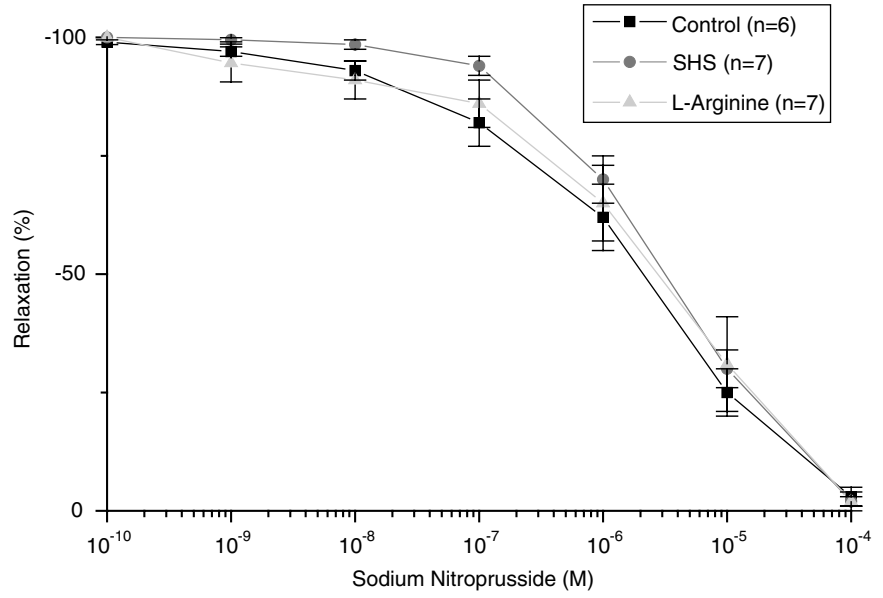


Figure 3 Sodium nitroprusside concentration–response curves in isolated rabbit corpus cavernosum strips precontracted with phenylephrine 10^{-6}M. Each point is expressed as a percentage of the contraction induced by phenylephrine, and is given as the mean ± standard error of the mean (s.e.m.). Number of rabbits in each group is shown in parentheses.

of endogenous relaxant(s) that exerts nonselective physiological antagonism against constrictor stimuli. The penis has the ability to synthesize various prostanoids and it has been suggested that arachidonic acid cascade products may be involved in the control of penile erection.[19,20] The mechanism of reduced contractile response to phenylephrine appears to be related to the increased production of cyclooxygenase vasodilator substances, such as prostacyclin. However, this possibility is

