Kefir administration reduced progression of renal injury in STZ-diabetic rats by lowering oxidative stress

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A B S T R A C T

This study aimed at assessing the effects of Kefir, a probiotic fermented milk, on oxidative stress in diabetic animals. The induction of diabetes was achieved in adult male Wistar rats using streptozotocin (STZ). The animals were distributed into four groups as follows: control (CTL); control Kefir (CTLK); diabetic (DM) and diabetic Kefir (DMK). Starting on the 5th day of diabetes, Kefir was administered by daily gavage at a dose of 1.8 mL/day for 8 weeks. Before and after Kefir treatment, the rats were placed in individual metabolic cages to obtain blood and urine samples to evaluate urea, creatinine, proteinuria, nitric oxide (NO), thiobarbituric acid reactive substances (TBARS) and C-reactive protein (CRP). After sacrificing the animals, the renal cortex was removed for histology, oxidative stress and NOS evaluation. When compared to CTL rats, DM rats showed increased levels of glycemia, plasmatic urea, proteinuria, renal NO, superoxide anion, TBARS, and plasmatic CRP; also demonstrated a reduction in urinary urea, creatinine, and NO. However, DMK rats showed a significant improvement in most of these parameters. Despite the lack of differences observed in the expression of endothelial NO synthase (eNOS), the expression of inducible NO synthase (iNOS) was significantly lower in the DMK group when compared to DM rats, as assessed by Western blot analysis. Moreover, the DMK group presented a significant reduction of glycogen accumulation within the renal tubules when compared to the DM group. These results indicate that Kefir treatment may contribute to better control of glycemia and oxidative stress, which is associated with the amelioration of renal function, suggesting its use as a non-pharmacological adjuvant to delay the progression of diabetic complications.

Introduction

Diabetes mellitus has become a serious public health problem that affects millions of individuals worldwide. The World Health Organization predicts that 439 million people will have this disease in 2030, and Brazil was listed 5th of 10 countries estimated to have the highest number of people with diabetes, affecting approximately 12.7 million Brazilians in 2030 [1].

Hyperglycemia and oxidative stress have been closely linked to diabetic complications, such as neuropathy, retinopathy and nephropathy. Additionally, excessively high blood glucose levels lead to the increased production of reactive oxygen species (ROS), such as hydrogen peroxide and superoxide radicals [2]. A chronic hyperglycemic state may also cause ROS increases via glucose auto-oxidation in various tissues, leading to high oxidative/nitrosative stress with subsequent impaired nitric oxide (NO) bioavailability. NO is a potent, endogenous vasodilator that modulates renal function and plays a key role in endothelial dysfunction [3].

High levels of ROS contribute to lipid peroxidation (LPO) in cellular membranes, increasing their fluidity and permeability. Specifically, high levels of ROS generate malondialdehyde (MDA), a highly toxic molecule, and its secondary product, thiobarbituric acid reactive substances (TBARS), which is used as marker of LPO [4]. ROS are also responsible for the activation of nuclear factor-kappa B (NF-κB), which increases the expression of pro-inflammatory biomarkers, such as tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6), which augments the expression of C-reactive protein (CRP) [5].
Kefir is a beverage made from milk that is fermented by a complex mixture of bacteria, including various species of lactobacilli and yeasts. It has been considered a probiotic due to its antioxidant and anti-inflammatory properties \cite{6,7}. In this study, we investigated the effects of Kefir on the production of nitric oxide and oxidative stress and renal damage in STZ-induced diabetic rats.

Material and methods

Animals

Male Wistar rats, 8 weeks of age, weighing ±250 g, were obtained from Central Animal Housing, Sao Paulo, Brazil. All protocols were approved by the Ethics Committee in Research of Universidade Federal de Sao Paulo (protocol #1335/09). The animals were maintained in the Animal Housing of Nephrology Division at a temperature of 22 ± 1 °C and a light–dark cycle of 12/12 h, beginning at 6:00 am. The animals were given free access to standard chow (Nuvital CR-1, Nuvilab, Colombo, PR, Brazil) and water. The rats were allocated into the four following groups: CTL (n = 9, control group); CTLK (n = 9, control group that received Kefir); DM (n = 12, diabetic group) and DMK (n = 12, diabetic group treated with Kefir).

