Oxidative stress is not associated with vascular dysfunction in a model of alloxan-induced diabetic rats

Estresse oxidativo não está associado à disfunção vascular em um modelo de diabetes induzida por aloxana em ratos

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ABSTRACT

Objectives: To verify if an experimental model of alloxan-diabetic rats promotes oxidative stress, reduces nitric oxide bioavailability and causes vascular dysfunction, and to evaluate the effect of N-acetylcysteine (NAC) on these parameters. **Methods:** Alloxan-diabetic rats were treated or not with NAC for four weeks. Plasmatic levels of malondialdehyde (MDA) and nitrite/nitrate (NOx), the endothelial and inducible nitric oxide synthase (eNOS and iNOS) immunostaining and the vascular reactivity of aorta were compared among diabetic (D), treated diabetic (TD) and control (C) rats. **Results:** MDA levels increased in D and TD. NOx levels did not differ among groups. Endothelial eNOS immunostaining reduced and adventitial iNOS increased in D and TD. The responsiveness of rings to acetylcholine, sodium nitroprusside, and phenylephrine did not differ among groups. **Conclusions:** NAC had no effect on the evaluated parameters and this experimental model did not promote vascular dysfunction despite the development of oxidative stress. Arq Bras Endocrinol Metab. 2010;54(6):530-9

Keywords
Oxidative stress; diabetes; nitric oxide; vascular function; N-acetylcysteine

RESUMO

Objetivos: Verificar se um modelo experimental de diabetes por aloxana promove estresse oxidativo, reduz a disponibilidade de óxido nítrico e causa disfunção vascular, e avaliar o efeito da N-acetilcisteína (NAC) nesses parâmetros. **Métodos:** Ratos diabéticos por aloxana foram tratados com NAC por quatro semanas. Níveis plasmáticos de malondialdeído (MDA) e nitrito/nitrato (NOx), imunomarcação para óxido nítrico sintase endotelial e induzida (eNOS e iNOS) e reatividade vascular da aorta foram comparados entre ratos diabéticos (D), diabéticos tratados (TD) e controles (C). **Resultados:** O MDA aumentou nos grupos D e TD. O NOx não diferiu entre os grupos. A marcação da eNOS no endotélio reduziu e a da iNOS na adventícia aumentou nos grupos D e TD. A responsividade dos anéis vasculares à acetilcolina, nitroprussiato de sódio e fenilefrina não diferiu entre os grupos. **Conclusões:** A NAC não teve efeito sobre os parâmetros avaliados. Esse modelo experimental não promoveu disfunção vascular apesar do desenvolvimento de estresse oxidativo. Arq Bras Endocrinol Metab. 2010;54(6):530-9

Descritores
Estresse oxidativo; diabetes; óxido nítrico; função vascular; N-acetilcisteína

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INTRODUCTION

Vascular diseases are the main causes of death and disability in people with diabetes (1). Oxidative stress is the ultimate cause of structural and functional changes to vascular cells in diabetic patients, and the superoxide anion ($\bullet O_2^-$) is responsible for the reduced nitric oxide (NO) bioavailability (2). The reaction between $\bullet O_2^-$ and NO forms peroxynitrite ($ONOO^-$) (3), which is able to promote lipid peroxidation (4). Moreover, $ONOO^-$ can oxidize tetrahydrobiopterin (BH_4),

leading to a phenomenon known as "nitric oxide synthase (NOS) uncoupling", which in turn results in $\bullet O_2^-$ synthesis instead of NO production by NOS (5).

Despite the overwhelming evidence of decreased NO bioavailability in diabetes (3,6), investigations regarding vascular reactivity exhibit quite conflicting results, thereby jeopardizing the exact participation of NO in diabetic dysfunctions. There are many reports showing impaired endothelium-dependent relaxation (7-10), while others demonstrate unaltered (11-13) and even augmented endothelium-dependent relaxation (14,15). Vascular reactivity experiments also provided controversial results when the endothelium-independent relaxation and the contraction responses of the smooth muscle were evaluated (8,10,12,16-19).

These conflicting data prompted us to investigate whether an experimental model of alloxan-diabetic rats promotes oxidative stress, reduces NO bioavailability, and causes vascular dysfunction. The effects of treatment with N-acetylcysteine (NAC) on the same variables were also examined. NAC, an antioxidant and glutathione precursor, was the drug of choice due to its wide capacity of attenuating oxidative stress in many diseases (20,21).

METHODS

Animals and induction of diabetes

Male Wistar rats (200-210 g) were housed on a 12h light-dark cycle, under constant temperature (22ºC), and were allowed free access to standard laboratory diet and drinking water. The investigation was approved by the Ethical Animal Committee of the University of São Paulo, Campus of Ribeirão Preto.

