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ORIGINAL ARTICLE

Long-term phosphodiesterase 5 inhibitor administration reduces inflammatory markers and heat-shock proteins in cavernous tissue of Zucker diabetic fatty rat (ZDF/fa/fa)

JE Toblli, G Cao, M Angerosa and M Rivero

Oxidative stress and nitrosative stress present in type 2 diabetes mellitus (T2DM) and metabolic syndrome (MS) induce inflammatory response in diverse tissues including cavernous tissue (CT). Heat-shock proteins (HSPs) have an important role in modulating and repairing tissue injury, although their participation in CT in T2DM is unclear. Beyond the specific action of phosphodiesterase type 5 inhibitors (PDE5i) on erectile function, it has been proposed that chronic administration of these agents improves endothelial function and ameliorates fibrotic changes. The aim of this study was to determine in CT of Zucker Diabetic Fatty (ZDF) rat, an experimental model of T2DM and MS: (1) the degree of oxidative stress and nitrosative stress; (2) the magnitude of inflammatory markers such as tumor necrosis factor-α (TNFα) and interleukin 6 (IL6); (3) immunoexpression of HSP70 and HSP27; (4) how a long-term PDE5i administration may modify these variables. For 6 months, (1) untreated ZDF; (2) ZDF+Sildenafil (Sil) and (3) control Lean Zucker Rat (LZR) received no treatment, were studied. Penises were processed for functional '*in vitro*' studies, oxidative and nitrosative stress evaluation and immunohistochemistry in CT using TNFα; IL6; nitrotyrosine, HSP70 and HSP27 antibodies. ZDF+Sil presented better relaxation in corporal strips versus untreated ZDF. Furthermore, ZDF+Sil presented less lipoperoxidation in CT versus untreated ZDF. The activity of antioxidant enzymes CuZn superoxide dismutase (CuZnSOD) and glutathione peroxidase (GPx) was also reduced in untreated ZDF in CT along with a decrease in glutathione versus untreated ZDF. Nitrotyrosine expression was increased in untreated-ZDF rats versus ZDF+Sil and LZR. TNFα and IL6 were decreased in CT in ZDF+Sil versus untreated ZDF. Additionally, the expression of HSP70 and HSP27 was reduced in CT in ZDF+Sil versus untreated ZDF. In conclusion, this study provides substantial evidence supporting a protective role of a long-term therapy with Sil on CT in a recognized animal model of T2DM and MS.

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INTRODUCTION

Patients with diabetes mellitus (DM) have an increased risk of future cardiovascular events comparable to that of patients with coronary artery disease.[1–3] Thus, the risk of future cardiovascular events is equivalent in DM and established coronary artery disease.[3] Endothelial dysfunction, defined as an abnormal response leading to a reduction in the bioavailability of nitric oxide (NO) and impaired vasodilatation, is thought to have a major role in the development of atherosclerosis and acute coronary syndromes, which are in turn frequent complications of DM. Recently, it has been suggested that endothelial dysfunction is often present in diabetic patients who do not have clinically evident atherosclerotic diseases.[4] In addition, type 2 DM, in both humans and rats, is also associated with severe alterations in vascular smooth muscle cells as well as in cavernous tissue together with an increase in fibrosis and increased oxidative stress.[5–10] Consequently, oxidative stress and also nitrosative stress, present in diverse tissues in DM, lead to an overproduction of a variety of cytokine and inflammatory molecules such as IL6 and tumor necrosis factor-α (TNFα) and trigger the apoptotic pathway.[11–13]

As a result, a number of mechanisms involved in modulating and repairing this injury acquire a major role to re-establish the normal structural and functional tissue behavior. In this sense, heat-shock proteins (HSPs), also known as molecular chaperones, comprise a group of highly conserved, abundantly expressed proteins with diverse functions. HSPs have an important role in protein–protein interactions, such as folding and assisting in the establishment of proper protein conformation.[14,15] These proteins appear to have a significant function in the responses and adaptation of the smooth muscle cell to stress, but the actual role of HSPs in the modulation of cavernous tissue (CT) in the metabolic syndrome (MS) scenario is unclear.

Sildenafil (Sil), the well-known first specific phosphodiesterase type 5 inhibitor (PDE5i), facilitates penis erection by increasing NO availability through the inhibition of cyclic-GMP (cGMP) break-down in endothelial cells.[16–18] Interestingly, as the PDE5 enzyme is widely expressed in the vasculature,[19] Sil enhances NO-mediated responses in vascular beds other than the penis. It is worth mentioning that the efficacy of PDE5i is lower in diabetic patients with erectile dysfunction (ED) compared with those without DM.[20]

Chronic Sil administration may regulate the transduction pathway leading to the activation of endothelial nitric oxide synthase, with no effect on NO bioavailability or on the cGMP pathway and without concerns for tachyphylaxis.[21] Furthermore, the chronic administration of PDE5i may improve endothelial

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function in the long term.[22] Therefore, the chronic administration of PDE5i should improve the responsiveness of the dysfunctional endothelium in patients with lipid alterations, high blood pressure and DM.

The Zucker Diabetic Fatty (ZDF) rat is a recognized animal model of obesity, type 2 DM, arterial hypertension and hyperlipidemia.[23–25] Thus, it is an attractive experimental model to investigate deleterious vascular effects related to the human MS.

Considering this background, the aim of the present study was to determine in CT of this representative animal model of MS: (1) the degree of oxidative stress and nitrosative stress; (2) the magnitude of inflammatory markers such as TNFα and IL6; (3) immunoexpresion of HSPs 70 and 27; (4) how a long-term PDE5i administration may modify these variables.

MATERIALS AND METHODS

All the experiments were performed in the laboratory of experimental medicine of our hospital after being approved by the Ethics Committee and the Teaching and Research Committee of our institution and in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Two groups of 10-week-old-male ZDF rats, group 1 ZDF no treatment ($n = 12$), group 2 ZDF+Sil ($n = 12$) and one group of age-matched group 3 LZR no treatment ($n = 12$) (Charles River Laboratories, Wilmington, MA, USA) were housed in individual cages at room temperature (21 ± 2 °C) and a 12-h light/darkness cycle (0700–1900 h).

