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LIPOPEROXIDATION AND VASCULAR REACTIVITY RESPONSE IN RAT MODELS OF STREPTOZOTOCIN-INDUCED DIABETES MELLITUS.

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Abstract:

The murine model of streptozotocin-induced diabetes mellitus has been widely used for many authors. However, there are meaningful discrepancies about the functional alterations reported; especially regarding vascular response. ***Objective:*** *The objective of the present study was to examine how the prolonged effects of diabetes mellitus induced by administering streptozotocin affects vascular reactivity, lipoperoxidation and endothelial function in aortic rings and if the administration of nicotinamide will partially protect those adverse outcomes. Performing parallel studies could contribute in part to clarify previous controversial results.*

Methods: *Four groups (n= 8 rats per group) were used; control, control + Nicotinamide, streptozotocin or streptozotocin + Nicotinamide. Animals were euthanized after 20 weeks of streptozotocin and/or Nicotinamide administration. Glucose, lipoproteins plasma levels or lipid peroxidation in liver, heart and thoracic aorta were determined. Thoracic aorta was dissected and vascular reactivity to phenylephrine, isoproterenol, sodium nitroprusside or carbachol was determined.* ***Results:*** *Diabetic rats ingested more food and water, and lost more weight than control rats. The lipid peroxidation was higher in heart, liver and aorta in diabetic rats compared to control rats. In diabetic rats, total cholesterol, Low Density Lipoprotein and Triglyceride were higher and High Density Lipoprotein was lower than in control rats. The vasoconstrictor response to phenylephrine in streptozotocin groups was lower than streptozotocin + Nicotinamide and control groups. Diabetic animals showed a lower vasorelaxation response to carbachol and isoproterenol, but there was no significant difference in vascular response to sodium nitroprusside among groups.* ***Conclusions:*** *The prolonged effect of streptozotocin -induced diabetes increases chronically glycemia and alters vasodilator responses via different from the effect of oxidative stress. The partial protection with nicotinamide reduced vasomotor responses, suggesting that the artery is a major cause of the hypertensive status that characterizes the disease.*

Keywords: *Diabetes; endothelial dysfunction, animal model.*

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INTRODUCTION:

The advance in both prevention and treatment of diabetes depends largely on the understanding of the pathophysiological mechanisms involved in the alterations produced by this disease [1] .The use of rats as animal models still occupies a prominent place in the study of diabetes mellitus. Streptozotocin is one of the diabetogenic agents widely used to mimic diabetes mellitus in animals. It is generally used at doses ≥ 45 mg / kg body weight, via i.v or i.p in rats to produce both type 1 diabetes mellitus and with previous administration of nicotinamide to simulate the type 2 diabetes mellitus disturbances [2]. Nicotinamide is a substance able to partially protect the pancreatic beta cells against the cytotoxic effect of streptozotocin [3]. However, there is a large discrepancy of results related to vascular response. Among the most common causes of these discrepancies, it can be mentioned the difference in the dose of the diabetogenic agent used, the elapsed time after inducing diabetes, the gender of the animals, the rat strain [4] [5] or the preparation of aortic rings which may generate a difference in the vascular response in the presence of both vasodilators and vasoconstricting agents. The endothelium is a dynamic tissue that is involved in modulating vascular response [6]. However, age is a factor contributing to the variability of its response regardless of pathologies that may present individuals [7]. Endothelial dysfunction is a key factor to develop vascular diseases which is attributed to hyperglycemia, the decreased production of NO and the increased production of free radicals in vascular tissue. On the other hand, it has been observed that the level of malondialdehyde (MDA) as a reference parameter of the oxidation / antioxidation balance may be associated with the increased damage to tissues caused by diabetes mellitus [8]. The aim of the present study was to examine the prolonged effects of streptozotocin-induced diabetes mellitus either without or with partial protection by nicotinamide on endothelial response in aortic rings and lipoperoxidation, using the same dose of streptozotocin during the same treatment time and similar experimental conditions.

