International Journal of Impotence Research (2012) 24, 185 – 190
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www.nature.com/ijir

ORIGINAL ARTICLE

Effects of chronic low- and high-dose ethanol intake on the nitrergic relaxations of corpus cavernosum and penile nitric oxide synthase in the rabbit

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Epidemiological evidence showed that chronic ethanol consumption is a major risk factor in the development of impotence. The present study investigated the effects of carbachol-, electrical field stimulation (EFS)-, sodium nitroprusside (SNP)- and papaverine-induced relaxant responses in the isolated corpus cavernosum tissues from rabbits submitted to an 12-week course of chronic low (5% v/v) or high ethanol intake (30% v/v). Increased carbachol- and EFS-induced relaxant responses but not SNP and papaverine, were observed in low ethanol-fed rabbits compared with controls. However, impaired carbachol- and EFS-induced relaxant responses were observed in high ethanol-fed rabbits compared with control rabbits. There were no significant differences in SNP- and papaverine-induced relaxant responses between control and high ethanol-fed rabbits. In addition, decreased neuronal nitric oxide synthase (nNOS) and endothelial NOS (eNOS) immunoreactivity in penile tissue were found in high ethanol-fed rabbits, but increased the immunoreactivity in low ethanol-fed group, compared with control group. These results suggest that alterations in nitric oxide (NO) production within the cavernous tissue in the high ethanol-fed rabbits are, at least in part, responsible for the erectile dysfunction.

International Journal of Impotence Research (2012) **24,** 185–190; doi:10.1038/ijir.2012.14; published online 10 May 2012

Keywords: alcohol consumption; carbachol; corpus cavernosum; impotence; nitric oxide

INTRODUCTION

Many epidemiological studies have shown that chronic ethanol consumption is associated with increased incidence of impotence.[1] Generally, low levels of alcohol ingestion increase male sexual activity by increasing libido; however, higher alcohol levels impair the penile tumescence and reduce sexual performance.[2] Nitric oxide (NO) is possibly the principal neurotransmitter for cavernous smooth muscle relaxation, the key step in the erectile process.[3] In recent years, impaired NO-mediated relaxant responses of corporal tissue has been recognized as an important cause of impotence in various pathological conditions such as diabetes mellitus,[4] aging,[5] hyperthyroidism,[6] pudental arterial occlusion,[7] hypercolesterolemia,[8] cavernous nerve denervation[9] and smoking.[10] Although chronic alcohol consumption results in impotence, Saito *et al.*[11] demonstrated that *in vivo* chronic ethanol administration (5% v/v) augmented acetylcholine- and electrical field stimulation (EFS)-induced relaxant responses of corporal smooth muscle. Also, many reports in the literature revealed that ethanol increases the synthesis or release of NO in isolated arteries and cultured endothelial cells.[12,13] On the other hand, some investigators noted that ethanol decreased endothelium-dependent relaxations in isolated arteries.[14–16] Recent studies on experimental animals and humans indicated that the effects of ethanol on NO availability are highly dependent on whether it is taken at low or high concentration and acute or chronic intake.[17] Therefore, the aim of the present study was to investigate the effects of chronic ethanol intake on the nitrergic-mediated relaxant responses of the rabbit corpus cavernosum and to determine penile endothelial nitric oxide synthase (eNOS) and neuronal NOS (nNOS) expression. To our knowledge, there are no similar studies examining the effect of chronic high dose of ethanol on the nitrergic relaxation of corpus cavernosum smooth muscle.

MATERIALS AND METHODS

Experiments were performed on mature male New Zealand White rabbits (2–2.5 kg) obtained from the Experimental Medical Research and Application Center (DETAB, Kocaeli University, Kocaeli, Turkey). The 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.

Ethical approval was granted by the Kocaeli University Animal Research Ethics Committee (Kocaeli, Turkey).

Treatment schedule

The rabbits were divided into three groups: 9 rabbits in group 1 received tap water ad libitum, and 9 each in groups 2 and 3 were fed 5% ethanol and 30% ethanol, respectively. The model of ethanol feeding was that described previously in which rabbits received 5% ethanol (vol/vol) in the drinking water for the first week, 10% for the next 2 weeks, 20% from weeks 4–6 and 30% from weeks 6–12 in the group 3.[18] In the group 2, rabbits were received 5% ethanol constantly for 12 weeks. All rabbits had constant access to standard laboratory rabbit chow.

