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Article

HISTOLOGICAL CHANGES IN RENAL TISSUE DURING THE ONSET OF DIABETES MELLITUS IN RATS AND ASSOCIATION WITH AGING

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Abstract

Diabetes Mellitus (DM) favors kidney remodeling, leading to diabetic kidney disease (DKD). Kidney histological alterations are identified after DKD installation, but have not been studied in the early stage of DM. We studied renal remodeling in Wistar rats at the early stage of DM, before DKD development, investigating alterations indicating a poor prognosis and whether age is an impact factor. Diabetes was induced with Alloxan. Groups: adult diabetic (DMA), elderly diabetic (DMI), adult sham control (CSA), and elderly sham control (CSI). After 30 days of confirmed diabetes, blood, urine, and kidney were collected. Both diabetic groups presented normal renal function. DMA and DMI showed tubular infiltration and hypertrophy, decreased Bowman's space, and reduced glomerular area. A decrease in the glomeruli number was observed in the DMI. An inverse correlation between the glomeruli number and the ApoB/ApoA ratio was observed on DMA. We conclude that histological changes characteristic of DKD can be observed in the early stages of DM, and age can have a role in the injury intensity. Tubular and glomerular alterations occur during DM installation and continue to evolve as DKD develops. Therefore, targeting the prevention of kidney remodeling could be a good therapeutic approach to prevent DKD.

Keywords: diabetes mellitus; diabetic kidney disease; inflammation; histology

1. Introduction

The progressive loss of pancreatic β -cells is a common characteristic of type 1 and type 2 Diabetes Mellitus (DM2), which will lead to less insulin availability and sustained hyperglycemia [1]. During hyperglycemia, increased formation of advanced glycation end products (AGEs) leads to greater generation of reactive oxygen species (ROS) [2,4]. ROS promotes the formation of free radicals, which induce oxidative stress and inflammation, leading to micro and macrovascular damage in target organs like the kidney [2,3]. Moreover, hyperglycemia stimulates greater extracellular matrix (ECM) formation, favoring tissue remodeling and diabetic kidney disease (DKD) development [5]. During

aging, this pathophysiological process may develop more prominently due to cellular senescence. Therefore, it is expected that the tissue damage may be more evident in advanced ages [6,7].

DKD is characterized by albuminuria and progressive decline in glomerular filtration rate (GFR), as well as morphological changes in renal tissue [8,9]. A study conducted in diabetic rats observed that after 6 weeks, the kidneys showed decreased renal function, along with glomerular and tubular damage. This was evidenced by expansion and thickening of Bowman's space, as well as tubular dilation and atrophy of tubular cells, respectively [8]. Glomerular and tubular lesions play a crucial role in the pathophysiology of DKD, leading to apoptosis, fibrosis development, and progressive loss of renal function [5,9]. Another study, also conducted in rats, observed that 15 days after diabetes induction, the animals exhibited mesangial expansion, acute tubular necrosis, thickening of the glomerular basement membrane, and inflammatory cell infiltration [10].

Inflammation involves the release of leukotrienes, which are chemotactic and stimulate immune cell infiltration, thereby increasing cytokine release. This promotes and initiates the inflammatory and fibrotic processes [11,12]. In a study conducted in diabetic mice, infiltration of T lymphocytes and macrophages, along with increased expression of pro-inflammatory factors, was observed in renal tissue after 8 weeks [11].

The harmful hyperglycemic stimulus also stimulates differentiation of pericytes into myofibroblasts, which migrate to the interstitium and increase collagen production. Collagen is the main component of the extracellular matrix (ECM) [13,14]. Therefore, increased formation of AGEs in the diabetic kidney is also associated with increased collagen deposition, triggering tissue remodeling and fibrosis [15,16]. A study with mice induced into diabetes observed that after 12 weeks, the animals showed changes in renal function, as well as increased glomerular deposition of collagen types 1, 4, and 6 [15]. Another study conducted in diabetic mice observed that after 16 weeks, the animals exhibited increased collagen deposition and higher expression of collagen types 1, 3, and 4 in the kidney [13].

Some studies suggest that in the early stages of renal injury, tubular changes precede glomerular changes, and both play independent roles in the progressive development of early renal function decline [9,17]. In obese rats induced into pre-diabetes, it was observed after 21 days that there were no histological changes in the kidney, despite increased tubular expression of the renal injury molecule NGAL and increased glomerular deposition of collagen 4 [18].

Most studies demonstrate renal histological changes alongside alterations in renal function parameters, reflecting the onset of DKD [8,9,11,15]. However, it is also important to elucidate at what point during diabetes onset the renal tissue remodeling begins, to provide more information about the early stages of DKD, before the loss of cellular function. Therefore, this study aimed to determine whether, in the early phase of diabetes onset, before the development of DKD, there are already renal morphological changes that may indicate a poor prognosis. Additionally, the study aimed to determine whether age is a factor impacting these alterations.

2. Results

The biochemical parameters show an increase in glycemia and HbA1c, demonstrating the installation of DM. All renal parameters are within the reference values, but there was a statistical difference between the DM groups when compared to their respective controls, for urea and plasmatic and urinary creatinine (Table 1).