MATERIALS AND METHODS:

All of the experimental procedures were conducted according to the recommendations of the Guide for the Care and Use of Laboratory Animals of the Mexican Council for Animal Care (NOM-062-ZOO-1999) and were approved by the National School of Biological Sciences Ethics and Biosecurity Committee.

Thirty-two rats, weighing between 250 -300g distributed in 4 groups (n=8 rats per group): 1) STZ (STZ-injected rats, 65 mg / kg body weight via ip), 2) control (sodium citrate buffer injected), 3) STZ + Nic (STZ-injected rats, 65 mg / kg body weight via ip after a partial protection by nicotinamide 150 mg / kg body weight via ip) or 4) control + Nic (sodium citrate buffer injected after a sodium nicotinamide injection) .

After 48 hrs, the concentration of blood glucose was measured with a glucometer via tail vein puncture to verify the induction of diabetes mellitus. Food and water were provided *ad libitum* and their intake was measured daily, while body weight was determined once per week during the twenty weeks of the experiment.

Glucose measurement and samples collection

At week twenty after STZ-induction of diabetes, blood samples were obtained from the tail of the rats in fasting condition (10 hrs). The glucose levels were determined in whole blood with a glucose meter (Optium medisense, Abbott Laboratories, Oxford, United Kingdom). After glucose measurements, blood samples (0.5 mL) were collected to determine total cholesterol, HDL-cholesterol and triglycerides (TG) levels using specific enzymatic-colorimetric kits. The samples were centrifuged at 3500 rpm for 15 min at 4°C. Plasma LDL-cholesterol levels were determined as reported in Keita et al. 2013 [9].

Lipid peroxidation assay:

Lipid peroxidation level in the aorta, heart and liver was determined by measuring the malondialdehyde (MDA) content, were determined with minor modifications which were detailed in Keita et al. 2013 [9].

Measurement of isometric force

At the end of the treatment, the rats were anesthetized with diethyl ether and euthanized by decapitation. The thoracic aorta was immediately dissected and placed in oxygenated, modified Krebs-Hanseleit solution (KHS) (NaCl, KCl, $NaHCO_3$, $CaCl_2$, $NaHPO_4$, $MgSO_4$, dextrose and EDTA). Briefly, it was cleaned free from connective tissue and cut into 3 mm long rings in an oxygenated (95% O_2, 5% CO_2) bath with 10 ml of KHS. The aortic rings were mounted on a transducer system (TSD125C-50 g, BIOPAC Systems Inc. Santa Barbara California, Model MP100). They were equilibrated for 45 min under a resting tension of 2.0 g. After contracting with phenylephrine (alpha-1-adrenergic agonist) (1×10^{-4} M) until reaching a stable plateau phase, carbachol (muscarinic agonist) (1×10^{-4} M) was added to the incubation media to verify the functionality of endothelium. The rings were precontracted with phenylephrine (1×10^{-4} M) until reaching again a stable plateau phase, then cumulative concentration-response curves to sodium nitroprusside (NO donor), isoproterenol (beta-

adrenergic agonist) or carbachol were determined from 1×10^{-8} to 1×10^{-4} M for the vasorelaxation studies. In another set of experiments, cumulative concentration-response curves to phenylephrine (1×10^{-8} to 1×10^{-4} M) were generated for the vasoconstriction studies**.**

Reagents:

(R)-(−)-phenylephrine hydrochloride, isoproterenol hydrochloride, sodium nitroprusside, streptozotocin and nicotinamide were all purchased from Sigma and Sigma-Aldrich, and carbachol was purchased from Research Biochemicals International. Total and HDL cholesterol were determined using commercially available enzymatic colorimetric assay kits (Randox Laboratories, Crumlin, United Kingdom).

Statistical analyses:

The results are presented as the means ± SEM, and one way ANOVA or two-way RM-ANOVA analyses were used to compare the mean values among the different groups, followed by further analysis with the Student-Newman–Keuls' post hoc test. In all cases, a P value of 0.05 or less (two-tailed) was considered statistically significant.

