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Research Article

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Chronic moderate alcohol consumption induces iNOS expression in the penis: An immunohistochemical study

Süheyla GONCA[1,*], Yusufhan YAZIR[1], Semil Selcan GÖÇMEZ[2], Ekim Nur DALÇIK[3], Tijen UTKAN[4], Hakkı DALÇIK[1]

[1]Department of Histology and Embryology, Faculty of Medicine, Kocaeli University, Kocaeli, Turkey
[2]Department of Pharmacology, Faculty of Medicine, Namık Kemal University, Tekirdağ, Turkey
[3]Cerrahpaşa Medical Faculty, İstanbul University, İstanbul, Turkey
[4]Department of Pharmacology, Faculty of Medicine, Kocaeli University, Kocaeli, Turkey

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Aim: To investigate the effect of moderate alcohol consumption on metabolic alterations, inducible nitric oxide synthase (iNOS), immunohistochemical distribution, and morphological damage to penile erectile tissue in rats.

Materials and methods: Male Wistar albino rats were divided into 2 groups. Group 1 rats (control group, n = 8) received tap water ad libitum, and group 2 rats (n = 8) were fed with 20% ethanol. Increasing levels of alcohol were given to the rats over 12 weeks. Immunohistochemistry was then performed using the avidin–biotin–peroxidase technique on 5-µm thickness tissue sections. Stained sections were examined by imaging microscope.

Results: Alcohol consumption resulted in a significant increase in iNOS immunoreactivity in the penile erectile tissue. Increased iNOS expression was determined in the tunica albuginea, cavernosal smooth muscle cells, trabeculae of connective tissue, arterioles, and the urethral epithelium. Moreover, chronic alcohol consumption resulted in decreasing serum testosterone and high density lipoprotein (HDL) levels with increasing cholesterol and triglyceride levels.

Conclusion: Chronic moderate alcohol consumption can affect penile erectile tissue by increasing iNOS immunoreactivity and induce histopathological damage such as penile fibrosis. These abnormalities are also related to the defense mechanism against morphological damage.

Key words: Chronic moderate alcohol consumption, iNOS, immunohistochemistry, penis, rat

1. Introduction

Alcohol is an important liquid that affects the functional aspects of various tissue components. Alcohol diffuses across membranes and distributes through all cells and tissues, and it can acutely affect cell function by interacting with certain proteins and cell membranes. In addition, the formation of damaging molecules, known as reactive oxygen species, is another negative effect of alcohol. Blood alcohol concentration is determined by how quickly alcohol is absorbed, distributed, metabolized, and excreted (1). Alcohol elimination rate varies among individuals and is influenced by factors such as chronic alcohol consumption, diet, age, smoking, and time of day (2,3). Alcohol metabolism also results in the generation of acetaldehyde, a highly reactive and toxic by-product that may contribute to tissue damage (4).

It is well known that nitric oxide (NO) is formed from L-arginine via catalysis by several nitric oxide synthase (NOS) isoforms, neuronal (nNOS), endothelial (eNOS), and inducible NOS (iNOS) (5,6). Potential sources of NO and NOS expression are found in the sinusoidal endothelium and corporeal smooth muscle cells (7). It is known that under certain physiological conditions NO is released in small amounts and activates soluble guanylyl cyclase, which increases 3',5'-cyclic guanosine monophosphate (cGMP) levels (8), acting as a second messenger molecule (9), with cGMP producing smooth muscle relaxation in the corpus cavernosum (10,11).

NO is an important mediator for controlling vascular resistance and is responsible for vasodilatation. The inhibition of NO production thus causes increased vascular resistance and increased arterial blood pressure (6,12). Moreover, NO exerts a significant role in penile function, operating chiefly as the principal mediator of intracavernosal pressure increase.

Alcohol consumption has been known to stimulate NO and NOS expression in different tissues. Recently, we

* Correspondence: suhgonca@gmail.com

showed that eNOS and nNOS expression in penile tissues is reduced by long-term high-dose alcohol consumption, while low-dose alcohol consumption increased eNOS and nNOS expression (13).

