Male Health

ORIGINAL ARTICLE

Effects of natural mineral-rich water consumption on the expression of sirtuin 1 and angiogenic factors in the erectile tissue of rats with fructose-induced metabolic syndrome

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Consuming a high-fructose diet induces metabolic syndrome (MS)-like features, including endothelial dysfunction. Erectile dysfunction is an early manifestation of endothelial dysfunction and systemic vascular disease. Because mineral deficiency intensifies the deleterious effects of fructose consumption and mineral ingestion is protective against MS, we aimed to characterize the effects of 8 weeks of natural mineral-rich water consumption on the structural organization and expression of vascular growth factors and receptors on the *corpus cavernosum* (CC) in 10% fructose-fed Sprague–Dawley rats (FRUCT). Differences were not observed in the organization of the CC either on the expression of vascular endothelial growth factor (VEGF) or the components of the angiopoietins/Tie2 system. However, opposing expression patterns were observed for VEGF receptors (an increase and a decrease for VEGFR1 and VEGFR2, respectively) in FRUCT animals, with these patterns being strengthened by mineral-rich water ingestion. Mineral-rich water ingestion (FRUCTMIN) increased the proportion of smooth muscle cells compared with FRUCT rats and induced an upregulatory tendency of sirtuin 1 expression compared with the control and FRUCT groups. Western blot results were consistent with the dual immunofluorescence evaluation. Plasma oxidized low-density lipoprotein and plasma testosterone levels were similar among the experimental groups, although a tendency for an increase in the former was observed in the FRUCTMIN group. The mineral-rich water-treated rats presented changes similar to those observed in rats treated with MS-protective polyphenol-rich beverages or subjected to energy restriction, which led us to hypothesize that the effects of mineral-rich water consumption may be more vast than those directly observed in this study.
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INTRODUCTION

Metabolic syndrome (MS) represents a cluster of conditions (hyperglycemia/insulin resistance/type 2 diabetes, hypertension, hypertriglyceridemia, low high-density lipoprotein cholesterol and abdominal obesity) that identifies subjects at high risk for cardiovascular (CV) diseases.[1,2] The exact etiology of MS remains unclear; nevertheless, genetic, metabolic and environmental factors, including diet composition, which strictly modulates metabolic status,[3–5] contribute to the onset of MS.

Rodent experimental models and human studies have demonstrated that fructose consumption may induce metabolic changes similar to those observed in MS.[4,6-9; Pereira *et al.* full manuscript under review.]* In addition, the deficiency of potassium, calcium and magnesium, which is quite frequent in MS-inducing diets, apparently strengthens the deleterious effects of fructose consumption and, conversely, mineral ingestion protects against MS and/or MS manifestations.[3,5,10–14] The effects of

fructose intake on rat metabolic, hormonal and redox status markers were also partially prevented by co-ingestion of natural mineral-rich water.[6,7,15; Pereira *et al.* full manuscript under review.]* This novel evidence was not unexpected because the beneficial effects of natural mineral-rich water ingestion on isolated parameters included in the MS definition have been reported previously.[16–18]

Natural mineral water that originates underground is protected from chemical and microbiological contamination and is characterized by highly bioavailable mineral content.[19–21] Although the exact effects of natural mineral-rich water on the metabolic status of organisms are far from being clarified, there is a strong belief that natural mineral-rich water presents antioxidant properties,[22,23] exerting protection against reactive oxygen species (ROS).[24]

ROS production is highly increased in MS,[25] which induces oxidative molecular modifications and accelerates the degradation of nitric oxide (NO) in tissues.[26] A decrease of NO bioavailability in the

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endothelium is particularly deleterious because it seriously compromises vasodilatation capability and leads to endothelial dysfunction, which always precedes atherosclerosis formation, the underlying pathology of CV disease. Endothelial dysfunction is thus highly favored in MS patients,[27] but hardly identified in the organs.[28] An early manifestation of endothelial dysfunction is erectile dysfunction (ED).[29,30] Endothelial nitric oxide synthase (eNOS) produces most of the NO during an erection and, although eNOS activity is regulated primarily by Akt-mediated phosphorylation, recent evidence suggests that deacetylation catalyzed by the NAD⁺-dependent deacetylase sirtuin 1 (Sirt1)[31] also contributes to eNOS activation. Sirt1 belongs to the family of mammalian homologues of silent information regulator-2 (Sir2) of the yeast *Saccharomyces cerevisiae* and intervenes in the modulation of the cell cycle, senescence, apoptosis, metabolism and vascular function by distinct mechanisms.[32,33] Although previous reports associate nutritional patterns, in particular energy restriction (ER), with Sirt1 expression and activity in the vascular system,[34] the effect of nutritional pattern on eNOS activity in the erectile tissue of the penis remains to be elucidated.