Diabetes was induced by a single intravenous tail vein injection of alloxan (40 mg/kg; Sigma, St. Louis, MO, USA) after a 24h fast with water ad libitum. Alloxan was freshly dissolved in 50 μL of sodium citrate (0.05 M, pH 4.5) immediately before use. An equivalent volume of citrate was administered by the same route to control animals. The rats were considered diabetic if they had glycemia ≥ 200 mg/dL at 7 days after alloxan injection. Blood glucose levels were determined using a glucometer and glucose test strips (Precision Plus Electrodes, Medisense Products, Bedford, USA) following a 12h fast.

Experimental design

The animals were randomly assigned to three groups (n = 10 each): control (C), diabetic (D), and treated diabetic (TD). NAC (Deg, São Paulo, Brazil) was administered to the TD group in the drinking water for 4 weeks. NAC was freshly dissolved in drinking water, to give a daily intake of 1.4 g/kg body weight (22) (average 1.270 ± 0.202 g/kg/day). The experimentation period was ended by anesthetizing the rats with thiopental sodium (40 mg/kg, intraperitoneal injection; Cristália, São Paulo, Brazil), laparotomy was then performed for blood sampling from the abdominal aorta. Finally, the animal was exsanguinated via abdominal aorta and thoracotomy was performed for thoracic aorta harvesting.

Glycemia and body weight measurements

Fasting blood glucose (determined as previously described) and body weight were measured immediately before alloxan injection, at 1 week after alloxan injection (when the diabetes was confirmed and the animals were randomly assigned to diabetic groups), and at 5 weeks after alloxan injection (end of the treatment period).

Plasma malondialdehyde measurement

Arterial blood samples were collected in tubes containing EDTA. After blood centrifugation (3000×g, 10 min, 4°C), plasma aliquots were removed and stored at –70°C until analysis. Plasma malondialdehyde (MDA) concentration was measured using a commercially available kit (Lipid Peroxidation Assay kit, Calbiochem, San Diego, CA, USA). The assay is based on the ability of a chromogenic agent to react with MDA, yielding a stable chromophore with maximal absorbance at 586 nm.

Plasma nitrite and nitrate levels

Arterial blood samples were collected in tubes containing heparin. After blood centrifugation (3000×g, 10 min, 4°C), plasma aliquots were removed and stored at –70°C. Samples were analyzed in duplicates for nitrite and nitrate (NOx) using an ozone-based chemiluminescence assay. Briefly, the plasma samples were treated with cold ethanol (1 volume of plasma: 2 volumes of ethanol for 30 min at –20°C) and centrifuged (4000×g, 10 min). NOx levels were measured by injecting 25 μL of the supernatant in a glass purge vessel containing 0.8% of Vanadium (III) in HCl (1 N) at 90°C, which reduces NOx to NO gas. A nitrogen stream was bubbled through the purge vessel containing Vanadium (III), then through NaOH (1 N), and then into an NO analyzer (Sievers® Nitric Oxide Analyzer 280, GE Analytical Instruments, Boulder, CO, USA).

Immunohistochemical assay for endothelial and inducible nitric oxide synthases

Thoracic aortic samples were immediately fixed in 10% buffered formalin solution for 24h and embedded in paraffin. Paraffin-embedded tissue blocks were sectioned at 3 μm. The sections were processed for endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) staining using a commercially available kit (DAKO LSAB®2 Kit, Peroxidase for use on RAT Specimens, DAKO Corporation, Carpinteria, CA, USA). In brief, the sections were fixed to slides pretreated with [3-aminopropyl]triethoxysilane (Sigma, St. Louis, MO, USA). Subsequently, the sections were deparaffinized and rehydrated through a descending alcohol series followed by distilled water. In sequence, the endogenous peroxidase activity was inactivated with hydrogen peroxide, and the sections were incubated with citrate buffer in a humidified heat chamber (Optisteam Plus, Krups North America Inc., New Jersey, USA) for antigen retrieval. The non-specific bindings were blocked with swine normal serum. The sections were incubated with polyclonal eNOS antibody (NOS3 (H-159): sc-8311, Santa Cruz Biotechnology, California, USA) at a dilution of 1:25 or monoclonal iNOS antibody (NOS2 (C-11): sc-7271, Santa Cruz Biotechnology, California, USA) at a dilution of 1:5. In sequence, the sections were incubated with secondary antibody of LSAB®2 kit and then with the streptavidin peroxidase of the same kit. Finally, the reactions were revealed with 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA), and the sections were counterstained with Harris' haematoxylin. As negative controls, sections were processed with the above procedures, but the primary or secondary antibodies were omitted.