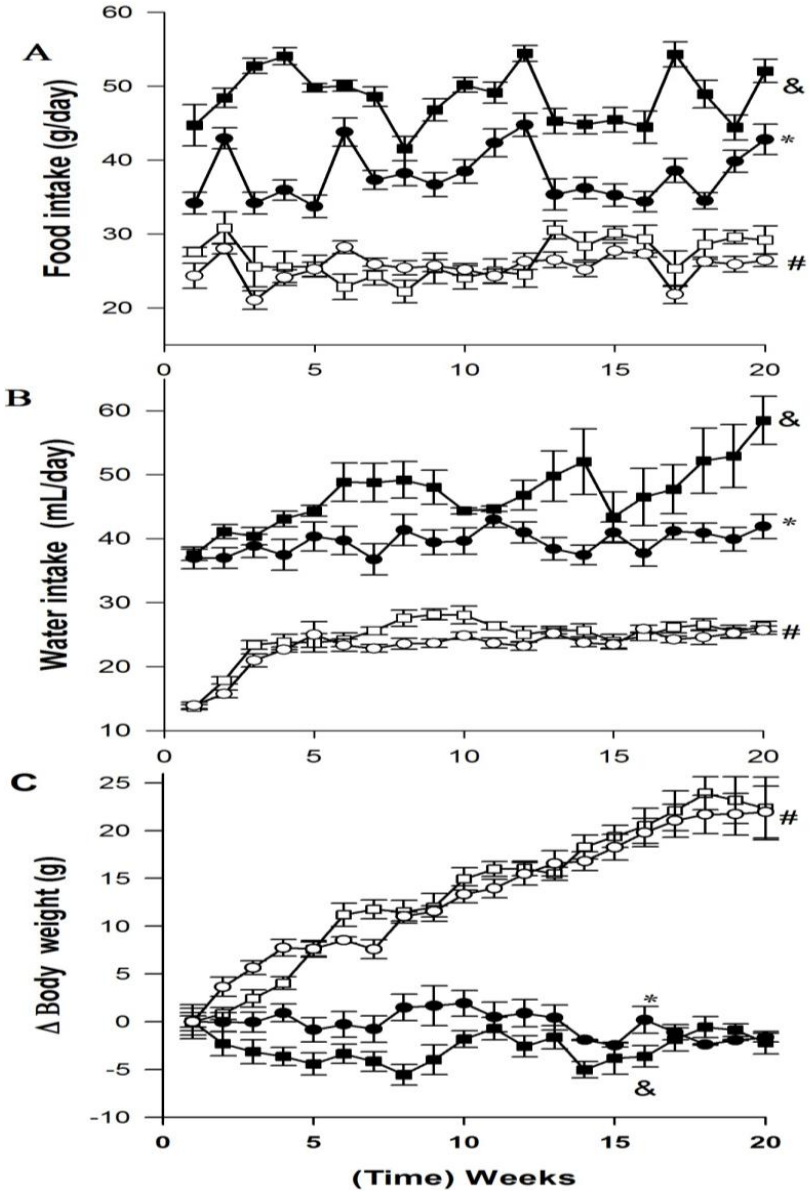

Figure 1: Food intake (A), water intake (B) and Δ body weight (C) of control (opened squares), control +Nic (opened circles), injected STZ (filled squares) or STZ + Nic(filled circles) rats during 20 weeks. # P < 0.05 significant difference compared with the control groups; & P < 0.05 significant difference compared with STZ group and * P < 0.05 significant difference compared with STZ+ Nic by 2 way RM ANOVA (n=8 per group).

RESULTS:

Ingestion of food, water and body weight gain.

Food intake (figure 1- A) and water consumption (figure 1- B) were higher in both STZ and STZ + Nic rats than in both control groups $p<0.05$. Both groups injected with STZ and STZ+Nic gained less weight than non-diabetic animals groups, $P \leq 0.05$ (figure 1- C).

Serum total cholesterol, lipoprotein cholesterol (HDL and LDL) and triglycerides (TG).

