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ORIGINAL ARTICLE

Energy restriction and exercise modulate angiopoietins and vascular endothelial growth factor expression in the cavernous tissue of high-fat diet-fed rats

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The purpose of the current study was to evaluate the effect of a high-fat (HF) diet, energy restriction and exercise on the expression of vascular endothelial growth factor (VEGF), angiopoietin (Ang) 1 and 2, and their receptors in rat corpus cavernosum (CC). Male Wistar rats were fed *ad libitum* with an HF diet for 8 or 16 weeks. After 8 weeks of the HF diet, a group of rats was subjected to energy restriction with or without exercise for 8 weeks. Control animals had free access to standard diet for the same period. After euthanasia, blood was collected and the penises removed for immunofluorescence assays (VEGF, VEGF receptor (VEGFR) 1 and 2, Ang1, Ang2 and Tie2) and semiquantification of VEGF, VEGFR1, VEGFR2, Ang1, Ang2, Tie2, endothelial nitric oxide synthase (eNOS) and Akt/phospho-Akt by Western blotting. HF diet-fed rats exhibited lower high-density lipoprotein cholesterol (HDL-c) levels, higher systolic blood pressure and an increased atherogenic index. A significant increase in Ang2 expression in the CC was verified and coupled to a decrease in VEGF and VEGFRs. The Akt pathway was activated by the HF diet. Energy restriction and exercise increased eNOS expression and restored most HF diet-induced modifications except for VEGFR2 expression. These results emphasize the role of diet on vascular function regulation, demonstrating that cavernous imbalance of VEGF/VEGFRs and Angs/Tie2 systems occurs before serum lipid changes and obesity onset, antedating structural atherosclerotic features.
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INTRODUCTION

Erectile dysfunction (ED) affects more than 152 million men worldwide[1] and often manifests in patients with vascular disease.[2] Moreover, ED is frequently associated with metabolic disturbances such as diabetes, atherogenic dyslipidemia and visceral obesity.[3] These disorders constitute major risk factors for endothelial dysfunction, a condition that precedes the earliest clinical manifestations of atherosclerosis.[4] Endothelial dysfunction is characterized by a deficient synthesis of nitric oxide (NO),[4] which results in impairment of endothelium-dependent smooth muscle relaxation and an inability to maintain penile erection.[5] Therefore, several authors consider endothelial dysfunction equivalent to ED.[6]

It is well established that chronic consumption of a high-fat (HF) diet strongly favours endothelial dysfunction.[7] However, the exact mechanism through which this dietary pattern contributes to the onset and progression of ED is not fully understood. Recent evidence suggests that HF diets induce secretion of pro-inflammatory molecules and cause an imbalance of expression of angiogenic factors, which together contribute to endothelial damage.[8] Furthermore, the impairment of endothelial function in HF feeding conditions decreases NO synthase activity, contributing to increase oxidative stress and decrease NO bioavailability.[9] In fact, the reduction in endothelial NO synthase (eNOS) expression is considered one of the main mechanisms that leads to endothelial dysfunction and, consequently, to atherogenesis.[10,11]

Vascular maintenance and repair in the corpus cavernosum (CC) is under the control of multiple angiogenic factors. Vascular endothelial growth factor (VEGF), a key angiogenic factor that regulates angiogenesis *in vivo*,[12] binds to the high-affinity tyrosine kinase membrane receptors, VEGF receptor (VEGFR) 1 and 2. VEGFR2 activation is responsible for most of the angiogenic properties of VEGF.[13] Furthermore, VEGF crosstalks *in vivo* with other angiogenic molecules, such as angiopoietins (Ang).[14] Ang1 and Ang2 are natural antagonists that compete for binding to their specific receptor, Tie2 (tyrosine-kinase with immunoglobulin-like loop and EGF homology domains).[14] Ang1 cooperates with VEGF in the stabilisation of vasculature, promoting interactions between endothelial cells (ECs) and mural cells, and the extracellular matrix.[14] On the other hand, Ang2, whose activity is tissue-specific, is involved in vessel growth and remodelling when sufficient VEGF is available.[15] In contrast, in the absence/inhibition of VEGF, Ang2 destabilizes pre-existing vasculature.[16]

The aim of this study was to evaluate the effect of a chronic intake of an HF diet on the expression of VEGF, angiopoietins and their

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transduction pathways in rat CC and whether it might be modified by energy restriction and exercise. As far as we know, this is the first study of VEGF/VEGFRs and Ang/Tie2 pathways in rat cavernous tissue under nutritionally defined conditions. It is our conviction that a better understanding of nutritional effects on cellular and molecular mechanisms involved in penile endothelial function and/or remodelling regulation will disclose new preventive and therapeutic approaches for ED.

MATERIALS AND METHODS

Animals

All animal procedures were undertaken according to the European Community Guidelines (86/609/EEC) and the Portuguese Act (129/92) for the use of experimental animals.

Adult male Wistar rats (8 weeks old, $n=42$) weighing 287.9±29.8 g (s.d.), obtained from the colony of the Instituto de Biologia Molecular e Celular da Universidade do Porto, were individually housed with free access to tap water and maintained under standard laboratory conditions (controlled 12-h light/dark cycle; 20–22 °C temperature; 40%–60% humidity). Animals had free access to a purified rodent diet with 45% of energy supplied by fat (#58V8 TestDiet, Purina Mills; LLC/PMI Nutrition International, Richmond, VA, USA), for 8 or 16 weeks (8- and 16-week HF diet (HFD) group, $n=18$ and $n=12$, respectively). After 8 weeks of the HF diet, groups of six randomly selected rats were subjected for an 8-week period to either energy restriction (the ER group, $n=6$) or energy restriction associated with exercise (the EREx group, $n=6$). These groups received a limited quantity of standard rat chow, corresponding to 60% energy restriction, based on daily HF-diet individual energy intake from 8 to 16 weeks. Diet portions were restored every 2 days. Exercise was carried out using a motorized rotarod (RotaRod ENV-576; MedAssociates Inc., Vermont, GA, USA), three times a week at 13.6×10^{-2} m s^{-1} for 12 min. Diet control rats had free access to a standard laboratory diet (A04; Panlab SL, Barcelona, Spain) for 8 or 16 weeks (8- and 16-week control groups, $n=6$ per group). The experimental group design and the composition of the diets are shown in **Figure 1** and **Table 1**, respectively.