All slides were observed and photographed with a high definition camera (AxioCam HRc, Zeiss, Germany) coupled to a microscope (Axioskop 2 plus, Zeiss, Germany). The immunostaining was graduated in 4 classes (0 – when the tissue was not marked, as in negative controls, 1 – when the tissue was marked lightly, 2 – when the tissue was marked moderately, and 3 – when the tissue was marked densely) by an observer blind to the animal group.

Morphometric analysis of the thoracic aorta medial layer

Thoracic aorta samples were immediately fixed in 10% buffered formalin solution for 24h and embedded in paraffin. Paraffin-embedded tissue blocks were sectioned at 3 μm, and sections were stained with Masson's trichrome. With the use of a high definition camera (AxioCam HRc, Zeiss, Germany) coupled to a microscope (Axioskop 2 plus, Zeiss, Germany), all slides were photographed in such a way that the vascular ring fitted a unique image. The area and the mean thickness of the thoracic aorta muscular layer were measured by an observer unaware of the animal group by means of the KS400 – Carl Zeiss version 2.0. To obtain the area of the muscular layer, each vascular ring was submitted to manual exclusion of the adventitia and perivascular tissues by the use of the "eraser" tool after zooming in. In sequence, the mean thickness of the muscular layer was obtained by choosing the lines (vertical, horizontal, or radial) that best fitted the vascular ring according to its orientation on the slide.

Vessel preparation and isometric tension recording

The thoracic aorta was carefully dissected free of connective tissue and immersed in a cooled and oxygenated Krebs solution (NaCl: 118.0, KCl: 4.7, $CaCl_2$: 2.5, KH_2PO_4: 1.2, $MgSO_4$: 1.66, glucose: 11.1, $NaHCO_3$: 25.0 (mM), pH 7.4). Ring segments of the thoracic aorta (4 mm in length) were prepared with great care, so as not to touch the intimal surface. In some segments the endothelium was removed by gently rubbing the intimal surface of the blood vessel with a pair of watchmaker's forceps. This procedure removes endothelium but does not affect the ability of the vascular smooth muscle to contract or relax.

Thoracic aorta segments were mounted in organ chambers (10 mL) filled with Krebs solution maintained at 37°C and bubbled with 95% O_2/5% CO_2 (pH 7.4). Each arterial ring was suspended by two stainless steel clips placed through the lumen. One clip was anchored to the bottom of the organ chamber, while the other was connected to a strain gauge for measurement of the isometric force using Grass FT03 (Grass Instrument Company, Quincy, MA, USA). The rings were placed at an optimal length-tension of 1.5 g (10,12) and allowed to equilibrate for 60 minutes with the bath fluid being changed every 15 to 20 minutes. Endothelial integrity was assessed qualitatively by the degree of relaxation caused by acetylcholine (Ach, 10^{-6} M; Sigma, St. Louis, MO, USA) in the presence of contractile tone induced by phenylephrine (Phe, 10^{-7} M; Sigma, St. Louis, MO, USA). For studies of endothelium intact vessels, the ring was discarded if relaxation with Ach was not 80% or greater. For studies of endothelium-denuded vessels,

rings were discarded if there was any measurable degree of relaxation. Sequentially, each ring was washed and re-equilibrated for 30 min. Aortic rings were then precontracted with Phe (10^{-7} M), and cumulative concentration-response curves were obtained after a stable plateau was reached. The endothelium-dependent relaxation was evoked by Ach (10^{-10} - 10^{-5} M) in intact aortic rings, while the endothelium-independent vasorelaxation was evoked by sodium nitroprusside (SNP, 10^{-10} - 10^{-5} M; Sigma, St. Louis, MO, USA) in denuded rings. Cumulative concentration-response curves for Phe (10^{-10} - 10^{-5} M) were determined on endothelium-intact and -denuded aortic rings. Concentration-response curves were also accomplished by pre-incubating the vascular rings with Nω-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M, a non-specific NOS inhibitor; Calbiochem, San Diego, CA, USA) for 50 minutes.

Statistical analysis

Statistical two-way repeated-measures ANOVA and Bonferroni post-test data evaluations were performed using the program SPSS 15.0 (Apache Software Foundation, 2000). Values were considered to be statistically significant at p values less than 0.05.

RESULTS

Glycemia and body weight

The initial glycemia and body weight did not differ among the groups. After the alloxan injection, diabetic and treated diabetic rats presented higher fasting blood glucose and lower body weight than control animals (p < 0.001). NAC treatment did not affect glycemia or body weight compared with diabetic animals without treatment (Table 1).