In addition to directly promoting endothelium-dependent vasodilatation, NO indirectly mediates vascular endothelial growth factor (VEGF)-induced angiogenesis,[35–37] which is fundamentally necessary to maintain the vascular integrity of the cavernous tissue of both rat and human origin.[38–40] VEGF is the primary angiogenic growth factor and essential to endothelial cell survival. VEGF binds specifically to tyrosine kinase membrane receptors VEGFR1, or fms-like tyrosine kinase-1 receptor (Flt-1), and VEGFR2, or kinase insert domain-containing receptor (KDR/Flk-1).[41] Other angiogenic factors, such as angiopoietins, modulate VEGF effects *in vivo*.[42–44] Angiopoietin-1 (Ang1) promotes vascular maturation by intervening in the stabilization of nascent capillaries. In contrast, angiopoietin-2 (Ang2) may destabilize vasculature in the absence of VEGF,[45] but in the presence of VEGF, which potently stimulates Ang2 mRNA expression and release,[46] endothelial cell migration and proliferation is promoted.[47] Ang1 and Ang2 compete to bind to the endothelial-specific tyrosine kinase with immunoglobulin-like loop and EGF homology domains (Tie2) receptor, which is tyrosine-phosphorylated in angiogenic and quiescent adult tissues, suggesting a dual function in vascular growth and maintenance. Our previous studies demonstrated that long-term consumption of beverages rich in antioxidants modifies the expression of vascular growth factors and receptors in the cavernous tissue of the rat, which could prevent atherosclerosis progression.[48,49]

Considering this evidence, we aimed (i) to characterize the effects of a fructose-rich diet on the expression of vascular growth factors and receptors in the *corpus cavernosum* (CC) of Sprague–Dawley rats (predominantly acquired MS model)[8] and (ii) to verify if the consumption of hypersaline sodium-rich naturally sparkling mineral water could counteract the effects of fructose, which, to the best of our knowledge, has not been reported.

MATERIALS AND METHODS

Chemicals/reagents
All chemical substances used were of analytical grade.

The natural mineral-rich water was kindly provided by Unicer Bebidas, S.A. (Leça do Balio, Matosinhos, Portugal) and presents a total mineralization content of 2855 mg l⁻¹, is primarily rich in sodium and bicarbonate, and contains a higher potassium, calcium and magnesium content than tap water (**Table 1**).

For the morphometric analysis of the CC, an anti-α-actin primary monoclonal antibody was purchased from Chemicon, Millipore

Table 1: Chemical characteristics of tap and natural mineral-rich waters

Characteristics	Tap water	Hypersaline sodium-rich naturally sparkling mineral water-Pedras Salgadas®
Total mineralization (mg l⁻¹)	148-151	2855
pH	6.5-9.0	6.16
Sodium (mg l⁻¹)	200	591
Calcium (mg l⁻¹)	30.5-40.2	92.5
Magnesium (mg l⁻¹)	3.6-9.2	26.2
Potassium (mg l⁻¹)	2.6	29.9
Copper (mg l⁻¹)	2	0.0013
Zinc (µg l⁻¹)	a	1.1
Selenium (µg l⁻¹)	10	<2.0
Bicarbonate (mg l⁻¹)	a	2013
Chloride (mg l⁻¹)	250	30.8
Sulfate (mg l⁻¹)	250	6.4

a: no need for control (Portuguese Act 306/2007, from 27 August)

Corporation (Billerica, MA, USA) and correspondent biotinylated secondary antibody and streptavidin-horseradish peroxidase (HRP) complex were purchased from Vectastain, Vector Laboratories Inc. (Burlingame, CA, USA). The same anti-α-actin primary antibody was used for the dual immunofluorescence labeling.

For the dual immunofluorescence labeling, antiplatelet endothelial cell adhesion molecule 1 (PECAM-1; endothelial cell marker), anti-Sirt1, anti-VEGFR2, anti-Tie2, anti-Ang1 and anti-Ang2 primary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA), and anti-VEGF and anti-VEGFR1 primary antibodies were purchased from R&D Systems (Minneapolis, MN, USA) and Lab Vision Corporation (Fremont, CA, USA), respectively. The secondary antibodies and 4'-6-diamino-2-phenylindole (DAPI) were purchased from Molecular Probes (Leiden, Netherlands).

For the Western blot analysis, some of the antibodies used in immunofluorescence were employed, anti-Sirt1 and anti-β-actin were acquired from ProteinTech (Chicago, IL, USA) and Abcam (Cambridge, UK), respectively. Although the anti-VEGF and anti-VEGFR2 antibodies used for the immunofluorescence assay and Western blot analysis were from the same commercial brand, the anti-VEGF and anti-VEGFR2 antibodies for the Western blot analysis were produced in different species (goat *vs* mouse and rabbit *vs* mouse, respectively). Nitrocellulose Hybond C-extra membrane was purchased from Amersham, GE Healthcare (Buckinghamshire, UK) and the non-fat dry powdered milk Molico® was produced by Nestlé, Portugal, S.A. (Linda-a-Velha, Portugal). SuperSignal West Pico chemiluminescent substrate was purchased from Pierce Biotechnology (Rockford, IL, USA). The Sirt1 and corresponding β-actin detection substrate was acquired from GE Healthcare, Amersham (Buckinghamshire, UK). The bicinchoninic acid protein assay kit was purchased from Pierce (Rockford, IL, USA).