During 6 months, sildenafil citrate (Sil) 10 mg kg^{-1} per day[26] was administered continuously in drinking water in the corresponding group, while the groups without treatment received regular tap water throughout the experiment.

At the end of the study, all the animals were killed by the administration of a lethal dose of intraperitoneal sodium thiopental. A midline abdominal incision was made, and the penises were excised in bulk from the crus to the distal aspect.

Biochemical procedures

After 14-h fasting, rat blood samples were collected from the tail vein in capillary tubes at baseline, and from the inferior cava vein before the rats being killed at the end of the experiment. Plasma glucose levels were measured by the glucose oxidase method with an Automatic Analyzer (Hitachi 911, Tokyo, Japan). Serum cholesterol and triglycerides were assessed according to standard methods. Serum insulin was determined by the sensitive rat insulin RIA kit (SRI-13, LINCO Research, St. Charles, MO, USA). Serum samples were stored before testing.

Blood pressure measurement

Systolic blood pressure (SBP) was measured monthly from baseline to the end of the experiment by tail cuff plethysmography. Measurements were obtained with the rats restrained in a plastic chamber without anesthesia. A pneumatic pulse transducer positioned on the ventral surface of the tail distal to the occlusion cuff detected the return of the pulse following a slow deflation of the cuff. Cuff pressure was determined by a Pneumatic Pulse Transducer, using a Programmed electro-sphygmomanometer PE-300 (Narco Bio-Systems, Austin, TX, USA), and pulses were recorded on a Physiograph MK-IIIS (Narco Bio-Systems). A minimum of three such determinations were taken at each session, and the SBP registered was the average of the three readings.[27,28]

Tissue processing and examination. The penises were removed and a portion of each one was destined for organ bath studies and the rest, fixed in phosphate-buffered 10% formaldehyde (pH 7.2). The tissue samples were embedded in paraffin. Three-micron sections were cut and stained with hematoxylin-eosin (H&E) and Masson's trichrome.

Organ bath studies. After being removed, the penises were immediately dissected. The tunica albuginea was carefully opened from the proximal extremity of the corpus cavernosum to the penile shaft and the erectile tissue within the corpus cavernosum was microsurgically dissected free. CSM strips (1–2 mm × 0.5 mm) were then transferred to ice-cold Krebs-bicarbonate solution (in mmol l^{-1}: 128 NaCl; 4.6 KCl; 2.5 $CaCl_2$; 1.2 KH_2PO_4; 1.2 $MgSO_4$; 11.1 glucose; 25 $NaHCO_3$; 0.1 ethylenediaminetetraacetic acid) at pH 7.4 and continuously gassed with a mixture of 95% oxygen and 5% carbon dioxide and the temperature maintained at 37 °C. The strips were mounted in a vertical organ bath system (MyoBath-4, World Precision Instrument, Sarasota, FL, USA) using chambers containing 5 ml of Krebs-bicarbonate solution. The tissues were connected to a force-displacement transducer (FORT10; World Precision Instrument). A pre-tension of 200 mg (optimum tension defined in preliminary experiments for both ZDF and LZR) was applied, and the strips were allowed to equilibrate for 45–60 min without additional mechanical manipulation. During the equilibration, the organ bath solution was replaced every 15 min. At the end of the equilibration period, a concentration–response curve to phenylephrine (Phe, $10^{-8} - 10^{-4}$ mol l^{-1}) was performed for each sample. Next, a concentration–response curve to acetylcholine (ACh, $10^{-8} - 10^{-4}$ mol l^{-1}) was conducted on Phe induced (10^{-4} mol l^{-1}). The contractile responses were expressed as the absolute change in maximal developed tension (mg), normalized per mg tissue weight, and the relaxation, as the percentage of changes in Phe-induced tone. The concentration inducing 50% of maximal effect was expressed as pD2.[29]

Oxidative stress parameters

Homogenates preparation. A fraction of the penile tissue was homogenized (1:3, weight:volume) in ice-cold 0.25 mol l^{-1} sucrose, then glutathione levels were determined in the 10 000 g supernatant following the methods previously described and the results were expressed as micromoles per milligram of protein.[30] Another fraction of the penile tissue was homogenized (1:10, w-v) in 0.05 M sodium phosphate buffer, pH 7.4 and was directly used for the determination of malondialdehyde (MDA) or centrifuged at 4 °C during 15 min at 9500 *g*. The peroxidation index was evaluated by the formation of MDA and determined as thiobarbituric reactive species.[31] Finally, another fraction of the penile tissue was homogenized (1:3, weight:volume) in ice-cold sucrose (0.25 mol l^{-1}). The supernatant obtained after centrifugation at 105 000 g for 90 min was used for measuring CuZn superoxide dismutase (CuZnSOD) and glutathione peroxidase (GPx) activity.[32–34] Protein concentration was determined by the method of Lowry *et al*.[35] Enzyme units (U) were defined as the amount of enzyme producing 1 nmol of product or consuming 1 nmol of substrate under the standard incubation conditions. Specific activity (Sp. Act.) was expressed as U mg^{-1} protein. One unit of CuZnSOD was defined as the amount of CuZnSOD capable of inhibiting the rate of NADH oxidation measured in the control by 50%.

Western blotting and densitometric analysis. Samples from penile tissue were solubilized in 1% triton detergent and analyzed by western blotting as described previously.[36] For assessing nitrosative stress, polyvinyl difluoride membranes with transferred proteins were probed with anti-nitrotyrosine antibody at 1:2000 dilution (AB5411, Millipore, Billerica, MA, USA). Inflammatory response was evaluated by probing for anti-TNFα (AF-510-NA, R&D Systems, Minneapolis, MN, USA) at 1:1000 dilution and IL6 with an anti-IL6 antibody (sc1265, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:1000. Repair response was assessed with anti-heat shock protein 27 (HSP 27; sc-1048, Santa Cruz Biotechnology) and with anti-HSP 70 (HSP 70, sc-1060, Santa Cruz Biotechnology), both at a dilution of 1:1000. After washing, membranes were probed with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody at dilution 1:20 000 (sc-2004, Santa Cruz Biotechnology).

