Caloric restriction increases internal iliac artery and penile nitric oxide synthase expression in rat: Comparison of aged and adult rats

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Because of the positive correlation between healthy cardiovascular system and sexual life we aimed to evaluate the effect of caloric restriction (CR) on endothelial and neuronal nitric oxide synthase (eNOS, nNOS) expression in cavernous tissues and eNOS expression in the internal iliac artery in young and aged rats. Young (3 mo, n = 7) and aged (24 mo, n = 7) male Sprague-Dawley rats were subjected to 40% CR and were allowed free access to water for 3 months. Control rats (n = 14) fed ad libitum had free access to food and water at all times. On day 90, rats were sacrificed and internal iliac arteries and penis were removed and paraffinized, eNOS and nNOS expression evaluated with immunohistochemistry. Results were evaluated semiquantitatively. eNOS and nNOS expression in cavernous tissue in CR rats were more strong than in control group in both young and old rats. eNOS expression was also higher in the internal iliac arteries of CR rats than in control in young and old rats. As a result of our study we can say that there is a positive link between CR and neurotransmitter of erection in cavernous tissues and internal iliac arteries. CR has beneficial effect to prevent sexual dysfunction in young and old animals and possible humans.

KEY WORDS: Rat; Caloric restriction; Nitric oxide synthase; Internal iliac artery; Penis.

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INTRODUCTION
The incidence of erectile dysfunction (ED) increases with age. It is reported that 35% of married men aged 60 years and older suffer from ED (1). From the prevalence rates reported in the Massachusetts Male Aging Study (MMAS), between the ages of 40 and 70 years, the probability of complete ED increased from 5.1% to 15%, moderate dysfunction increased from 17% to 34%, and mild ED remained constant at about 17% (1). According to the study of the National Health and Social Life Survey (NHLSLS) the following prevalence rates for ED were reported (responses to questions regarding obtaining and maintaining erection): 7% for ages 18 to 29 years, 9% for ages 30 to 39, 11% for ages 40 to 49, and 18% for 50 to 59 (1). Obesity has become a worldwide public health problem, it may decrease life expectancy by 7 years at the age of 40 years: excess bodyweight is now the sixth most important risk factor contributing to the overall burden of disease through the world. Overweight and obesity may increase the risk of ED by 30-90% as compared with normal subjects. Moreover, women with the metabolic syndrome have an increased prevalence of sexual dysfunctions as compared with matched controls. Lifestyle changes reducing body weight induces amelioration of both erectile and endothelial functions in obese men (2). Patients with ED show a higher body mass index (BMI), waist circumference (WC), and insulin-resistance (IR) and lower levels of total testosterone (TT) and bioavailable testosterone (BT). There is a negative correlation between erectile function and IR and abdominal obesity. The TT levels are lower in patients with increased BMI,
WC and IR. Negative correlation was shown only between BT and abdominal obesity (3). Androgen deficiency together with endothelial dysfunction may be responsible from ED in obesity (4).

In animal experiments penile endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS) expression were found decreased in hypercholesterolemic cavernous tissue due to decrease activity of AMP-activated protein kinase (AMPK), which increases the expression of neuronal (n) NOS and endothelial (e) NOS (5).

In another experiment we showed that mild to moderate exercise increases penile eNOS and nNOS expression as well as serum total testosterone levels in young and aged rats (6).

Modifiable lifestyle factors such as obesity, lack of exercise and smoking play a role in the development, progression or remission not only of erectile dysfunction (ED), but also in cardiovascular disease and the metabolic syndrome. One-third of obese men with ED can regain their sexual activity after 2 years of adopting health behaviors, mainly regular exercise and reducing weight. Western societies actually spend a huge part of their health care costs on chronic disease treatment and interventions for risk factors. The adoption of healthy lifestyles can reduce the prevalence of obesity and the metabolic syndrome, and hopefully the burden of sexual dysfunction (7).

Mediterranean-style diets and a reduction in caloric intake have been found to improve erectile function in men with the aspects of the metabolic syndrome. In addition, both clinical and experimental studies have confirmed that combining the two interventions provides additional benefit to erectile function, likely via reduced metabolic disturbances (e.g., inflammatory markers, insulin resistance), decreased visceral adipose tissue, and improvement in vascular function (e.g., increased endothelial function) (8).

Mediterranean-style diet might be effective in ameliorating sexual function in women with metabolic syndrome. Lifestyle changes, mainly focusing on regular physical activity and a healthy diet, are effective and safe ways to reduce cardiovascular diseases and premature mortality in all population groups; they may also prevent and treat sexual dysfunctions in both sexes (9).

Taking all of these background into account, the objective of the work was to investigate the role of caloric restriction on eNOS as well as nNOS and eNOS expression in the internal iliac artery and cavernous tissue of young and aged rats, respectively.