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Received 14 June 2011; revised 5 October 2011; accepted 11 April 2012; published online 10 May 2012

Strip preparation and organ bath studies

The rabbits from each group were anesthetized with ketamine (25 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.) and exsanguinated 12 weeks post treatment, as peviously described.[19] Briefly, the penis was dissected free and removed at the level of the crural attachments to the puboischial bones. The specimen was immediately placed in Kreb's bicarbonate buffer composed of (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25, $MgSO_4$ 1.2, KH_2PO_4 1.2 and glucose 11. The tunica albuginea was cleared of the overlying tissue and opened. The proximal half of the corporal body was dissected free from the tunica and harvested en bloc as previously described.[5,7,9] Each corporal body was cut transversely to obtain two longitudinal strips. Each strip was mounted in a 20-ml organ bath containing Kreb's bicarbonate buffer equilibrated with a gas mixture of 95% O_2 and 5% CO_2 and maintained at 37 °C. Recording of isometric strip tension were made using a transducer (MAY-COM, FDT 10 A, COMMAT Iletisim, Ankara, Turkey) and recorded online on a computer via a four channel transducer data acquisition system (TDA 94, COMMAT ILETİSİM, Turkey) using appropriate software (Polywin 95 1.0 COMMAT ILETİSİM, Turkey). The resting load was set at 2 g, a value that has been previously found to be optimal for the measurement of changes in the tension of rabbit corpus cavernosal tissue preparations.[5,7,9] The preparations were allowed to equilibrate in Kreb's bicarbonate buffer for 1 h and during this time Kreb's bicarbonate buffer was replaced every 15 min with fresh solution.

The corpus cavernosum strips in organ chambers were contracted with 10^{-6} M phenylephrine and added to carbachol (10^{-8}–10^{-4} M), sodium nitroprusside (SNP; 10^{-8}–10^{-4} M) and papaverine (10^{-5}–10^{-4} M). Transmural EFS was provided by a stimulator (ST95 PT, COMMAT ILETISIM, Turkey) and applied via two platinum wire electrodes set vertically within the organ bath on the opposite sides of the suspended tissue. Before electrical stimulation, the tissue was treated with guanethidine (5 μM; an adrenergic nerve blocker) and atropine (1 μM; a muscarinic receptor blocker) for 30 min. Strips were precontracted with phenylephrine at 10^{-5} M, EFS was performed. Square-wave pulses of 10 V with a 0.5-ms duration in 10-s trains with varying frequencies (2–32 Hz) were applied at 5-min intervals. The strips were allowed to return to baseline precontractile tension at each frequency.

Immunohistochemistry

Cavernous specimens were fixed in 10% neutral-buffered formalin. The specimens were embedded in regular paraffin wax and cut into 3-μm thick sections. Tissue sections were deparaffinized in xylene and rehydrated in ethanol. Antigen retrieval was performed by microwave oven at 600W for 3 × 5 min in the citrate buffer. Endogenous peroxidase was blocked by 3% H_2O_2 in methanol 15 min and again washed three times in phosphate-buffered saline. Afterwards, sections were incubated in a blocking serum (Histostatin plush kit broad spectrum, Invitrogen, CA, USA) for 10 min at room temperature to block non-specific binding. Subsequently, sections were incubated over night at room temperature with primary goat anti-nNOS polyclonal antibody (abcam-ab1376) at 2 μg/ml and 20 min at room temperature with primary anti-eNOS polyclonal antibody (abcam-ab87750) at 5 μg/ml in a humidified chamber. Negative control incubations were performed by replacing the primary antibody with the appropriate non-immune immunoglobulin G in the same concentrations. Sections were washed three times in phosphate-buffered saline and incubated with the biotinylated secondary antibodies (Histostatin plush kit broad spectrum, Invitrogen, CA, USA) for 20 min at room temperature. After three washes with phosphate-buffered saline, the sections were incubated with peroxidase labeled streptavidin (Histostatin plush kit broad spectrum, Invitrogen, CA, USA) for 10 min. Peroxidase activity was visualized with 3-amino-9-ethylcarbazol chromogen in large-volume 3-amino-9-ethylcarbazol substrate (AEC red kit 00-2007, Invitrogen, CA, USA) for nNOS and with 3, 3′-diaminobenzidine substrate (DAB kit 88-2014, Invitrogen, CA, USA) for eNOS. All incubations were performed in a moist chamber at room temparature using phosphate-buffered saline for washes between incubation steps. The sections were counterstained with Mayer's hematoxylin (Invitrogen, CA, USA) and mounted with Clearmount (Invitrogen, CA, USA) on glass slides. Slides were examined under light microscope (Olympus BX 50, Tokyo, Japan) and photomicrographs were taken with Leica DM 100 (Leica DFC 290HD, Wetzlar, Hessen, Germany). All samples were treated with exactly the same protocol. Two independent observers graded the staining intensity on a semi-quantitative scale ranging from no expression (−), very weak (+), moderate (++), strong (+++) to very strong (++++) expression.