Induction of type 1 diabetes

After seven days of adaptation, 9-week-old animals received a single intravenous administration of STZ 45 mg/kg dissolved in 0.1 M cold citrate buffer, pH 4.5. The controls rats only received citrate buffer. After 72 h of induction, glycemia was measured in blood samples collected from the tail vein and the values were determined using a glucometer. DM was defined in this study as fasting blood glucose ≥200 mg/dL and animals that failed to meet this criteria were excluded. The fasting blood glucose (3 h) of each animal was measured during 8 weeks protocol for monitoring diabetes.

Production and preparation of Kefir

The ingredients used were as follows: milk: skim milk powder Molico (Nestle, Sao Paulo, Brazil); lactic culture: Kefir (10^{10} CFU/g of lactic acid bacteria and 10^4–10^5 CFU/g of yeast), containing Lactococcus lactis subsp., Leuconostoc sp., Streptococcus thermophilus, Lactobacillus sp., Kefir yeast, and Kefir grains microflora that are not genetically modified, according to European Parliament (Danisco Biolacta, Olstyn, Poland). The preparation of the matrix was carried out as follows: skim milk powder was reconstituted to 10% with distilled water and was incubated at 85 °C for 15 min (Lauda Wobser GMBH & CO. KG type A100, Lauda-Königshofen, Germany) using a mechanical shaker (Quimis Q250M1, Diadema, SP, Brazil). Subsequently, 20 mg freeze-dried Kefir culture was added to 100 mL of the treated milk and fermentation was carried out at 23 °C for 16 h. This was monitored by the Cinac System (Cynetique dacification, Ysebaert, Frépillon, France) \cite{8}. When the desired pH of 4.6 was reached, the fermentation was stopped by cooling the flasks in ice bath, and storing them under refrigeration at 4 °C until utilization. Kefir was prepared as described above, once a week, thus ensuring freshness of the product. All procedures were performed at the Laboratory of Food Technology, Faculty of Pharmaceutical Sciences, Universidade de Sao Paulo, Sao Paulo, SP, Brazil.

Kefir treatment

The CTLK and DMK groups received Kefir starting on the 5th day after diabetes induction. Administration was carried out for 8 weeks by gavage, at a dose of 1.8 mL/day. The other groups, CTL and DM, received water as a control. All animals were placed in individual metabolic cages for 24 h prior to and after the treatment protocol, with water and food ad libitum, for urine collection. After this period, the rats were fasted for 3 h, after which a blood sample was collected from the retro-orbital plexus while the rats were under anesthesia. For anesthesia, intramuscular injection of ketamine chloride (67 mg/kg) and xylazine chloride (8 mg/kg) was used. All samples were stored in freezer at −20 °C. At the end of protocol, the animals were killed by CO₂, followed by exsanguination and the removal of the kidneys.

Oral glucose tolerance test (OGTT)

After 8 weeks of Kefir treatment, OGTT was performed in the animals. After 12 h of fasting, the animals were orally fed a 20% glucose solution (1 g/kg) and their blood glucose concentrations were determined at 0, 30, 60 and 120 min intervals using a glucometer.

Renal function assessment

The plasma and urinary urea concentrations were estimated by urea kit CE. Creatinine was measured by colorimetric assay creatinine kit and proteinuria was evaluated by sensiprot kit.

Kidney histology

The kidneys were fixed in 10% formaldehyde, embedded in paraffin, sectioned at 4 μm thickness and stained with periodic acid–Schiff reagent (PAS). The histological changes in the stained sections were assessed by a nephrologist under a light microscope at 400× magnification. This was carried out under blinded conditions and for each kidney, 6 randomly selected areas of cortex were photographed. The area of each renal cortex was digitalized by an imaging program and a count was made according to changes in the tubules.