Total cholesterol, cholesterol LDL and TG were significantly higher in both STZ and STZ + Nic groups in comparison with the control groups ($P \leq 0.05$). HDL plasma levels were lower in the STZ groups as compared with the control groups ($P \leq 0.05$). No significant difference was observed between STZ and STZ + Nic groups ($P \geq 0.05$) (table 1).

Plasma glucose level:

Twenty weeks after streptozotocin injection, plasma glucose level was significantly higher in the streptozotocin injected groups in comparison with the control groups ($P \leq 0.05$). However, STZ +Nic group has lower glucose levels than STZ group (table 1).

Table 1: Plasma levels of total cholesterol, TG, HDL, LDL (mmol / L), and glucose (mg / dL) at 20 weeks after streptozotocin-induction of diabetes in rat.

	Control	Control+Nic	STZ	STZ+Nic
Total Colesterol (mmol/L)	1.17 ± 0.1^{a}	1.21 ± 0.1^{a}	1.74 ± 0.11^{b}	1.78 ± 0.24^{b}
TG (mmol/L)	0.51 ± 0.0^{a}	0.43 ± 0.0^{a}	0.74 ± 0.1^{b}	0.71 ± 0.1^{b}
HDL (mmol/L)	0.18 ± 0.02^{a}	0.19 ± 0.0^{a}	0.11 ± 0.0^{b}	0.13 ± 0.0^{b}
LDL (mmol/L)	0.82 ± 0.9^{a}	0.79 ± 1.1^{a}	1.21 ± 1.7^{b}	1.13 ± 1.7^{b}
Glucose (mg/dL)	85.5 ± 2.9^{a}	84.90 ± 2.2^{a}	366.20 ± 18.1^{b}	265.6 ± 18.5^{c}

Different letters $P \leq 0.05$, significant difference among the groups in each determination by one-way ANOVA (n = 8 per group). Values are expressed as the means ± SEM.

Lipid peroxidation

Malondialdehyde levels in the thoracic aorta, heart and liver of both STZ treated rats groups were significantly higher than the levels found in the control groups ($P \leq 0.05$; Table 2). Nicotinamide administration did not modify MDA levels.

Table 2: Comparison of malondialdehyde concentration in aorta, heart and liver

Group	Control	Control+ Nic	STZ	STZ+Nic
Arterial MDA (mM/mg protein)	$1.83 + 0.2^{a}$	1.48 ± 0.1^{a}	3.22 ± 0.4^{b}	3.05 ± 0.3^{b}
Heart MDA (mM/mg protein)	1.31 ± 0.3^{a}	1.27 ± 0.2^{a}	2.19 ± 0.1^{b}	2.00 ± 0.2^{b}
Hepatic MDA (mM/mg protein)	2.12 ± 0.2^{a}	1.85 ± 0.2^{a}	3.11 ± 0.4^{b}	2.83 ± 0.1^{b}

Values are expressed as the means ± SEM (n = 8 per group). MDA malondialdehyde. Different letters $P \leq 0.05$, significant difference compared with the control groups according to one-way ANOVA.

Constriction response to phenylephrine *in vitro*

In the concentration-response curves for phenylephrine, the aortic rings of STZ administered rats showed a significantly lower response to the vasoconstrictor α-agonist when compared to the response elicited in all other groups ($P \leq 0.05$) (Figure 2 A).

Relaxation response to isoproterenol, carbachol or sodium nitroprusside

The addition of phenylephrine (1×10^{-4} M) to the incubating medium induced a maximum contraction that reached a plateau. Subsequently, the concentration- response curves for isoproterenol, carbachol (1×10^{-8} to 1×10^{-4} M) or sodium nitroprusside (1×10^{-9} to 1×10^{-5} M) were determined for each group. The relaxing effect of isoproterenol and carbachol was significantly lower in the diabetic groups compared with the control groups ($P \leq 0.05$) (Figure 2 B and C). However, the relaxing effect of the two drugs was significantly lower in STZ groups compared with the STZ + Nic group ($P \leq 0.05$) (Figure 2 B and C). The relaxation response to sodium nitroprusside was similar among the experimental groups ($P \geq 0.05$) (Figure 2 D).