Animals and treatments
Sprague–Dawley CD rats (*n* = 21, male, 388–483 g) were purchased from Charles River Laboratories (Chatillon/Chalaronne, France). The rats were housed individually in an enriched environment and were maintained on 12 h: 12 h light: dark schedule (20–22°C) with free access to standard laboratory pellet food (2014 Teklad Global 14% Protein Rodent Maintenance Diet from Harlan Interfauna Iberica, S.A., Barcelona, Spain) and tap water. The animal experimental protocol was authorized by the Veterinary National Department of the Portuguese Ministry of Agriculture, Rural Development and Fisheries. The handling and care of the animals were conducted in conformity

with the European Community Council Guidelines for the use of experimental animals (86/609/EEC) and Portuguese Act 129/92.

After 10 days of acclimatization in the local animal facility, the animals were divided randomly into 3 groups ($n = 7$) and maintained for 8 weeks with free access to the above mentioned chow diet and to the following different drinking solutions: (i) tap water (CONT), (ii) 10% fructose in tap water (FRUCT) or (iii) 10% fructose in a hypersaline sodium-rich naturally sparkling mineral water (FRUCTMIN; Pedras Salgadas®). The FRUCTMIN group was pretreated with the natural mineral-rich water for 3 weeks (while the other rats drank tap water) to allow the rats to adjust to the water flavor and sparkles. At the end of the treatment, all of the animals were anesthetized deeply with sodium pentobarbital (80 mg kg^{-1} body weight, intraperitoneal) and blood was collected from the left ventricle into heparinized syringes. After a transcardial perfusion with an ice-cold isotonic saline solution, the penis was dissected from the skin and the surrounding fat, excised and divided in two fragments: one fragment was immediately fixed in 10% buffered formaldehyde and the other fragment was frozen at -80 °C until the molecular analysis.

Computer-assisted morphometric analysis of the corpus cavernosum

Penis fragments were fixed for 24 h and were embedded (oriented along the transversal axis) in paraffin. Sections (5 μm thick) were cut with a Leica RM2145 microtome (Leica Microsystems GmbH, Wetzlar, Germany) and placed onto 0.1% poly-*L*-lysine coated microscopy slides for immunodetection of α-actin, a smooth muscle cell (SMC)-specific marker. The sections were deparaffinized, hydrated, treated with 3% H_2O_2 in methanol to block endogenous peroxidase activity, exposed to 1 mol l^{-1} HCl for 30 min (for epitope retrieval) and neutralized with borax for 5 min. Then, all of the slides were incubated overnight with a mouse monoclonal anti-α-actin primary antibody. After a 30-min incubation with biotinylated secondary antibody and streptavidin-HRP complex, the sections were reacted with 3,3'-diaminobenzidine tetrahydrochloride/H_2O_2 and counterstained with hematoxylin. All of the stained slides were observed and the images were captured using an optical microscope connected to a digital camera (Carl Zeiss MicroImaging GmbH, Göttingen, Germany). To assess the proportion of the smooth muscle (SM) to total erectile tissue area by pixel classification (red for SM), the images were subjected to computer-assisted color histomorphometric analysis employing ImageJ® software (NIH, Bethesda, Maryland, USA). The areas belonging to tissues other than the CC were excluded from the analysis. The ratio of SM area to total tissue area was calculated in four randomly selected cases per group.

Dual immunofluorescence labeling of α-actin/PECAM-1, α-actin/ Sirt1, VEGF/VEGFR1, VEGF/VEGFR2, Ang1/Tie2 and Ang2/Tie2

The immunofluorescence detection of α-actin/PECAM-1, α-actin/ Sirt1, VEGF/VEGFR1, VEGF/VEGFR2, Ang1/Tie2 and Ang2/Tie2 was performed in CC sections that had been previously deparaffinized, hydrated and exposed to 1 mol l^{-1} HCl for 30 min (for epitope retrieval) as described above. The tissue sections were incubated overnight with the appropriate mix of primary antibodies (mouse anti-α-actin and goat anti-PECAM-1 or rabbit anti-Sirt1, goat anti-VEGF with rabbit anti-VEGFR1 or rabbit anti-VEGFR2 and rabbit anti-Tie2 with goat anti-Ang1 or goat anti-Ang2) followed by a suitable mix of secondary antibodies (Alexa™ 568 (red)-conjugated anti-goat with Alexa™ 488 (green)-conjugated anti-rabbit or Alexa™ 488-conjugated anti-mouse or Alexa™ 488-conjugated anti-rabbit with Alexa™ 568-conjugated anti-mouse) and then observed using an Apotome

microscope (Zeiss, Göttingen, Germany). The nuclei counterstaining was achieved by incubating the sections in DAPI for 5 min at room temperature and then visualized at 350 nm (blue). The AxionVision 3.0 program (Zeiss System) was used for acquiring the images.