Specific bands were detected by enhanced chemiluminescence (Amersham, Piscataway, NJ, USA). Autoradiographs were scanned and band intensities were quantified by digital densitometry using Gel-Pro Analyzer 4.0 software (Media Cybernetics, Silver Spring, MD, USA). The data were recorded as mean optical density intensity.

Equal protein loading of samples was confirmed by stripping polyvinyl difluoride membranes with a commercial re-blot solution (Chemicon, Billerica, MA, USA) and then re-probing with anti-β-actin primary antibody (A5316, Sigma-Aldrich, St. Louis, MO, USA) at 1:5000 dilution; and horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:20 000 dilution; cat. no. sc-2005, Santa Cruz Biotechnology) followed by chemiluminescent detection and densitometric quantification as described earlier.

Immunolabeling and light microscopy. Immunolabeling of specimens was performed through a modified avidin-biotin-peroxidase complex technique. Following deparaffinization and rehydration, the sections were washed in phosphate-buffered saline (PBS) for 5 min. The quenching of endogenous peroxidase activity was achieved by incubating the sections

in 1% hydrogen peroxide in methanol for 30 min. After being washed in PBS, pH 7.2, for 20 min, the sections were incubated with blocking serum for 20 min. Thereafter, the sections were incubated with the primary antibody overnight, rinsed in PBS, and incubated with biotinylated universal antibody for 30 min. After being washed in PBS, the sections were incubated with Vectastain® Elite ABC reagent (Vector Laboratories, Birlingame, CA, USA) for 40 min and exposed to 0.1% diaminobenzidine (Polyscience, Warrington, PA, USA) and 0.2% hydrogen peroxide in 50 mM Tris buffer, pH 8, for 5 min.

With the aim of detecting inflammatory as well as repair tissue markers in CT, the following antibodies were used: (1) antibody against rat TNFα (AF-510-NA, R&D Systems) at a dilution of 1:50; (2) antibody against interleukin 6 (IL6) (sc1265, Santa Cruz Biotechnology) at a dilution of 1:100; (3) anti-heat shock protein 27 (HSP27; sc-1048, Santa Cruz Biotechnology) and (4) anti-HSP 70 (HSP70, sc-1060, Santa Cruz Biotechnology), both at a dilution of 1:100. Nitrosative stress in tissue was evaluated using a rabbit polyclonal antibody anti-nitrotyrosine (AB5411, Millipore) at a dilution of 1:100.

Table 1. Blood pressure recording and biochemical parameters

Mean ± s.d.	*ZDF* (n = *12*)	*ZDF+Sil* (n = *12*)	*LZR* (n = *12*)
a. Baseline data			
Body weight (g)	405 ± 22	401 ± 24	231 ± 16*
SBP (mm Hg)	154.1 ± 2.7	150.9 ± 3.5	118.2 ± 2.2*
Glycemia (mmol l^{-1})	14.7 ± 1.3	14.9 ± 1.4	4.9 ± 0.3*
Insulin (ng ml^{-1})	45.9 ± 6.0	46.4 ± 5.8	5.0 ± 1.1*
Cholesterol (mmol l^{-1})	5.2 ± 1.0	5.2 ± 1.1	1.5 ± 0.3*
Triglycerides (mmol l^{-1})	7.7 ± 1.2	7.8 ± 1.0	0.5 ± 0.1*
b. At the end of the experiment (6 months)			
Body weight (g)	612 ± 15	599 ± 18	339 ± 12*
SBP (mm Hg)	156.8 ± 3.4	152.9 ± 3.8	119.8 ± 3.0*
Glycemia (mmol l^{-1})	29.1 ± 5.9	28.3 ± 6.2	5.6 ± 0.5*
Insulin (ng ml^{-1})	4.8 ± 0.8	5.1 ± 0.7	5.2 ± 0.6
Cholesterol (mmol l^{-1})	7.3 ± 0.5	7.1 ± 0.6	1.6 ± 0.2*
Trygliceride (mmol l^{-1})	7.8 ± 1.7	7.3 ± 2.0	0.6 ± 0.2*

Abbreviations: LZR, Lean Zucker Rat; SBP, systolic blood pressure; ZDF, Zucker Diabetic Fatty. *$P < 0.01$ versus all groups.

Morphometric analysis. Between six and eight transverse histology sections from the penis of each animal were studied by an image analyzer (Image-Pro® Plus, version 4, Media Cybernetics). To compare similar segments of the corpus of all rats, sections were taken from the proximal middle portion of the penis of each animal. The morphological analyses were performed with the observer blinded to the animal group, and the data were averaged. The CT was delineated by the tunica albuginea, and then a quantification of the extent of immunostaining was performed. In every sample, positive immunostaining for TNFα, IL6, HSP70, HSP27 and Nitrotyrosine was assessed and the data were expressed as percentage per area. Control sections used for the determination of antibody specificity were also qualitatively evaluated by the presence of positive or negative staining. All data were averaged in the final result.

Statistical analysis

Values were expressed as the mean ± s.d. All the statistical analyses were performed using absolute values and processed through GraphPad Prism, version 2.0 (GraphPad Software, San Diego, CA, USA). For parameters with non-Gaussian distribution, such as histology data, comparisons were made by the nonparametric analysis of variance and Dunn's multiple comparison test. A value of $P < 0.05$ was considered as significant.