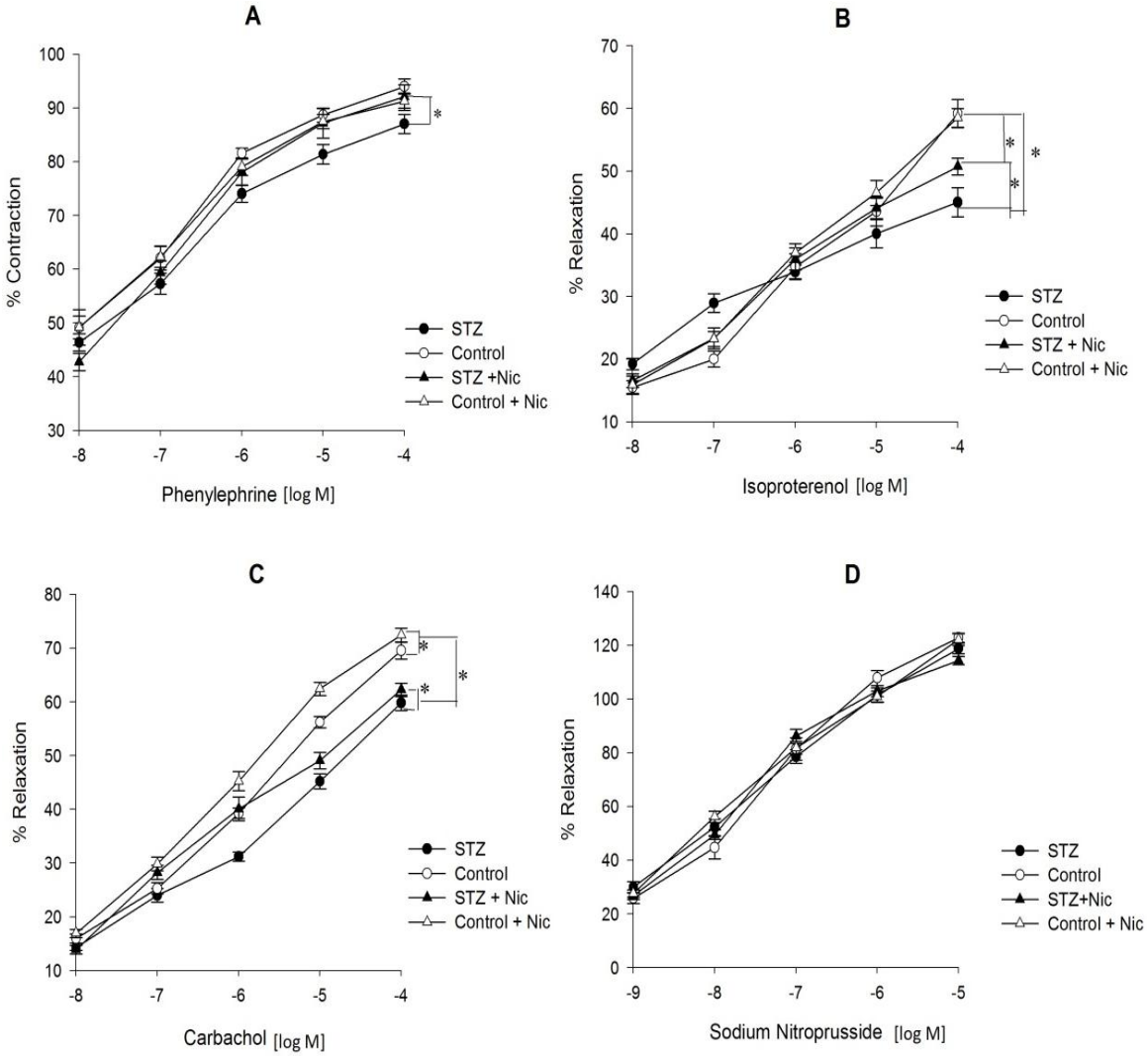

Fig 2: Effect of STZ-induced diabetes on responses to vasoconstrictor and vasodilator agonists or potent hypotensive agent. Concentration-response curves for phenylephrine (A), Isoproterenol (B), carbachol (C) or sodium nitroprusside (D) of thoracic aortic rings obtained from age-matched control, (n = 8 per group). Responses were measured as a percentage of the maximal contraction induced by phenylephrine (10 -8 to 10-4 M) and the relaxation as a percentage of the effect induced by isoproterenol or carbachol (1 × 10-8 to 1 × 10-4 M) or sodium nitroprusside (1 × 10-9 to 1 × 10-5 M). * $P \leq 0.05$ significant difference among groups, by 2-way RM ANOVA. Values are the means ± SEM.

DISCUSSION:

Experimental models of diabetes have played an important role in the understanding of the fundamental aspects of the pathophysiology, prevention, and/or treatment of this disease [1]. The STZ is a drug widely used in laboratory animals to induce experimental diabetes, which is characterized by hyperglycemia, polyuria, polydipsia and polyphagia as a result of the partial or total destruction of the pancreatic beta cells responsible for insulin production [10]. The results of this study coincide with those characteristic signs of diabetes mellitus including the loss of body weight. Several authors suggest that the body weight loss observed in streptozotocin-induced diabetes is associated with insulin deficiency and lack of energy at the cellular level which causes that the body undergoes through an intense catabolic process of macronutrients like protein and fatty acids to generate new energy sources. And the differences in food intake, water intake or in weight loss can be explained by the magnitude of destruction of pancreatic beta cells by STZ [11]. After several weeks of STZ administration, and in the absence of treatment, the damage to the pancreatic cells is intensified due to hyperglycemia, high levels of free radicals and/or hyperlipidemia [12]. In coincidence with many studies, it was noticed that the concentration of glucose is significantly higher in the STZ group compared to the STZ+Nic group at week 20 after their administration and it is well established that the elapsed time after inducing diabetes, is a critical factor in determining the degree of alterations observed in individuals with type 2 diabetes mellitus untreated [13]

STZ does not destroy all the population of pancreatic beta cells to a certain dose and the remaining beta-cells viable are capable to continue producing insulin but to a level that do not maintain effectively the homeostasis of plasma glucose [14]. Although insulin levels were not determined, the results suggest that remaining beta cells from STZ administered rats perform an extra effort trying to maintain glucose homeostasis. The increased plasma levels of cholesterol, TG, LDL or the reduced HDL plasma levels shown by STZ treated rats coincide with previous reports from this model [15], which are associated with cardiovascular disease in diabetic subjects. It has been shown that the high concentration of glucose in blood increases the levels