Lard and/or canola oil-rich diets induce penile morphological alterations in a rat model[1]

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ABSTRACT

PURPOSE: To investigate the effect of dietary lipid quantity and/or quality on penis morphology in adult rats.

METHODS: Thirty-eight male Wistar rats were divided into 4 groups: normal lipid diet (NL), high-fat diet rich in saturated fatty acids (HF-S), high-fat diet rich in polyunsaturated fatty acids (HF-P), and high-fat diet rich in saturated and polyunsaturated fatty acids (HF-SP). Blood samples were collected and the penises were removed for histomorphometrical and immunohistochemical analysis.

RESULTS: All high-fat diets promoted an increase in the body mass ($p<0.0001$). The HF-S and HF-SP groups presented hyperglycemia ($p=0.0060$), hyperinsulinemia ($p=0.0030$), and hypercholesterolemia ($p=0.0020$). Concerning the penis, the high-fat diets led to an increase in the collagen fibers ($p<0.0001$) and smooth muscle cell density area ($p=0.0027$), and a decline in the sinusoidal space density area ($p<0.0001$) and corpus cavernosum cell proliferation ($p=0.0003$).

CONCLUSION: Diets rich in saturated and/or polyunsaturated fatty acids promoted overweight and induced penile changes in rodent models, which may lead to the development of erectile dysfunction.

Key words: Canola oil. High-fat diet. Lard. Morphology. Penis. Rats.

Introduction

The incidences of overweight and obesity have increased in the Western world, reaching pandemic proportions. The excessive accumulation of fat is considered a chronic disease of multifactorial character, where the interaction between genetic and environmental factors such as diet and sedentary lifestyle is one of the main mechanisms responsible for its prevalence[1]. Large prospective epidemiological studies including the Framingham study[2] indicate that overweight and obesity are strongly associated with an increased incidence of death from cardiovascular diseases. Recently, erectile dysfunction (ED) has been proposed to be a good marker of the risk of cardiovascular events[3]. By definition, ED is characterized by the inability to achieve and/or maintain a penile erection sufficient for sexual intercourse. The prevalence of this disease increases with age, and can be considered a serious public health problem given that ED often coexists with other diseases, such as diabetes mellitus, hypertension, and hyperlipidemia[4].

Few studies have evaluated the role of diet on ED and more specifically in the modification of penile morphology. It is known that unhealthy diets (rich in cholesterol, for example) are associated with inflammation and the presence of cellular adhesion molecules, which causes injury to the endothelial cells by oxidative stress[5]. On the other hand, the replacement of saturated fatty acid (SFA) with polyunsaturated fatty acid (PUFA) seems to be a great alternative in the prevention of metabolic diseases and endothelial dysfunction[6], which could diminish the risk of development of ED.

Until now, there have been no reports on the effect of these types of lipids on the morphology and extracellular matrix components of the penis. Qiu *et al*.[7] was one of the first to show that high-fat diet-induced hyperlipidemia causes hyperplasia of the smooth muscle of the corpus cavernosum (CC), which could be associated with ED development. Thus, the purpose of the present study was to investigate the effect of dietary lipid quantity and/or quality on the morphology of the penis in adult rats.

Methods

All procedures were approved by the Institutional Animal Care and Use Committee of our institution (IBRAG, CEUA/027/2012).

Thirty-eight male Wistar rats were studied from 3 to 7 months of age and housed in the Urogenital Research Laboratory (Rio de Janeiro, RJ, Brazil) in a temperature-controlled room (21 ± 1ºC) with a 12 h:12 h light-dark cycle. At 3 months of age, the animals were randomized into 4 groups according the lipid content of the diet: normal lipid diet (NL; n=8), high-fat diet rich in saturated fatty acid (HF-S; lard; n=10), high-fat diet rich in polyunsaturated fatty acid (HF-P; canola oil; n=10), and high-fat diet rich in polyunsaturated and saturated fatty acids (HF-SP; n=10). The composition of the diet was detailed in a previous publication[8]; it followed the recommendations of the AIN-93M[9] and was made by PragSoluções (www.pragsolucoes.com.br)

Before the euthanasia (7 months old), all rats were fasted overnight (12 h), and approximately 5 mL of blood was drawn from the right atrium. Blood was centrifuged at room temperature for 8 min (3000 rpm) and plasma was separated and frozen in an 80ºC freezer to conduct future analyses (triacylglycerol, serum total cholesterol, serum glucose, insulin, and testosterone). Then, a bilateral thoracotomy was performed and the penises were removed for histological analysis.

