

# Unraveling vascular inflammation in diabetes mellitus: experimental evidence to guide clinical care

*Desvendando a inflamação vascular no diabetes melito: evidências experimentais para guiar o cuidado clínico*

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## ABSTRACT

**Introduction:** Endothelial dysfunction, a precursor of vascular diseases, is modulated by systemic inflammation, intensified in type 2 diabetes mellitus (T2DM). Streptozotocin (STZ)-induced models allow investigating the relationship between systemic inflammation and early vascular changes, especially in the aorta.

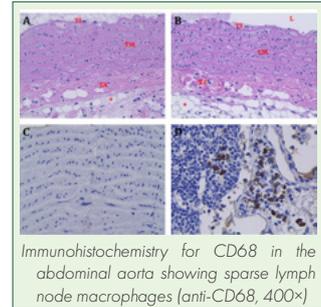
**Objective:** To evaluate systemic and vascular inflammation in STZ-induced diabetic Wistar rats by measuring plasma IL-6 and TNF- $\alpha$  and histological/immunohistochemical analysis (CD68) in different aortic segments.

**Method:** Experimental study with 20 male rats distributed as control (n = 10) and diabetic (n = 10). Plasma samples were analyzed for IL-6 and TNF- $\alpha$ , and aortic segments were subjected to histology and immunohistochemistry.

**Result:** Diabetic rats showed a significant increase in TNF- $\alpha$  (p < 0.05), confirming systemic inflammation. IL-6 remained unchanged. There was no detectable vascular inflammation in any of the aortic segments studied (histology and CD68).

**Conclusion:** The model demonstrated systemic inflammation associated with T2DM. There was no evidence of detectable vascular inflammation in any of the aortic segments studied. These findings reinforce the usefulness of systemic markers, such as TNF- $\alpha$ , in the early detection of cardiovascular risk in diabetics.

**KEYWORDS:** Inflammation. Diabetes mellitus. Endothelium. TNF- $\alpha$ , CD68.



## Central Message

In an experimental model of type 2 diabetes mellitus, increased systemic inflammation (elevated TNF- $\alpha$  levels), but no signs of detectable vascular inflammation, is observed in different segments of the aortic wall. These findings indicate that vascular aggression in diabetes may begin silently, before the onset of visible atherosclerosis, reinforcing the importance of early diagnosis and intervention.

## Perspective

The results of this study suggest that systemic inflammation in diabetes precedes any detectable vascular inflammation, analyzing several segments of the aorta. Thus, it opens the opportunity for therapies that aim to modulate the inflammatory response before the development of atherosclerosis. The experimental model of type 2 diabetes, with the use of streptozotocin (STZ), can contribute to the identification of early markers and guide strategies for the prevention of cardiovascular complications in diabetic patients.

## RESUMO

**Introdução:** A disfunção endotelial, precursora de doenças vasculares, é modulada pela inflamação sistêmica, intensificada no diabetes melito tipo 2 (DM2). Modelos induzidos por estreptozotocina (STZ) permitem investigar a relação entre inflamação sistêmica e alterações vasculares precoces, especialmente na aorta.

**Objetivo:** Avaliar inflamação sistêmica e vascular em ratos Wistar diabéticos induzidos por STZ, por meio da dosagem plasmática de IL-6 e TNF- $\alpha$  e da análise histológica/imuno-histoquímica (CD68) em diferentes segmentos da aorta.

**Método:** Estudo experimental com 20 ratos machos distribuídos em controle (n = 10) e diabético (n = 10). Amostras plasmáticas foram analisadas para IL-6 e TNF- $\alpha$ , e segmentos da aorta submetidos à histologia e imuno-histoquímica.

**Resultado:** Ratos diabéticos apresentaram aumento significativo de TNF- $\alpha$  (p < 0,05), confirmando inflamação sistêmica. IL-6 permaneceu inalterada. Não houve inflamação vascular detectável em nenhum dos segmentos aórticos estudados (histologia e CD68).