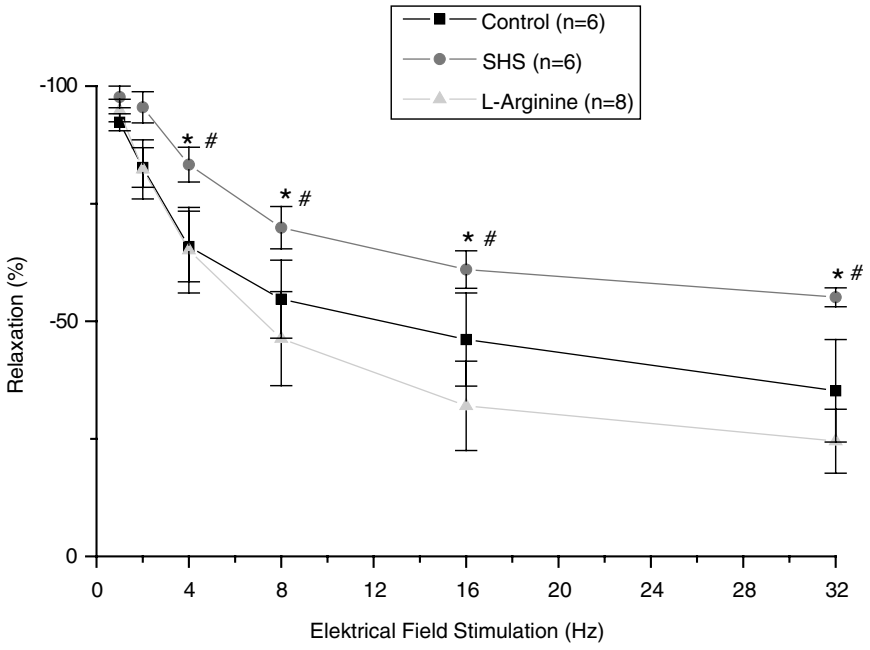


Figure 4 The relaxation responses evoked by electrical field stimulation (EFS) of isolated rabbit corpus cavernosum strips precontracted with phenylephrine 10^{-6}M. Each point is expressed as a percentage of the contraction induced by phenylephrine, and is given as the mean $\pm$ standard error of the mean (s.e.m.). Number of rabbits in each group is shown in parentheses. $^{*}P<0.05$ statistically different from the response of the strips from control rabbits.

unlikely, since cigarette smoke impairs prostacyclin release in cultured human endothelial cells.[21] Also, the reduced contractile responses to phenylephrine in the present secondhand smoking groups could not be explained simply by nonselective mechanisms because contraction produced by K^+ was not inhibited by secondhand smoking. Contractile response of corpus cavernosum smooth muscle to K^+ depolarization depends on an opening of voltage-gated Ca^{2+} channels spanning the sarcolemmal membrane, with the resulting influx of Ca^{2+} activating the contractile protein assembly. Since secondhand smoking did not affect K^+ responses in corpus cavernosum smooth muscle, it seems unlikely that secondhand smoking exerted a major deleterious effect either on voltage-dependent sarcolemmal Ca^{2+}-channels or subsequent Ca^{2+}-dependent activation of corpus cavernosum smooth muscle contractile filaments. Rather, since phenylephrine receptors share activation of phospholipase C with its resulting effects on inositol triphosphate and intracellular Ca^{2+}, it seems plausible that secondhand smoking in some way adversly affects this transduction cascade in rabbit corpus cavernosum smooth muscle.

In the present study, secondhand smoking exposure affected neither the potency (EC_{50}) nor the efficacy (maximal response) of sodium nitroprusside, resulting in concentration-dependent relaxant curves in secondhand smoking tissue indistinguishable from control curves. We interpret the sodium nitroprusside data as evidence that this secondhand smoking model failed to effect either the ability of NO to activate guanylyl cyclase-cGMP or the sensitivity of corpus cavernosum smooth muscle contractile apparatus to cGMP-mediated actions.

In contrast to the direct corpus cavernosum smooth muscle relaxant action of sodium nitroprusside, the relaxant mechanism for acetylcholine requires a muscarinic receptor-dependent release of endothelium-derived NO synthesized from an N^G-guanidino nitrogen of L-arginine through the action of NOS.[22] In the penis, NO is also released from nonadrenergic noncholinergic nerves, mediating relaxation of penile erectile tissue necessary for erection.[1,2] NO diffuses readily from the synthesizing cell to the corpus cavernosum smooth muscle, where it exerts its activity without requiring a specific membrane receptor. Binding of NO to the heme group of soluble guanylate cyclase induces the formation of cGMP from guanosine triphosphate.[5] Since secondhand smoking inhibited relaxant responses to acetylcholine or to EFS but did not alter relaxant effects of the directly acting cGMP-dependent sodium nitroprusside, present data are consistent with the interpretation that secondhand smoking inhibited the synthesis or availability of NO in corpus cavernosum smooth muscle tissue rather than affecting NO-guanylyl cyclase interactions or subsequent cGMP-dependent mechanism in the corpus cavernosum smooth muscle cells. This

is consistent with the results of previous reports on secondhand smoking-exposed rats. Authors reported that secondhand smoking exposure shows a reduction in penile basal NO synthesis.[23] It has been well documented that the major causative factor contributing to erectile dysfunction in elderly or diabetic patients is the reduction in the amount of NO synthesis in the penis, resulting in alterations of normal penile homeostasis.[24,25]

An increasingly popular theory is that formation-advanced glycosylation-end (AGEs) products have an important pathophysiologic role in the erectile dysfunction in aging diabetes and smoking.[26–28] Alteration of endothelium-mediated relaxation may be the result of quenching and inactivation of NO by AGEs.[27] Nonenzymatic glycosylation of proteins, often referred to as the Maillard reaction, has been proposed to play a role in age- and diabetes-related process by forming protein and DNA links may contribute to erectile dysfunction by scavenging NO, which is needed for erection.[29,30] In addition to these, endogenously formed glycosylation-end products can also be introduced into the body from exogenous sources. Tobacco smoke, for example, is a well-known exogenous source of AGEs. Serum AGEs or LDL-linked AGEs are significantly elevated in cigarette smokers.[31] These evidence provide new insight into the pathogenesis of impotence associated with tobacco usage.