Plasma MDA and NOx levels

At the end of the 4-week treatment, plasma MDA levels were significantly higher in diabetic (p = 0.003) and treated diabetic animals (p = 0.015) than those in the control group. At the end of the same period, plasma NOx levels did not differ among the groups. Administration of NAC did not change plasma MDA or NOx levels compared with diabetic rats without treatment (Table 2).

eNOS and iNOS immunohistochemical analysis

The intensity of positive eNOS immunostaining decreased in the thoracic aorta endothelium of diabetic and treated diabetic rats compared with age-matched control rats (p = 0.007). However, NAC treatment did not affect the eNOS immunostaining in the endothelium. In the muscular layer, the eNOS immunostaining ranged from light to moderate and there were no differences among the three groups. In the adventitia, no positive immunostaining for eNOS was observed in any group (Table 3, Figure 1A).

In the endothelial and smooth muscle layers of thoracic aorta, the iNOS immunostaining was not evident in any group. However, the intensity of positive iNOS immunostaining increased in the adventitia of diabetic and treated diabetic rats compared with their age-matched control rats (p < 0.001). Again, NAC treatment did not affect the iNOS immunostaining (Table 3, Figure 1B).

Thoracic aorta muscular layer area and mean thickness

The area of the thoracic aorta smooth muscle of diabetic and treated diabetic animals decreased compared with control (p = 0.001 and p < 0.001, respectively). Moreover, NAC treatment promoted a reduction in the area of aorta medial layer of treated diabetic compared with non-treated diabetic rats (p = 0.029) (Table 4).

Table 1. Fasting blood glucose and body weight in control, diabetic and treated diabetic rats

		Control	Diabetic	Treated diabetic
Fasting glucose (mg/dL)	Day of alloxan injection	92.60 ± 8.87	90.10 ± 8.99	87.40 ± 16.15
	1 week after alloxan injection	90.10 ± 8.99	364.47 ± 53.18*	352.33 ± 56.94*
	5 weeks after alloxan injection	85.27 ± 8.73	435.83 ± 53.00*	401.67 ± 54.28*
Body weight (g)	Day of alloxan injection	190.70 ± 16.81	199.80 ± 11.99	198.36 ± 6.45
	1 week after alloxan injection	312.60 ± 20.66	216.42 ± 36.97*	200.39 ± 26.73*
	5 weeks after alloxan injection	514.71 ± 24.72	220.21 ± 67.97*	205.54 ± 42.52*

Values were obtained previous and after alloxan-induced diabetes in diabetic and treated diabetic rats or vehicle administration in control rats. All values are means ± SD (n = 10). * p < 0.001 *vs.* control (One-way ANOVA, Bonferroni's Multiple Comparison Test).

Table 2. Plasma MDA and NOx levels in control, diabetic and treated diabetic rats

	Control	Diabetic	Treated diabetic
MDA (µM)	4.45 ± 1.57	8.71 ± 2.42*	9.44 ± 3.31#
NOx (µM)	30.28 ± 4.67	28.68 ± 11.06	48.55 ± 31.00

Values were obtained 5 weeks after alloxan-induced diabetes in diabetic and treated diabetic rats or vehicle administration in control rats. All values are means ± SD (n = 10). * p = 0.003 *vs.* control, # p = 0.015 *vs.* control (One-way ANOVA, Games-Howell's Multiple Comparison Test).

Table 3. Degree of eNOS and iNOS immunostaining in the three layers of thoracic aorta of the control, diabetic and treated diabetic rats

		Control	Diabetic	Treated diabetic
Degree of eNOS immunostaining	endothelium	2.56 ± 0.53	1.61 ± 0.66*	1.64 ± 1.01*
	muscle	1.78 ± 0.83	1.65 ± 0.65	1.50 ± 0.52
	adventitia	0	0	0
Degree of iNOS immunostaining	endothelium	0	0	0
	muscle	0	0	0
	adventitia	0	3#	3#