**Conclusão:** O modelo demonstrou inflamação sistêmica associada ao DM2. Não houve evidência de inflamação vascular detectável em nenhum dos segmentos da aorta estudados. Os achados reforçam a utilidade de marcadores sistêmicos, como TNF- $\alpha$ , na detecção precoce do risco cardiovascular em diabéticos.

**PALAVRAS-CHAVE:** Inflamação. Diabetes melito. Endotélio. TNF- $\alpha$ , CD68.

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## INTRODUCTION

Chronic low-grade inflammation is recognized as one of the main pathophysiological mechanisms associated with diabetes mellitus (DM), particularly in regard to increased cardiovascular risk. Patients are more likely to develop atherosclerosis, a phenomenon that results from the complex interaction between metabolic, immunological, and hemodynamic factors. In this scenario, pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), play a central role by modulating processes of insulin resistance, endothelial dysfunction, and vascular remodeling.<sup>1-3</sup>

At the same time, the activation of macrophages in the vascular wall is a critical event in the progression of local inflammation. The CD68 marker, classically used as an indicator of the presence of tissue macrophages, allows the evaluation of the intensity and regional distribution of the inflammatory response in different segments of the arterial tree. The heterogeneity of the aorta, with structural and hemodynamic variations among its segments, can influence cellular infiltration and inflammatory response, configuring potential regional patterns of vulnerability to vascular damage.<sup>4-6</sup>

The simultaneous investigation of systemic inflammatory mediators and cellular markers of vascular inflammation in an experimental model of diabetes provides important subsidies for understanding the interface between systemic inflammation and local inflammation. In addition, the study of these variables in different regions of the aorta can contribute to clarify the initial mechanisms of atherogenesis in diabetic individuals, offering perspectives for the identification of therapeutic targets and strategies for the prevention of cardiovascular complications.<sup>7-9</sup>

Thus, the present study aimed to evaluate the presence of systemic inflammation by means of plasma IL-6 and TNF- $\alpha$  assay, as well as to investigate the expression of CD68 in different segments of the aorta of diabetic rats, seeking to identify possible regional patterns of vascular inflammation associated with diabetes.

## METHOD

This is an experimental study, approved by the Ethics Committee on the Use of Animals of the Biological Sciences Sector (CEUA-UFPR), protocol No. 23075.065293/2022-16, in accordance with Law No. 11,794/2008 and current animal experimentation standards (Figure 1).

Twenty male Wistar rats (*Rattus norvegicus*, Var. *Albinus*), from previous mating, and kept in standard plastic cages, under a 12-h light/dark cycle, temperature of  $23 \pm 1^\circ\text{C}$ , with free access to water and commercial feed (Nuvital Nutrients Ltda, Curitiba, PR). The animals were randomly distributed into 2 groups: control (CG,  $n = 10$ ) and diabetic (GD,  $n = 10$ ).

Induction occurred on the 5th postnatal day, by single intraperitoneal injection of streptozotocin (STZ, 100 mg/kg), dissolved in 10 mM citrate buffer (pH 4).<sup>51</sup> The animals in the control group received only the citrate

buffer. Confirmation of diabetes was performed at 80 days of life, by glucose tolerance test (GTT).

During the experiment, the animals were weighed weekly and their growth was evaluated monthly by the Lee index. At 90 days, they were euthanized by decapitation, with blood collected in heparinized tubes, centrifuged at 4528 g for 7 min. The plasma obtained was stored at  $-80^\circ\text{C}$ . The aorta artery was removed and segmented (arch and abdominal), fixed in formalin and subsequently stored in 70% alcohol for histological and immunohistochemical analyses.

Plasma concentrations of interleukin-6 (IL-6) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) were determined by enzyme-linked immunosorbent assay (ELISA), as instructed by the manufacturers (Biomatik, Ontario, Canada). Histological analysis (hematoxylin-eosin and Gomori's trichrome) and immunohistochemistry for CD68 were performed at the Ioshii Pathology Diagnostics and Consulting LTDA Laboratory, Curitiba-PR.

## Statistical analysis

The data were evaluated for normality using the Shapiro-Wilk test. Comparisons between groups were made using Student's t-test or the Mann-Whitney test, considering  $p < 0.05$  as significant.