Table 1. Biochemical paramathers in serum, plasma and urine. Values presented as mean ± DSVP and number of samples. Mann-Whitney and Student's t-test *P <0.05. 95% CI. Reference values for Wistar rats: Glycemia: 72 – 193 mg/dL; HbA1c: 4 – 6%; Urea: 45 - 80 mg/dL; Plasma Crea.: 0.24 – 1.20 mg/dL; Urine Crea.: 32 – 97.4 mg/dL; Creatinine clearance: 0.2 – 1.8 mL/min; Proteinuria: 8.8 – 33.2 mg/dL; Apo B/A Ratio: 0.7 – 1.2 [49-54].

Parameters	CSA	DMA	*p	CSI	DMI	*p
Glycemia (mg/dl)	132,4 ± 45, n= 5	462,7 ± 98, n= 9	0,001*	106,8 ± 9,2, n= 9	447,4 ± 125,7, n= 7	0,0002*

HbA1c (%)	4,5 ± 0,2, n= 5	9,0 ± 2, n=8	0,0016*	4,6 ± 0,05, n= 5	9,3 ± 1,3, n= 4	0,008*
Urea (mg/dL)	48,5 ± 6,9, n= 5	135,5 ± 38,3, n= 6	0,0043*	45,4 ± 9,1, n= 9	93,2 ± 53,8, n= 7	0,023*
Plasmatic Crea. (mg/dL)	0,6 ± 0,1, n= 5	0,7 ± 0,1, n= 6	0,0065*	0,5 ± 0,1, n= 9	0,7 ± 0,1, n= 7	0,01*
Urinary Crea. (mg/dL)	60,7 ± 11,2, n=5	21,7 ± 10,3, n= 9	0,001*	63,2 ± 40,8, n= 9	26,4 ± 14,5, n= 6	0,0360*
Creatine clearance	0,3 ± 0,2, n= 5	0,2 ± 0,0, n= 4	0,1	0,6 ± 0,2, n= 5	0,3 ± 0,1, n= 4	0,0317*
Proteinuria (mg/dl)	16 ± 3,1, n= 5	12,5 ± 2,3, n= 9	0,0460*	28,3 ± 15,7, n= 9	19,5 ± 11,4, n= 6	0,11
Apo B/A ratio	1,2 ± 0,7, n= 5	0,8 ± 0,2, n= 6	0,6623	0,8 ± 0,3, n= 9	0,4 ± 0,2, n= 6	0,1
Ureia/creatinine ratio	33,6 ± 4,6, n= 5	63 ± 20, n= 4	0,0145*	63,5 ± 23,8, n= 5	43,7 ± 24, n= 5	0,54

Histological analysis confirmed the validity of the experimental model by revealing significant morphological alterations in the pancreas of both the DMA and DMI groups. In the DMA group (Figure 1A), the exocrine parenchyma shows moderate inflammatory infiltrate and islet atrophy, when compared to the CSA group (Figure 1B), which exhibited preserved exocrine parenchyma, with large and well-defined islets. Similarly, pancreatic sections from the DMI group (Figure 1C) show in the exocrine parenchyma a moderate inflammatory infiltrate and islet atrophy, in contrast to the CSI group, where the exocrine parenchyma is preserved and the islet are preserved (Figure 1D).