Biochemical parameters

Blood glucose, triglyceride, total cholesterol and high-density lipoproteins (HDL) levels were measured in the rabbit's blood using Abbott Architect c16000 autoanalyzer (Abbott Laboratories, IL, USA). Also testosterone levels were measured in blood using Roche Analytics E170 Immnunology Analyzer (Roche, Tokyo, Japan).

Analysis of data

Experimental values are expressed as the mean ± s.e.m. The relaxant effects of the agonists are expressed as percantage of the precontraction response to phenylephrine. To evaluate the effects of the agonists, pD_2 (that is, the negative logarithm of the concentration for the half-maximal response; EC_{50}) and maximum response (E_{max}) values 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 the groups was performed using analysis of variance followed by Tukey's test. The score of immunoreactivity was assessed by the Kruskal–Wallis Test. Probabilities of $P < 0.05$ were considered significant.

Drugs

The following drugs were all obtained from Sigma Chemical (St. Louis, MO, USA): carbachol chloride, phenylephrine hydrochloride, SNP, atropine sulfate, guanethidine sulfate and papaverine hydrochloride. All drugs dissolved in distilled water and were freshly prepared on the day of the experiment.

RESULTS

The contractions elicited by KCl (124 mM) were similar in all the three groups (Table 1).

Effects of low- and high-dose ethanol consumption on endothelium-dependent relaxation

Carbachol (10^{-8}–10^{-4} M) produced concentration-dependent relaxation in submaximally precontracted (10^{-6} M phenylephrine) corpus cavernosum strips obtained from control, low-dose and high-dose alcohol-fed rabbits. In the low-dose alcohol consumption group, the carbachol concentration–response curve was

Table 1. Maximum responses (E_{max}) for carbachol, sodium nitroprusside (SNP), papaverine and KCl in corpus cavernosum strips obtained from control and alcohol-fed groups

	Control	*5% Alcohol-fed*	*30% Alcohol-fed*
Carbachol (%)	67.9 ± 4	90.4 ± 4.2*	42.8 ± 4.4*
EFS (%)	52.1 ± 8.5	83.7 ± 5.1*	33.6 ± 1.9*
SNP (%)	98.8 ± 0.5	98.7 ± 0.4	97.3 ± 0.7
Papaverine (%)	98.6 ± 1.4	97.5 ± 3.4	98.0 ± 2.7
KCl (mg)	3144.6 ± 466.3	3352.6 ± 402.6	3422.6 ± 266.1

Abbreviations: EFS, electrical field stimulation; KCl, potassium chloride.
*$P < 0.05$, statistically different from the response from control rabbits ($n = 9$ in each group).
E_{max} (Percentage of 10^{-6} M phenylephrine contractile response) values for carbachol, EFS, SNP, papaverine and E_{max} (mg of contractile response) values for KCl. Values are arithmetic means ± s.e.m, n = the number of preparations used.