Estimation of oxidative stress and inflammatory parameters

Kidney tissue homogenate

Homogenates of the renal cortex were prepared as previously described \cite{9}. The homogenates were used for NO, superoxide anion and LPO assays and the protein content was analyzed by Bradford assay.

NO measurement

NO was measured to evaluate the magnitude of vascular damage and oxidative stress of diabetic rats in plasma, urine and renal cortex. Because NO is extremely unstable, we used a method in which the stable NO metabolites, nitrite and nitrate, were re-converted to NO through a reaction with vanadium. The NO that was generated was quantified by a chemiluminescence method using the Nitric Oxide Analyzer (Sievers Instruments, Boulder, USA), a high-sensitive detector of NO in liquid samples (~1 pmol) that is based on the gas-phase chemiluminescent reaction between NO and ozone \cite{10}.

Western blot analysis

The protein expression of endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) were assessed in the kidney samples that were individually homogenized with a Polytron in K-HEPES buffer containing a mixture of protease inhibitors. After incubation at 4 °C for 15 min, the samples were centrifuged at 2000g. The protein concentrations were quantified using the Bradford assay method and the 80 μg of protein from each sample was separated on an 8% polyacrylamide gel and transferred to a nitrocellulose membrane. Subsequently, the membranes were probed with a mouse monoclonal eNOS (1:1000) or a
rabbit polyclonal iNOS (1:200) primary antibody, followed by anti-mouse (1:1000) or anti-rabbit (1:5000) secondary antibody, respectively. The bands were visualized using a chemiluminescence substrate and analyzed by gel documentation Alience 4.7 Uvitec (Cambridge, Cambs, UK). The relative expression of NOS proteins in each kidney were normalized using actin antibody.

Superoxide anion
The level of the superoxide anion in the renal cortex was detected indirectly according to the adapted nitroblue tetrazolium (NBT) protocol. The optical density (OD) was read in microplate reader at 560 nm [11].

LPO estimation
The LPO was estimated in terms of MDA by using the TBARS method. The MDA concentration was calculated using a molar extinction coefficient of 1.56 × 10^5 mol⁻¹ cm⁻¹ in plasma, urine and renal cortex [12] at the end of the Kefir treatment.

CRP measurement
To determine the plasmatic CRP, we utilized the turbidimetric technique [13].

Reagents
Streptozotocin was purchased from Sigma Chemical (St. Louis, MO, USA). Citric acid was acquired from LabSynth (Sao Paulo, SP, Brazil) for preparation of the citrate buffer. For the OGTT test, the Accu-chek advantage II glucometer strips were purchased from Roche Diagnostics (Mannheim, Baden-Württemberg, Germany) and glucose D anidra was obtained from LabSynth (Sao Paulo, SP, Brazil). The Dopalen (ketamine chloride) and Anasedan (xylazine chloride) anesthetics were obtained from Sespo (Sao Paulo, SP, Brazil). The creatinine, urea and proteinuria assay kits were obtained from Labtest (Lagoa Santa, MG, Brazil). Vanadium was obtained from Labtest (Lagoa Santa, MG, Brazil). Vanadrum was purchased from Sigma Chemical (St. Louis, MO, USA). Trichloroacetic acid was obtained from LabSynth (Sao Paulo, SP, Brazil) and thiobarbituric acid was purchased from J.T. Baker Chemical (Phillipsburg, NJ, USA). Nitroblue tetrazolium chloride (NBT) was obtained from Amresco (Solon, OH, USA). HEPES was purchased from USB Corporation (Cleveland, OH, USA) and the K-HEPES buffer contained 200 mM mannitol, 80 mM HEPES and 41 mM KOH, pH 7.5. Protease inhibitors cocktail was acquired from Millipore (Billerica, MA, USA). Mouse anti-eNOS was obtained from Abcam (Cambridge, Cambs, UK), rabbit anti-iNOS and actin were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The creatinine, urea and proteinuria assay kits were obtained from Amresco (Solon, OH, USA). HEPES was purchased from USB Corporation (Cleveland, OH, USA) and the K-HEPES buffer contained 200 mM mannitol, 80 mM HEPES and 41 mM KOH, pH 7.5. Protease inhibitors cocktail was acquired from Millipore (Billerica, MA, USA). Mouse anti-eNOS was obtained from Abcam (Cambridge, Cambs, UK), rabbit anti-iNOS and actin were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The enhanced chemiluminescence (ECL) Western blotting analysis system was obtained from Amersham International, Plc (Little Chalfont, Bucks, UK).