Body weight (BW) and *ad libitum* food intake were monitored weekly. The mean weekly energy intake (kJ week^{-1}) was calculated as follows: food intake (g)×energy value of diet (kJ g^{-1}). Plasma glucose concentrations were determined monthly using a glucose

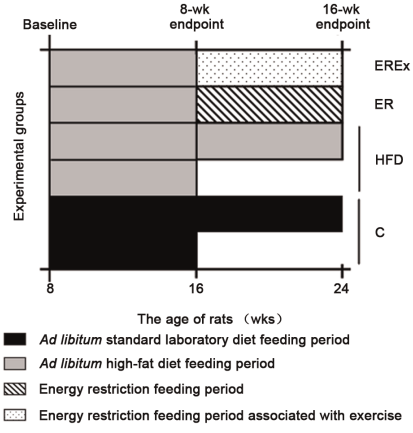

Figure 1 The time course of experimental procedures for the animal groups studied. C, control animal group; ER, energy-restricted animal group; EREx, energy-restricted and exercised animal group; HFD, high-fat-diet fed animal group.

Table 1 Major macronutrient constituents and total energy of the high-fat and standard laboratory diets

	High-fat diet	*Standard diet*
Protein (g 100 g^{-1}) (%)[a]	21.3 (18.3)	14.6 (20.2)
Carbohydrate (g 100 g^{-1}) (%)[a]	41.9 (36.0)	55.1 (76.0)
Lipid (g 100 g^{-1}) (%)[a]	23.6 (45.7)	1.2 (3.8)
Fiber (g 100 g^{-1})[a]	5.8	3.9
Total energy (kJ g^{-1})[b]	19.5	12.1

[a] Percentage of energy content as protein, carbohydrate and fat (w/w).
[b] Total energy (kJ g^{-1}): sum of decimal fractions of protein, carbohydrate and lipid, calculated on the basis of 16.7 kJ g^{-1} protein and carbohydrates and 37.7 kJ g^{-1} lipid.

analyser (OneTouch Ultra; Lifescan Inc., Milpitas, CA, USA). Systolic (SBP) and diastolic blood pressures (DBP) were measured using the tail-cuff method in conscious rats (LE5008-05PL; Panlab S.I., Barcelona, Spain) three times. Measurements were taken 10–15 min after acclimatisation and under restraining conditions 2 days before euthanasia.

Rats were decapitated and the trunk blood was collected in heparinized tubes. Plasma fractions were separated by centrifugation at 1000 *g* for 30 min at 4 °C and maintained at −80 °C until analysis. Plasma testosterone and insulin levels were determined using commercially available RIA kits (Testo-RIA-CT; Biosource Europe S.A., Nivelles, Belgium, and Sensitive Rat Insulin RIA Kit #SRI-13K; Millipore Co., Billerica, MA, USA, respectively). Plasma total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c) and triglyceride (TG) concentrations were determined using enzymatic colorimetric tests in an auto-analyser (Cobas Mira Plus; HORIBA-ABX Diagnostic, Shefford, UK) employing commercial kits (Cholesterol, HDL Cholesterol, Triglycerides Direct; HORIBA-ABX Diagnostics, Shefford, UK). Atherogenic index (AI) was calculated for all animals according to the formula TC×(HDL-c)$^{-1}$.

The penises were dissected from skin and surrounding fat and excised. Each penis was divided into two fragments. One was immediately stored at −80 °C for molecular analysis, and the other was fixed in 10% buffered formaldehyde and embedded in paraffin oriented along its transversal axis. Five-micron-thick sections were cut in a microtome (RM2145; Leica Microsystems GmbH, Wetzlar, Germany) and placed on 0.1% poly-*L*-lysine-covered microscopy slides for immunofluorescence studies.

Immunofluorescence

Dual-immunolabelling of VEGF/VEGFR1, VEGF/VEGFR2, Ang1/Tie2 and Ang2/Tie2 was performed. Briefly, sections were deparaffinized, rehydrated, exposed to 1 mol l^{-1} HCl for epitope retrieval and neutralized with 0.1 mol l^{-1} borax, followed by incubation for 1 h with blocking solution (1% bovine serum albumin in phosphate-buffered saline). Sections were incubated overnight at 4 °C with a mixture of primary antibodies: goat anti-VEGF (R&D Systems, Minneapolis, MN, USA) with rabbit anti-VEGFR1 (Lab Vision Corporation, Fremont, CA, USA) or with mouse anti-VEGFR2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); and rabbit anti-Tie2 with goat anti-Ang1 or with goat anti-Ang2 (Santa Cruz Biotechnology). Then the slides were incubated with appropriated secondary antibodies: anti-goat conjugated with Alexa 568 (red) with anti-rabbit or anti-mouse both conjugated with Alexa 488 (green) (Molecular Probes, Leiden, The Netherlands). Negative controls were performed by primary antibodies omission. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Molecular Probes), for

5 min. Sections were mounted in a buffered glycerol solution and observed in an Apotome fluorescence microscope (Imager.Z1; Carl Zeiss MicroImaging GmbH, Göttingen, Germany). Images were captured with an Axiocam MRm camera and digitalized using the AxionVision software (Carl Zeiss MicroImaging GmbH).