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shifted to the left and E_{max} values were increased compared with control rabbits ($P<0.05$; Figure 1, Table 1). There were no significant changes in pD_2 values (Table 2). However, in the high-dose alcohol consumption group, the carbachol concentration–response curve was shifted to the right with significantly lower pD_2 value and E_{max} values were decreased compared with control rabbits ($P<0.05$; Figure 1, Tables 1 and 2).

Effects of low- and high-dose ethanol consumption on endothelium-independent relaxation

In precontracted strips, SNP (10^{-10}–10^{-4} M) and papaverine (10^{-5}–10^{-4} M) produced concentration-dependent relaxation. The relaxation elicited by both SNP and papaverine was similar in low-dose alcohol consumption, high-dose alcohol consumption and control groups, and there were no significant changes in the pD_2 and E_{max} values (Figure 2, Tables 1 and 2).

Effects of low- and high-dose ethanol consumption on neurogenic relaxation

In precontracted strips, EFS (2–32 Hz) evoked frequency-dependent relaxations. In the low-dose alcohol-fed group, EFS responses were higher than those in the control group ($P<0.05$; Figure 3, Table 1). But in the high-dose alcohol-fed group, EFS responses were inhibited compared with the control group ($P<0.05$; Figure 3, Table 1).

Blood glucose, triglyceride, total cholesterol, HDL and testosterone levels in control and alcohol-fed rabbits

Levels of blood glucose, triglyceride, total cholesterol, HDL and testosterone in control and alcohol-fed rabbits are shown in Table 3. The blood glucose (mg/dl) and HDL levels (mg/dl) of 5% alcohol-fed and 30% alcohol-fed rabbits were similar to the control group. Cholesterol (mg/dl) and triglyceride (mg/dl) levels were significantly higher in 30% alcohol-fed rabbits compared with the control group ($P<0.05$). Additionally, testosterone level (ng/dl) was significantly lower in 30% alcohol-fed rabbits compared with that of the control group ($P<0.05$).

Effects of low- and high-dose ethanol consumption on penile nNOS and eNOS expression

Immunopositive staining was found after nNOS or eNOS immunohistochemistry in penile tissue. Weak immunostaining was observed in penile tissue in high ethanol-fed group (Figures

Table 2. pD_2 values for carbachol and sodium nitroprusside (SNP) in corpus cavernosum strips obtained from control and alcohol-fed groups

	Control	*5% Alcohol-fed*	*30% Alcohol-fed*
Carbachol	5.92 ± 0.24	5.79 ± 0.24	4.82 ± 0.28*
SNP	5.50 ± 0.17	5.45 ± 0.18	5.60 ± 0.08

*$P<0.05$, statistically different from the response from control rabbits ($n=9$ in each group).
Values are arithmetic means ± s.e.m.

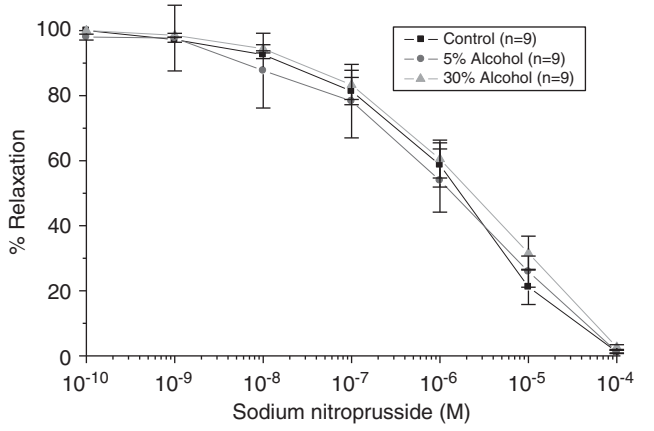

Figure 2. Sodium nitroprusside concentration–response in isolated rabbit corpus corpus cavernosum strips precontracted with phenylephrine 10^{-6} M. Each point is expressed as a percentage of the contraction induced by phenylephrine and is given as the mean ± s.e.m. Numbers in parentheses indicate the number of preparations used.