Statistical analysis
The results were expressed as the mean ± standard error media (SEM). The differences among the four groups were examined for statistical significance using one-way analysis of variance (ANOVA) followed by Newman–Keuls Multiple Comparison post test or by Kruskal–Wallis followed by Dunn’s Multiple Comparison post test. The differences between two groups were analyzed by unpaired t or Mann Whitney tests, as appropriate. The correlation was analyzed by Sperman rank coefficient. Values were considered statistically significant when p < 0.05.

Results

Metabolic profile, renal function, and oxidative stress before Kefir treatment
The parameters of the rats after the 5th day of diabetes induction are shown in Table 1. DM animals demonstrated significant differences in all parameters, except for plasmatic TBARS. These animals showed significant increases in water and chow intake, diuresis, fasting blood glucose, plasmatic urea and creatinine, and excretion of proteinuria and TBARS. In contrast, DM rats had a decrease in body weight, urinary urea and creatinine, and plasmatic and urinary NO when compared to CTL rats.

The changes in metabolic variables, renal function, and oxidative stress after 8 weeks of Kefir treatment are shown in Table 2. Kefir administration did not cause difference in any of these parameters in the CTLK group. The control and diabetic groups showed significant differences in the following parameters: water and chow intake, diuresis and weight gain. However, in the DMK group, these parameters were significantly decreased when compared to the DM group; an exception was weight gain, which was higher in DMK animals than in DM animals.

Glycemia
The levels of glycemia after Kefir treatment are shown in Fig. 1A. On the 5th day after diabetes induction (0), the fasting blood glucose level was increased in DM rats when compared to CTL rats (293 ± 21 vs 89 ± 5, p < 0.001) and in DMK rats when compared to CTLK rats (294 ± 14 vs 95 ± 5, p < 0.001). After the 8th week of Kefir treatment, these levels were reduced in DMK rats when compared to DM rats (325 ± 32 vs 457 ± 31, p < 0.001). In contrast, during OGTT, as shown in Fig. 1B, it was observed that glycemia in the DMK group was decreased after 30, 60 and 120 min when compared to DM, although this was only significant after 30 min (344 ± 37 vs 221 ± 51, p < 0.05).

Renal function
As shown in Table 2, the levels of plasmatic urea were increased in DM rats when compared with CTL rats and in the DMK group when compared to the CTLK group. However, this was reduced in DMK rats when compared with DM rats. The concentration of urinary urea was four times lower in the DM group than in the