Western blotting

For protein analysis, penile fragments were mechanically homogenized in 50 mmol l^{-1} Tris pH 7.2, 0.1 mol l^{-1} NaCl, 5 mmol l^{-1} EDTA, 0.5% (v/v) Triton X-100, and 2% (v/v) Protease Inhibitor Cocktail P8340 (Sigma-Aldrich Co., Hertfordshire, UK) and sonicated for 15 cycles (30 s ON/OFF) with the Bioruptor (Diagenode, Liège, Belgium). After spectrophotometric quantification of total protein, 40 μg of protein from each sample was loaded into an 8% or 12% SDS–PAGE. Electrophoresis-separated peptides were transferred to a nitrocellulose membrane with a pore size of 0.45 μm (BioRad Laboratories, Hercules, CA, USA). Membranes were incubated with blocking solution (5% non-fat dried milk Molico; Nestlé Portugal S.A., Linda-a-Velha, Portugal, with 0.1% Tween-20 in Tris-buffered saline) and immunoreacted overnight with monoclonal mouse anti-VEGF (R&D Systems), goat anti-VEGFR1, mouse anti-VEGFR2, goat anti-Ang1, goat anti-Ang2, rabbit anti-Tie2, rabbit anti-eNOS (Santa Cruz Biotechnology), rabbit anti-Akt (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-phospho-Akt (Cell Signaling Technology) or rabbit anti-β-actin (Abcam, Cambridge, UK). After extensive washing and incubation with the appropriate secondary antibody coupled to horseradish peroxidase, labelled bands were visualized using chemiluminescent substrate (Kit SuperSignal; Pierce Biotechnology, Rockford, IL, USA). Results were quantified by densitometry using the ScionImage software (Scion Corporation, National Institutes of Health, MN, USA) and represent the variation in the mean value and s.d. of the density units of bands for each studied protein and treatment group (HFD, ER and EREx) compared with age-matched controls (C group). The total levels of VEGF, VEGFR1, VEGFR2, Ang1, Ang2, Tie2, eNOS and total-Akt were normalized to β-actin, which was used as the loading control. Phospho-Akt was normalized to the semiquantified total-Akt in each sample. Each experiment was repeated three times and performed in four samples of each experimental group.

Statistical analysis

Results are expressed as mean values with s.d. Data obtained from biometrical, serum biochemical tests and semiquantification of proteins were statistically analysed using one-way analysis of variance (ANOVA), followed by Bonferroni multiple comparison test, or unpaired *t*-test when appropriate. Statistical analysis was performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA). A probability value of $P<0.05$ was considered statistically significant.

RESULTS

Animal weight, energy intake, blood pressure and biochemical results

All the studied animals presented a linear increase in BW during the study (**Table 2**). Despite the diet and nutritional differences, HFD animals did not show significant differences in final BW compared to control animals after 8 ($P=0.574$) or 16 weeks of an HF diet ($P=0.819$). By contrast, energy restriction decreased the BW gain of ER and EREx rats ($P=0.028$ and $P=0.028$, respectively).

The weekly measured food intake of HFD animals was lower than that observed for C groups ($P=0.017$ and $P<0.001$, for 8 and 16 weeks, respectively). However, because an HF diet has a higher energy value than the standard diet (19.5 *vs.* 12.1 kJ g^{-1}), the total energy intake of the HFD animals was greater than that observed for rats that were exclusively fed the standard diet ($P=0.012$ and $P<0.001$, for 8 and 16 weeks, respectively).

Table 2 Mean values of final and body weight gain, food and energy intake, systolic and diastolic blood pressures, and biochemical results (serum concentrations of glucose, testosterone, insulin, total cholesterol, high-density lipoprotein cholesterol, atherogenic index and triglycerides) (values are expressed as mean±s.d.)

	8 weeks		*16 weeks*			
	C group	*HFD group*	*C group*	*HFD group*	*ER group*	*EREx group*
Final BW (g)	522.3±32.3	537.2±53.6	632.7±50.9	625.3±68.9	512.0±44.2[c,d]	543.3±29.4[c,d]
BW gain (g)	250.0±27.9	237.3±27.7	345.0±39.2	356.3±39.1	201.7±35.3[c,d]	236.3±22.5[c,d]
Food intake (g $week^{-1}$)	183.1±7.8	156.5±10.8[a]	189.8±7.3	138.7±7.3[b]	135.3±27.4*	145.8±6.4*
Energy intake (kJ $week^{-1}$)	2223.6±74.9	3047.6±212.4[a]	2304.0±104.1	2700.4±145.1[b]	1644.2±333.4*	1770.2±64.7*
SBP (mmHg)	125.3±4.4	103.0±9.1	129.5±1.2	134.7±2.4[b]	129.0±6.1[e]	111.7±5.6[c]
DBP (mmHg)	75.8±5.6	84.0±4.7	80.5±1.2	73.0±12.1	76.7±2.2	76.5±2.9
Glycaemia (mg dl^{-1})	135.3±10.3	136.8±16.2	144.8±13.9	138.8±7.6	130.7±18.1	134.7±18.1
Testosterone (ng ml^{-1})	1.8±0.6	4.9±3.5	1.0±0.4	1.9±0.9	1.2±0.6	0.8±0.3
Insulin (ng ml^{-1})	1.4±0.1	1.5±0.1	1.5±0.1	1.4±0.1	1.3±0.0[d]	1.3±0.2[d]
TC (mg dl^{-1})	101.8±18.1	91.2±6.7	101.1±23.5	112.6±20.5	95.7±8.4	98.8±14.8
HDL-c (mg dl^{-1})	31.4±6.0	28.3±2.3	33.8±5.2	29.9±2.6[b]	28.5±1.2	29.0±2.8
AI	3.3±0.7	3.3±0.5	3.0±0.2	3.8±0.7[b]	3.4±0.2	3.4±0.2
TG (mg dl^{-1})	257.0±54.8	165.7±43.9[a]	282.3±91.4	183.1±55.2[b]	285.0±94.2	225.2±77.0

Abbreviations: AI, atherogenic index, calculated according to the formula: TC×(HDL-c)−1; BW, body weight; C, control animal group; TC, total cholesterol; DBP, diastolic blood pressure; ER, energy-restricted animal group; EREx, energy-restricted and exercised animal group; HDL-c, high-density lipoprotein cholesterol; HFD, high-fat-diet-fed animal group; SBP, systolic blood pressure; TG, triglycerides.