**Materials and Methods**

**Animals and diet**

3-month-old young and 24-month-old aged male Sprague-Dawley rats were divided into four experimental groups (n = 7 rats per group). Control rats in each group were fed ad libitum with pelleted standard diet. Another two groups were subjected to 40% caloric restriction for three months (10).

Rats were housed individually with free access to water in wire-bottom cages and acclimated at 22°C with a 12-h light/dark cycle. Caloric restricted rats were fed on a daily basis at the beginning of the dark cycle and the amount of food was weekly updated. All animal experiments were approved by the Animal Ethics Committee.

Isolation of samples and immunohistochemical staining

At the end of 3 months rats were sacrificed and the and internal iliac arteries and penises were quickly removed, washed with saline and paraffinized. All procedures were performed under general anaesthesia with 50 mg kg ketamine HCL administered intraperitoneally. eNOS, nNOS expression in all tissues was evaluated with immunohistochemistry using specific antibodies.

For the immunohistochemical evaluation, specimens were processed for light microscopy and sections incubated at +4°C overnight and then de-waxed in xylene for 30 min. After rehydrating in a decreasing series of ethanol, sections were washed with distilled water and phosphate buffered saline (PBS) for 10 min. Sections were then treated with 2% trypsin in 50 mm Tris buffer (pH 7.5) at 37°C for 15 min and washed with PBS. Sections were delineated with a Dako pen (Dako, Glostrup, Denmark) and incubated in a solution of 3% H2O2 for 15 min to inhibit endogenous peroxidase activity. Then, sections were incubated with eNOS Ab-I (RB-9279-R7, Neomarkers, Labvision, Fremont, CA, USA) and nNOS (sc-648, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) antibodies.

The Ultra-vision (Labvision) horseradish peroxidase/3-amino-9-ethylcarbazole staining protocol was used at this stage.

Sections prepared for each case were examined by light microscopy. Positive and negative controls were conducted in parallel with NOS stained sections. Staining of sections with commercially available antibodies served as the positive control.

Negative controls included staining tissue sections with omission of the primary antibody. The sections were evaluated for diffuseness and staining. Penile eNOS and nNOS were evaluated according to the diffuseness and intensity of staining in penile cavernous smooth muscle.

According to the diffuseness of the staining, sections were graded as 0 = no staining; 1 = staining < 25%; 2 = staining 25-50%; 3 = staining 50-75%; 4 = staining > 75%. According to staining intensity, sections were graded as follows: 0 = no staining; 1 = weak but detectable staining; 2 = distinct; 3 = intense staining (11, 6).

Immunohistochemical values were obtained by adding the diffuseness and intensity scores.

**Results**

eNOS expression in internal iliac artery: In control young and aged rat internal iliac arteries there was focal mild to moderate eNOS expression, but diffuse in caloric restricted young and aged rats (Figure 1A-D).

eNOS and nNOS expression in cavernous tissue: eNOS, nNOS expression were weak in the cavernous tissues of control rats. In caloric restricted group eNOS, nNOS expression were more evident than in control young and aged rats (Figure 2A-H).
Figure 1A-C.
eNOS expression in internal iliac artery.

A-B: Young Control Group: Focal mild internal iliac artery eNOS staining (IHC 400X).

C-D: Young Caloric Restriction Group: Diffuse internal iliac artery eNOS staining (IHC 400X).

Figure 2A-H.
eNOS and nNOS expression in cavernosal tissue.

A: Young Control Group: Focal mild penile eNOS staining (IHC 400X).

C: Young Caloric Restriction Group: Diffuse penile eNOS staining (IHC 400X).

E: Young Control Group: Focal mild penile nNOS staining (IHC 400X).

G: Young Caloric Restriction Group: Diffuse penile nNOS staining (IHC 400X).

B: Aged Control Group: Focal mild penile eNOS staining (IHC 400X).

D: Aged Caloric Restriction Group: Diffuse penile eNOS staining (IHC 400X).

F: Aged Control Group: Focal mild penile nNOS staining (IHC 400X).

H: Aged Caloric Restriction Group: Diffuse penile nNOS staining (IHC 400X).


**DISCUSSION**

Erectile function is a multi-system phenomenon involving vascular, neuronal and endocrine system. In this process nitric oxide (NO) released from nerve endings and endothelial cells plays a key role. NO is produced from L-arginine through an enzymatic reaction in which the enzyme nitric oxide synthase is involved. In the cavernosal tissue NO stimulates guanylate cyclase enzyme present in the smooth muscle cells. Guanylate cyclase induces the formation of cyclic guanosine monophosphate (GMPC) from guanosine triphosphate (GTP). Phosphorilation of GMPC, results in cytoplasmic calcium release causing smooth muscle relaxation of the corpus cavernosum, with the subsequent penile tumescence (12, 13).