RESULTS

At baseline, body weight, SBP, glycemia, serum triglycerides and cholesterol were all significantly increased in both ZDF groups together with fasting serum insulin level, without differences between them in comparison with control LZR, as described in Table 1a. At the end of the experiment (6 months), ZDF groups presented significant hyperglycemia compared with control LZR, but with a low fasting insulin level. Furthermore, body weight, SBP, serum triglycerides and cholesterol were equally increased in both ZDF groups with respect to the baseline level and also consequently higher versus LZR, as shown in Table 1b. All these variables suggest the progression of the diabetic disease as it frequently observed in human beings in clinical practice.

'In vitro' functional studies

Developed tensions to Phe (10^{-8}–10^{-4} mol l^{-1}) in corporal strips were not different between groups (ZDF = 760 ± 34 mg mg^{-1} ww; ZDF+Sil = 758 ± 32 mg mg^{-1} ww and LZR = 759 ± 39 mg mg^{-1} ww at Phe 10^{-4} mol l^{-1}) as represented in Figure 1a. The mean pD_2 value calculated for Phe presented no difference between groups (ZDF = 5.8 ± 0.1; ZDF+Sil = 5.8 ± 0.2; and LZR = 5.7 ± 0.2).

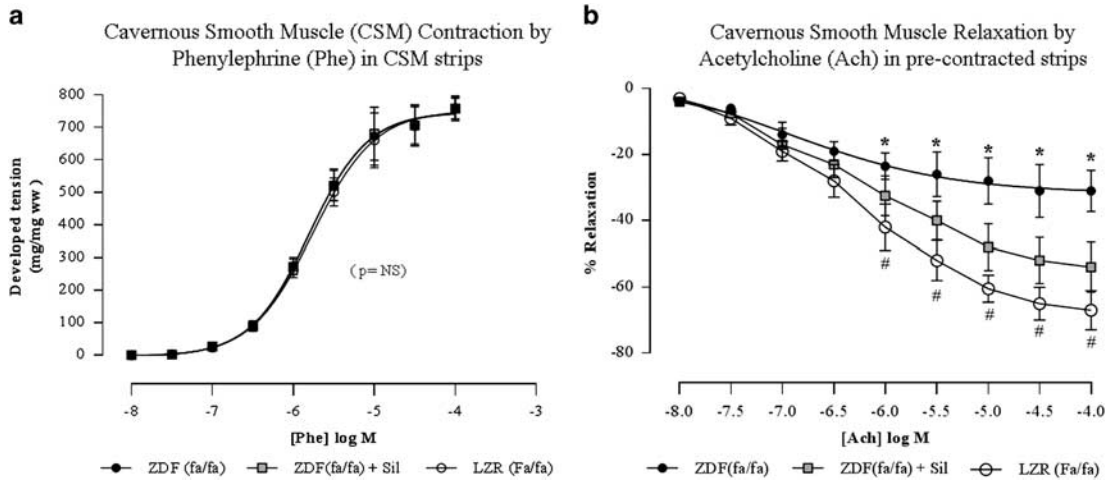

Figure 1. (**a**) Concentration–response curve to Phenylephrine (Phe, 10^{-8}–10^{-4} mol l^{-1}) in corporal strips from all groups. Results are expressed as the maximal developed tension values induced by Phe normalized by the wet weight (ww) of the strip. (**b**) Concentration–response curve to acetylcholine (ACh, 10^{-8}–10^{-4} mol l^{-1}) in corporal strips from all groups. Results are expressed as a percentage of relaxation from the initial developed tension obtained with Phenylephrine (10^{-5} mol l^{-1}). *ZDF versus all groups $P < 0.01$: # LZR versus ZDF +Sil $P < 0.01$. LZR, Lean Zucker Rat; Sil, Sildenafil; ZDF, Zucker Diabetic Fatty.

Table 2. Oxidative stress parameters in cavernous tissue

Mean ± s.d.	*ZDF* (n = *12*)	*ZDF+Sil* (n = *12*)	*LZR* (n = *12*)
MDA (nmol mg^{-1} protein)	6.7 ± 1.1*	2.3 ± 0.8	2.1 ± 0.6
Glutathione (μmol mg^{-1} protein)	137.6 ± 10.7*	179.8 ± 12.5	186.4 ± 15.1
CuZnSOD (U mg^{-1} protein)	52.3 ± 6.0*	90.1 ± 7.7	96.7 ± 10.5
GPx (U mg^{-1} protein)	9.8 ± 0.5*	13.9 ± 0.7	14.8 ± 0.4

Abbreviations: CuZnSOD, CuZn superoxide dismutase; GPx, glutathione peroxidase; LZR, Lean Zucker Rat; MDA, malondialdehyde; ZDF, Zucker Diabetic Fatty. *$P < 0.01$ versus all groups.

Table 3. Immunohistochemistry in cavernous tissue

Mean ± s.d.	*ZDF* (n = *12*)	*ZDF+Sil* (n = *12*)	*LZR* (n = *12*)
Nitrotyrosine[a]	8.4 ± 1.6*	1.1 ± 0.3	0.9 ± 0.5
Tumor necrosis factor-α[a]	12.2 ± 3.9*	1.2 ± 0.4	1.0 ± 0.3
Interleukin 6[a]	11.7 ± 2.5*	1.1 ± 0.2	1.0 ± 0.2
Heat-shock protein 70[a]	9.9 ± 1.7*	1.3 ± 0.2	1.1 ± 0.3
Heat-shock protein 27[a]	8.5 ± 1.9*	1.2 ± 0.3	1.0 ± 0.3

Abbreviations: LZR, Lean Zucker Rat; Sil, Sildenafil; ZDF, Zucker Diabetic Fatty. *$P < 0.01$ versus all groups. [a]Expresses as percentage of positive immunostaining per area.