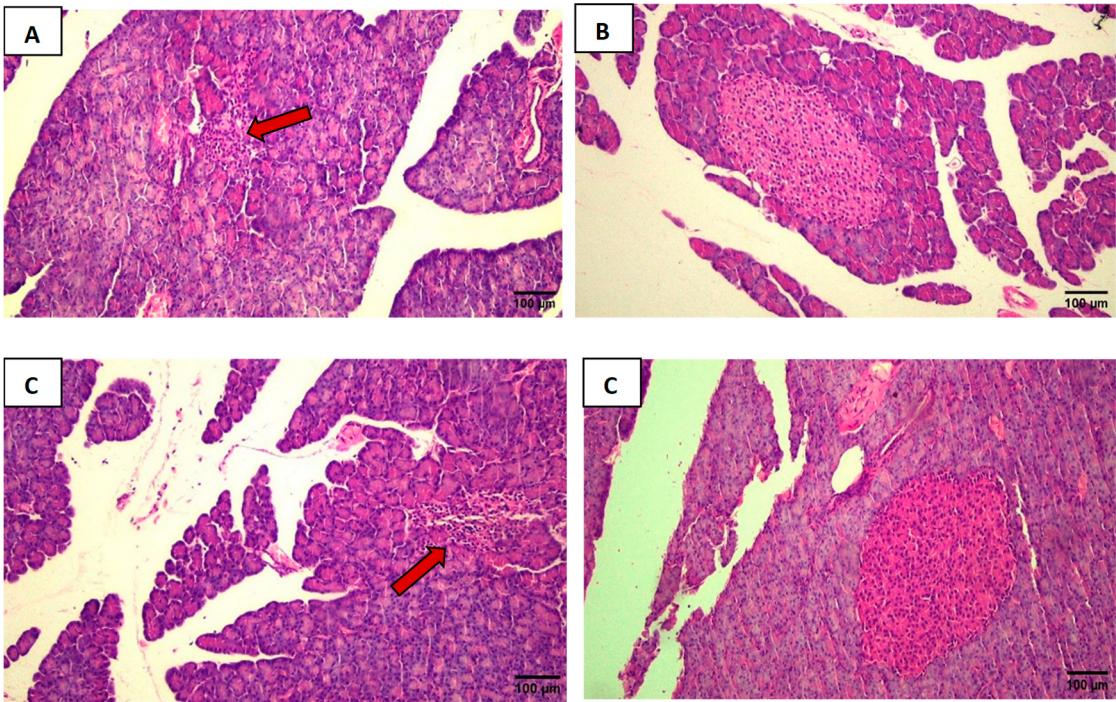


Figure 1. The black arrows indicate the islets of Langerhans. It is possible to note the lesion of the islets of Langerhans and a reduction in cellular density in the DMA group (A) compared to the CSA group (B). The same lesion is observed in the DMI group (C) when compared to the CSI group (D). Photos were captured using a Nikon Eclipse E200 microscope at 100x magnification.

In the morphological analysis of DMA renal tissue, 20 fields were analyzed, and 92% showed intense inflammatory infiltrate. The same was observed in the DMI group in 82% of the 20 analyzed fields. Additionally, the analyzed fields of the DMA and DMI groups presented tubular hypertrophy (Figures 2A and 2C), which is characterized by the expansion of the cytoplasmic volume and, consequently, enlargement of the tubule. The respective controls were used for the comparison

(Figures 2B and 2D). 26% of the 20 analyzed fields in the DMA group, both tubular hypertrophy and tubular lesions were observed. The tubular lesions were evidenced by the tubules with big tubular lumens with thin tubular epithelium. The same was noted in 10% of the 20 analyzed fields in the DMI group (Figures 2E and 2G) compared to their respective controls (Figures 2F and 2H), where renal tissue with preserved parenchyma and tubules, and glomeruli showed regular size.