Sex-specific vascular effects by chronic ethanol consumption in rats indicated that mRNA levels for eNOS and iNOS were not altered by ethanol consumption, whereas ethanol intake reduced eNOS protein levels and increased iNOS protein levels in the aorta from female rats (14). Lizarte et al. (15) demonstrated that despite the overexpression in corpus cavernosum smooth muscles of eNOS and iNOS in ethanol-treated rats, the impaired relaxation induced by acetylcholine may suggest that chronic ethanol consumption induces endothelial dysfunction. However, whether chronic moderate alcohol consumption affects biochemical markers (i.e. blood alcohol, glucose levels), iNOS immunoexpression, and penile morphological changes is not known yet.

The purpose of this study was to investigate whether moderate alcohol consumption induces morphological degeneration and changes in iNOS expression in the penile erectile tissue of rats.

2. Materials and methods

2.1. Animals

The experiments reported in this study were conducted in accordance with the Regulation of Animal Research Ethics Committee in Turkey (6 July 2006, Number 26220). Ethical approval was granted by the Kocaeli University Animal Research Ethics Committee (Project Number: AEK – 192-5, Kocaeli, Turkey). Adult male Wistar rats (200–250g) were obtained and housed in the Experimental Medical Research and Application Unit (DETAB, Kocaeli University, Kocaeli, Turkey) in a temperature and humidity controlled room (22 ± 3 °C and 62 ± 7%, respectively) in which a 12-12 h light-dark cycle was maintained (0800–2000 h light).

2.2. Treatment schedule

The rats were divided into 2 groups (n = 8 per group). The 8 rats in group 1 received tap water ad libitum, while the 8 rats in group 2 were fed with 20% ethanol. The model of ethanol feeding was that described previously, in which rats received 5% ethanol (vol/vol) in the drinking water for the first week, 10% for the next 2 weeks, and 20% from weeks 4 to 12 (16). All rats had constant access to standard laboratory rat chow.

2.3. Immunohistochemistry

After being perfused transcardially with 500 mL of 4% paraformaldehyde in 0.1 mol/L phosphate buffer, obtained rat penis specimens were post-fixed overnight in the same fixative, then washed in running water for at least 4 h, and dehydrated in increasing alcohol series (70%, 80%, 90%, and 100%) and xylene, prior to embedding in paraffin wax. Embedded tissues were sectioned (5–6 µm thickness) on a microtome and were then deparaffinized and hydrated by sequential incubations in xylene and ethanol. After washing in 3 × PBS for 5 min, the sections were blocked with 3% H_2O_2 for 10 min to quench endogenous peroxidase activity. Sections were then washed in PBS-Triton X 100 (Tx). Heat-induced epitope (antigen) retrieval methods were performed using antigen unmasking solution (antigen retrieval solution: 0.01 M sodium citrate buffer, pH 6.0, 600 W) in a microwave oven (5 min, 3 times). Sections were then washed (3 × 5 min) in PBS-Tx. Immunocytochemistry was performed using the avidin–biotin–peroxidase method (Zymed, San Francisco, CA, USA). To eliminate the nonspecific binding, sections were pretreated with normal 10% nonimmune goat serum. Sections were incubated in prediluted liquid rabbit polyclonal iNOS primary antibody (1/100, Neomarkers, Fremont, CA, USA) for 24 h at 4 °C in a humidified chamber. Following washing in PBS-Tx, biotinylated anti IgG secondary antibodies (Zymed, San Francisco, CA, USA) were applied for 15 min at room temperature. Following washing in PBS-Tx, streptavidin–peroxidase conjugate (Zymed, San Francisco, CA, USA) was applied to the sections for 15 min at room temperature. Sections were then washed in PBS-Tx, and the tissue was immunoreacted with a chromogen solution (Liquid DAB-Black Substrate Kit, Zymed, San Francisco, CA, USA) for 5 min at room temp. As a control, the primary antibody was omitted and replaced with nonimmune serum. Reactions were stopped by rinsing the sections several times in PBS-Tx. Sections were placed on poly-l-lysine–coated glass coverslips, air dried, dehydrated, and mounted. The coverslipped sections were then photographed under a bright field using an imaging microscope (BX50F; Olympus, Tokyo, Japan). The degree of staining was evaluated as follows: 0 (no staining), + (weak staining), ++ (moderate staining), and +++ (strong staining).