As well known, cigarette smoke contains large amounts of free radicals and prooxidants such as superoxide anions or hydrogen peroxide.[32] It is reported that secondhand smoking impairs endothelium-dependent relaxation of isolated rabbit arteries and the impaired relaxations of arteries mediated through the degradation of released NO by superoxide anion derived from cigarette smoke.[9,33,34] Therefore, it is speculated that secondhand smoking-induced impaired neurogenic and endothelium-dependent relaxation may be due to the increase in oxygen-derived free radicals production in smoke, as reported by Ota *et al*,[33] which may inactivate NO continuously released from endothelial cells.

In the present study, we have also demonstrated that chronic L-arginine supplementation for 10 weeks prevents secondhand smoking-induced neurogenic and endothelial dysfunction, but not phenylephrine-induced contractility. The fact that L-arginine failed to normalize the secondhand smoking-induced decrease in phenylephrine-induced constriction may suggest that differences in NO activity do not underlie the differences in phenylephrine-induced constriction. Our results are consistent with previous findings in blood vessels from cholesterol-fed rabbits.[13] In this study, it has been shown that dietary supplementation with L-arginine protects cholesterol-fed rabbits from endothelial dysfunction associated with exposure to environmental tobacco smoke, suggesting that impaired endothelial production of NO may be pathogenically important in this animal model of atherosclerosis related to environmental tobacco smoke.[13] In addition to this evidence, some studies report good clinical results under oral L-arginine medication in the treatment of erectile dysfunction. Klotz *et al*[35] reported that oral L-arginine is not better than placebo as a first-line treatment for mixed-type impotence. In contrast, another study has shown that oral administration of L-arginine in high doses seems to cause significant subjective improvement in sexual function in men with organic ED only if they have decreased NO excretion or production.[36] Furthermore, Yildirim *et al*[37] showed that diabetes mellitus reduced endothelium-dependent acetylcholine-induced relaxation of corpus cavernosum smooth muscle from rabbits and L-arginine blocked this adverse effect, whereas neither L-arginine nor insulin treatment restored the impared NANC neurogenic relaxation. The mechanisms of the beneficial effects of L-arginine on impaired relaxation in isolated corpus cavernosum smooth muscle from secondhand smoked rabbits are unknown. In recent studies, it has been found that the infusion of L-arginine was associated with increased plasma cGMP and L-citrulline levels,[38] and orally administered L-arginine increased plasma nitrate levels in healthy men.[39] Furthermore, Moody *et al*[40]showed that long-term oral L-arginine doubled the circulating and penile levels of L-arginine in rats. They showed that supersaturation of the animal with L-arginine and the subsequent increase in penile L-arginine neither stimulated nor reduced the NOS content of the tissue, whereas the concentrations of L-arginine found in the penile cytosol preparations from rats receiving long-term oral L-arginine were able to clearly stimulate NOS activity. These authors concluded that exogenous oral arginine in rats could activate NOS in a typical substrate saturation manner by increasing the intracellular pool of L-arginine available to NOS. Therefore, previous results and our findings suggest that this protective effect of L-arginine on the relaxations of corpus cavernosum may result in increased penile NOS activity.

In conclusion, our findings suggest that secondhand smoking may impair both neurogenic and endothelium-dependent relaxation of corpus cavernosum smooth muscle, leading to impotence. The impaired relaxations of corpus cavernosum smooth muscle can be markedly improved by administration of L-arginine. This observation strongly supports the concept that decreased production and/or availability of NO contribute to impotence in secondhand smoking.

Acknowledgements

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