## RESULT

Body mass gain was monitored weekly from 4 to 13 weeks of age. No significant differences were observed between the groups in body mass gain or in the area under the curve (AUC) of the Lee index.

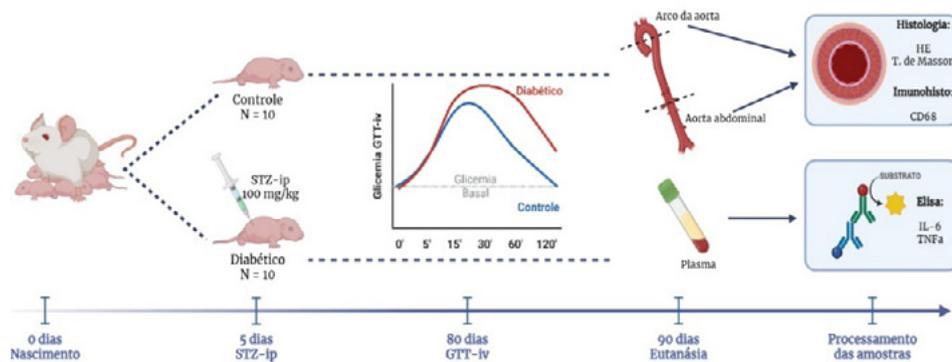
In the intravenous glucose tolerance test (ivGTT), performed at 80 days, the diabetic animals showed marked glucose intolerance compared to controls ( $p < 0.0001$ , Figure 2).

ELISA analysis revealed significantly higher plasma levels of TNF- $\alpha$  in the diabetic group ( $p < 0.0001$ ), with no differences in IL-6 concentrations between the groups (Figure 3).

The histopathological evaluation of the aortic segments showed exuberant endothelial cells, myxoid deposits and vacuolar degeneration ranging from mild to severe in diabetic animals. No macrophages were detected in histology or immunohistochemistry for CD68, except for a few macrophages in the lymph node of 1 sample (Table and Figure 4). The control group showed no alterations. There was no statistical difference between the segments of the aortic arch and the abdominal aorta.

## DISCUSSION

Experimental models of streptozotocin (STZ)-induced diabetes are essential tools to understand the inflammatory and atherosclerotic mechanisms associated with chronic hyperglycemia. Intraperitoneal administration of STZ on the 5th postnatal day, as used in this study, reproduces relevant characteristics of type 2 diabetes mellitus (DM2) and allows early evaluation of vascular alterations. This route of administration presents



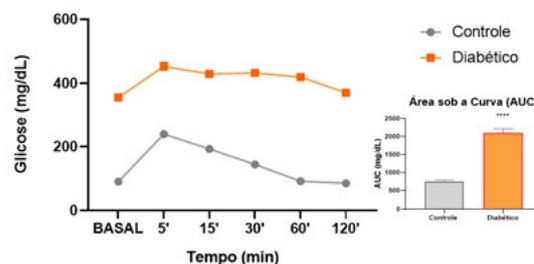
Legend: STZ-ip: Intraperitoneal Streptozotocin; GTT-iv: Intravenous glucose tolerance test.