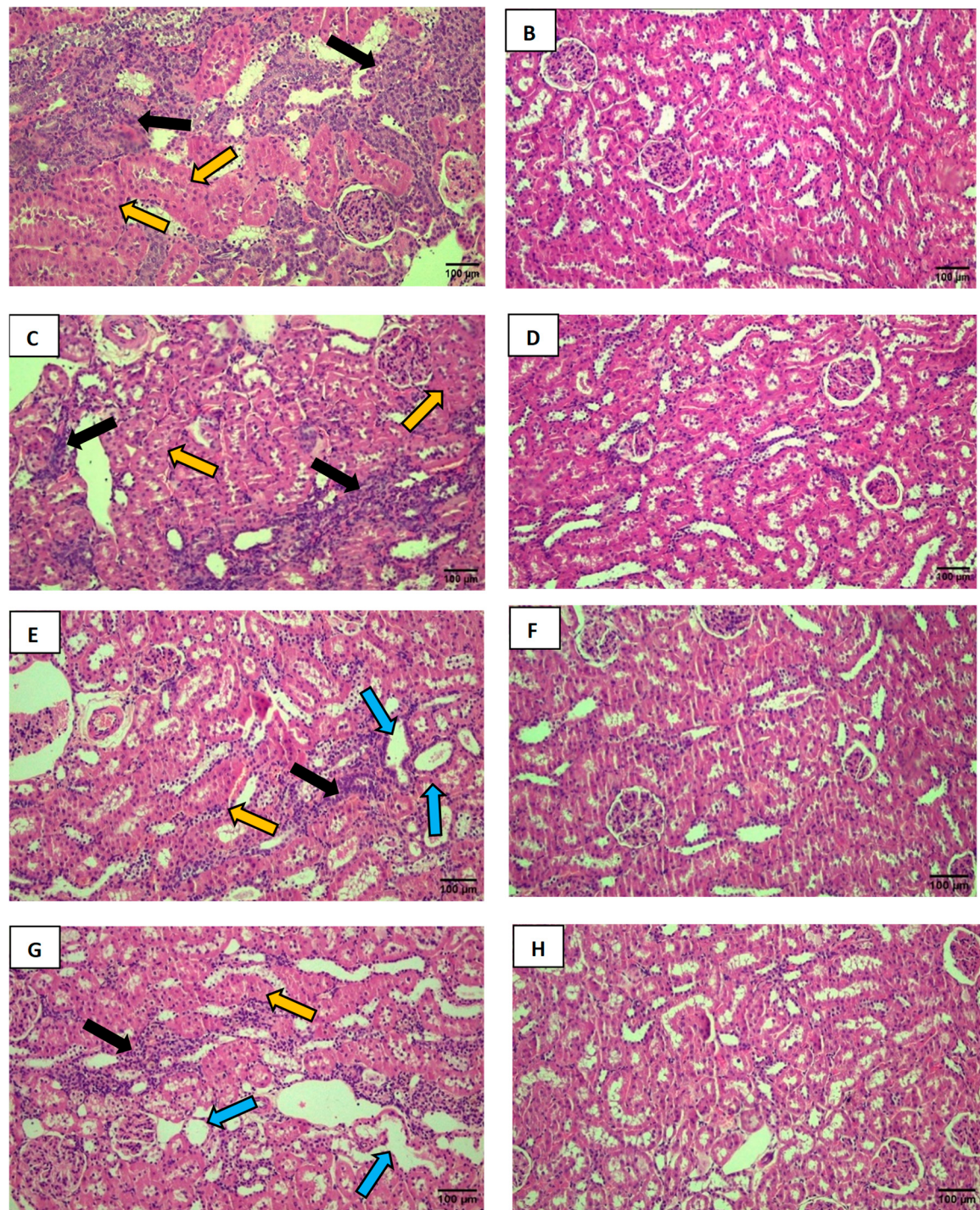


Figure 2. The black arrows indicate inflammatory infiltration. The yellow arrows show hypertrophied tubules with size enlargement. The blue arrows point to areas of tubular damage, where it is possible to see tubules with big tubular lumens with thin tubular epithelium. Intense cellular infiltration and tubular hypertrophy are visible in the DMA group (A) compared to the CSA group (B). The same observations were made in the DMI group (C) compared to the CSI group (D). In panels E and G: In addition to cellular infiltration and tubular hypertrophy,

it is possible to observe in the DMA and DMI groups that some tubules exhibit atrophic characteristics compared to their respective controls (F and H). Photos were captured using a Nikon Eclipse E200 microscope at 100x magnification.

A 19. of the 20 analyzed fields in the DMA group compared to the CSA group (Figures 3A, 3B). The same alteration was observed in 32% of the 20 analyzed fields in the DMI group (Figure 3C) when compared to the CSI group (Figure 3D). This observation is characterized by limited visualization of the region between the glomerular capillary tuft and Bowman’s capsule (Bowman’s urinary space). Due to the intense inflammatory infiltrate in certain field regions, the observation of tubular structure is considerably impaired. This was more pronounced in the analyzed fields of the DMA group (Figure 3E) compared to its control (Figure 3F).