*Energy restriction after 8 weeks of the HF diet.

[a] $P<0.05$ compared with 8-week C rats.

[b] $P<0.05$ compared with 16-week C rats.

[c] $P<0.05$ compared with 16-week HFD rats.

[d] $P<0.05$ between ER and 16-week C rats.

[e] $P<0.05$ compared with EREx rats.

Blood pressure and biochemical results obtained for all the experimental groups are also summarized in **Table 2**. With regard to blood pressure, EREx rats had lower SBP levels than 16-week HFD ($P<0.001$) or ER ($P=0.002$) rats. No significant differences were found between the EREx and 16-week C group ($P=0.074$) or between ER and 16-week HFD rats ($P=0.095$). DBP values remained constant.

Glycaemia levels did not vary between the experimental groups. Likewise, testosterone mean values did not vary between the groups, mostly because of the elevated inter-individual variation. Eight-week HFD rats displayed the highest testosterone levels (4.9 ± 3.5 ng ml^{-1}), which dropped to 1.9 ± 0.9 ng ml^{-1} during the experiment. This trend was also observed in C animals. The rats submitted to energy restriction (ER and EREx) exhibited the lowest concentrations of insulin in the blood, lower than for rats observed after 16 weeks of the standard diet ($P=0.028$ and $P=0.046$, respectively). We also observed that insulinemia increased in 8-week HFD rats when compared with respective controls, a condition that reversed in each group after 16 weeks of treatment; nevertheless, none of these variations was significant.

The plasma levels of TC, HDL-c and TG were also quantified and the AI was calculated. Cholesterolemia levels did not vary between groups ($P=0.210$). Also, HDL-c levels showed no differences between the ER and EREx groups ($P=0.753$) or between the ER and EREx groups and the HFD or C groups. Conversely, HFD groups exhibited lower HDL-c levels than the C groups; however, this was observed only after 16 weeks of HF-diet ingestion ($P=0.046$). Sixteen-week HFD rats displayed the maximum AI of all the experimental groups, particularly when compared with age-matched C rats ($P=0.004$). Plasma TG levels were unexpectedly high in all experimental groups, with the lowest levels in HFD animals.

Simultaneous immunofluorescence detection of VEGF and Angs with receptors

Dual immunolabelling of VEGF (red) and VEGFR1 (green) showed colocalisation (yellow) in the smooth muscle cell (SMC) of the CC of all experimental groups (**Figure 2**). Nevertheless, VEGF and VEGFR1 expression were less intense in the 16-week HFD group. A low intensity of this receptor was also found in 8-week HFD animals. VEGF and VEGFR2 (green) coexpression was detected in the EC (yellow), with reduced intensity for VEGFR2 in HFD, ER and EREx groups (**Figure 3**).

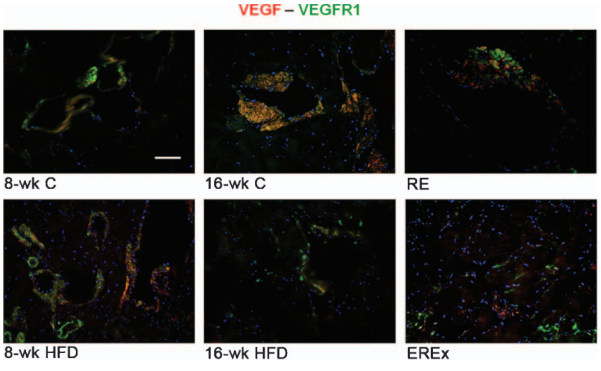

Figure 2 Dual immunofluorescence labelling of VEGF (red)/VEGFR1 (green) in the rat cavernous tissue of all experimental groups. DAPI (nuclear staining), blue; C, control animal group; ER, energy-restricted animal group; EREx, energy-restricted and exercised animal group; HFD, high-fat fed animal group. Scale bar=100 μm. DAPI, 4′,6-diamidino-2-phenylindole; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

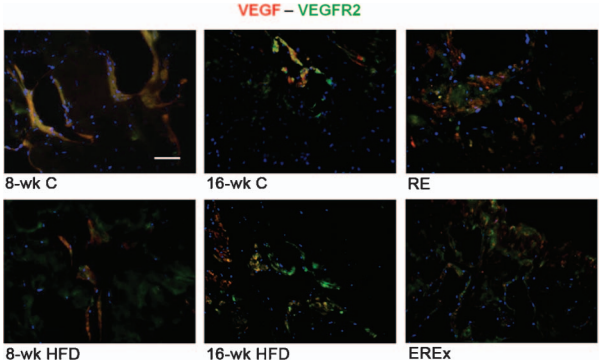

Figure 3 Dual immunofluorescence labelling of VEGF (red)/VEGFR2 (green) in the rat cavernous tissue of all experimental groups. DAPI (nuclear staining), blue; C, control animal group; ER, energy-restricted animal group; EREx, energy-restricted and exercised animal group; HFD, high-fat-diet-fed animal group. Scale bar =100 μm. DAPI, 4′,6-diamidino-2-phenylindole; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

Dual immunofluorescent labelling detection of Ang1 (red) and Tie2 (green) revealed a confined expression of both in the EC, but the two proteins were not colocalized (**Figure 4**). A sparse labelling of Ang1 in the SMC was also observed. Very little Ang2 (red) was observed in all groups studied, except for the HFD groups, which presented evident endothelial expression (**Figure 5**).