Semiquantitative Western blot analysis of Sirt1, VEGF, VEGFR1, VEGFR2, Ang1, Ang2 and Tie2

For the protein analysis, the CC fragments were mechanically homogenized in 50 mmol l^{-1} Tris-HCl pH 7.2, 0.1 mol l^{-1} NaCl, 5 mmol l^{-1} ethylenediaminetetraacetic acid and 0.5% TritonX-100 supplemented with 2% protease inhibitor cocktail P8340 and 0.2% phosphatase inhibitor cocktails 1 and 2 (P2850 and P5726, respectively, from Sigma-Aldrich Co (Dorset, UK)) and sonicated for 15 cycles (30 s on/off) with a Bioruptor (Liège, Belgium). The total protein levels were determined using a bicinchoninic acid assay. After denaturation (65 °C for 30 min with gentle agitation) and centrifugation (16 438 g, 5 min), 40 μg of each sample was loaded per lane and separated using 8% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), to analyze the expression of VEGF receptors, Tie2 and angiopoietins or Sirt1 and VEGF, respectively, using a discontinuous buffer system[50] and transferred to a nitrocellulose membrane for 1 h. The membranes were incubated in blocking solution (5% non-fat dry powdered milk Molico® in Tris-buffered saline (10 mmol l^{-1} Trizma-base and 150 mmol l^{-1} NaCl) with 0.1% Tween-20 (TBST)) for 1 h at room temperature, and the membranes were immunoreacted with the following primary antibodies: rabbit anti-Sirt1, mouse monoclonal anti-VEGF, rabbit anti-VEGFR1, mouse anti-VEGFR2, goat anti-Ang1, goat anti-Ang2, rabbit anti-Tie2 and mouse monoclonal anti-β-actin. All of the antibodies (except Sirt1) were diluted in 5% non-fat dry powdered milk Molico® in TBST and immunoreacted for 48 h at 4 °C with gentle shaking. Sirt1 was diluted in 5% bovine serum albumin in TBST and immunoreacted for 96 h. After washing extensively with TBST, the membranes were incubated with the appropriate HRP-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 h at room temperature with gentle agitation. The immunoreacted bands were visualized using a chemiluminescent substrate. The total protein in each band and the corresponding loading control were quantified by densitometry using the Visionworks LS software UVP (Upland, CA, USA). Sirt1 and the corresponding loading control were quantified with ImageLab® software (version 4.0.1; Bio-Rad Laboratories, Hercules, CA, USA). For the normalization of the studied proteins, β-actin was used as the loading control. The mean values of the studied proteins in each experimental group are shown after the quantification of each protein in all of the CC samples.

Quantification of plasma oxidized low-density lipoprotein (oxLDL) and plasma testosterone

oxLDL levels were evaluated using an ELISA kit (CSB-E07932r) purchased from Cusabio Biotech (Wuhan, China), and the total testosterone levels were determined using a commercial kit (Testosterone II, #05200067) from Roche Diagnostics GmbH that employed an electrochemiluminescent immunoassay using a Cobas® e411 automated analyzer (Roche Diagnostics GmbH, Mannheim, Germany).

Statistical analysis

The values are expressed as the crude means ± standard error of the mean (GraphPad Prism® software version 6.00; La Jolla, CA, USA). To achieve a significance level of 5% and a power of 80%, if the difference between groups is 5/3 of the standard deviation value, seven animals for each group would be the required sample size. The outcomes

were evaluated using analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test (IBM SPSS Statistics® software version 20.0; Armonk, NY, USA). For these outcomes, we used the analysis of covariance (ANCOVA) statistical tests to control the results for weight. A value of $P < 0.05$ was considered significant, after body weight adjustments.

RESULTS

Periendothelial SMC density of cavernous tissue

After the immunohistochemical staining of α-actin in SMC, the proportion of the labeled area to the total cavernous tissue area was evaluated using a morphometric assay. Rodent CC SM fibers are restricted to the endothelium periphery.[48,49] Here, the SM fiber relative area did not vary in the CONT rats compared with the FRUCT (11.3% ± 1.1% *vs* 8.3% ± 1.2%, respectively, $P = 0.261$) or the FRUCTMIN rats (14.7% ± 1.2%, $P = 0.313$). However, the proportion of cavernous SM significantly increased in the rats treated with natural mineral-rich water compared with the FRUCT rats ($P = 0.010$, global $P = 0.011$).

Dual immunofluorescence labeling of α-actin/PECAM-1, α-actin/Sirt1, VEGF/VEGFR1, VEGF/VEGFR2, Ang1/Tie2 and Ang2/Tie2

The organization of the cavernous tissue of the rats in each of the experimental groups was observed after dual immunofluorescence labeling of the endothelium and SMC with antibodies that bind PECAM-1 and α-actin, respectively. No differences were noted among the groups; the endothelium lined the vascular lumen and the muscle layer was restricted to the endothelium periphery (**Figure 1a–1c**).