The most common causes of ED are organic such as cardiovascular and endocrine diseases including obesity, type-2 diabetes mellitus (DM2) and metabolic syndrome. Depression, hormonal changes and vascular or neurological damage alter trauma or surgery are other factors associated with ED (3, 14).

Obesity causes insulin resistance and cardiovascular system diseases through disrupting in the signaling pathways required for nitric oxide production with subsequently endothelial dysfunction. Nowadays obesity is a major health problem through the world, especially in Western countries. Type 2 diabetes mellitus, hyperension, hyperlipidemia are comorbidities associated with obesity that cause cardiovascular disease and endothelial dysfunction. These abnormities are frequently clustered in the so called “metabolic syndrome”. Obesity and metabolic syndrome may lead directly to endothelial dysfunction and subsequently erectile dysfunction (15).

Villalba et al. reported that endothelial relaxant responses were impaired in penile arteries of obese Zucker rats (16). Enhanced superoxide production and reduced basal NO activity are the proposed underlying mechanism in this process. In human, obesity causes impaired indices of endothelial function and increases circulating concentrations of the proinflammatory cytokines interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-18 (IL-18), as well as C-reactive protein (CRP) and NO bioavailability (17). NOS expression in high-fat-fed obese rats has been found lower and restored by metformin (18). In our experiment we found e NOS and n NOS expression lower in hypercholesterolemic young and aged rats. Caloric restriction restriction restores NOS expression in both group. Reduced caloric intake decreases arterial pressure in healthy individuals and improves endothelium vasodilatation in obese and overweight individuals. In literature it is reported that caloric restriction promotes endothelium-dependent vascular relaxation by activating e NOS activity in mice through SIRT1 (19). In our experiment we found that caloric restriction increases e NOS expression in the internal iliac artery of rats. Because penile arterial supply comes from internal iliac artery we can say that caloric restriction improves penile blood supply by increasing internal artery vasodilatation. In vitro experiments are required to demonstrate the effect of caloric restriction on the endothelial relaxant response of caloric restriction in the internal iliac artery. Weight loss resulting from CR improves endothelium-dependent vascular relaxation in obese and overweight individuals with hypertension (20, 21). In this experiment, Authors show that SIRT1 promotes endothelium-dependent vasodilation by targeting endothelial nitric oxide synthetase (eNOS) for deacetylation. SIRT1 and eNOS co-localize and co-precipitate in endothelial cells, and SIRT1 deacetylates eNOS, stimulating eNOS activity and increasing endothelial nitric oxide (NO). These mechanisms may be effective in the internal iliac arteries and cavernosal endothelial cells. Further studies are needed to confirm this suggestion.

Caloric restriction improves cardiovascular system through increase of systemic NO release, increase of NO bioavailability, upregulation of sirtuin-1 as well as reducing oxidative stress in animal models. Recently, it is reported that 8 weeks 30% caloric restriction reverses vascular endothelial dysfunction in old mice by restoring NO bioavailability, reducing oxidative stress (via reduced NADPH oxidase-mediated superoxide production and stimulation of anti-oxidant enzyme activity) and upregulation of sirtuin-1 (22). In another study it was found that, CR reduce blood pressure by elevating NO production and lowering ACE activity in rats (23). Shinnura et al. reported that prolonged (6 months) CR improves myocardial ischemic tolerance and restores the ischemic preconditioning effect in middle-aged rats through nitric oxide-dependent increase in nuclear SIRT1 content (24).

Nisoll et al. report that caloric restriction for either 3 or 12 months induced endothelial nitric oxide synthase (eNOS) expression and 3’,5’-cyclic guanosine monophosphate formation in various tissues of male mice. Other Authors stated that this was accompanied by mitochondrial biogenesis, with increased oxygen consumption, adenosine triphosphate production and enhanced expression of sirtuin 1 (25). In different experiments it was shown that CR increases aortic eNOS and NO release as well as improves endothelium-dependent vasorelaxation to acetylcholine (26).

In a clinical study caloric restriction improves endothelial-dependent vasodilatation through an increased release of nitric oxide in obese hypertensive patients (27).

As a conclusion, as it shown in literature, CR improves cardiovascular system by increasing NO levels, NO bioavailability as well as decreasing ROS, proinflammatory and inflammatory cytokines. Our study is the first to demonstrate the local effect of caloric restriction in the pathophysiology of ED at molecular level. Further in vitro studies are needed to evaluate the contraction-relaxation responses of cavernous and internal iliac artery strips in CR rats. In clinical practice we think that CR improves response to phosphodiesterase-5 inhibitors in aged and young subjects. Further clinical studies are also needed to confirm this suggestion.

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