Semiquantification of VEGF, VEGFRs, Angs, Tie2, eNOS, phospho- and total-Akt, by Western blotting

Figure 6 shows representative Western blotting bands of VEGF, VEGFR1, VEGFR2, Ang1, Ang2 and Tie2 detected in the CC of all the experimental groups (semiquantitative analysis was detailed in the graphs). The expression of the studied proteins in the 8-week HFD animals and the 16-week HFD, ER and EREx groups is indicated as a percentage, with 100% representing the 8- and 16-week control groups for the respective groups. VEGF (**Figure 6a**) appeared as a single band of 25 kDa, and no differences were detected between experimental groups, with the exception of 16-week HFD rats, which exhibited a decrease in VEGF (70%, $P=0.017$). Regarding VEGF receptors, 180 kDa bands were observed for both VEGFR1 (**Figure 6b**) and VEGFR2 (**Figure 6c**). A marked decrease in VEGFR1 expression was detected in HFD groups (45%, $P=0.019$

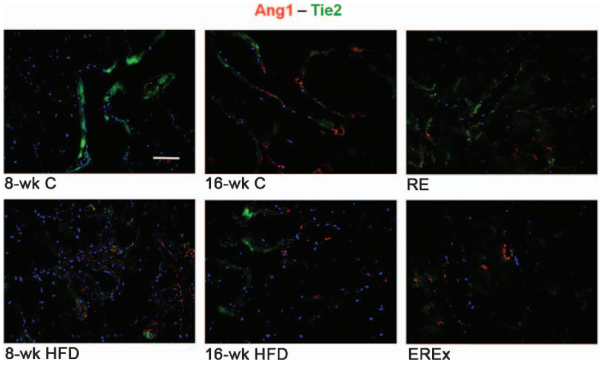

Figure 4 Dual immunofluorescence labelling of Ang1 (red)/Tie2 (green) in the rat cavernous tissue of all experimental groups. DAPI (nuclear staining), blue; C, control animal group; ER, energy-restricted animal group; EREx, energy-restricted and exercised animal group; HFD, high-fat-diet fed animal group. Scale bar =100 μm. Ang, angiopoietin; DAPI, 4′,6-diamidino-2-phenylindole.

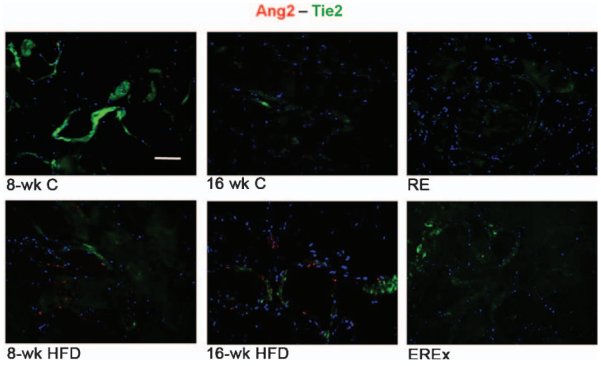

Figure 5 Dual immunofluorescence labelling of Ang2 (red)/Tie2 (green) in the rat cavernous tissue of all experimental groups. DAPI (nuclear staining), blue; C, control animal group; ER, energy-restricted animal group; EREx, energy-restricted and exercised animal group; HFD, high-fat-diet-fed animal group. Scale bar =100 μm. Ang, angiopoietin; DAPI, 4′,6-diamidino-2-phenylindole.

and 60%, $P=0.005$, for 8- and 16-week HFD rats, respectively). VEGFR1 expression did not vary among the ER, EREx and C groups. Nevertheless, ER rats revealed an increased in VEGFR1 expression compared to the 16-week HFD group ($P=0.017$). A reduction in VEGFR2 expression was also detected in HFD animals (77%, $P=0.019$ and 87%, $P=0.054$, for the 8- and 16-week HFD rats, respectively); this was maintained after energy restriction or exercise.

Ang1 (60 kDa) expression did not vary in the CC of HFD rats compared to controls (**Figure 6d**). However, taking Ang1 expression of 16-week HFD animals as 100%, energy restriction solely or in combination with ER led to an increase in Ang1 expression (182%, $P=0.039$ and 154%, $P=0.042$, for ER and EREx rats, respectively). Conversely, Ang2 (66 kDa) (**Figure 6e**) expression increased in HFD groups (204% for 8-week, $P=0.026$ and 300% for 16-week HFD rats, $P=0.043$) that persisted after energy restriction (204% in ER, $P=0.018$ and 240% in EREx, $P=0.030$). Compared with the 16-week HFD group (100%), ER and EREx Ang2 levels were diminished (80%, $P=0.017$ and 81%, $P=0.035$, respectively). No alterations were observed in Tie2 protein expression (140 kDa) despite the observed increase of this receptor in all treated groups (**Figure 6f**).