of oxidative stress [12] which might disrupt the cell membrane by modifying its lipid composition [16] .In this study, the results show an increase in lipoperoxidation in the diabetic groups, which indicates an association between hyperglycemia and damage to the cell membrane. The cardiovascular disease is a complication of diabetes mellitus which is characterized by the impairment of endothelial function [17] that is commonly evaluated in studies *in vitro* on aortic rings from diabetic animals. The lower vasoconstrictor response to phenylephrine shown on aortic rings of STZ rats or STZ + Nic rats coincides with other reports [18]. Also, there are studies that did not show differences in the vascular response to phenylephrine in the STZ-diabetic rat [19] .Additionally, there are reports that support the increasing vasoconstrictor response [12] .The lower vasoconstrictor response evoked by phenylephrine of aortic rings from STZ rats may be explained by the insulin deficiency in this model of diabetes, in which it has been reported that the expression of the mRNA for alpha adrenergic receptor(α_{1B} or α_{1D}) was downregulated [20].The vasodilator response to isoproterenol of diabetic rats (STZ and STZ + Nic groups) compared to control groups is reduced. Previous studies support that the beta adrenergic receptors may be diminished by the limited availability of insulin [21]. In addition, it is known that the beta adrenergic receptors decrease as the individual gets older, At the beginning of the study, when STZ or STZ-Nic were administered, the rats had an average weight of 275g, which corresponds to 10 weeks of age, the age at which the animals were used to perform the endothelium studies were in adulthood, during week 30 of age. This condition may partly explain the decreasing response of beta-adrenergic receptors in smooth muscle to a maximum contraction of 60%, including control groups [17].The increase in the vasorelaxing response to carbachol elicited in the aortic rings from Nic rats (control + Nic or Nic + STZ groups) can be explained, on the one hand by the lowering effect of Nic on destruction of pancreatic beta cells and decreased both insulin secretion and hyperglycemia caused by STZ [20], which also contributed to protect the smooth muscle and endothelium by decreasing the levels of free radicals. It is also known that nicotinamide reduces plasma levels of VLDL and LDL [3]. Furthermore, the hyperglycemia observed in the groups STZ or STZ+ Nic, might be related in the decrease in vasorelaxing response to carbachol which is a drug that induces NO production in an endothelium dependent manner. The difference evoked by aortic rings from STZ+Nic group compared to those from STZ group may be associated to the antioxidant effect of nicotinamide [3]. In addition, the decreasing response to carbachol at endothelium level might be associated with the age effect also [17]. The vasodilation mediated by sodium nitroprusside did not change among groups. As in many studies, it is considered that in these models of diabetes the nitric oxide transduction pathway is not affected. However, it is considered that the