The values were attributed according to immunostain intensity. All values are means ± SD (n = 10). * p = 0.007 *vs.* control, # p < 0.001 *vs.* control (Kruskal-Wallis Test).
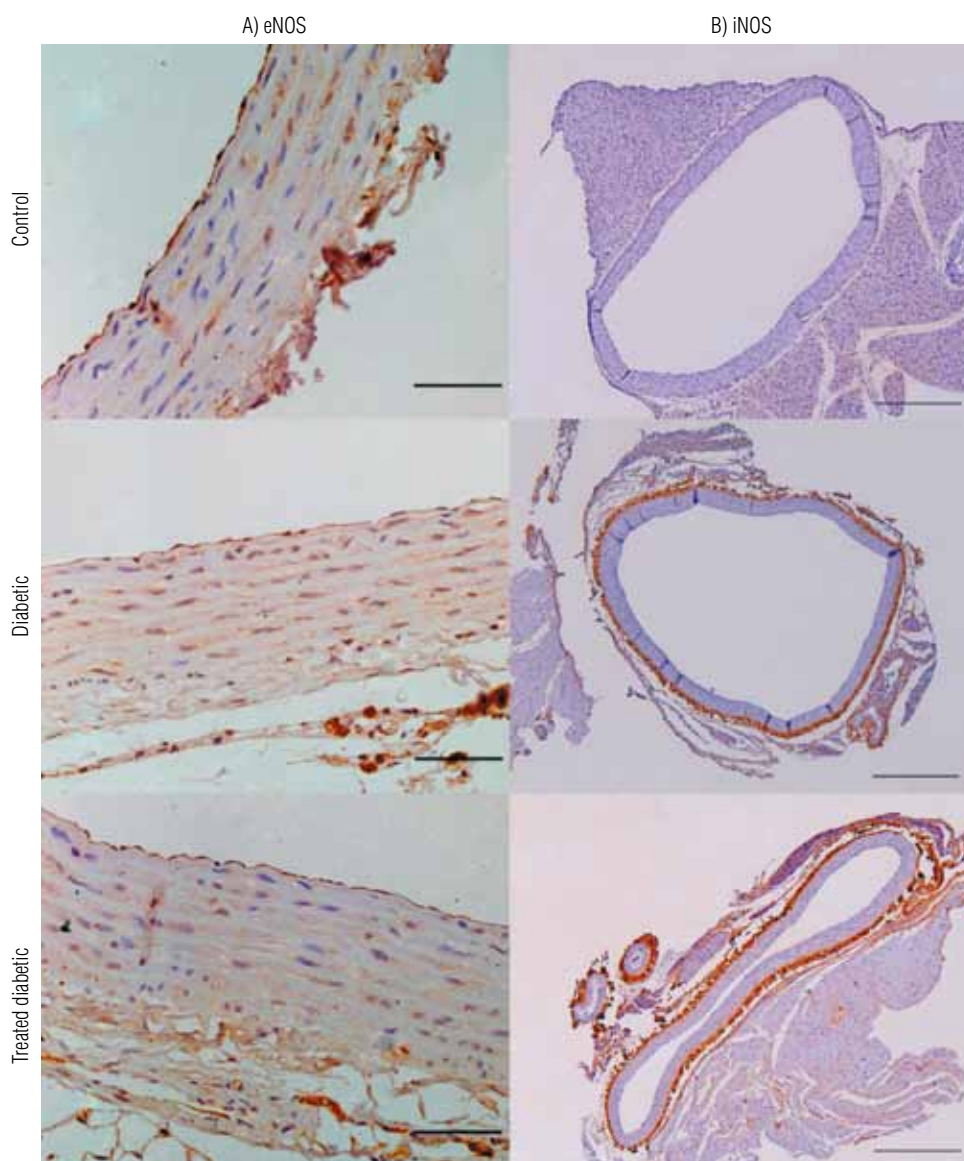

Figure 1. Photomicrography of thoracic aorta of control, diabetic, and treated diabetic animals. A) Endothelial nitric oxide synthase (eNOS) immunostaining: note that the three vessel layers were stained; however; the eNOS immunostaining is decreased in the endothelium of diabetic rats, treated or not (scale bar = 100 µm). B) Inducible nitric oxide synthase (iNOS) immunostaining: observe that only the adventitia of diabetic and treated diabetic rats was stained (scale bar = 0.5 mm).

Table 4. Area and mean thickness of thoracic aorta muscular layer in control, diabetic and treated diabetic rats

	Control	Diabetic	Treated diabetic
Area (mm²)	2.48 ± 0.48	1.62 ± 0.18[†]	1.08 ± 0.50[*#]
Mean thickness (mm)	0.14 ± 0.02	0.09 ± 0.01[*]	0.09 ± 0.00[*]

All values are means ± SD (n = 10). [†] p = 0.001 *vs.* control, [*] p < 0.001 *vs.* control, [#] p = 0.029 *vs.* diabetic (One-way ANOVA, Games-Howell's Multiple Comparison Test).

The mean thickness of the thoracic aorta muscular layer was also lower in diabetic and treated diabetic animals compared with age-matched controls (p < 0.001). However, administration of NAC had no effect on the mean thickness when diabetic rats were compared with treated diabetic animals (Table 4).