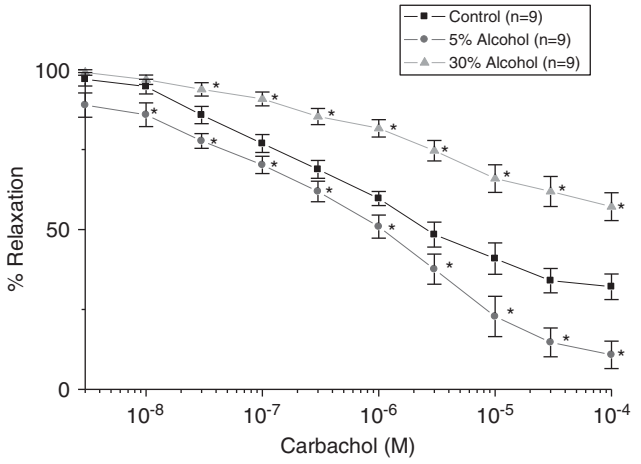

Figure 1. Carbachol concentration–response in isolated rabbit corpus corpus cavernosum strips precontracted with phenylephrine 10^{-6} M. Each point is expressed as a percentage of the contraction induced by phenylephrine and is given as the mean ± s.e.m. Numbers in parentheses indicate the number of preparations used. *$P<0.05$, statistically different from the response from control rabbits.

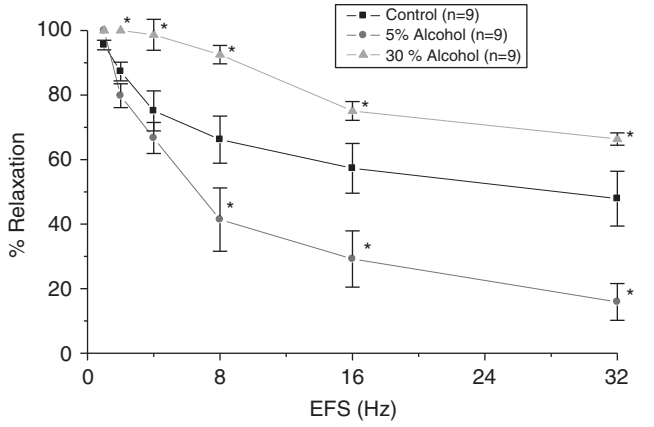

Figure 3. Relaxation responses evoked by EFS of isolated rabbit corpus cavernosum strips precontracted with phenylephrine 10^{-6} M. Each point is expressed as a percentage of the contraction induced by phenylephrine and is given as the mean ± s.e.m. Numbers in parentheses indicate the number of preparations used. *$P<0.05$, statistically different from the response from control rabbits. EFS, electrical field stimulation.

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4c and f) compared with control (Figures 4a and d; $P<0.05$), indicating the NO generation through nNOS or eNOS is impaired in the penile tissue of the high-dose ethanol-fed rabbits. Contrarily, in low ethanol-fed group (Figures 4b and e), increased immunostaining was observed compared with the control group (Figures 4a and d; $P<0.05$).

DISCUSSION

The present study addresses the changes in neurogenic and endothelium-dependent relaxation to carbachol in the corporal smooth muscle and its relationship with blood alcohol levels in rabbit-ingested ethanol at different doses for 12 weeks. In most of the human studies, chronic alcohol consumption is a major risk factor in the development of impotence. Also, it is well known that the concentration and duration of ethanol ingestion have an important impact on erectile outcomes in animal models. Experiments on alcohol and erectile response showed that high doses of alcohol attenuate erectile response; however, light-to-moderate alcohol consumption increases male sexual activity.[20–22] The results obtained with the corpus cavernosum were consistent with previous studies showing that light-to-moderate alcohol consumption is inversely associated with cardiovascular diseases.[23–25] It has been proposed that increased NO production is a major physiological system for the protective action of moderate alcohol consumption.[12,13] However, experimental and epidemiological evidence show that chronic high-dose ethanol consumption increases mortality and causes cardiovascular complications, including hypertension.[26] It is hypothesized that chronic alcohol-induced hypertension is associated with the downregulation of antioxidants and NO production in the aorta of rats.[14–16]

Table 3. Blood glucose, triglyceride, total cholestrol, HDL and testosterone levels in control and alcohol-fed rabbits