The concentrations of triacylglycerol (monoreagent-K117), serum total cholesterol (monoreagent-K083), and serum glucose (monoreagent-K082) were measured by a colorimetric assay (Bioclin Systems II, Quisaba, Bioclin, Belo Horizonte, MG, Brazil). Insulin and testosterone levels were measured using the following commercially available enzyme-linked immunosorbent assay kits: rat/mouse insulin kit (Millipore - Cat. EZRMI-13 k; St Charles, MO, USA) and general testosterone kit (Uscn - Cat. E90458Ge; Wuhan, China). All samples were analyzed in duplicate with an intra-assay coefficient of variation of 1.4%.

Penile samples were fixed in 1.27 mol/L of formaldehyde in 0,1M phosphate buffer, pH 7.2 for 48 h, at room temperature. Then, the specimens were embedded in paraffin. Sections were cut at 5 µm and stained for the evaluation of tissue integrity (hematoxylin and eosin), for analysis of elastic fibers (Resorcin-fuchsin Weigert), and for determining the percentage of collagen fibers (Picro Sirius Red). For immunohistochemistry stain, tissue sections were subjected to antigen retrieval with Tris-EDTA buffer (proliferating cell nuclear antigen [PCNA]) and incubated with trypsin for 20 min at 37˚C (alpha smooth muscle actin). Endogenous peroxidase activity was blocked by incubating the slides with 3% H_2O_2 in methanol for 15 min followed by applying a protein block (10% dry milk for 10 min). After draining this solution from the tissue section, the slides were incubated for 1 h at 37ºC with mouse polyclonal primary antibodies to PCNA (1:100; Invitrogen, Carlsbad, CA, USA 13-3900) and alpha smooth muscle actin (1:50; Invitrogen, 08-0106). Next, sections were treated with a goat anti-mouse secondary antibody (1:500; Thermo Scientific, Wilmington, DE, USA 31800) for 20 min

and the reaction was amplified with a Streptavidin system (1:500; EMD Millipore Corporation, CA, USA 18-152) for 10 min, and 3,3 diaminobenzidine tetrachloride (Invitrogen, 859643) was used as the chromogen. After incubation, the sections were counterstained with Mayer's hematoxylin. Control tissue sections were obtained from the replacement of the primary antibody with 1% phosphate-buffered saline/ bovine serum albumin.

The stained tissues were observed with an Olympus BX51 optical microscope and photographed with an Olympus DP71 digital camera. Computerized histomorphometric analysis (25 images per animal) was performed using ImageJ® software (Image Processing and Analysis in Java) and ImagePro-plus® 4.5 software (Media Cybernetics). The content of smooth muscle, sinusoids, and elastic fibers in penile tissues was estimated by density area (Sv) using the test-system (grid of 100 points) on bright field images captured at a final magnification of ×400, ×400, and ×600, respectively. The Sv of proliferating cells was performed by positive cells labeling the anti-PCNA immunohistochemistry / area of the CC (mm^2) and expressed as positive cells/mm. Concerning the total area (A_T) of the penis and the CC with and without tunica albuginea, we used the freehand selection tool (ImageJ® software) at a magnification of ×200. Collagen area in the CC was measured by the colorimetric method using ImagePro-plus ® 4.5 software[10].

Data were analyzed with Prism 5 (GraphPadPrism software, San Diego, CA, USA). One-way analysis of variance followed by Bonferroni's post hoc test was used to determine the differences among the groups. The values were considered to be significantly different at $p<0.05$.