Concentration-response relaxation curves were evoked by cumulative addition of ACh (10^{-8}–10^{-4} mol l^{-1}) on corporal strips precontracted with Phe (10^{-4} mol l^{-1}), and untreated ZDF showed the lower value. During Phe-induced contraction, endothelium-dependent relaxation to ACh was impaired significantly ($P < 0.01$) in untreated ZDF corporal strips versus the other groups (ZDF = 31.0 ± 6.2%; ZDF+Sil = 54.2 ± 7.5% and LZR = 67.0 ± 5.9%), as shown in Figure 1b. Although ZDF+Sil presented better relaxation response to Ach versus untreated ZDF, this group showed significant differences compared with LZR (Figure 1b). The mean pD_2 value calculated for ACh presented no difference between groups (ZDF = 6.4 ± 0.1; ZDF+Sil = 6.3 ± 0.2 and LZR = 6.3 ± 0.2).

Oxidative stress and nitrosative stress evaluation in CT

A remarkable high level of lipoperoxidation in CT was observed in untreated ZDF rats when compared with ZDF+Sil and control LZR, which presented no differences between them, as the significant increase in MDA indicates (Table 2). Additionally, the activity of the antioxidant enzymes, CuZnSOD and GPx, was reduced in untreated ZDF in CT along with a substantial decrease in glutathione content, as shown in Table 2. On the other hand, ZDF+Sil showed no differences between LZR relative to CuZnSOD and GPx activity as well as to the glutathione content (Table 2).

Nitrotyrosine expression, the well-known marker of nitrosative stress, was significantly ($P < 0.01$) increased in untreated ZDF rats in comparison with ZDF+Sil and LZR, which presented no differences between them, as represented in Table 3 and Figure 2.

Evaluation of molecules linked to the inflammatory process and repair in CT

TNFα as well as IL6 were similarly increased ($P < 0.01$) in CT in untreated ZDF with respect to ZDF+Sil and LZR, which presented no differences between them, as displayed in Table 3 and illustrated in Figures 3 and 4. In line with this finding, the expression of HSP70 and HSP27, both molecular markers of the repair mechanism in CT, was similar increased in untreated ZDF when compared with LZR. On the contrary, ZDF+Sil presented remarkably lower ($P < 0.01$) immunoexpression of these two molecular chaperones and showed no differences versus LZR, as indicated in Table 3 and Figures 5 and 6.

DISCUSSION

In the present study, ZDF rats showed severe alterations in metabolic parameters such as overweight, hyperglycemia, hypertriglyceridemia, hypercholesterolemia, as well as moderate high blood pressure. Furthermore, functional evaluations in strips of CT revealed impaired relaxation in ZDF rats. Inflammatory markers as TNFα and IL6, and molecules involved in repair (HSP70 and HSP27) were dramatically increased in ZDF rats. All these alterations were associated with a superlative degree in nitrosative and oxidative stress. In contrast to these results, a long-term treatment with Sil for 6 months was able to improve significantly functional and morphological variables studied in ZDF rats despite the fact that they presented no modifications in metabolic parameters and blood pressure with respect to untreated ZDF rats.

Among the various mechanisms responsible for the pathogenesis of ED, hyperglycemia has been recognized as an important risk factor. Hyperglycemia induces the overproduction of superoxide anion, which can potentially cause the inactivation of Akt and the activation of poly (ADP-ribose) polymerase pathways, thereby contributing to diabetic complications by inducing apoptosis or cell death.[37–40] Moreover, increased oxidative stress, which is induced by several hyperglycemia-activated pathways, is considered as a major etiologic factor in the pathogenesis of diabetic complications.[41] In normal conditions, there is a balance between the production of oxidative stress by pro-oxidants and the scavenging by antioxidants of excess reactive oxygen species (ROS). However, in pathological circumstances as occurring during permanent hyperglycemia, this balance is broken and oxidative stress occurs. ROS generated during regular oxygen molecule metabolism primarily in the vascular endothelium, include, H_2O_2 and peroxynitrite ($ONOO^-$). The increased production of $ONOO^-$ reduces the available NO concentration in CT, this leading to hindering the normal relaxation of cavernous muscle and contributing to developing ED.

$ONOO^-$ is a strong oxidant and nitrating agent that may be generated by a variety of cells, including activated macrophages, neutrophils and endothelial cells.[42,43] Furthermore, $ONOO^-$ has been shown to have multiple pathways of reactivity, oxidize sulfhydryl and induce lipid peroxidation.[44] The formation of the specific product 3-nitrotyrosine through the nitration of tyrosyl residues has been taken as an accurate biomarker of $ONOO^-$ production. In the present experiment, both nitrotyrosine expression, a representative marker of nitrosative stress, as well as the MDA surrogated marker of lipid peroxidation, were significantly ($P < 0.01$) increased in CT of ZDF rats when compared with control LZR, this indicating a substantial increase in $ONOO^-$ and lipid peroxidation in erectile tissue. Furthermore, the antioxidant enzymes CuZnSOD and GPx were also dramatically increased ($P < 0.01$) in the CT of ZDF rats together with a significant reduction ($P < 0.01$) in the glutathione content in this group relative to control LZR. In concordance with all these findings, the endothelium-dependent relaxation to ACh was impaired significantly in strips of CT in ZDF rats.