	Control	*5% Alcohol-fed*	*30% Alcohol-fed*
Glucose (mg/dl)	143.33 ± 1.47	141.67 ± 23.64	149 ± 7.07
Cholesterol (mg/dl)	42 ± 10.14	31.83 ± 4.64	93.3 ± 11.74*
HDL (mg/dl)	11 ± 3.24	17 ± 4.15	19.83 ± 5.05
Triglyceride (mg/dl)	57.83 ± 8.31	74.5 ± 8.50	181.55 ± 21.30*
Testosterone (ng/dl)	4.36 ± 0.56	3.79 ± 0.62	0.95 ± 0.24*

Abbreviation: HDL, high-density lipoprotein.
*$P<0.05$, statistically different from the response from control rabbits ($n=9$ in each group).
Values are arithmetic means ± s.e.m.

NO plays an essential role in the erection of penis. It is well known that both the autonomic nerves that innervate corpus cavernosum are independent sources of NO.[27,28] In this study, EFS and exogenous application of SNP, papaverine and carbachol produced relaxation responses in both alcohol-treated and control groups. There were no significant differences in response to SNP and papaverine in all the groups. However, the relaxation response to EFS and carbachol were significantly increased in low-dose alcohol-treated rabbits. Therefore, it is speculated that low-dose alcohol consumption increased production of NO. It is also possible that low-dose alcohol consumption may increase relaxation of trabecular smooth muscle or increase the ability to relax via the NO/cGMP pathway; however, this possibility is unlikely as the strips relaxed well to papaverine or SNP. Similarly, we have previously shown increased endothelium-dependent relaxant response to acetylcholine in isolated aortas from 8-week ethanol-treated (7.5%) rats.[29] Our findings are consistent with previous studies demonstrating that chronic moderate alcohol ingestion enhanced NO production by vascular cells *in vitro*.[13] Our evidence indicates that chronic light alcohol ingestion increases trabecular NO production and causes increased trabecular smooth muscle relaxation leading to increase in sexual performance.

Also, in this study, EFS- and carbachol-induced relaxation responses were significantly decreased in the higher-dose alcohol-treated group. The results of the present study further showed that penile nNOS significantly decreased in high ethanol consumption, indicating erectile dysfunction. It is reported that, when rats treated with graded concentrations of alcohol for 12 weeks, higher concentrations of alcohol were associated with hypertension and reduced plasma levels of NO metabolites. Authors recently clarified that daily oral treatment with ethanol for 12 weeks caused significant decreases in aortic NO production, reduced endothelium-dependent vasorelaxation and increased blood pressure that were due to, in part, alcohol-induced reductions in eNOS.[14] This finding is consistent with our results, therefore we suggest that impaired NO production have an important pathophysiological role in the erectile dysfunction after high-dose alcohol consumption in rabbits.

In addition, several mechanisms have been proposed to account for the pathogenesis of impotence associated with chronic alcohol consumption such as impairment of hypotalamic function.[30] Androgens are essential in the maintenance of libido

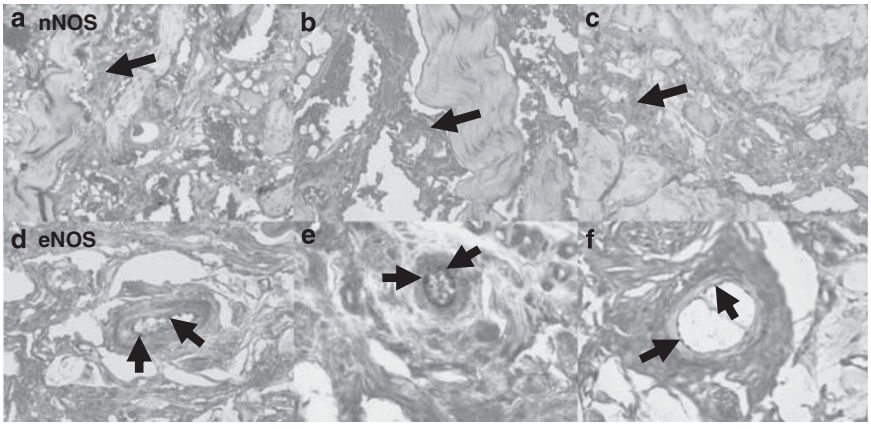

Figure 4. Representative light microscopy of control and chronic ethanol-fed groups in penile tissue. Decreased (**c**) neuronal nitric oxide synthase (nNOS) and (**f**) endothelial NOS (eNOS) immunoreactivity at corpus cavernosum in high-dose ethanol-fed rabbits and increased (**b**) nNOS and (**e**) eNOS immunoreactivity at penile tissue in low-dose ethanol-fed rabbits compared with (**a, d**) control groups.