Results

Regardless of the diet lipid quality, all animals that received the high-fat diets showed an elevation in body mass. After the first week of the diet, the animals in the HF-SP group (386.00 ± 44.02 g) were heavier than those in the NL group (328.00 ± 28.22 g; p=0.0050). The HF-S (398.20 ± 36.12 g) and HF-P (401.80 ± 24.46 g) groups showed a significant increase of body mass from the second week of the diet supply in relation to the NL group (335.20 ± 29.52 g, p=0.0005). This pattern was maintained until the end of the experiment. At 7 months of age, the body mass values of the HF-S (529.30 ± 57.39 g), HF-P (546.40 ± 40.13 g), and HF-SP groups (532.90 ± 48.27 g) were 25%, 29%, and 26% higher than the body mass of the NL group (424.22 ± 40.29 g, p<0.0001), respectively.

Concerning serum biochemistry, the triacylglycerol and testosterone levels did not differ between the groups (data not shown). However, the animals from the HF-S and HF-SP groups developed hypercholesterolemia (104.80 ± 12.95 mg/dL; 105.20 ± 19.65 mg/dL), hyperinsulinemia (2.75 ± 0.45 µlU/mL; 2.85 ± 0.92 µlU/mL), and hyperglycemia (10.62 ± 2.36 mmol/L; 11.09 ± 1.65 mmol/L) at the end of the experiment. The NL and HF-P groups presented normal levels of cholesterol (80.56 ± 11.75 mg/dL; 100.10 ± 10.38 mg/dL, p=0.0020), insulin (1.49 ± 0.41 µlU/mL; 2.15 ± 0.83 µlU/mL, p=0.0030), and glucose (7.87 ± 1.61 mmol/L; 9.85 ± 1.61 mmol/L, p=0.0060).

Histomorphometrical analysis showed that the Sv of elastic fibers (in the CC), the A_T of the penis and CC (with and without tunica albuginea) did not differ between the groups (Table 1). Nevertheless, an increase of 28%, 27%, and 40% was observed in the Sv of collagen fibers in the HF-S, HF-P, and HF-SP groups when compared with the NL group (p<0.0001), respectively (Table 1). The HF-SP group presented an elevation in this parameter in comparison with HF-S and HF-P groups (Table 1)

Additionally, the Sv of smooth muscle cells was higher in the HF-S, HF-P, and HF-SP groups than in the NL group (p=0.0027), corresponding to increases of 43%, 43%, and 44%, respectively (Table 1 and Figure 1). Inversely, the Sv of sinusoids was lower in the HF-S (27%), HF-P (28%), and HF-SP (32%) groups than the NL group (p<0.0001) (Table 1 and Figure 1). Similarly, the high-fat diet groups showed a reduction on the cell proliferation in the CC in comparison with the NL group (HF-S: 35%, HF-P: 46%, HF-SP: 34%; p=0.0003) (Table 1 and Figure 1).

TABLE 1 - Histomorphometrical analysis of the penis in the experimental groups.

Data	NL		HF-S		HF-P		HF-SP		ANOVA
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	*p* value
Sv elastic fibers, %	1.61	0.18	1.62	0.27	1.54	0.34	1.68	0.32	0.7727
Sv collagen fibers, %	38.32	2.01	49.11	2.90[a]	48.65	2.26[a]	53.59	2.42[a,b,c]	<0.0001
Sv smooth muscle cells, %	11.56	2.90	16.51	2.43[a]	16.50	3.29[a]	20.51	4.96[a]	0.0027
Sv sinusoids, %	25.35	3.27	18.54	2.63[a]	18.22	1.88[a]	17.20	2.18[a]	<0.0001
Cell proliferation, µm/mm²	388.60	101.90	253.20	63.44[a]	209.40	85.69[a]	225.50	64.70[a]	0.0003
A_T pênis, mm²	7.90	1.10	7.85	0.49	7.54	0.41	8.21	0.82	0.3690
Area of Corpus cavernosum (+), mm²	5.28	0.72	4.46	0.46	5.04	0.34	5.60	0.49	0.1734
Area of Corpus cavernosum (-), mm²	2.46	0.41	2.52	0.21	2.46	0.24	2.65	0.28	0.5073

NL, normal lipid diet; *HF-S,* high-fat diet rich in saturated fatty acid (lard); *HF-P,* high-fat diet rich in polyunsaturated fatty acid (canola oil); *HF-SP,* high-fat diet rich in saturated and polyunsaturated fatty acids; Sv, area density; A_T, total area; (+) with tunica albuginea; (-) no tunica albuginea. The values are presented as the means and standard deviations (SD). The symbol [a] indicates a result that is different from the *SC* group; [b] indicates a result that is different from the *HF-S* group; [c] indicates a result that is different from the *HF-P* group (one-way ANOVA and Bonferroni's post hoc test, $p<0.05$).