**FIGURE 1** – Scheme of the experimental design

**TABLE** – Histopathological and immunohistochemical findings in the aortic segments of diabetic rats

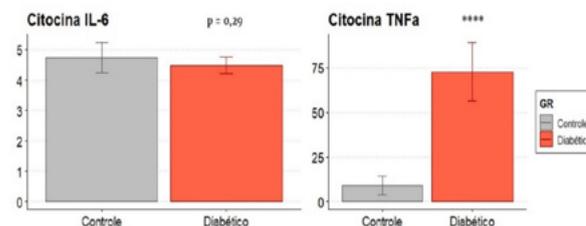
DIABETIC GROUP	Thickness	Cells Endothelial Lush	Deposits Myxoids	Degeneration Vacuolar	Cells CD68
Animal 11					
Sample 1 AA	0,3531	N	S	2	
Sample 2 AA	0,2568	S	S	2	
Sample 3 AAB	0,1947	N	N	1	
Sample 4 AAB	0,1605	N	N	0	
Animal 12					
Sample 1 AA	0,321	S	S	3	
Sample 2 AA	0,2889	N	S	2	
Sample 3 AAB	0,1798	N	N	1	
Sample 4 AAB	0,1605	S	S	2	
Animal 13					
Sample 1 AA	0,2054	N	N	1	
Sample 2 AA	0,1926	S	S	2	
Sample 3 AAB	0,1477	N	N	1	
Sample 4 AAB	0,1412	N	N	1	
Animal 14					
Sample 1 AA	0,2568	S	S	3	
Sample 2 AA	0,1926	N	S	2	
Sample 3 AAB	0,1605	N	N	1	
Sample 4 AAB	0,1605	N	S	2	
Animal 15					
Sample 1. AA	0,3852	S	S	3	
Sample 2 AA	0,2568	S	S	3	
Sample 3 AAB	0,1733	N	N	1	
Sample 4 AAB	0,1605	N	N	1	
Animal 16					
Sample 1. AA	0,2247	N	N	1	
Sample 2 AA	0,1926	N	S	2	
Sample 3 AAB	0,1605	N	N	1	
Sample 4 AAB	0,1541	S	S	2	
Animal 17					
Sample 1 AA	0,2568	S	S	3	
Sample 2. AA	0,2375	N	N	1	
Sample 3 AAB	0,1605	N	N	1	
Sample 4 AAB	0,1605	N	S	2	
Animal 18					
Sample 1 AA	0,3338	S	S	3	
Sample 2 AA	0,2531	S	S	3	
Sample 3 AAB	0,1669	N	N	1	
Sample 4 AAB	0,1412	N	N	1	
Animal 19					
Sample 1 AA	0,2375	S	S	2	
Sample 2 AA	0,2054	N	S	3	
Sample 3. AAB	0,1605	N	N	1	
Sample 4 AAB	0,1541	N	N	1	
Animal 20					
Sample 1 AA	0,244	S	S	3	
Sample 2 AA	0,1798	S	S	2	
Sample 3 AAB	0,1605	N	N	1	
Sample 4 AAB	0,1605	N	N	1	

Legend: 4 samples from each animal were analyzed (2 from the aortic arch and 2 from the abdominal aorta). Endothelial cell hyperplasia, myxoid deposits, vacuolar degeneration (grades 1 to 3) and presence of CD68-positive macrophages were evaluated. Abbreviations: AA, aortic arch; AAB, abdominal aorta; N, no; S, yes; grade 1, mild; grade 2, moderate; grade 3, intense.



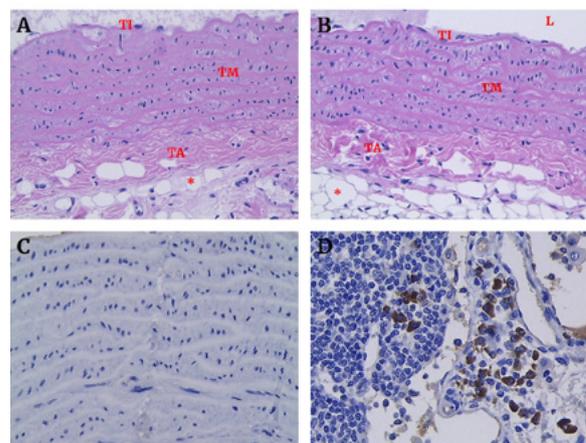
Caption: Blood glucose was measured at 5, 15, 30, 60 and 120 min after glucose injection. AUC values were calculated for each group. Normality was verified using the Shapiro-Wilk test and statistical comparison was performed using Student's t-test (\*\*\*\* p < 0.0001).

**FIGURE 2** – Intravenous glucose tolerance test (ivGTT) in control and diabetic rats at 80 days of age.



Legend: Normality was confirmed by the Shapiro-Wilk test and the comparison between the groups was performed by the Student's t-test (\*\*\*\* p < 0.0001).