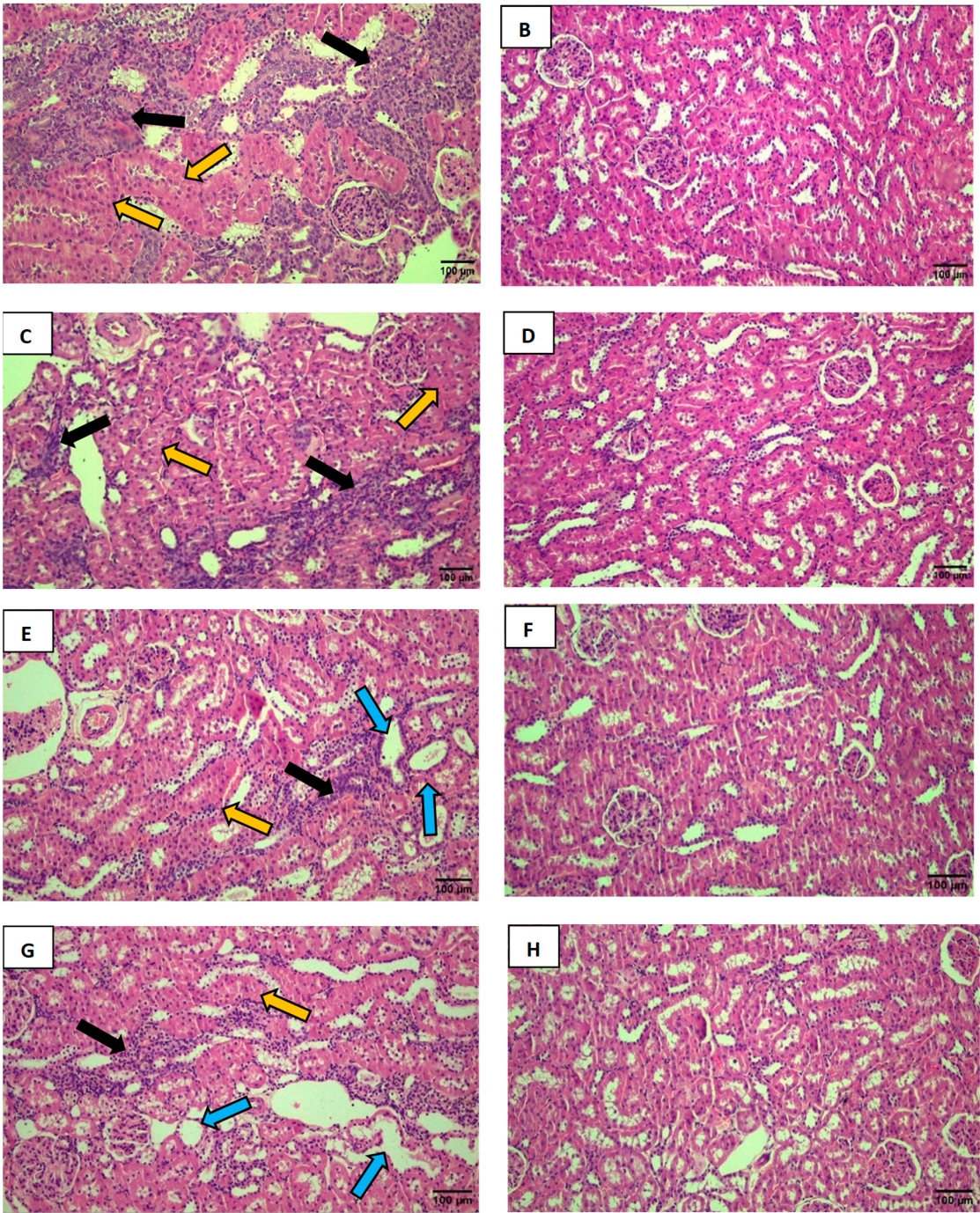


Figure 3. The black arrows indicate a reduction in Bowman's space. The yellow arrows show the loss of visual delineation of the tubules. A decrease in Bowman's space is visible in the DMA group (A) compared to the CSA group (B). The same observation was made in the DMI group (C) compared to the CSI group (D). Excessive cellular infiltration with a loss of visual delineation of the tubules is observed in the DMA group (E) compared to the CSA group (F). Photos were captured using a Nikon Eclipse E200 microscope at 100x magnification.

Glomerulus counting, performed per field and per slide, revealed a numerical decrease only in the DMI group compared to the CSI group (Table 2)

Table 2. Number of glomeruli per field and per slide and glomerular area of the studied groups. Mann-Whitney test and Student's t-test. *P < 0.05. 95% CI.

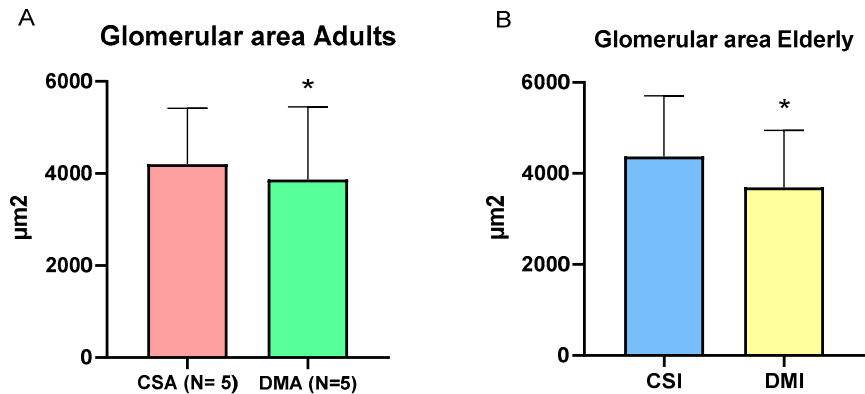
	Age	Number of Glomeruli ± SD		P value	Variations
		Sham Control	Diabetics		
Glomeruli per field	Adult	49,5 ± 13; n= 5	40,1 ± 8,8; n= 5	0,0619	-
	Elderly	60,5 ± 11,7; n= 5	44,7 ± 10,9; n= 5	0,0071*	↓
Glomeruli per slide	Adult	2,4 ± 0,6; n= 5	2 ± 0,4; n= 5	0,0619	-
	Elderly	3 ± 0,6; n= 5	2,2 ± 0,5; n= 5	0,0071*	↓

We also observed in the correlation analysis with biochemical parameters that there was an inverse correlation between the number of glomeruli and the ApoB/ApoA ratio in the DMA group compared to the CSA group (Table 3)

Table 3. Correlations between glomerulus count and ApoB/ApoA ratio. Spearman correlation test. *P < 0.05.

Group	Correlation	R value	P value
Adult	Glomeruli per slide X ApoB/ApoA, n= 5	-0,9750	0,0047*
	Glomeruli per field X ApoB/ApoA, n= 5	-0,9750	0,0047*

The evaluation of glomerular area demonstrated a decrease in both diabetic groups compared to their respective controls (DMA 3884 ± 113.4, n = 188 vs CSA 4201 ± 74.9, n = 263, p = 0.0003; DMI 3751 ± 85.3, n = 204 vs CSI 4370 ± 79.9, n = 280, p < 0.0001) (Figures 4A and B). In the renal tubule area analysis, the histological observation of tubular hypertrophy was confirmed. Both DMA and DMI groups showed an increase in tubular area compared to their respective control groups. This alteration was observed in the diameter (µm) of both longitudinal (CSA 29.6 ± 4, n = 263 vs DMA 42.2 ± 9.3, n = 242, p < 0.0001; CSI 26.4 ± 4, n = 250 vs DMI 35 ± 5.1, n = 237, p < 0.0001) and transverse (CSA 29.7 ± 3.3 / 33.4 ± 4.3, n = 227 vs DMA 36.1 ± 6 / 40.4 ± 6.5, n = 242, p < 0.0001; CSI 27.2 ± 3.4 / 29.8 ± 3.6, n = 242 vs DMI 34 ± 4 / 37.6 ± 4.6, n = 237) tubules (Figures 4C and D).