Thoracic aorta vascular function

In the relaxation study, changes in vascular wall tension were expressed as a percent of the relaxation of the maximal contraction following exposure to Phe, a convention that corrects inter-animal variability in the response of the tissue to the drug.

In the contraction study, changes in vascular wall tension were expressed as g of tension/dry weight (mg) of the aorta ring that generates the respective curve. This convention avoids measurement errors that may arise from inter-animal variability to the amount of tissue.

Endothelium-dependent relaxation

Ach caused concentration-dependent relaxation of the endothelium-intact aortic rings of the 3 groups in the absence of any inhibitor (p < 0.001) (Figure 2A) and in the presence of L-NAME (C: p = 0.007; D: p = 0.001; TD: p < 0.001) (Figure 2B).

In inter-group comparisons, no statistical differences were observed among groups when the concentration-response curves were achieved without NOS inhibition (Figure 2A). However, after L-NAME incubation, the endothelium-intact rings of diabetic and treated diabetic rats relaxed more than the rings obtained from control animals (p = 0.020 and p = 0.025, respectively); moreover, there was no statistical difference between treated and non-treated diabetic animals (Figure 2B).

Endothelium-independent relaxation

SNP caused concentration-dependent relaxation of endothelium-denuded aortic rings irrespective of group (p ≤ 0.001), with no inter-group statistical differences (Figure 3).
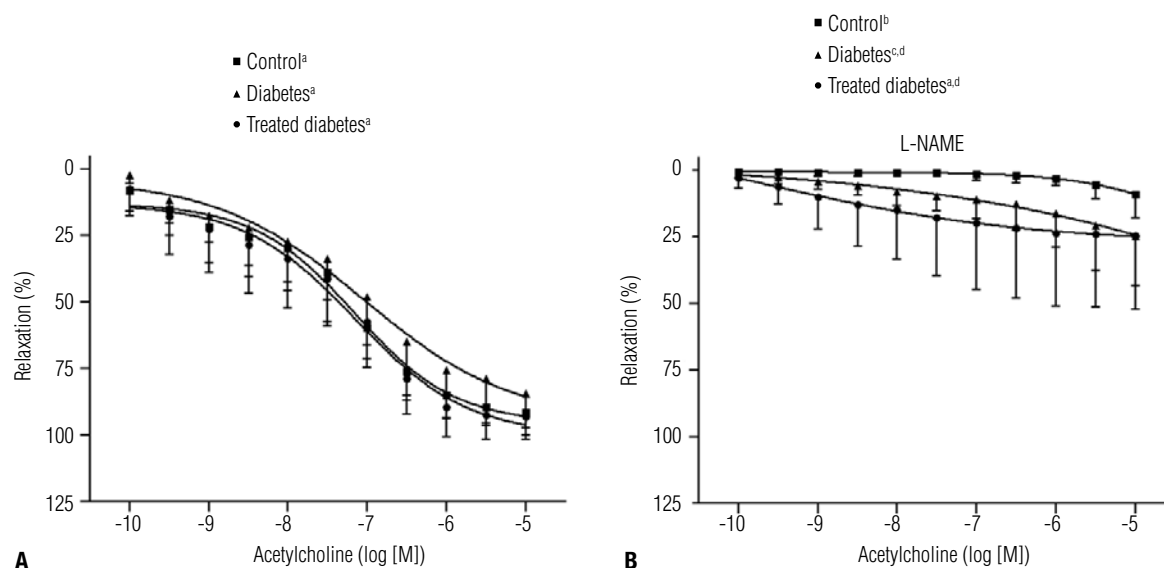

Figure 2. Vasodilator responses of rat thoracic aorta segments of control, diabetic, and treated diabetic groups. Phenylephrine-preconstricted aorta rings with endothelium were relaxed with cumulative concentrations of acetylcholine in the absence (A) and in the presence of L-NAME (B). All values are means + SD (n = 10). [a] p < 0.001 in within-group comparison; [b] p = 0.007 in within-group comparison; [c] p = 0.001 in within-group comparison; [d] statistical difference compared to control (C *vs.* D: p = 0.020 and C *vs.* TD: p = 0.025). Repeated measures two-way ANOVA, Greenhouse-Geisser Test (intra-group comparison), Games-Howell Test (inter-group comparison).