**FIGURE 3** – Statistical analysis of plasma levels of TNF-α and IL-6 in control and diabetic rats.



Legend: A) Hematoxylin-eosin (HE) staining of the abdominal aorta; B) HE of the aortic arch; C) immunohistochemistry for CD68 in the aortic arch showing absence of macrophages; D) immunohistochemistry for CD68 in the abdominal aorta showing sparse macrophages in the lymph node (anti-CD68, 400x).

**FIGURE 4** – Histological and immunohistochemical images representative of aortic segments of diabetic rats.

safety, efficacy, and applicability in experimental protocols.<sup>10-12</sup>

In the present study, diabetic induction resulted in persistent hyperglycemia at 80 days, confirming the effectiveness of the model. Despite the glycemic alteration, there was no significant weight loss, suggesting that, at this stage, the systemic metabolism of the animals was not yet severely impaired, a finding compatible with studies that describe the gradual progression of metabolic alterations in young models of STZ-induced DM.<sup>13,14</sup>

Serological analysis showed a significant increase in TNF- $\alpha$  in the diabetic group, while IL-6 levels remained unchanged. This inflammatory profile suggests immune imbalance: TNF- $\alpha$ , recognized as a central mediator of vascular inflammation, stimulates the expression of endothelial adhesion molecules, promotes leukocyte recruitment, and intensifies oxidative stress. The absence of IL-6 elevation may reflect immunologic exhaustion from chronic inflammation or early stage of disease. Studies indicate that IL-6 and TNF- $\alpha$  often act synergistically in atherogenesis, and the mismatch in this interaction can influence the chronology of vascular injury.<sup>15-17</sup>

From the morphological point of view, the presence of exuberant endothelial cells, myxoid deposits, and vacuolar degeneration, without detection of macrophages or foamy cells, suggests an initial structural alteration, prior to the inflammatory infiltrate typical of atherosclerosis. Negative immunostaining for CD68 reinforces the absence of significant macrophage infiltration. These findings are in line with studies that describe endothelial dysfunction as an early, potentially reversible event, in the sequence that culminates in inflammation and atherosclerotic plaque formation.<sup>18,19</sup>

The absence of detectable vascular inflammation at this stage does not invalidate the hypothesis of future progression to advanced atherosclerotic lesions. On the contrary, the isolated increase in TNF- $\alpha$  suggests systemic inflammatory activation capable of impacting the vascular wall over time. This moment may represent a critical window for therapeutic interventions aimed at the early modulation of the inflammatory response.<sup>20,21</sup>

Thus, this study contributes to the characterization of an experimental model of T2DM with potential for future investigations on the interaction between systemic inflammation and vascular remodeling. The integration of temporal analyses, including additional markers such as IL-1 $\beta$ , CRP, and VCAM-1, combined with the functional assessment of vascular reactivity, may deepen the understanding of the sequence of events that link hyperglycemia to inflammation and arterial damage. These data can support the development of earlier and more effective diagnostic and therapeutic strategies in the context of cardiovascular complications of diabetes mellitus.

## CONCLUSION

The induction of type 2 diabetes mellitus with STZ on the 5th postnatal day proved to be effective, resulting

in persistent hyperglycemia and a significant increase in TNF- $\alpha$ , with no change in IL-6 levels and no loss of body mass. No tissue inflammation was detected in the aortic segments evaluated, suggesting that the model represents an initial stage of endothelial dysfunction. These results reinforce the potential of the model for studies on early mechanisms of vascular injury in diabetes mellitus type 2.

### Authors' contributions

Luciane Bittencourt Carias de Oliveira – Conceptualization, methodology, validation, writing, revision and editing.

Oswaldo Malafaia – Supervision.

Fernando Issamu Tabushi – Project management, writing – proofreading and editing.

Stephanie Rubianne Silva Carvalhal – Methodology.

Sérgio Ossamu Ioshii – Methodology, validation.

Rodrigo Schuh – Methodology, validation.

Laís Soares Rodrigues – Methodology, validation.

Thomas Horlem – Validation.

Nicolau Gregori Czezko – Project Management.

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