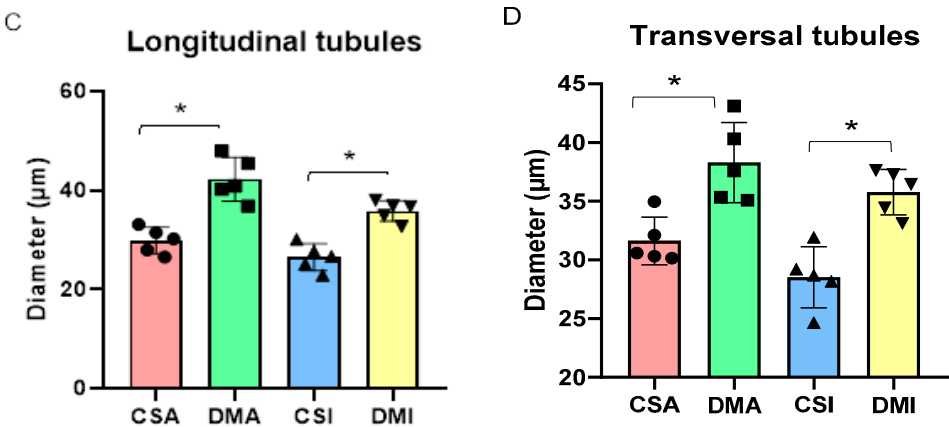


Figure 4. Comparative graph of glomerular area between the CSA and DMA groups (A) and between the CSI and DMI groups (B). Mann-Whitney test *P < 0.05, 95% CI. Comparative representation of the mean diameter of longitudinal (C) and transverse (D) tubules between the studied groups. Tukey's Multiple Comparison Test *P < 0.0001, 95% CI.

3. Discussion

In this study, we showed in diabetic rats that before DKD installation, the kidney already presents marked signs of tissue remodeling. As we can see in the renal biochemical parameters, although there is some significant increase in plasmatic creatinine, creatinine clearance, proteinuria, and urea/creatinine ratio in diabetic rats, all renal parameters are within the reference values described in the literature [49 – 54]. Therefore, according to the biochemical parameters used in the medical clinics nowadays, the diabetic rats used in this study had not developed DKD yet.

In the pathophysiology of diabetes mellitus, the decrease in insulin secretion by the pancreas is one of the mechanisms of hyperglycemia. Thus, the injury to the Langerhans islets and β-cells is a histological alteration frequently reported [24]. The lesions on the Langerhans islets are a common characteristic between DM types 1 and 2, although they appear more frequent in type 1. In this case, it is necessary to introduce the insulin therapy [25]. As confirmation of the experimental model of diabetes with alloxan, besides the hyperglycemia, we also observed in the pancreas of diabetic rats the atrophy of the Langerhans islets with reduced cellular density.

We also showed that there are some differences between the kidneys of adult and elderly mice in the early stages of DM. The aging demonstrated some calcification in the pancreas from CSI rats, which has already been related in human biopsies [53]. Also, the CSI kidneys showed a few inflammatory infiltrations that are also associated with the aging process [54]. Only the DMI group presented a decrease in the glomeruli number in comparison with CSI. This data indicate that elderly diabetic rats may exhibit greater susceptibility to glomerular injury and loss in the early stages of DM, and corroborate with the knowledge that there is a negative correlation between aging and the number of glomeruli [41,42].

The histology alterations were present in both diabetic groups, being more prominent in the DMA group. One of the characteristics of nephropathy is the infiltration of macrophages into the tubular epithelium, due to the inflammatory hyperglycemic stimulus and the release of leukotrienes that have chemotactic action. Activated macrophages release pro-inflammatory cytokines, which stimulate apoptosis [26,27]. In our study, we observed excessive cellular infiltration in the tubules of adult and elderly diabetic rats even before any renal function changes. In some fields of the DMA group, the infiltration was so massive that there was a loss of visual delineation of the tubules. Studies in diabetic mice have noted intense tubular infiltration of macrophages after 3 to 4 months of diabetes induction, but by then, the animals already exhibited renal function changes [26,28].

Another morphological finding in this study was that in the diabetic groups DMA and DMI, there was hypertrophy of the tubules in the analyzed fields compared to the respective controls,

which was confirmed by the tubular measurements. In some fields, both tubular hypertrophy and atrophy were observed, always accompanied by intense cellular infiltration. It is known that tubular hypertrophy occurs in the early stages of nephropathy due to tubular dilation [29]. Hyperglycemic stress stimulates epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1), and activates the renin-angiotensin-aldosterone system. Additionally, the induction of the p27Kip1 protein, which modulates cell cycle transition, leads to a G1 phase arrest and triggers cellular hypertrophy [30,31]. Initially, hypertrophy is compensatory; however, with time, it will lead to tubular atrophy and fibrosis [32]. Hyperglycemia-induced apoptosis also stimulates hypertrophy and accumulation of ECM, being an important factor in renal remodeling [33]. Therefore, our model demonstrated that in the early stages of diabetes mellitus, even before the onset of nephropathy, significant renal alterations are already underway.

Regarding glomerular morphology, we observed a reduction in Bowman's space in some fields of the DMA and DMI groups compared to the CSA and CSI controls, respectively. Most studies report Bowman's space enlargement as a characteristic of the onset and progression of nephropathy [34,36]. In a study conducted in rats induced to diabetes mellitus with streptozotocin, a reduction in Bowman's space was observed [36]. However, in our study, the animals did not exhibit the onset of nephropathy. It is possible that in the early stages of diabetes mellitus, there is initially capillary hypertrophy. After the onset of nephropathy, it is known that inflammatory and fibrotic processes eventually trigger glomerular capillary atrophy, leading to an increase in Bowman's space³⁷. However, further experiments would be needed to confirm this observation.