Sirt1 expression was detected in the endothelium and in the SMC, which was verified by the simultaneous detection of Sirt1 and α-actin. A thin green line, representing Sirt1 labeling, was evident in the endothelium in close proximity to the SMC staining (yellow). Sirt1 labeling was more intense in the FRUCTMIN treated animals (**Figure 1d–1f**).

VEGF expression was visualized in the SMC of the rats in each experimental group and co-localized with VEGFR1 (**Figure 1g–1i**), which presented a lower intensity in the control animals (**Figure 1g**). In contrast, VEGFR2 was restricted to the endothelium (**Figure 1j–1l**). An apparent decrease in VEGFR2 expression was observed in the FRUCTMIN rats (**Figure 1l**).

Regarding the angiopoietins/Tie2 system, Tie2 expression was restricted to the endothelium without changes among the experimental groups (**Figure 1m–1r**). Ang1 was expressed slightly in the periendothelial SMC (**Figure 1m–1o**) and Ang2 was expressed in the endothelium without co-localizing with Tie2 (**Figure 1p–1r**). These observations agree with previous findings in the CC of rats under different experimental conditions.[49] In agreement with the absence of differences in Tie2 labeling among the groups, no apparent differences were observed in angiopoietin expression.

The specificity of each of the primary antibodies was confirmed by the absence of staining in the negative controls.

Semiquantitative Western blot analysis of Sirt1

Sirt1 protein expression in the cavernous tissue was not altered in rats that ingested fructose-containing tap water compared with the control rats. However, Sirt1 expression in the FRUCTMIN group demonstrated a strong increasing trend compared with both the CONT and FRUCT groups (global $P = 0.045$, with $P = 0.101$ for FRUCTMIN *vs* CONT and $P = 0.068$ for FRUCTMIN *vs* FRUCT) (**Figure 2**).
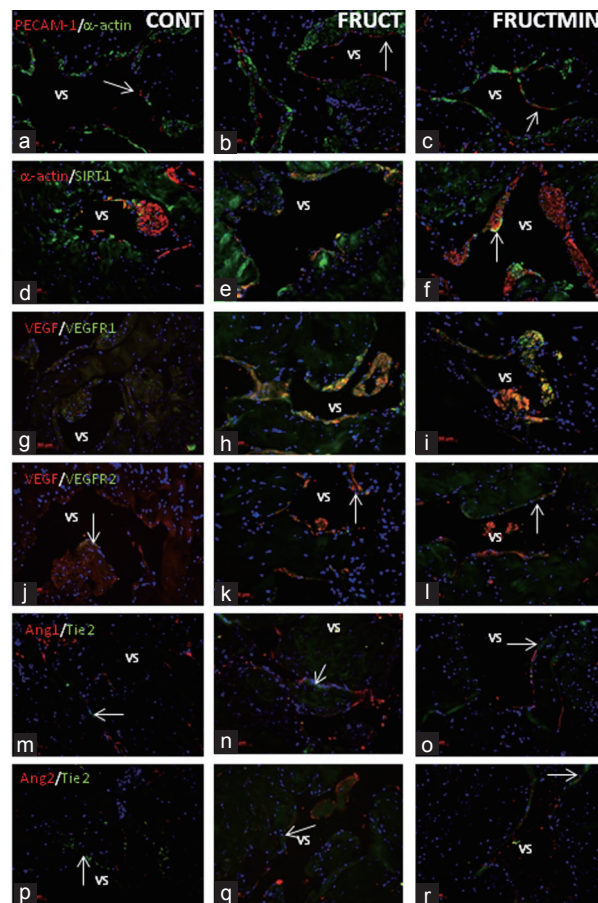
Figure 1: Dual immunofluorescence labeling of PECAM-1 (red) and α-actin (green), specific markers of the endothelium (Et) and smooth muscle cells (SMC) in the *corpus cavernosum* (**a-c**), respectively. Et (arrow) lines the vascular spaces (VS) and SMC form a layer surrounding Et. No differences in the cellular organization of the tissue were observed among the experimental groups. Detection of Sirt1 (green) expression was observed in the Et, as a thin line, and in SMC, where Sirt1 co-localized with α-actin (red) resulting in yellow labeling in all of the experimental groups (**d-f**). Sirt1 labeling was more intense in the FRUCTMIN (**f**) animals (arrow). VEGF (red) expression was visualized in the SMC of all experimental groups (**g-i**) and co-localized with VEGFR1 (green) (**g-i**), which resulted in yellow labeling, with lower intensity in the control animals (**g**). In contrast, VEGFR2 (green) was restricted to the Et (**j-l**) (arrow), exhibiting reduced labeling in the FRUCTMIN rats (**l**). Tie2 expression (green) was detected with low intensity in the Et without changes among experimental groups (**m-r**) (arrow). No apparent differences were observed either in the detection of angiopoietins (red). Ang1 was expressed slightly in the periendothelial SMC (**m-o**) and Ang2 was detected in the endothelium (**p-r**). The nucleus was stained blue (DAPI). Ang: angiopoietin; CONT: control; DAPI: 4'-6-diamino-2-phenylindole; FRUCT: 10% fructose in tap water; FRUCTMIN: 10% fructose in natural mineral-rich water; VEGF: vascular endothelial growth factor; VEGFR: VEGF receptor; Sirt1: sirtuin 1.