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CTL</th>
<th>DM</th>
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<tr>
<td></td>
<td>n = 18</td>
<td>n = 24</td>
</tr>
<tr>
<td>Water intake (mL/24h)</td>
<td>29.9 ± 0.9</td>
<td>80.2 ± 4.9*</td>
</tr>
<tr>
<td>Chow intake (g/24h)</td>
<td>19.1 ± 0.4</td>
<td>23.9 ± 0.9*</td>
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<td>Diuresis (mL/24 h)</td>
<td>13.0 ± 0.7</td>
<td>61.1 ± 4.6*</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>269.8 ± 5.2</td>
<td>253.7 ± 4.0*</td>
</tr>
<tr>
<td>Fasting blood glucose (mg/dL)</td>
<td>91.8 ± 3.5</td>
<td>293.5 ± 12.3*</td>
</tr>
<tr>
<td>Plasmatic urea (mg/dL)</td>
<td>29.2 ± 1.9</td>
<td>55.6 ± 4.6*</td>
</tr>
<tr>
<td>Urinary urea (mg/dL)</td>
<td>7,556 ± 444</td>
<td>2,403 ± 129*</td>
</tr>
<tr>
<td>Proteinuria (mmol/24 h)</td>
<td>0.70 ± 0.04</td>
<td>0.90 ± 0.02*</td>
</tr>
<tr>
<td>Plasmatic creatinine (mg/dL)</td>
<td>160.8 ± 28.0</td>
<td>62.8 ± 14.7*</td>
</tr>
<tr>
<td>Urinary creatinine (mg/dL)</td>
<td>11.2 ± 0.6</td>
<td>21.4 ± 1.0*</td>
</tr>
<tr>
<td>Proteinuria (µM)</td>
<td>89.7 ± 12.2</td>
<td>58.9 ± 9.0*</td>
</tr>
<tr>
<td>NO excretion (µmol/24 h)</td>
<td>15.9 ± 2.7</td>
<td>1.4 ± 0.2*</td>
</tr>
<tr>
<td>TBARS (nmol/mg)</td>
<td>3.03 ± 0.06</td>
<td>3.17 ± 0.06</td>
</tr>
<tr>
<td>TBARS excretion (nmol/24 h)</td>
<td>86.9 ± 5.3</td>
<td>192.4 ± 10.4*</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SEM. Unpaired t or Mann Whitney test. Control (CTL); diabetic (DM).

* p < 0.05.

* p < 0.001 vs CTL.
CTL group, while in the DMK group, it was 2.8 times lower than in the CTLK group, demonstrating that this parameter was increased in the DMK group when compared to the DM group. Moreover, plasmatic creatinine was not different among control and diabetic animals, and urinary creatinine and proteinuria were not different between DMK and DM groups.

In relation to renal histology, the diabetic groups presented with an accumulation of glycogen in the renal tubules. This was reduced in the DMK group when compared to the DM group (9 ± 1 vs 13 ± 1, p < 0.001), as demonstrated in Fig. 2.

Oxidative stress and inflammatory state

According to Table 2, there was no difference in plasmatic NO levels in the animals studied. However, the excretion of NO in DM rats was significantly reduced by 7 times when compared to CTL rats and in DMK rats, this parameter was higher compared to DM rats, but did not show any difference when compared to CTLK rats. Interestingly, the data demonstrated a strong and inverse correlation between NO and proteinuria excretion, which is an early marker of renal lesion, among the control and diabetic groups ($r = -0.83$, p < 0.001), as shown in Fig. 3A.