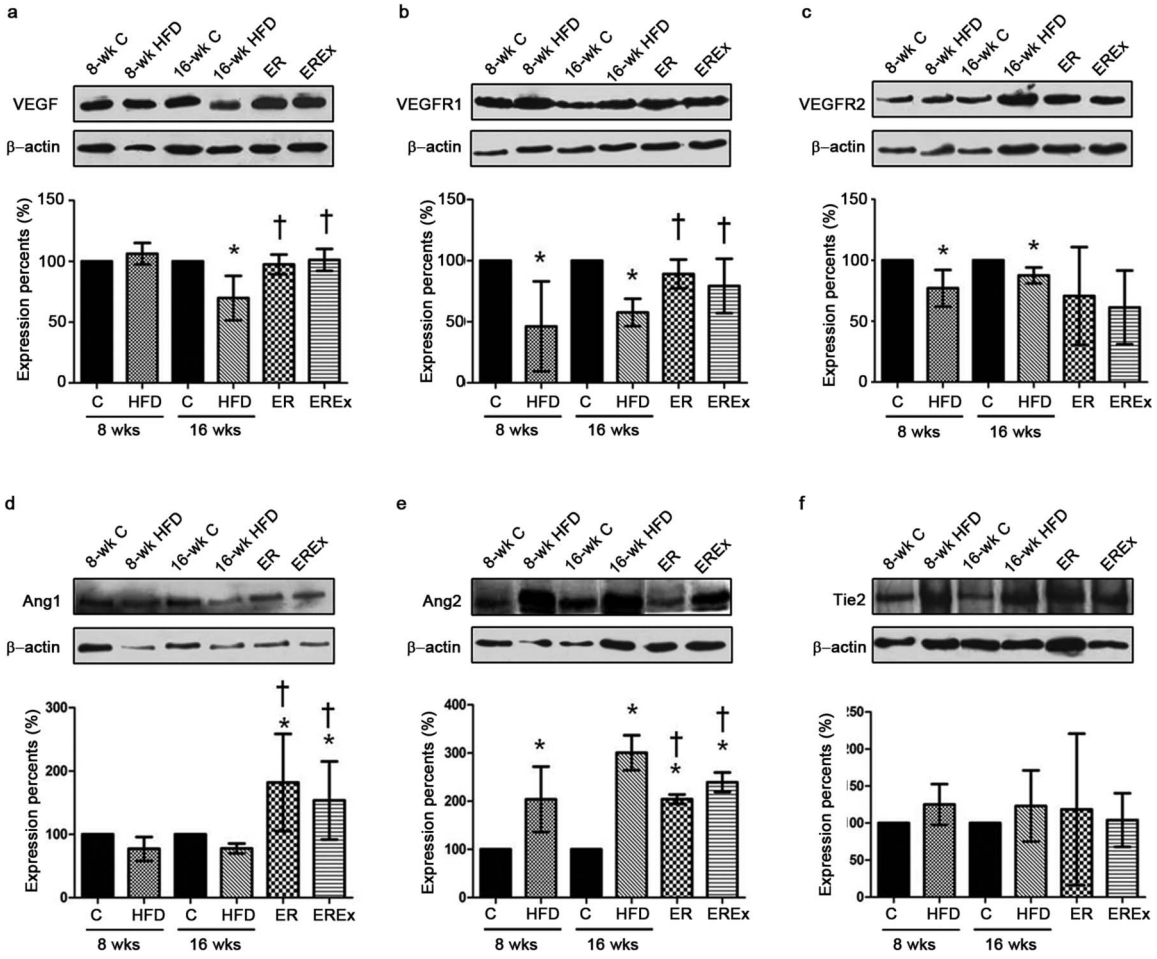

Figure 6 Western blot analysis of VEGF, VEGFR1, VEGFR2, Ang1, Ang2 and Tie2. Representative bands obtained by Western blot analysis of VEGF (**a**), VEGFR1 (**b**), VEGFR2 (**c**), Ang1 (**d**), Ang2 (**e**) and Tie2 (**f**), in rat cavernous tissue of all experimental groups. The graphs below represent the semiquantitative analysis of the aforementioned proteins ($n=4$ for each group), calculated by the fraction of pixels presented by each band relative to β-actin band used as the loading control. Error bars represent standard deviation. C, control animal group; ER, energy-restricted animal group; EREx, energy-restricted and exercised animal group; HFD, high-fat diet-fed animal group. *$P<0.05$ between HFD and the corresponding 8- and 16-week control groups (black bars); †$P<0.05$ between ER or EREx, and 16-week HFD group; *†$P<0.05$ between ER or EREx, and the 16-week control and HFD groups. Ang, angiopoietin; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

Regarding eNOS expression (140 kDa) (**Figure 7a**), the semiquantitative analysis (detailed in the graph) demonstrated no differences between the HFD and C groups: 93% (P=0.409) and 89% (P=0.856), respectively, for 8- and 16-week HFD rats. However, an increase was exhibited by both ER (134%, P=0.048) and EREx (125%, P=0.033) groups compared to the 16-week C and HFD groups (P=0.025 and P=0.042, for ER and EREx, respectively).

Total Akt (56 kDa) expression did not exhibit statistical differences among experimental groups (P=0.984) (**Figure 7b**), in contrast to phospho-Akt (**Figure 7c**). An increase in phospho-Akt was verified in 16-week HFD rats (279%, P<0.001) but was not evident after 8 wks of the HF diet (84%, P=0.031). Despite the decrease in phospho-Akt observed in the ER and EREx groups (P=0.007 and P=0.001, respectively) compared to the 16-week HFD group, it remained higher than that observed in the 16-week controls (145%, P=0.229 and 125%, P=0.068, respectively).

DISCUSSION

The present experiments demonstrate that consumption of an HF diet early in life, before marked changes in blood lipids occur, leads to an increased AI and SBP and abnormal expression of angiogenic factors in rat CC. We also confirmed that such changes are reversed by energy restriction and exercise, strongly suggesting that endothelial function in the CC is modulated by dietary composition and lifestyle.

Endothelial dysfunction, which has been recognized as a marker of early atherosclerosis,[17] is the leading cause of cardiovascular disease and vasculogenic ED.[18,19] Penile erection is a vascular process that is dependent on appropriate blood perfusion of the small vessels of the penis; any functional and structural changes in cavernous tissue, particularly atherosclerotic occlusion, could manifest as ED.[18,19]

Several factors, including HF diet and sedentariness, contribute to the progression of endothelial dysfunction. Unquestionably, prolonged consumption of an HF diet, commonly associated with low physical activity, results in endothelial dysfunction,[20,21] a condition that reverses upon ending the HF diet.[22] However, the mechanism behind this effect is largely unknown.