2.4. Blood ethanol, glucose, testosterone, triglyceride, cholesterol, and HDL determination

Blood ethanol concentration was determined in a drop of whole blood collected at the time of death, using the alcohol dehydrogenase method (Bayer opeRA Chemistry Analyzer, Bayer Diagnostics, Tarrytown NY, USA). Triglyceride, total cholesterol, and HDL levels were measured in blood using a Beckman LX 20 autoanalyzer (Beckman Coulter, Inc, Brea, CA, USA). Total testosterone levels were measured in blood using a Roche Analytics E170 Immnunology Analyzer (Roche, Tokyo, Japan). Blood glucose was also determined using a commercial glucose meter and glucose sensitive dipsticks (Accutrend Alpha glucometer, Boehringer, Manheim, Germany).

2.5. Statistical analysis
Blood alcohol concentration results are expressed as mean ± SEM where n equals the number of animals. Statistically significant differences between the groups were calculated by Student's t test. Probabilities of less than 5% ($P < 0.05$) were considered significant.

3. Results
3.1. Blood ethanol, glucose, testosterone, cholesterol, triglyceride, and HDL levels
The mean blood ethanol level was 28.13 ± 3.02 mg/dL at the time of sacrifice. No ethanol was detected in the control group. Levels of blood glucose, triglyceride, total cholesterol, HDL, and testosterone in control and alcohol-fed rats are shown in the Table.

The blood glucose levels (mg/dL) of 20% alcohol-fed rats were similar to those of the control group. Cholesterol (mg/dL) and triglyceride (mg/dL) levels were significantly higher in 20% alcohol-fed rats compared to the control group ($P < 0.05$). Additionally, testosterone (ng/mL) and HDL levels (mg/dL) were significantly lower in 20% alcohol-fed rats than in the control group rats ($P < 0.05$).

3.2. INOS immunohistochemistry
Cross-sections of the penile shaft obtained from control and alcoholic rats were stained with a polyclonal anti-iNOS antibody for immunohistochemistry. We performed immunohistochemical staining to localize iNOS in the penises of each group.

The penile tissue from the control rats showed moderate iNOS immunostaining (++) in the corporeal and tunica areas (Figures 1a, 1b, 1c). The iNOS immunostaining of the urethral epithelium was strong compared to other penile areas in the control penises (Figure 1a). Weak (+) or moderate (++) iNOS immunoreactivity was seen in the

Table. Blood glucose, triglyceride, total cholesterol, HDL, and testosterone levels in control and alcohol-fed rats.

	Control	20% Alcohol-fed
Glucose (mg/dL)	125.3 ± 16.7	146.1 ± 19.8
Cholesterol (mg/dL)	58.8 ± 2.84	118.63 ± 7.08*
HDL mg/dL	30.34 ± 1.41	13.75 ± 1.70*
Triglyceride (mg/dL)	71.30 ± 7.36	182.00 ± 25.19*
Testosterone (ng/mL)	1.72 ± 0.08	0.84 ± 0.02*

Note: values are arithmetic means ± SEM. * $P < 0.001$, significantly different from the response from control rats (n = 8 in each group).