Contraction study

Phe caused concentration-dependent contraction of denuded aortic rings (Figure 4A) and endothelium-intact rings, pre-incubated or not with L-NAME (Figure 4B) irrespective of group (p < 0.001) and with no inter-group statistical differences (Figures 4A and 4B).
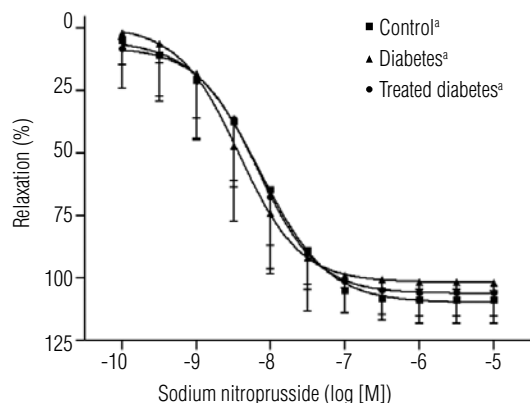

Figure 3. Vasodilator responses of rat thoracic aorta segments of control, diabetic, and treated diabetic groups. Phenylephrine-preconstricted aorta rings without endothelium were relaxed with cumulative concentrations of sodium nitroprusside. All values are means + SD (n = 10). [a] p ≤ 0.001 in within-group comparison. Repeated measures two-way ANOVA, Greenhouse-Geisser Test (intra-group comparison), Games-Howell Test (inter-group comparison).

DISCUSSION

The alloxan-induced diabetes model in rats has been widely employed (23-26), and the present study reinforces the ability of this diabetogenic drug to promote experimental diabetes in rats since the animals presented hyperglycemia and reduced body weight, as observed by many authors (8,10,12,18).

As already mentioned, hyperglycemia induces oxidative stress. This relationship has been demonstrated in many investigations, including our study. Increased levels of MDA or other biomarkers of oxidative stress have been observed in humans with type 1 (27) and type 2 diabetes (28) and in experimental models of type 1 (12,18,25,29) and type 2 diabetes (16,30,31). Taking this into consideration, NAC treatment aiming at reduced MDA levels represents a promising therapeutic opportunity. But, in spite of many reports reassuring its beneficial effect on oxidative stress (22,32-34), our study has failed to reduce MDA concentrations. We do not have a clear understanding about the reason why NAC treatment did not restore normal MDA levels, but we hypothesize that this failure could be related to NAC dose or late onset of NAC treatment. However, lower doses of NAC normalized ROS production in streptozotocin-diabetic rats (35), and NAC treatment initiated one week after diabetes induction also nor-
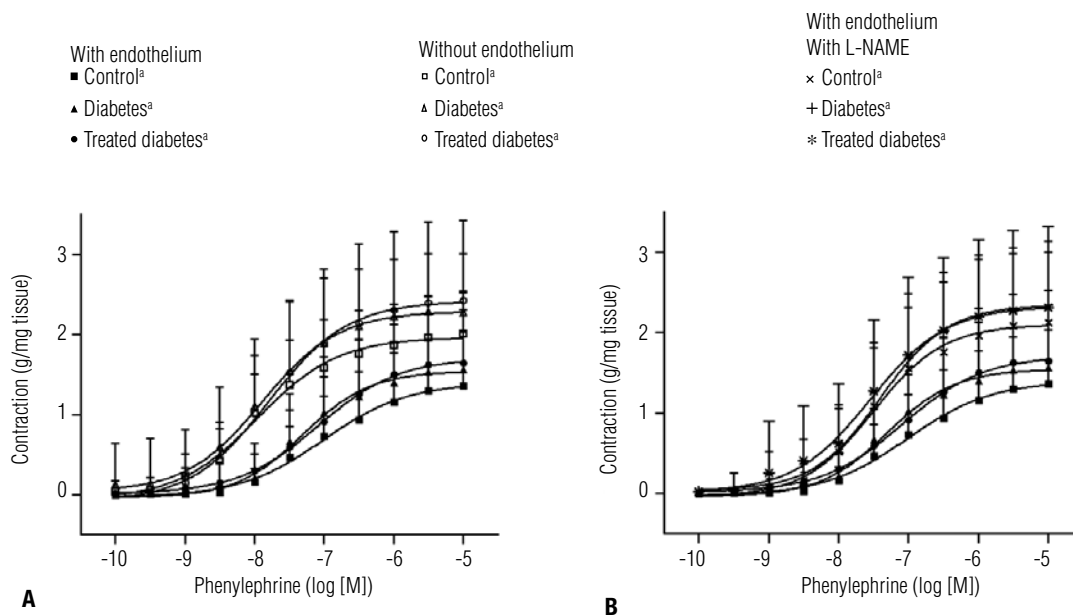
Figure 4. Concentration-response curves for phenylephrine in rat thoracic aorta segments of control, diabetic, and treated diabetic groups. A) Concentration-response curves were obtained from endothelium intact (full symbols) and denuded-rings (open symbols). B) Concentration-response curves were obtained from endothelium intact-rings before and after L-NAME incubation. All values are means + SD (n = 10). [a] p < 0.001 in within-group comparison. Repeated measures two-way ANOVA, Greenhouse-Geisser Test (intra-group comparison), Games-Howell Test (inter-group comparison).