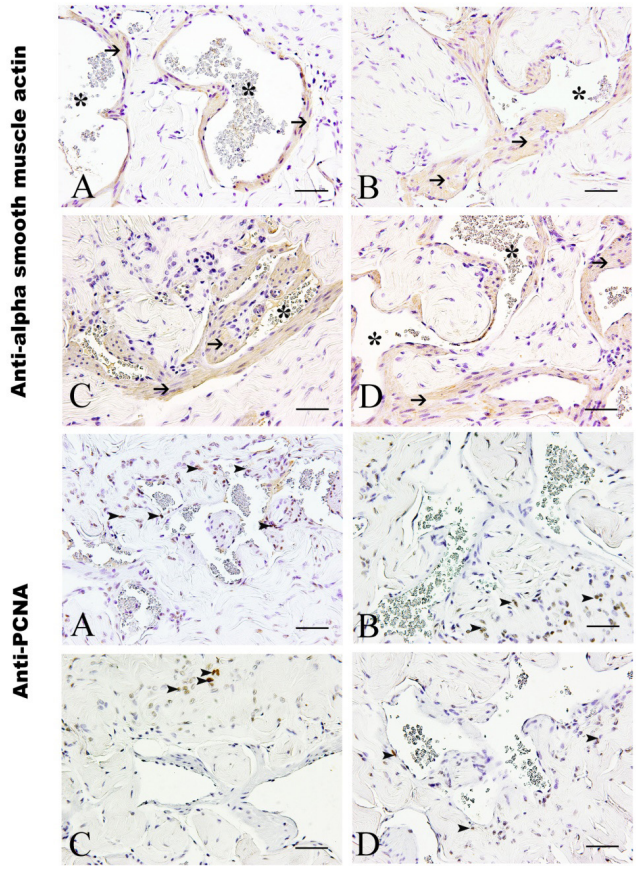

FIGURE 1 - Photomicrographs illustrating the distribution of alpha smooth muscle actin and PCNA-positive cells in the rat corpus cavernosum. (A) *NL*, normal lipid diet presented a decrease in the immunolabeling the smooth muscle cells (upper image marked with arrows) and an increase in the sinusoids (upper image marked with asterisk) and in the cell proliferation (lower image marked with arrow head) when compared with another groups (B,C and D); (B) *HF-S*, a high-fat diet rich in saturated fatty acid (lard); (C) *HF-P*, a high-fat diet rich in polyunsaturated fatty acid (canola oil); (D) *HF-SP*, a high-fat diet rich in saturated and polyunsaturated fatty acids. All images were captured at a final magnification of ×400.

Discussion

In the present study, we investigated the effect of the quantity and/or the quality of dietary lipid on the morphology of the penis in adult rats. Rats demonstrated significant morphological alterations in the penis, regardless of the quality of dietary lipid. These findings indicate that obesity itself can be considered an important factor to induce penile modifications and ED. Although we have not evaluated the functionality of the penis (this is one of the limitations of the present study) part of our results indicated that chronic poor nutrient intake may supersede many of the traditional metabolic risk factors at predicting risk of ED development in this rodent model.

Consistent with the hypothesis, obesity has been shown to adversely affect blood pressure, lipid profile, and glycemic state, which have an impact on the progression of metabolic syndrome[11]. Metabolically, even with equivalent values of triacylglycerol and testosterone, the rats that received the animal fat-rich diet (HF-S and HF-SP groups) became obese, hyperglycemic, hypercholesterolemic, and hyperinsulinemic, which increases the risk for the development of insulin resistance and atherosclerosis. On the other hand, chronic intake of canola oil, rich in PUFAs of the n-3 series (HF-P group), proved to be effective in preventing these alterations in the metabolism of lipids and carbohydrates.