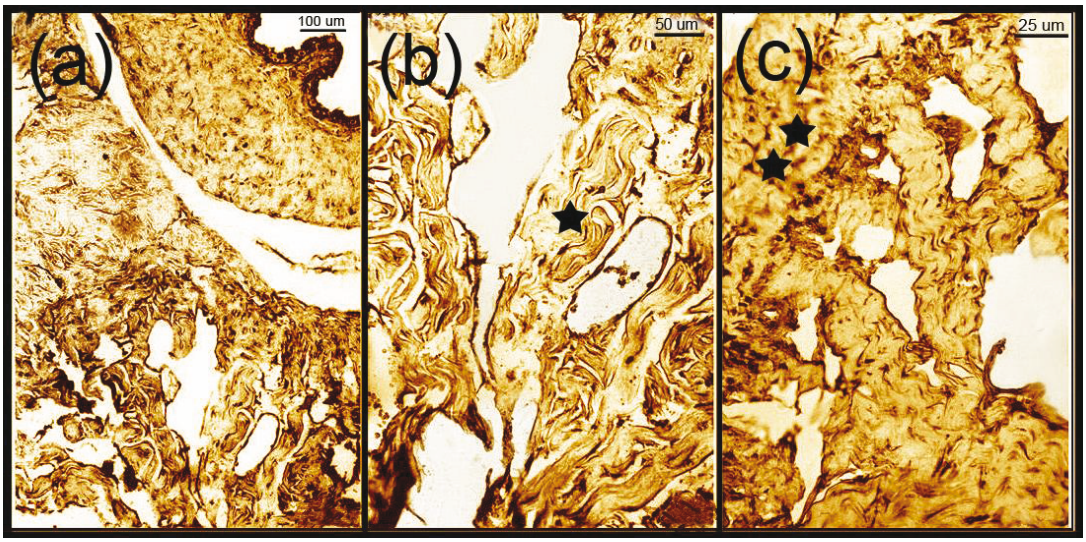

Figure 1. Immunohistochemical detection of iNOS expression in the control rat penile shaft (Figures 1a, 1b, 1c). Section showed more intense iNOS immunostaining in the urethral epithelium (Figure 1a). Weak (*) iNOS immunoreactivity in the corporeal areas (Figure 1b). Moderate intensity (**) of iNOS immunoreactivity in the corporeal areas (Figure 1c).

corporeal areas (Figures 1b, 1c). In the alcoholic-treated group, the intensity of the iNOS immunostaining was higher compared to the control group (Figures 2a, 2b, 2c). The intense iNOS immunostaining (+++) was mainly present in the tunica albuginea, the smooth muscles of the corpora cavernosa, and trabecular connective tissue. Alcohol-fed rats had increased iNOS expression and thickening of the tunica albuginea (Figures 2a, 2b). The smooth muscle cells and cavernosal areas showed strong immunoexpression of iNOS (Figure 2c).

4. Discussion

Erectile dysfunction is a common, multifactorial disorder that is associated with a range of organic and psychogenic conditions, including alcohol consumption (17), smoking (18), hypercholesterolemia (19), diabetes mellitus (20), cardiovascular disease (21), arterial occlusion (22), and hormonal impairments (23,24).

In the current study, we determined the iNOS expression of penile tissue using specific immunocytochemical staining with prediluted liquid rabbit polyclonal iNOS primary antibody. INOS immunoreactivity was found in both examined groups. In the experimental alcoholic rats, increased iNOS immunoreactivity was markedly demonstrated in the tunica albuginea, corpora cavernosa smooth muscle cells, and trabeculae of connective tissue. However, iNOS expression was limited in the control rats. INOS may be induced in the corpora cavernosa smooth muscles during the development of penile fibrosis associated with aging (25), and atherosclerosis (26), and Peyronie's disease (27). Moreover, aging associated erectile dysfunction is primarily caused by a reduction in smooth muscle cells and an increase in collagen within the corpora cavernosa. This is accompanied by the expression of iNOS to produce nitric oxide that scavenges reactive oxygen species and inhibits collagen deposition (28). Thus, iNOS can be used as an important immunohistochemical marker to determine the degree of penile fibrosis.

Taken together, our results suggest that alcohol-induced endogenous iNOS expression may have a role in maintaining endothelial and erectile tissue function. Increased NO synthesis, due to iNOS activity, may be a defense mechanism against penile fibrosis, in agreement with the inhibitory effects of NO on collagen synthesis and the development of fibrosis (29). These results are in parallel with ours.