HF foods are characterized primarily by high-energy density and high palatability, which favours its consumption.[22] In the present study, despite the higher-energy intake, the BW gain of HF diet-fed rats did not differ from that observed for control animals. However, this finding does not preclude the development of endothelial dysfunction, which can be induced by voluntary ingestion of a highly palatable HF diet[20] independently of weight gain.[23] Thus, our experimental model constitutes a valuable tool for the study of HF diet-induced vascular changes in CC.

In order to study how a change in lifestyle might benefit animals on an HF diet, we submitted the rats to energy restriction solely or in combination with an 8-week exercise program. It is known that reduced energy intake and enhanced physical activity at a relatively low-intensity level of training lead to improvement in endothelial function, which enhances sexual performance;[24–26] by contrast, an intense exercise regimen results in genotoxic, apoptotic and unwanted cardiovascular effects,[27] which argued against its use in the current research. Both ER and EREx regimens resulted in minimal BW variations, suggesting an adaptation to the reduced quantity of available food. The finding agrees with those of other authors who showed that homeostatic mechanisms triggered in the rat restored the energy balance after low-energy diet or exercise periods.[28]

Regarding blood biochemical analysis, consumption of an HF diet did not increase glycaemia or insulinemia, a likely result of a long-term compensatory effect. In fact, studies have previously demonstrated a progressive normalisation of insulinemia after a transitory hyperinsulinemic period induced by HF diet consumption.[29,30] Although no differences were observed in TC levels between groups, the HFD animals showed low levels of HDL-c, which favours endothelial dysfunction[31] and directly increases the risk of ED.[32] Unexpectedly, the ER animals, which had previously been on the HF diet, did not have varying HDL-c levels compared to the HFD group. A similar result was found in the EREx group, suggesting that 8 weeks of low- to moderate-intensity exercise did not markedly affect their HDL-c metabolism, which is consistent with previous reports.[33] TG blood concentrations were evaluated under non-fasting conditions, justifying the high plasma TG levels found in ER, EREx and C animals. Compared to HFD animals, the increased triglyceridemia of the ER, EREx and C animals might be attributed to the high-carbohydrate content of standard diet (76.0%), as low-fat/high-carbohydrate diets

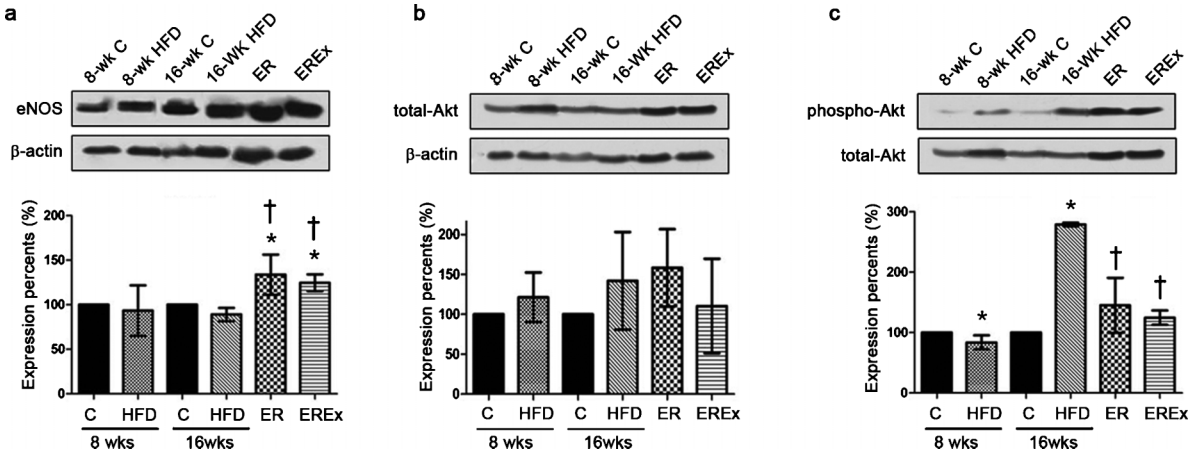

Figure 7 Western blot analysis of total eNOS, phospho-Akt and total-Akt. Representative bands obtained by Western blot analysis of total eNOS (**a**), total-Akt (**b**) and phospho-Akt (**c**) in the rat cavernous tissue of all experimental groups. The graphs below represent the semiquantitative analysis of the aforementioned proteins (n=4 for each group). The total eNOS and total-Akt were normalized to β-actin, used as the loading control. Phospho-Akt levels were normalized to semiquantified total-Akt in each sample. Error bars represent standard deviation. C, control animal group; ER, energy-restricted animal group; EREx, energy-restricted and exercised animal group; HFD, high-fat diet-fed animal group. *P<0.05 between HFD animals and the corresponding 8- and 16-week control groups (black bars); †P<0.05 between ER or EREx, and 16-week HFD; *†P<0.05 between ER or EREx and 16-week control and HFD groups. eNOS, endothelial nitric oxide synthase.

led to an increase in TG levels.[34] Conversely, after 16 weeks of an HF diet, rats presented high levels of AI associated with significantly elevated values of SBP, relatively to the 16-week controls. Corroborating previous reports,[35] SBP decreased with exercise.