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and have an important role in regulating erectile capacity in man. In hypogonadal patients, it is known that exogenous testosterone administration stimulates erectile function.[31] Previous studies have shown that alcohol generally decreases circulating testosterone levels in animals and humans, but many man who are alcohol dependent have normal testosterone and estrogen levels.[32–34] Cicero and Badger[32] reported that low doses of ethanol significantly increased serum testosterone, whereas high doses decreased serum levels of testosterone, in male Spraque–Dawley rats. Moreover, it has been demonstrated that androgens are essential in maintenance of NO-mediated erectile activity in the rat.[35] Animal studies have also revealed that androgens regulate the expression of NOS in the penis.[36,37] These findings are consistent with our results. Although this study was not designed to explore the effects of testosterone on the corpus cavernosal smooth muscle function, their decrease after heavy alcohol consumption may have contributed to the impaired relaxation response to both EFS and carbachol as observed in our study.

Epidemiological evidence suggests that alcohol ingestion modulates the development of cardiovascular disease.[38] Moderate alcohol consumption may prevent atherosclerotic vascular disease, whereas excessive intake may enhance cardiovascular disease.[39] The mechanism which underlie the protective effects of moderate alcohol consumption on cardiovasular disease risk are not fully understood. However, moderate alcohol consumption affects lipoprotein metabolism.[40] Epidemiological[41] and experimental studies[42] have demonstrated that alcohol consumption increases HDL concentration, which is inversely associated with cardiovascular disease risk. In addition, chronic alcohol intake has been shown to increase,[43] decrease[44] or not modify[45] serum cholesterol levels. Furthermore, Azadzoi and Saenz de Tejada[8] reported that hypercholesterolomia impairs endothelium-dependent relaxation of rabbit corpus cavernosum smooth muscle. Hence, in our study, changes in blood total cholestrol, HDL and trigliyceride levels in chronic high-dose alcohol consumption seem to be important parameters for impaired nitrergic relaxations in corpus cavernosum.

Another popular theory is that acetaldehyde inhibits NO formation, which leads to impaired NO-mediated relaxation in chronic high-dose of alcohol consumption. It is well known that after ingestion, ethanol is metabolized sequentially to acetaldehyde, acetate, CO_2 and water. Previously, Kim *et al*[46] showed that acetaldehyde suppressed neurogenic relaxation induced by transmural EFS in rabbit corpus cavernosum smooth muscle, and noted that increasing acetaldehyde level seen in chronic alcoholism may contribute to male erectile dysfunction mainly by the inhibition of NO formation. Taking these findings together, we speculated that chronic high dose of alcohol consumption impairs the synthesis or availability of NO in corpus cavernosum smooth muscle.

In conclusion, the present study showed that chronic low-dose ethanol consumption leads to increased nitrergic-mediated corpus cavernosum smooth muscle relaxation as well as increased immunostaining in nNOS and eNOS. Our findings suggest that this increment appears to be related to increased NO production and/or release. Also, we demonstrated that nitrergic-mediated relaxations decreased in strips of corpus cavernosum from chronic high-dose ethanol-treated rabbits as well as decreased immunostaining in nNOS and eNOS. This suggests that treatment with high-dose ethanol under the conditions that were used in the study impaired both the neurogenic and endothelial functions. Therefore, we speculated that impaired nitrergic relaxations during high-dose ethanol treatment may contribute to erectile dysfunction through reduced NO production and/or release.

ACKNOWLEDGEMENTS

This study was supported by a grant from Kocaeli University Research Fund (Project number: 200319). Research Foundation had no further role in the study design, in the collection, analysis and interpretation of data, in the writing of the report and in the decision to submit the paper for publication.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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