Semiquantitative Western blot analysis of VEGF, VEGFR1 and VEGFR2

VEGF protein expression was not altered among the three groups of rats (global $P = 0.711$).

However, VEGFR1 and VEGFR2 demonstrated opposite expression pattern tendencies according to the experimental treatment. VEGFR1 and VEGFR2 tended to increase and to decrease, respectively, in the rats that ingested the fructose-containing tap water compared with the controls. The natural mineral-rich water co-ingestion strengthened the fructose effect on the expression

of both receptors; however, the effect did not reach statistical significance (global $P = 0.198$ for VEGFR1 and global $P = 0.285$ for VEGFR2) (**Figure 3a–3c**).

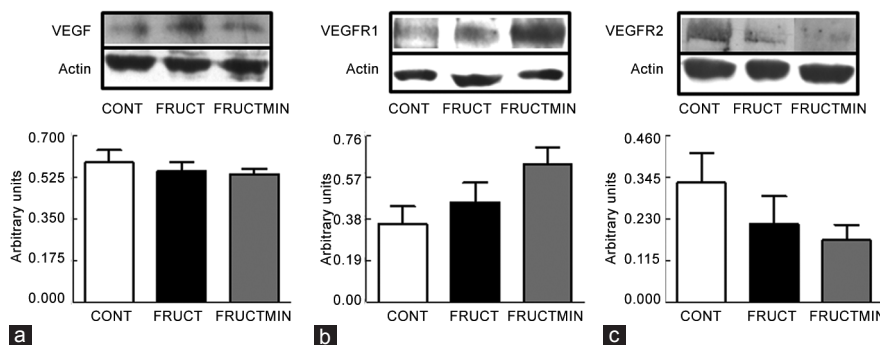### Semiquantitative Western blot analysis of Ang1, Ang2 and Tie2

Neither the fructose nor the natural mineral-rich water treatment modified the expression of Ang1 (global $P = 0.578$), Ang2 (global $P = 0.744$) and their receptor Tie2 (global $P = 0.931$) compared with the control group (**Figure 4a–4c**).

Quantification of plasma oxLDL and plasma testosterone

The oxLDL level in the rats that ingested fructose-containing natural mineral-rich water tended to increase slightly: 4.4 ± 0.5, 5.0 ± 1.2 and 6.5 ± 2.7 pg ml^{-1} for the CONT, FRUCT and FRUCTMIN groups, respectively (global $P = 0.805$). Neither the fructose nor the natural mineral-rich water treatment modified the total testosterone levels: 2.7 ± 0.7, 2.5 ± 0.7 and 2.4 ± 0.3 pg ml^{-1} for the CONT, FRUCT and FRUCTMIN groups, respectively (global $P = 0.703$).
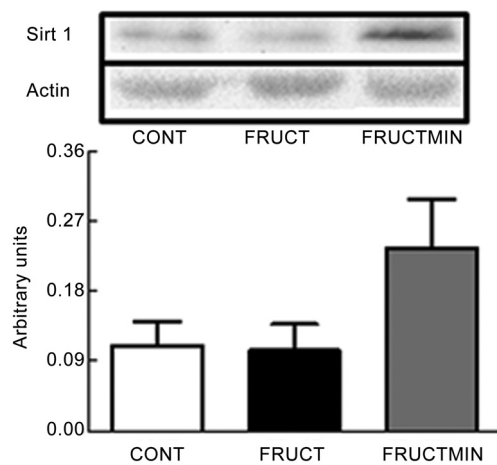
Figure 2: Representative bands obtained by semiquantitative Western blot analysis of Sirt1 in the rat cavernous tissue homogenates of the experimental groups. CONT: control; FRUCT: 10% fructose in tap water; FRUCTMIN: 10% fructose in natural mineral-rich water; Sirt1: sirtuin 1. The graph indicates, for each experimental group, the mean values of the Sirt1 bands in arbitrary units after normalization with the respective β-actin density. Error bars represent the standard error of the mean.
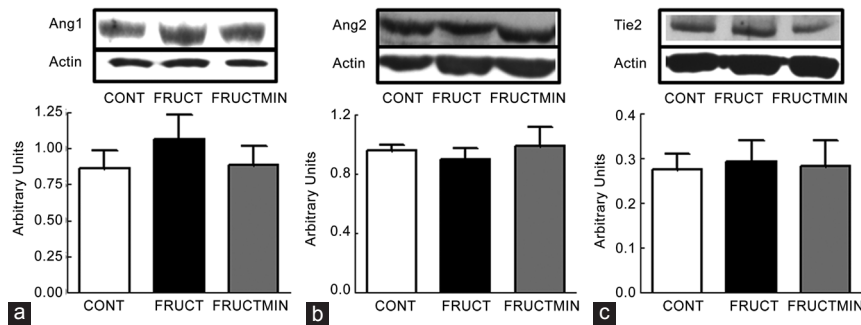
DISCUSSION