Overall, the concomitant AI and SBP increases observed in animals on 16 weeks of an HF diet favour progression of endothelial dysfunction. This finding is supported by other reports that showed development of endothelial dysfunction prior to the onset of obesity and/or insulin resistance[20,21] and by a previous study with a similar design that demonstrated increased perivascular lipid deposition and SMC layer thickness, major contributors to atherosclerotic disease development in CC.[36]

HF diets lead to an increase in oxidative stress, endothelial early injury and decreased availability of NO, an eNOS-generated anti-atherosclerotic molecule of paramount importance for endothelial integrity maintenance and erectile function.[37] In the current investigation, HFD animals did not evidence a change in eNOS expression, compared to controls, but when they were submitted to ER and/or exercise, an increase in eNOS expression was noted. This finding, which was similar to results for other tissues,[38] provides evidence that diet and exercise can modulate CC endothelial function. Moreover, although it was different from controls, eNOS expression in the ER-plus-exercise group was not different from that in the ER-alone group. It suggests that, similarly to regular physical activity, which exerts an independent upregulatory effect on genetic expression of eNOS,[33,39] ER independently has a beneficial effect on the CC endothelial function.

Endothelial dysfunction obligatorily precedes atherosclerosis of the major penile arteries.[4] Therefore, therapies aimed at neovascularisation for the recovery of CC function have recently been proposed,[40–41] although with limited applicability and success.

Angiogenesis is a highly regulated mechanism of new blood vessel formation from pre-existing ones, physiological vascular repair, regeneration and remodelling.[13] As in other tissues, the cavernous vessels depend on angiogenic factors that paracrinally regulate EC and vascular smooth muscle cell (VSMC) proliferation and migration.[42] In effect, changes in the expression of angiogenic factors were found in the CC of animal models of type 2 diabetes with increased risk of ED.[43] Here, we hypothesized that consumption of an HF diet modifies vascular growth factors expression in the rat CC too. In this setting, the observation of VEGF expression in EC and perivascular VSMC in all groups' CC emphasizes its autocrine/paracrine intervention.[12] It was further supported by VEGF/VEGFR1 and VEGF/VEGFR2 colocalisation in the VSMC and EC, respectively, corroborating previous studies in rat,[44] and human.[45,46] Interestingly, 16-week HFD rats presented a marked decrease in cavernous VEGF, VEGFR1 and VEGFR2 expression, as evidenced by Western blotting assays. By contrast, energy restriction or exercise substantially reverted the VEGF and VEGFR1 decrease, but was unable to restore levels of VEGFR2, suggesting that VEGFR1 expression is more responsive to metabolic conditions. Effectively, VEGFR1 or VEGFR2 had both been identified as putative targets for controlling the progression of atherosclerosis.[47] However, further molecular studies will be necessary to clarify the role of VEGFR1 in the angiogenic mechanisms of the CC. Reduction in VEGFR2 expression limits the pro-angiogenic effects of VEGF[13] and strongly compromises endothelial NO production,[43] which supports the hypothesis that HFD rats are at increased risk for ED progression. Despite our results suggesting that consumption of an HF diet for a mere 8 weeks can definitively compromise the CC endothelial mechanisms of response to VEGF, the increased eNOS expression in rats subjected to energy restriction after HF diet consumption led us to hypothesize that VEGF modulation of NO production does not depend directly on VEGFR2.

In the present study, Ang1 and receptor Tie2 were predominant in the EC of the rats' CC. Interestingly, no coexpression was evident, which could be explained by Ang1 entrapment in the extracellular matrix, while Tie-2 localizes at the plasma membrane.[14] Conversely, Ang2 was detected only in the EC of the HFD animals, which expresses a higher level of this protein. Ang2 accumulates in the Weibel–Palade bodies in EC cytoplasm and is released only after stimulation,[48] which hinders its immunodetection, as previously observed in human CC.[45] On the other hand, Western blotting assays demonstrated that either energy restriction or exercise led to increased levels of Ang1 expression, which results in endothelium protection owing to its anti-inflammatory and anti-atherosclerotic properties.[49] These two groups of animals also exhibit a reduction of Ang2 expression, highly increased in HFD rats. Ang2 is expressed mainly at sites of vascular remodelling,[14] in association with active angiogenesis, but it promotes vascular destabilisation when VEGF is inhibited or absent.[16] Taken together, these results indicate that an HF diet favours endothelial molecular changes: downregulation of VEGF and VEGFR2 coupled to upregulation of Ang2, which possibly contributes to local loss of vascular stabilisation. By contrast, energy restriction seems to have an important role in reversing these conditions.

We also analysed the downstream Akt signalling pathway activation in the present animal model. Akt could be activated by either VEGFR2 or Tie2,[50] and its phosphorylation stimulates the expression and activity of eNOS, consequently increasing NO availability.[51] Despite the concomitant increase observed for eNOS expression and Akt activation in ER animals, we found a significant increase in phospho-Akt in HFD rats that was not accompanied by an increase in eNOS protein expression. Considering that Ang2 and phospho-Akt levels varied equally in our experimental groups, and that Akt is a major signalling pathway activated by Tie2 receptor, we hypothesize that Ang2 is involved in the Tie2-dependent Akt activation in the CC. Supporting that, recent reports have shown that Ang2 can act as a Tie2 agonist *in vitro*.[48] Nevertheless, considering that Akt is a signalling pathway that mediates erectile function,[51] apoptosis inhibition[52] and angiogenesis promotion,[53] further studies will be necessary to clarify the exact role of Akt activation in the CC of rats consuming an HF diet and rats undergoing energy restriction.

Overall, these results emphasize the role of dietary modulation of vascular function and support the point that reduction in consumption of dietary fat is beneficial for endothelial integrity, which is fundamental for early prevention of vasculogenic ED. However, measurements of endothelial function and intracavernous pressure for erectile functional evaluation will be necessary to elucidate whether low-fat diets are useful prophylactic tools in the prevention of vascular events that contribute to ED.

AUTHOR CONTRIBUTIONS

IT, NT, HA and DN contributed substantially to the conception and design of the study; IT, HA and DN contributed to acquisition of data. All authors participated in the analysis and interpretation of data and the drafting and revision of the manuscript for intellectual content, and all have read and approved the final manuscript.

COMPETING FINANCIAL INTERESTS

The authors have no financial or commercial interests related to the study.

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