Western pattern diet has been used in rodent models to investigate the biochemical characteristics and erectile function[5,7]. A positive correlation has been observed between this dietary pattern and the development of insulin resistance, type 2 diabetes mellitus, and dyslipidemia[12]. Hyperlipidemic men, especially diabetics, are predisposed to injury in endothelial cells, and are

therefore, more susceptible to less availability of nitric oxide, an essential molecule in maintaining the erectile function[6].

In contrast, a substantial body of knowledge shows that a PUFA-rich diet inhibits the increase in serum cholesterol[13] and contributes to the reduction of oxidative stress and the state of subclinical inflammation, improving the endothelial dysfunction and insulin sensitivity[14]. Thus, the presence of a dietary pattern containing nutrients with anti-inflammatory properties could protect the individual for the development of chronic and metabolic diseases[15].

Nevertheless, independent of this beneficial effect, this study is the first to demonstrate that the excess administration of SFAs and/or PUFAs encourage important penile modifications in Wistar rats. However, the mechanism through which this occurs is poorly understood. We saw that the supply of high-fat diets led to an increase in the collagen fibers and Sv of smooth muscle cells and a decline in the Sv of the sinusoidal space and CC cell proliferation. Although the diets have not promoted changes in the Sv of elastic fibers, in the penis A_T and CC A_T, we speculate that the morphological alterations mentioned previously could be considered as the first step in the development of ED.

In humans, the CC of the penis is composed of 3 types of collagen: I, III, and IV, where type I and IV are the most prevalent in this organ[16]. Collagen type I and type III are primarily located in endothelial areas in the CC, and an excess may trigger the formation of fibrosis[17]. Moreover, the augment of collagen fibers in the erectile tissue could be linked to the increase in the transforming growth factor-beta (TGF-beta-1) levels. In the penis, this protein inhibits endothelial cell proliferation and activates some genes (Smad2 and Smad3), which are important in the induction of the fibrotic process. Even though we did not evaluate this protein expression in the penis, we suggest that the excessive accumulation and collagen fiber disorganization may have been induced by TGF-beta-1. The literature suggests that the cavernous fibrosis induced by TGF-beta-1 may be able to decrease the oxygen tension, having a negative effect on the normal penile erection mechanism[18].

Additionally, we identified that PUFAs were unable to minimize and/or stabilize the effects of saturated fat in the Sv of smooth muscle fibers. All high-fat diet groups showed an increase in the percentage of smooth muscle cells in the CC, which can compromise penile functionality. In rodents, ED is associated with a decline in the smooth muscle cell content[19]. However, Qiu *et al*.[7] reported that hyperlipidemic rats presented an elevation in the content of smooth muscle cells, which confirms our results. This CC enlargement, known as smooth muscle hyperplasia, is one of the triggering factors of ED. It is noteworthy that the increase in the Sv of smooth muscle cells in the canola oil-rich diet group was independent of the development of hypercholesterolemia, showing the direct effect of PUFAs on the penis.

Another important finding in this study was the decrease in the Sv of sinusoids in the CC of the penis in the HF-S, HF-P, and HF-SP groups. Once again, the diet rich in PUFAs did not affect this parameter, although n-3 fatty acids have anti-inflammatory and vasodilatory characteristics[20]. In fact, the overweight of the animals linked with penile morphological changes may have contributed to the decrease in the expandability of sinusoids (less blood flow), resulting in veno-occlusive dysfunction and ED. It is known that hypoxia can modify the different isoforms of the nitric oxide synthase enzyme, and thus, diminish the availability of nitric oxide, an important vasodilator[6].

In light of these findings, the high-fat diet administration, independent of the lipid quality, promoted an increase of body mass and penile morphological changes in rodent models. Polyunsaturated fatty acids and/or saturated fatty acids, when administered in excess, contributed to the increase in the number of collagen fibers and smooth muscle cells, and induced a reduction of the sinusoids, which may lead to ED. Certainly, more studies are needed to confirm these results, given the scarcity of the literature on the subject.

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