Initial cellular injuries caused by hyperglycemic harmful stimuli disrupt cellular autophagy and activate apoptosis and fibrosis mechanisms, leading to various complications (Gupta et al. 2023). The injury will trigger primary homeostatic responses (coagulation) as well as the attraction of immune system cells, such as macrophages and monocytes [38]. Persistent monocyte activation leads to continuously higher production of cytokines, triggering tissue damage [39]. Indeed, our histological findings indicate intense cellular infiltration in both diabetic groups compared to their respective controls. Further studies would be necessary to differentiate the cells from the immune infiltrate.

The glomerular area in both diabetic groups showed a reduction compared to their respective controls. Most studies report an increase in glomerular area in diabetic animals; however, in all cases, the animals already had established nephropathy [34,36,43]. As nephropathy progresses, atrophy of glomerular capillaries due to inflammation and fibrosis triggers an increase in Bowman's space, causing glomerular distension [37]. Studies show that during the onset of nephropathy, there is a progressive loss of the podocyte barrier, which also stimulates hypertrophy [44,45]. However, as in our model, the animals did not present installed DKD, it is possible that in the very early stages of diabetes mellitus, there is initially a transient reduction in glomerular area.

Additionally, the DMA group showed an inverse correlation between the number of glomeruli and the ApoB/A ratio. This ratio represents the balance between atherogenic (ApoB) and anti-atherogenic (ApoA) circulating cholesterol and is related to carotid atherosclerosis in diabetic patients⁴⁶. In general, there is a relationship between a lower number of nephrons and hypertension, as well as an increased cardiovascular and renal risk [47,48]. To our knowledge, this is the first study to relate that the number of nephrons correlates with ApoB/ApoA ratio, revealing an important pathway for cardiovascular risk in diabetic adults with a lower number of nephrons. However, further studies are needed to validate this hypothesis.

The limitations of this study include the induction of the disease only in male rats, which restricts the generalizability of the results. It is recommended to use both male and female animals in the methodology for assessing DM in laboratory animal experiments. Even so, our study was able to show, for the first time, strong evidence that the histological changes characteristic of DKD can already be observed in the early stages of DM, making the present data valuable for future studies targeting DKD prevention. However, to deeper understand the impact of the histology alterations described in the present study, further studies using podocyte markers and other techniques like

immunohistochemistry and immunofluorescence would strengthen the hypotheses proposed in this work.

4. Material and Methods

Animals and experimental models

Male *Rattus norvegicus* Wistar were housed in the Animal Facility of the Centro Universitário FMABC under the following conditions: a) 12-hour light/dark cycle; b) ambient temperature of 21 ± 2 °C; c) ad libitum access to water and rodent chow. Before experiments, animals underwent a minimum of five days of acclimatization. The animals were divided into the following experimental groups: DMA: adult animals approximately 3 months old, evaluated 30 days after induction of diabetes mellitus (DM) with alloxan. DME elderly animals, approximately 7 months old, were evaluated 30 days after induction of DM with alloxan. CSA: adult animals approximately 3 months old, evaluated 30 days after injection of physiological saline solution. CSI: elderly animals approximately 7 months old, evaluated 30 days after injection of physiological saline solution.

This research project was approved by the Ethics Committee on Animal Use (CEUA) at the Centro Universitário FMABC, under protocol number 20/2018. All experimental procedures were conducted in strict accordance with the guidelines outlined in the Manual of Good Laboratory Practices. The handling of Wistar rats adhered rigorously to the standards established by the Ethics Committee on Animal Use.

Induction of DM and glycemic monitoring

After the animal's adaptation period, an intraperitoneal injection of alloxan at a dose of 120 mg/kg was administered [19,20]. Animals with blood glucose levels above 250 mg/dL were selected 7 days after induction of diabetes mellitus (DM). They were monitored for 30 days without treatment, during which their weight and glucose levels were measured every 7 days using a commercial glucometer via tail vein puncture (Accu-chek Advantage, Roche Diagnostics, Indiana, USA). Alloxan specifically exhibits cytotoxicity towards pancreatic β -cells, causing damage to pancreatic islets, blood vessels, and cell death. The effectiveness of alloxan in inducing DM depends on dosage, infusion rate, administration route, diets, fasting time, and animal weight [21,22].

Metabolic cage

Following a 30-day monitoring period, the animals were housed individually in metabolic cages for 24 hours to facilitate the collection of urine for biochemical analyses.

Urine collection was performed using recipients containing RNA later, which were positioned in the conical outlet located below the cage. Animals underwent a fasting period of 12 hours before collection to minimize urine contamination by the feed. Water intake (mL) and urine excretion (mL) were quantified. Before collection at 24 hours, animals underwent 2 days of habituation in which they remained 12 hours in the metabolic cages.