malized the plasma and tissue levels of oxidative stress markers in streptozotocin-diabetic rats (34). Thus, another hypothesis to explain NAC failure in this study would be the diabetogenic agent used here, since all researches reviewed by us demonstrated reduction in oxidative stress after NAC treatment when streptozotocin was employed as a diabetogen. Furthermore, the activity of the γ-glutamylcysteine syntethase, the enzyme responsible for glutathione synthesis, was reduced in liver alloxan-diabetic rabbits, accounting for reduced glutathione concentrations (36). If this is so, reduced synthase activity may blunt the NAC antioxidant effect.

Increased oxidative stress is known to reduce NO bioavailability in diabetes (3,6). Indeed, researches that measure NO or its products and $\bullet O_2^-$ in endothelial cell culture (37), vessels (16,30), or plasma (38) have demonstrated an inverse relationship between NO and ROS in hyperglycemic conditions. However, plasma NOx levels did not differ among groups in our study, despite the presence of enhanced plasma MDA concentrations in diabetic rats. One reasonable explanation for this apparent contradiction of normal NOx levels in the presence of increased oxidative stress in diabetic rats is the incomplete inactivation of NO by ROS. This hypothesis is based on the inherent capacity of iNOS to produce greater amounts of NO (in the order of nanomols to micromols) (39) and on the fact that the aorta from our diabetic animals, treated or not, presented increased intensities of immunostaining for iNOS. Therefore, despite a reduction in eNOS immunostaining in the endothelium from the aorta of diabetic rats, which was observed by other authors (40,41), the increase in iNOS immunostaining could have made sufficient NO available to exert its biological effects. These ideas were also defended by Bojunga and cols., who had similar results (42).

Another result that reinforces our suggestion that NO bioavailability was not reduced in diabetic animals is the normal response of endothelium-intact rings from these animals to Ach, even after NOS inhibition. We also verified that after L-NAME incubation the endothelium-intact rings from diabetic rats, treated or not, presented a higher relaxing response to Ach than the control rings. However, we do not have sufficient data to affirm that this enhanced endothelium-dependent relaxation of the aorta from diabetic rats can be due to larger NO bioavailability, which was suggested as a possible vascular damaging agent in experimental diabetes (42). In addition, we cannot forget that other factors, such as the prostacyclin and the endothelium-derived hyperpolarizing factor can participate in relaxation.

Our results also show that this model of diabetes does not induce vascular smooth muscle dysfunction, neither endothelium-independent relaxation nor contraction aortic capability presented alterations. Previous reports also demonstrated unchanged vascular smooth muscle responsiveness to NO and (nor)adrenergic agonists in diabetic rings (10,12,43). Indeed, the investigations that observed enhanced vascular smooth muscle responsiveness to contracting agents related it to impaired endothelial NO synthesis during the evocation of a contraction (18,19). However, our graphs show that both NOS inhibition and endothelium removal resulted in the same effect: an increase in contracting response, leading to the supposition that NO availability was not impaired in this model of experimental diabetes.

We also observed that the area and the mean thickness of the muscular layer of the aorta were decreased in diabetic rats, treated or not, which is compatible with the lower body weights of these animals and emphasizes the need for adopting a correction factor for concentration-response curve comparisons. The treated diabetic animals presented lower aortic smooth muscle area than non-treated diabetic rats, but we believe that this difference can be assumed as an artifact. This suggestion is based on the fact that all vascular segments obtained from treated diabetic animals collapsed when immersed in formalin. However, the mechanism or the reaction that leads to this collapse is unknown.

The possible explanations given for the development or not of vascular dysfunction in diabetes can be related to different glucose concentrations in the media during the vascular reactivity study (44), the duration of diabetes, the vascular bed (44-46), and the studied species (44,45). We credit the failure in the vascular dysfunction development of our model to insufficient diabetes duration, which was reinforced by a recent report showing that diabetes lasting 4 weeks does not promote vascular dysfunction, whereas diabetes lasting 8 weeks causes endothelium-dependent and independent relaxation impairment (17).

CONCLUSION

In summary, the experimental model of alloxan-induced diabetes in rats lasting 5 weeks is effective for the development of diabetes and oxidative stress but fails to promote vascular dysfunction. NAC administration for

4 weeks to these diabetic animals does not reduce oxidative stress, change NOx levels, or alter vascular function.

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