MS is a highly prevalent condition that affects more than 20% of the population in the United States. Nevertheless, a decrease was observed during the last decade due to the use of therapies for MS manifestations.[51] In addition to medicinal products, nutritional patterns, mostly through the increased ingestion of plant-derived polyphenols, have emerged as an alternative or complementary intervention for the amelioration of MS.[52,53] Polyphenols are natural antioxidant molecules that are present in food and produce recognized beneficial effects that counteract the increase in oxidative stress, which is strongly associated with MS,[25,52,53] intervene in the prevention of CV diseases, significantly reduce mortality that is associated with CV diseases[54] and ameliorate erectile function.[55,56]

ED, defined as the inability to develop and maintain an erection for satisfactory sexual intercourse or activity, is an early manifestation of systemic CV disease. Among the possible causes, endothelial dysfunction is considered the main contributor to ED onset.[29,30]

Our previous studies in rodent models demonstrated that long-term (6 months) ingestion of beverages naturally rich in polyphenols, such as green tea or red wine, prevents the cavernous deposition of lipids[48] and strongly downregulates the expression of VEGF and VEGFR2,[48,49] which is the main effector of VEGF-induced mechanisms.[41] Although uncertain, the decrease in components of the VEGF/VEGFR2 system could affect the survival of the endothelial cells and the overall angiogenic capacity of the cavernous tissue.[48,49] Red wine-treated animals exhibit a compensatory significant increase in the expression of components of the angiopoietins/Tie2 system.[49] Additional differences were observed in the effects induced by the treatments with these beverages, as green tea[48] but not red wine[49] consumption strongly decreases the blood levels of total testosterone. Although we reported no noticeable cavernous structural modifications in green tea-treated rats,[48] other studies have demonstrated an association between hypogonadism and a decrease in cavernous NO synthesis,[57] an increase in ED prevalence in MS patients[58] and a shift in the cellular proportion in cavernous tissue similar to natural aging.[59] In fact, androgen chemical deprivation of young men induces a decrease in SM content in cavernous tissue coupled with an increase in connective tissue content, which is a pattern similar to that observed in chronological aging. During the aging process, a physiological decay in total testosterone levels occurs.[60] However, despite the relative advantage of red wine *vs* green tea consumption, ethanol imparts toxicity on several organs and systems[61,62] when ingested regularly

Figure 3: Representative bands obtained by semiquantitative Western blot analysis of VEGF (**a**), VEGFR1 (**b**) and VEGFR2 (**c**) in the rat cavernous tissue homogenates of the experimental groups. CONT: control; FRUCT: 10% fructose in tap water; FRUCTMIN: 10% fructose in natural mineral-rich water; VEGF: vascular endothelial growth factor; VEGFR: VEGF receptor. The graph indicates, for each experimental group, the mean values of the VEGF, VEGFR1 and VEGFR2 bands in arbitrary units after normalization with the respective β-actin density. Error bars represent the standard error of the mean.

Figure 4: Representative bands obtained by semiquantitative Western blot analysis of Ang1 (**a**), Ang2 (**b**) and Tie2 (**c**) in the rat cavernous tissue homogenates of the experimental groups. Ang: angiopoietin; CONT: control; FRUCT: 10% fructose in tap water; FRUCTMIN: 10% fructose in natural mineral-rich water; Tie2, endothelial-specifi c tyrosine kinase with immunoglobulin-like loop and EGF homology domains receptor. The graph indicates, for each experimental group, the mean values of the Ang1, Ang2 and Tie2 bands in arbitrary units after normalization with the respective β-actin density. Error bars represent the standard error of the mean.

and for long periods,[63] which should be considered when addressing erectile function.

Due to their antioxidant properties, natural mineral-rich waters[22,23] have emerged as a healthy alternative to the consumption of green tea or red wine for the protection of the cavernous tissue in conditions that induce the onset of MS. To clarify if the regular ingestion of hypersaline sodium-rich naturally sparkling mineral water could be beneficial for the maintenance of the expression of vascular growth factors in the cavernous tissue, we employed a rodent model of fructose-induced MS.[6–8,15] Fructose-induced MS compromises endothelial function through the decrease of eNOS expression coupled with an increase in oxidative stress and inducible NOS expression, as demonstrated in rat aortas.[64,65] Inducible NOS is expressed under inflammatory conditions and strongly compromises normal erectile function.[66]

In agreement with our previous findings in animals treated with green tea or red wine, natural mineral-rich water ingestion did not affect the cellular distribution of angiogenic growth factors and their receptors in the CC, as observed by immunofluorescence. In addition, VEGFR2 expression tended to decrease and VEGFR1 expression tended to increase. We speculate that because the VEGFR2 downregulation tendency could not be enough to interfere in the normal angiogenic processes in the cavernous tissue and because of the short extent of the treatment (8 weeks), no compensatory effects in the expression of Ang1, Ang2 or their receptor Tie2 were observed. Interestingly, these data also agree with our previous observations in 6-month-old rats subjected to 2 months of ER, after 2 months on a high-fat diet, which exhibited a similar change in the expression of vascular growth factors, a tendency for decreased expression of the components of the VEGF/VEGFR2 system and no differences in angiopoietins or Tie2 expression, when compared with age-matched controls.[67]