Collection and Preparation of Tissues for Histological and Histochemical Analysis

After the 24-hour urine collection, the animals were euthanized using thiopental. Following euthanasia, the left kidneys of each animal were collected and fixed in 10% buffered formalin for 24 hours. Subsequently, the kidneys were processed in a 70% ethyl alcohol solution, dehydrated in increasing ethanol series, diaphanized in xylol, and embedded in paraffin. The material is sectioned at a thickness of 5 μ m using a rotary microtome (Luptec MRP 2015 model), and the sections were transferred to standard and salinized slides for morphological evaluations using Hematoxylin and Eosin (HE) staining.

HE Staining was performed according to Paulete & Beçak (1976). This technique involves the use of Hematoxylin, a basophilic reagent that stains the cell nucleus blue, and Eosin, an acidophilic

reagent that stains the cell cytoplasm pink. To perform this technique, the slides were submerged in xylene to remove the paraffin, then hydrated in a series of alcohol solutions (100°, 95°, and 70° GL), followed by distilled water for 1 minute, stained with hematoxylin for 5 minutes, rinsed in running water for 4 minutes, stained with eosin for 1 minute, and finally dehydrated in a series of alcohol solutions (70°, 90°, and 100° GL) and xylene [23].

A total of 20 slices were examined: 5 slices from each of the 4 groups, where 20 fields were analyzed per kidney section. Morphological analyses of renal tissue were performed using light microscopy (Nikon Eclipse E200 microscope at 100x magnification). Further evaluations, including the analysis of the number of glomeruli and glomerular area, were performed utilizing ImageJ software (version 1.47, National Institutes of Health).

Determination of biochemical and renal function evaluations

Glycemia and HbA1c

Blood glucose was determined weekly using the photometric method and confirmed using fluoridated plasma by the automated enzymatic-colorimetric method, BioSystems Glucose Ref. COD 12503. HbA1c was determined using the immunoturbidimetry method, BioSystems Hemoglobin A1C-Turbi kit Ref. COD 13044. Dosages were performed using a COBAS 8.000 apparatus.

Plasma urea

Urea was determined by the enzymatic/colorimetric method using the Urea UV SL Elitech kit Cat. n° URSL - 0500 (Elitech Group Clinical Systems, France) according to the manufacturer's protocol, in a COBAS 8,000 device at an absorbance of 600 nm.

Serum and urine creatinine

Biochemical analyses of serum and urine creatinine were determined using the kinetic-colorimetric Jaffé alkaline picric method, Creatinine Jaffe Elitech Cat. n° CRCO-0600 (Elitech Group Clinical Systems, France), according to the manufacturer's protocol, in a COBAS 8,000 device at an absorbance of 510 nm. Urine was previously homogenized and centrifuged at 2500 rpm for 5 minutes at room temperature to precipitate the sediment. The supernatant was diluted (1:25) in distilled water using glass tubes.

Creatinine clearance

Creatinine clearance was calculated using the formula $Ccr = (Ucr \times Vu / Pcr) \times Ma$. Where: Ucr = urine creatinine concentration (mg/dl); Vu = urine volume (ml/min); Pcr = plasma creatinine concentration (mg/dl); Ma = animal weight (kg).

Proteinuria

Determination of urinary protein excretion was performed through the reaction with copper (II) ions in an alkaline medium, resulting in a colored complex whose intensity was quantified in a COBAS 8,000 device at an absorbance of 545 nm. The kit used was Protein (Total) BioSystems (BioSystems S.A., Spain) Cat. No. COD 12500.

ApoA, ApoB in plasma and urine

Determination of ApoB/ApoA was performed using the turbidimetry method, with BioSystems reagents Apolipoprotein B Turbidimetry Ref. COD 31099 and BioSystems Apolipoprotein A-I Turbidimetry Ref. COD 31096, respectively. All dosages were determined using a COBAS 8000 device, using control sera to verify assay performance.

Cystatin C

Cystatin C was quantified using the Enzyme-Linked Immunosorbent Assay (ELISA) method, Kit Cystatin C, catalog ALX-850-292, brand Enzo Life Sciences. The test is based on the identification of antigens by antibodies marked with an enzyme, which acts on its substrate and causes a change in the color of the chromogen (a colorless substance that changes color when oxidized by the enzyme).

Statistical analysis

The biochemical values were expressed as mean $\pm$ standard deviation (SD). A Student's t-test was performed for the CSA versus (vs) DMA group and between the CSI vs DMI group for parametric data, and the Mann-Whitney test was used for non-parametric data. Spearman correlation analysis was conducted in the DMJ and DMI groups, assessing the relationship between the number of glomeruli and the biochemical measurements. For multivariate comparison analysis across all studied groups, a one-way Kruskal-Wallis analysis of variance test and Tukey's Multiple Comparison Test were used. All analyses were performed using the GraphPad Prism software (GraphPad, version 7.0, USA). The level of significance was set at 5% ($p < 0.05$), with a 95% confidence interval (CI).

5. Conclusion

In this article, we bring new evidence that early histological changes, including the tubular and glomerular damage, are present already in the initial stages of DM, before renal dysfunction becomes evident. This represents a promising therapeutic target strategy to delay and/or prevent the progression to DKD. Interventions targeting the kidney remodeling associated with DKD have the potential to preserve renal function and mitigate long-term complications of the disease.

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