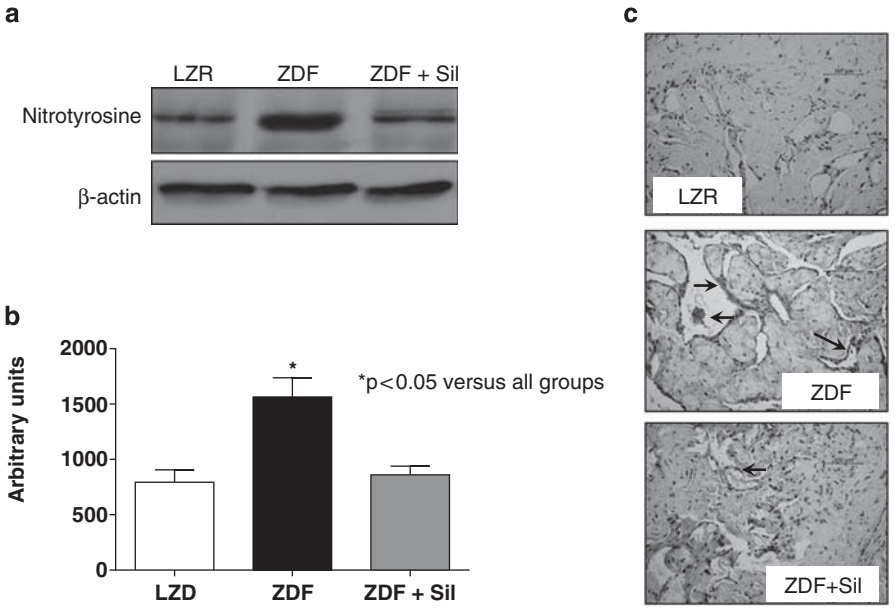

Figure 2. Evaluation of nitrosative stress in cavernous tissue (CT). (**a**) Representative immunoblot photographs for nitrotyrosine. (**b**) Densitometric analysis of the protein studied. (**c**) Localization of nitrotyrosine (arrows) in CT. ZDF showed a marked increase in nitrosative stress in CT as expressed by increased in abundance of Nitrotyrosine. On the other hand, ZDF+Sil and LZR present no significant difference between them. LZR, Lean Zucker Rat; Sil, Sildenafil; ZDF, Zucker Diabetic Fatty.

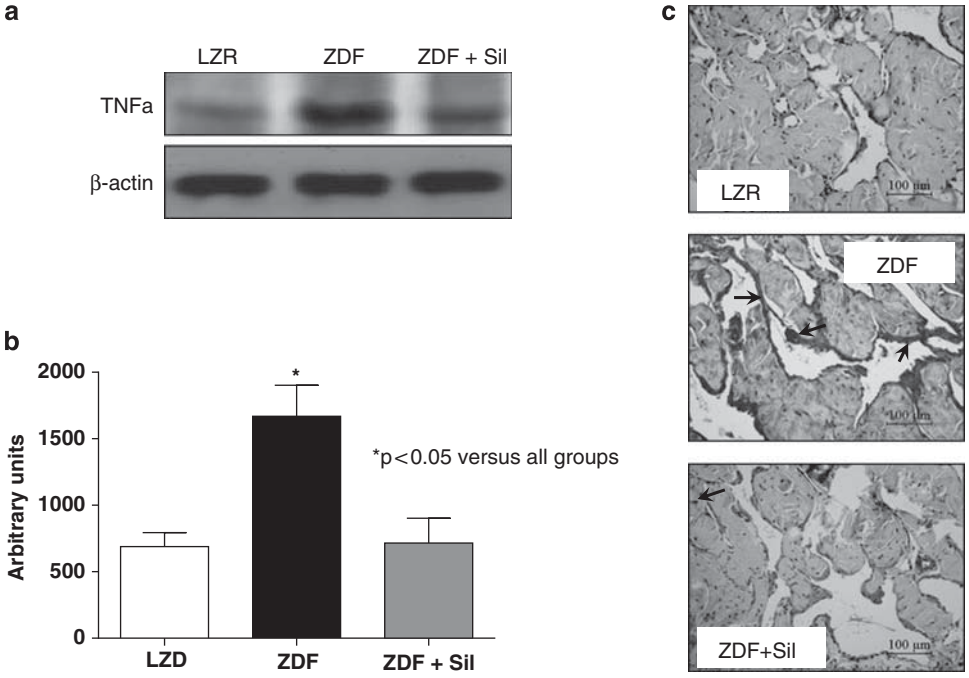

Figure 3. Evaluation of inflammatory response by tumor necrosis factor α (TNFα) in cavernous tissue (CT). (**a**) Representative immunoblot photographs for TNFα. (**b**) Densitometric analysis of the protein studied. (**c**) Localization of TNFα (arrows) in CT. Note a significant increase in TNFα in ZDF with respect to ZDF+Sil and LZR, which present similar expression of TNFα. LZR, Lean Zucker Rat; Sil, Sildenafil; ZDF, Zucker Diabetic Fatty.

Notably, in a rabbit model of high fat diet induces MS, reduced responsiveness to acetylcholine in penis, and therefore, alteration in the erectile function was associated with steatohepatitis,[45] this currently considered as the hepatic hallmark of MS.[46]

In agreement with these findings, previous studies from our group in obese Zucker rats have indicated liver inflammation with steatohepatitis.[47]

Some evidence indicates that diabetes, MS and increased insulin resistance are associated with increased levels of proinflammatory proteins such as IL6 and TNFα.[48] The activation of inflammatory signaling by TNFα has been also suggested to contribute to the development of cardiovascular diseases[49] and have a detrimental role in erectile function.[50,51] In line with this, a high immunoexpression of TNFα in the aorta and corpus