availability of NO is limited due to alteration of endothelial function found in these models [22].
Vasodilator responses in smooth muscle and endothelium were altered more importantly than vasoconstriction in diabetic rats and nicotinamide partially protected from these alterations, suggesting that the lower relaxation in the artery is a major cause of the hypertensive status that characterizes the disease. In this study, it has been shown that after five months of STZ-induced diabetes, the group of rats whose beta cell destruction was partially prevented by administration of Nic showed a lower hyperglycemia associated to a higher endothelial response than STZ treated rats. On the other hand, lipid peroxidation was not different among STZ groups, suggesting that oxidative stress is not the only cause of decreased endothelial-dependent relaxation in response to carbachol or isoproterenol that we found in the aorta from diabetic animals. Because glucose uptake in these cells is determined by glucose plasma levels, cytosolic glucose increases, accelerating its metabolism by nonoxidative glucose pathways (NOGPs) like the aldose reductase (AR) pathway and the production of advanced glycation end products (AGEs) specifically methylglyoxal and 3-deoxyglucose [23]. Cameron and Cotter (1992) [18]have found that endothelium-dependent relaxation to acetylcholine was reduced in aorta from rats after three months of STZ-induced diabetes which was prevented by treatment with the AR inhibitor (4-amino-2,6-dimethylphenyi-sulphonyil) nitromethane. Recently, it has been shown that glucose metabolism through this pathway was significantly increased in aortic homogenates and the endothelial-dependent relaxation (EDR) in response to acetylcholine was also impaired in aged rats compared to young rats [24] suggesting that the elapsed time after STZ induced diabetes also contributes to the severity of endothelium damage found in aged rats. Further studies are needed to demonstrate that AR pathway is less active in STZ-diabetic rats treated with Nic.

CONCLUSION:

This study demonstrates that STZ-induced diabetes at long term produces chronic hyperglycemia which increases lipoperoxidation in aorta and affects vascular reactivity. Aortic vasodilator responses in smooth muscle and endothelium were altered and the partial protection with nicotinamide attenuated these alterations.

Competing interests: The authors declare no competing interests.

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Abbreviations: HDL: High-density lipoprotein cholesterol; LDL: Low-density lipoprotein cholesterol; VLDL: very low-density lipoprotein; TG: Triglycerides. IP: Intraperitoneal; IV: Intravenous; KHS: Krebs Hanseleit solution; MDA: Malondialdehyde; NO: Nitric Oxide.

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