The tendency for a marked increase in Sirt1 expression was observed in the cavernous tissue in the FRUCTMIN group, which agrees with the Sirt1 levels observed in the liver (significantly increased *vs* the CONT and FRUCT groups) of the same animals.[68] This finding is important considering that the liver is the main contributor organ for rodent fructose-induced MS.[69]

Sirt1 is an enzyme that possesses NAD+-dependent deacetylase activity in multiple organs in mammals and is localized and active both in the nucleus and in the cytoplasm of cells.[33] Sirt1 expression and activity varies in a tissue-specific pattern during the aging process and in response to diet composition or Sirt1 activators/inhibitors.[70,71] Our laboratory previously detected Sirt1 in rat and human cavernous tissue, primarily in the SMC,[59,72] as observed here. Interestingly, we have also observed an increase in Sirt1 expression in the CC of 6-month-old rats subjected to ER for 4 months compared with controls and high-fat diet-treated animals.[72] This observation agrees with previous studies that demonstrate the upregulation of Sirt1 expression and activity in ER animals, supporting that the protective role of this diet pattern (which increases longevity and reduces metabolic and age-related disturbances incidence) is partially mediated through Sirt1.[70,73] Moreover, recent studies have demonstrated that Sirt1 plays a protective role in the vascular system due to its deacetylation-dependent upregulatory effect of eNOS activity, which leads to an increase in NO synthesis.[31] In particular, resveratrol-induced upregulation of Sirt1 activity protects against fructose-induced vascular dysfunction.[64] Sirt1 reduces the level of oxygen consumption, which is associated with decreased ROS production,[74] and consequently, contributes to the amelioration of fructose-induced vascular dysfunction. Sirt1 also prevents endothelial adhesion molecule expression, which impairs leukocytes transmigration to the subendothelial space and reduces the expression of lectin-like oxLDL receptor 1, a macrophage oxLDL receptor.[75] Therefore, the observed tendency for increased plasma oxLDL levels in the FRUTMIN group could be due to the downregulation of the expression of the oxLDL vascular receptor, supporting a protective role in the progression of atherosclerosis. Additionally, the oxLDL level in the FRUCTMIN group did not associate with an increase in oxidative stress, based on the observations in the liver of these animals. While fructose ingestion significantly decreases hepatic glutathione peroxidase activity and oxidized glutathione content and increases catalase activity compared with control animals, natural mineral-rich water co-ingestion partially counteracts these fructose-induced effects.[15; Pereira *et al.* full manuscript under review.*]

In the present study, a significant increase in the density of the SMC was observed in the FRUCTMIN group, which could be explained partially by the increase in Sirt1[76] observed in these animals. Alternatively, the SMC content increase itself could be responsible for the increase of Sirt1.[59] Once again, this finding aligns with features observed in 6-month old ER rats which also exhibit an increase in the SM layer compared with high-fat diet-treated counterparts.[77] This finding is very relevant considering the

importance of SMC in the relaxation of the cavernous vasculature and the protective effect of a moderate increase in the thickness of the periendothelial muscle layer.

However, this study presents some limitations. With the inclusion of an additional animal group, the effects of natural mineral-rich water on adult normal rat conditions and the evaluation of the effects of natural mineral-rich water with and without fructose ingestion could be explored. Furthermore, to elucidate the effects of natural mineral-rich water consumption on the prevention of deleterious vascular events associated with ED, the measurement of intracavernous pressure to evaluate erection functional capability is also necessary.

CONCLUSIONS

Taking together, these data indicate that the consumption of natural mineral-rich water induces changes in the cavernous expression of VEGF, VEGFR1, VEGFR2, Ang1, Ang2 and Tie2 in a trend related to those previously observed in rats treated with MS-protective polyphenol-rich beverages. In particular, the VEGF/VEGFR2 system tended to be down-regulated in all of the treatments. However, compared with the polyphenol-rich beverages, no adverse secondary effects, such as a decrease in blood testosterone or toxicity, were noted. Moreover, the cavernous tissue of the rats treated with natural mineral-rich water presented structural and molecular features (the vascular growth factor and receptor expression profiles, the increase in the SM layer and the upregulatory trend in Sirt1 expression) equivalent to those previously observed in ER rats.

Considering the recognized metabolic and anti-aging intervention of ER in a large set of organisms, these data prompted us to speculate that the beneficial effects of natural mineral-rich water consumption could be more vast than those directly observed in this study.

AUTHOR CONTRIBUTIONS

MJM designed the research protocol; CDP and LR performed the research protocol and collected and organized the obtained data; CDP, MS, MJM and DN analyzed the data; and CDP, MJM and DN wrote the paper. All authors read and approved the final manuscript.

COMPETING INTERESTS

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