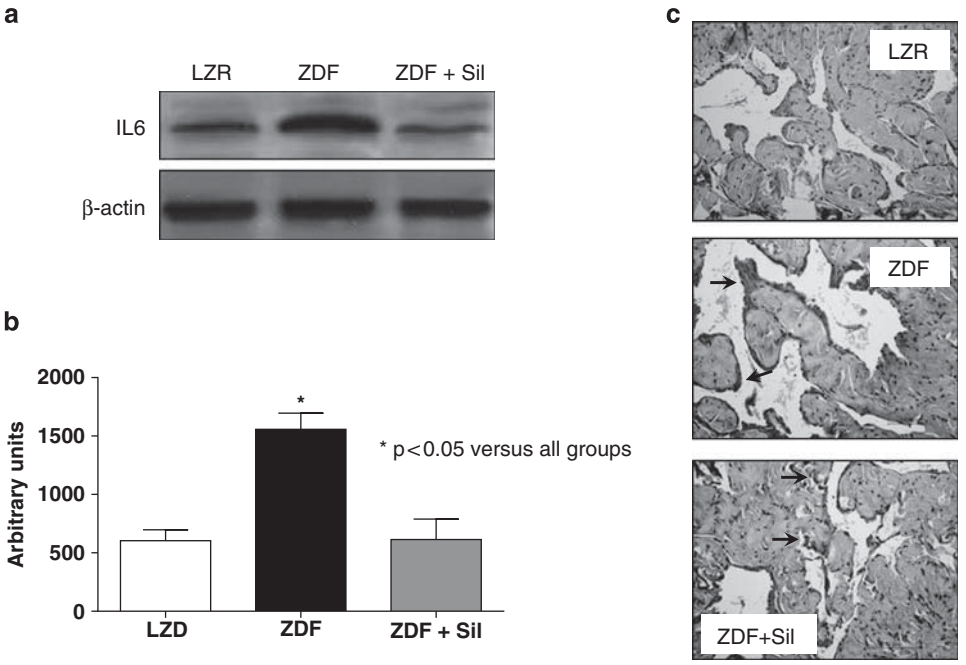

Figure 4. Evaluation of inflammatory response by interleukin 6 (IL6) in cavernous tissue (CT). (**a**) Representative immunoblot photographs for IL6. (**b**) Densitometric analysis of the protein studied. (**c**) Localization of IL6 (arrows) in CT. Note a significant increase in IL6 in ZDF with respect to ZDF+Sil and LZR, which present similar expression of IL6. LZR, Lean Zucker Rat; Sil, Sildenafil; ZDF, Zucker Diabetic Fatty.

cavernosum has been reported in streptozotocin induced diabetic (type I diabetes) rats.[52] Nevertheless, in a high fat diet-induced animal model of MS, Vignozzi *et al.*[45] have recently reported a significant increase in circulating levels of TNFα. Moreover, these investigators have also highlighted that circulating levels of TNFα were significantly related to TNFα mRNA expression level within the liver, but not with expression in other tissues. Interestingly, although computer-assisted quantitative immunohistochemistry for TNFα protein indicated that the relative levels were increased by at least five-fold in these animals in penile sections (both endothelial and smooth muscle cells of the vascular bed and cavernous spaces), the TNFα mRNA penile expression was not affected in that model of MS. These data suggesting that in such particular metabolic scenario, the penis is a target of inflammation but it is not the origin.

TNFα upregulates PDE5 expression in cavernosal vascular smooth muscle cells through an *a priori* upregulation of NADPH oxidase and formation of O_2^-. On the other hand, the treatment with Sil has demonstrated to reverse this effect by inhibiting O_2^- formation and normalizing PDE5 in cavernosal vascular smooth muscle cells.[53] In agreement with this, in the current study, ZDF rats treated with Sil reduced the immunoexpression of TNFα and IL6 in CT compared with untreated ZDF. This observation suggests a positive modulation of inflammatory response by Sil in penile structures in a diabetic scenario.

Sil as well as other PDE5is are agents that inhibit PDE5, thereby diminishing the degradation of cGMP and improving erectile function. Sil is known to be effective via the NO-cGMP pathway and is widely used in the treatment of diabetic ED.

Although the antioxidative stress pathway of PDE5is in diabetic ED is not well defined, data from experiments with tadalafil in STZ-induced diabetic rats indicate a local antioxidant mechanism.[54] In agreement with this information, in the current experiment, a long-term treatment with Sil was able to modulate favorably nitrosative and oxidative stress in ZDF rats. Moreover, Sil substantially improved endothelium-dependent relaxation to ACh in strips of CT in ZDF rats. Our findings are in concordance with those of Behr-Roussel *et al*,[21] who suggested that a chronic treatment with Sil potentiates ACh-induced endothelium-dependent cavernosal responses and enhances erectile responses in normal rats.

A reduction in endothelial nitric oxide synthase activity together with an increase in superoxide anion, which results in a decrease in NO bioavailability, has been proposed as the main mechanism by which penile vascular dysfunction occurs. PDE5i therapy successfully may restore erectile responses by diminishing superoxide anion, increasing penile endothelial nitric oxide synthase activity and restoring endothelial-derived NO in the penis, as demonstrated in mice.[55] Therefore, in the present study, it is not surprising that ZDF rats showed severe functional abnormalities in penile structures associated with a clear pattern of superlative oxidative and nitrosative stress in CT, and that the ZDF rats treated with Sil displayed a significant better outcome regarding these variables. These data are in line with two important recent studies which clearly demonstrated in prostate tissue, not only in a high fat diet-induced animal model of MS but also in human beings, anti-inflammatory and anti-fibrotic capacity of other PDE5is such as tadalafil and vardenafil.[56,57] Importantly, PDE5 inhibition showed significantly blunted prostate inflammation (increased CD45 immunopositivity), fibrosis (reduced muscle/fiber ratio) and hypo-oxygenation, thus suggesting a potential curative effect of PDE5 inhibition on MS-related prostate alterations.[56] These studies have demonstrated that PDE5 inhibition either by tadalafil or by vardenafil ameliorates inflammatory response induced by metabolic, as well as inflammatory stimuli, likely via the activation of cGMP/PKG signaling. These data together give a new insight into the comprehension of the mechanism of action of PDE5is in male genital structures.[56,57]

After metabolic stress, HSPs are expressed at high levels in all tissues and cells, and some of them such as HSP70 and HSP27 directly protect cells against damage-induced entry into death pathways. The elevation of HSP levels during the metabolic stress response was shown to inhibit stress-mediated cell killing and recent experiments indicate a highly versatile role for these proteins as inhibitors of programmed cell death.[58]