Actually, ethanol consumption and cigarette smoking are common in societies worldwide and have been identified as injurious to human health. It is suggested that prolonged exposure to alcohol and nicotine produces similar, and in some cases additive, oxidative tissue injuries in rats. (30). Furthermore, the antifibrotic, antioxidative, and smooth muscle protective roles of iNOS in the penile corpora cavernosa were confirmed in the iNOS knock out (iNOS KO)/streptozotocin (STZ)-induced diabetic mouse model (31). Long-term oral administration of supra-physiologic doses of L-arginine improves the erectile response in the aging rat (32). Gonzalez et al. (33) indicated that pharmacological iNOS induction, alternate NO donors, or L-arginine may constitute a valid approach to prevent or treat penile fibrosis and vasculogenic erectile dysfunction.

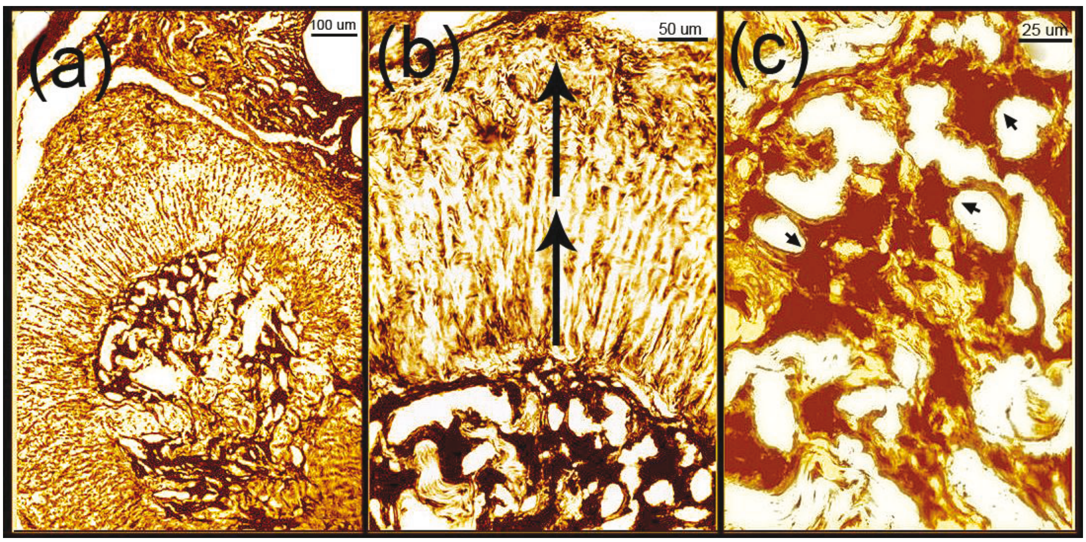

Figure 2. Immunohistochemical detection with a polyclonal antibody against iNOS stain in the alcoholic rat penile shaft (Figures 2a, 2b, 2c). Stained sections from alcoholic rats indicated markedly increased iNOS expression and thickening of the tunica albugenia (arrows) (Figures 2a, 2b). The smooth muscle cells and cavernosal areas showed a preferential expression of iNOS (arrow heads) (Figure 2c).

In summary, according to the results of the present study and other studies, a basal level of NO synthesis is still required for activation and relaxation of the corporeal smooth muscle. Establishing the NO-dependent regulatory system for penile erection could be a gateway for considering novel therapeutic approaches for erectile dysfunction now and in the future.

Furthermore, low levels of testosterone and high levels of lipids are associated with sexual dysfunction and altered reactivity of corpus cavernosum smooth muscle to different agents (34,35). Our data suggest that alcohol-induced decreases in testosterone levels and increases in lipid levels could be a possible mechanism underlying the erectile dysfunction associated with chronic alcohol consumption, and significantly contribute to endothelial dysfunction and consequent erectile dysfunction and oxidative stress.

In conclusion, the present study showed that chronic alcohol consumption contributes to or causes pathological consequences involving the penis. Therefore, we proposed that increased iNOS synthesis caused by chronic alcohol consumption may initiate a protective effect on erectile tissue and also may act as a defense mechanism against penile dysfunction and fibrosis.

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