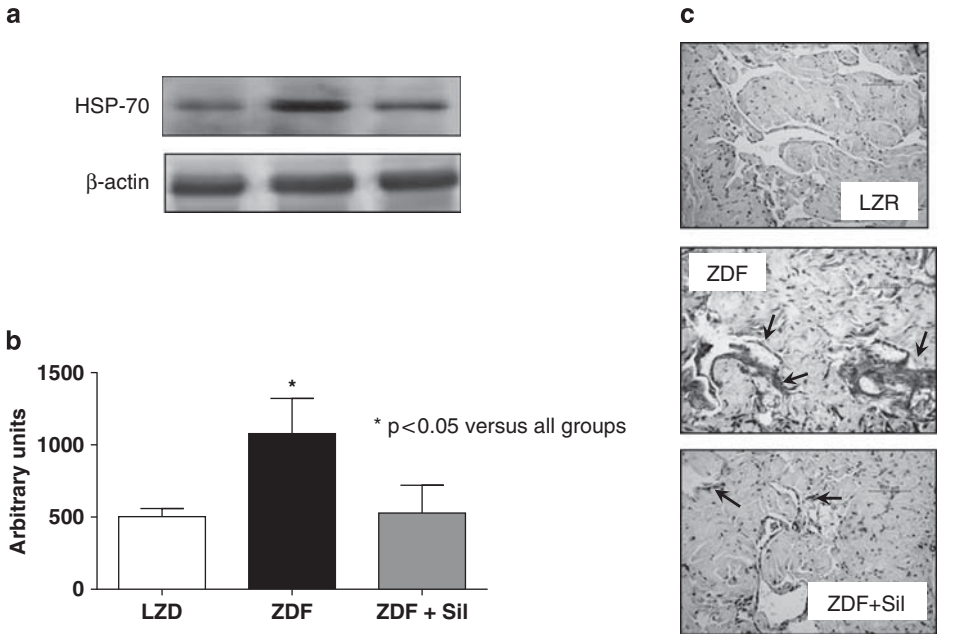

Figure 5. Assessment of repair response by heat-shock protein 70 (HSP70) in cavernous tissue (CT). (**a**) Representative immunoblot photographs for HSP70. (**b**) Densitometric analysis of the protein studied. (**c**) Localization of HSP70 (arrows) in CT. Note a significant increase in HSP70 in ZDF with respect to ZDF+Sil and LZR, which present similar expression of HSP70. LZR, Lean Zucker Rat; Sil, Sildenafil; ZDF, Zucker Diabetic Fatty.

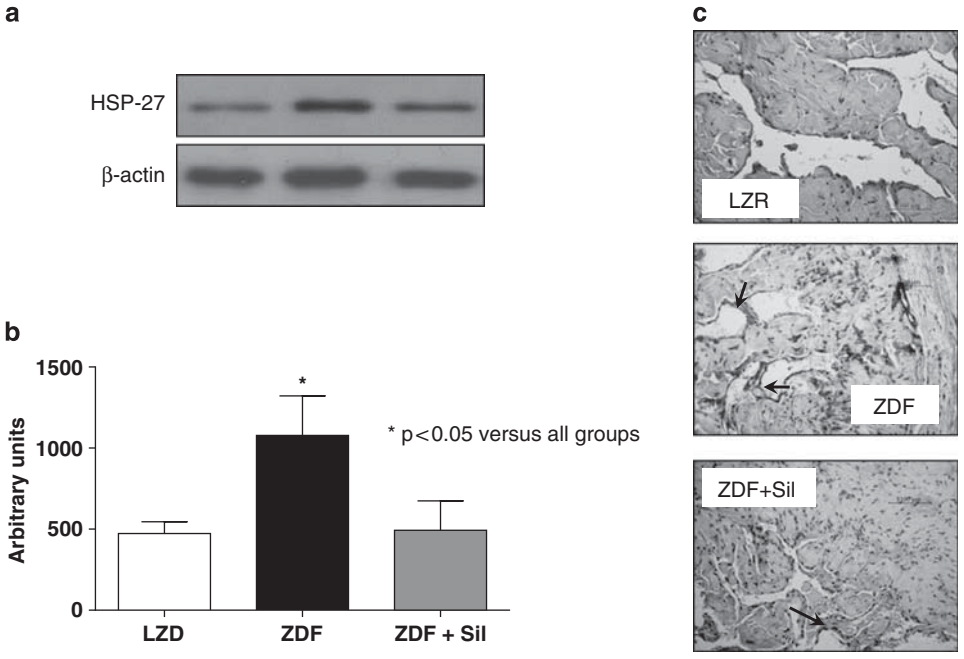

Figure 6. Assessment of repair response by heat-shock protein 27 (HSP27) in cavernous tissue (CT). (**a**) Representative immunoblot photographs for HSP27. (**b**) Densitometric analysis of the protein studied. (**c**) Localization of HSP27 (arrows) in CT. Note a significant increase in HSP27 in ZDF with respect to ZDF+Sil and LZR which present similar expression of HSP27. LZR, Lean Zucker Rat; Sil, Sildenafil; ZDF, Zucker Diabetic Fatty.

Studies in HeLa cells by Hall and Martinus[59] demonstrated that hyperglycaemic conditions and oxidative stress can lead to the induction of HSPs expression, suggesting that the increased levels of these molecular stress proteins observed in T2DM patients could also be due to uncontrolled hyperglycemia and oxidative stress. In addition, they observed a significant and concomitant increase in the intracellular levels of ROS generated during the exposure of the HeLa cells to the stressors being investigated, this suggesting that the induction of HSPs was related to ROS-mediated processes. In concordance with this background, in the present study, untreated ZDF rats presented a significant increased expression of HSP70 and HSP27 in CT when compared

with control LZR. Furthermore, the magnitude of this increase was in line with the proportion of the increase in the inflammatory proteins such as TNFα and IL6. These findings suggest some correspondence between the injury and repair process in CT in ZDF rats. Interestingly, the treatment with Sil was able to modify this process favorably. The most suitable approach to justify this outcome is the recognized positive modulation of PDE5i therapy on inflammatory response, which may support the attenuation of metabolic injury in CT, this leading to less tissue expression of HSPs, as observed in the ZDF rats in the current study.

In conclusion, this study provides substantial evidence supporting the protective role in CT of a long-term therapy with Sil in a recognized genetic animal model of MS and diabetes mellitus type 2.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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