**Table 2**

<table>
<thead>
<tr>
<th>Variables</th>
<th>CTL</th>
<th>CTLK</th>
<th>DM</th>
<th>DMK</th>
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</thead>
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<tr>
<td>Water intake (mL/24 h)</td>
<td>24.8 ± 1.8</td>
<td>25.8 ± 3.7</td>
<td>124 ± 12.4</td>
<td>94.1 ± 14.4</td>
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<td>Chow intake (g/24 h)</td>
<td>17.3 ± 0.6</td>
<td>19.1 ± 1.0</td>
<td>36.9 ± 2.0</td>
<td>30.6 ± 2.3</td>
</tr>
<tr>
<td>Diuresis (mL/24 h)</td>
<td>13.1 ± 1.0</td>
<td>13.7 ± 0.7</td>
<td>90.9 ± 9.0</td>
<td>70.9 ± 9.4</td>
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<tr>
<td>Weight (A)</td>
<td>67.5 ± 4.4</td>
<td>69.3 ± 1.6</td>
<td>25.3 ± 4.5</td>
<td>35.3 ± 5.9</td>
</tr>
<tr>
<td>Plasmatic urea (mg/dL)</td>
<td>31.9 ± 1.4</td>
<td>36.2 ± 2.5</td>
<td>58.5 ± 3.6</td>
<td>47.6 ± 2.4</td>
</tr>
<tr>
<td>Urinary urea (mg/dL)</td>
<td>8,691 ± 343</td>
<td>8,421 ± 229</td>
<td>2,006 ± 142</td>
<td>2,996 ± 322</td>
</tr>
<tr>
<td>Plasmatic creatinine (mg/dL)</td>
<td>0.71 ± 0.05</td>
<td>0.72 ± 0.04</td>
<td>0.75 ± 0.03</td>
<td>0.78 ± 0.05</td>
</tr>
<tr>
<td>Urinary creatinine (mg/dL)</td>
<td>131.7 ± 9.2</td>
<td>127.9 ± 4.5</td>
<td>33.2 ± 5.6</td>
<td>34.8 ± 7.8</td>
</tr>
<tr>
<td>Proteinuria (nmol/24 h)</td>
<td>10.4 ± 0.8</td>
<td>11.2 ± 0.7</td>
<td>25.5 ± 3.7</td>
<td>21.0 ± 2.8</td>
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<tr>
<td>Plasmatic NO (µM)</td>
<td>66.6 ± 4.3</td>
<td>77.8 ± 6.6</td>
<td>79.2 ± 5.0</td>
<td>76.5 ± 5.4</td>
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<tr>
<td>NO excretion (µmol/24 h)</td>
<td>14.9 ± 3.6</td>
<td>17.5 ± 3.8</td>
<td>2.1 ± 0.7</td>
<td>16.4 ± 4.9</td>
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<tr>
<td>Plasmatic TBARS (nmol/mL)</td>
<td>3.32 ± 0.06</td>
<td>3.16 ± 0.08</td>
<td>3.79 ± 0.10</td>
<td>3.58 ± 0.17</td>
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<tr>
<td>TBARS excretion (nmol/24 h)</td>
<td>81.6 ± 2.1</td>
<td>84.4 ± 4.3</td>
<td>300.4 ± 18.9</td>
<td>248.9 ± 19.2</td>
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</tbody>
</table>

Values are expressed as mean ± SEM. One-way ANOVA followed by Newman–Keuls Multiple Comparison post test. Control (CTL); control Kefir (CTLK); diabetic (DM); diabetic Kefir (DMK); n = 9–12/group.

- $^a$ p < 0.001 vs CTL.
- $^b$ p < 0.01 vs CTLK.
- $^c$ p < 0.05 vs DM.

![Fig. 1](image1.png)

(A) Glycemia levels after 0, 2, 4 and 8 weeks during Kefir treatment, n = 9–12. (B) Glycemia levels during oral glucose tolerance test (OGTT), n = 4–6/group. Control (CTL); control Kefir (CTLK); diabetic (DM); diabetic Kefir (DMK). Values are expressed as means ± SEM. One-way ANOVA followed by Newman–Keuls Multiple Comparison post test. **p < 0.05, ***p < 0.001 vs CTL; †p < 0.05, ††p < 0.01, †††p < 0.001 vs CTLK; †⁄p < 0.05, †⁄⁄p < 0.01 vs DM.

![Fig. 2](image2.png)

(A) Renal histology after Kefir treatment. Kidneys showed glycogen storage in diabetic rats tubules (shown by arrows). (B) Average glycogenated tubules represented graphically in each group. Control (CTL); control Kefir (CTLK); diabetic (DM); diabetic Kefir (DMK), n = 3/group. Values are expressed as means ± SEM. One-way ANOVA followed by Newman–Keuls Multiple Comparison post test. $&^a$p < 0.05, $&&^b$p < 0.001 vs CTL; $^*^c$p < 0.05, $^*^*^d$p < 0.01, $^*^*^*^e$p < 0.001 vs CTLK; $^*^f$p < 0.05, $^*^*^g$p < 0.01 vs DM.
The level of NO in the renal cortex was higher in DM rats when compared to CTL rats (13 ± 2 vs 3 ± 0.4, p < 0.05), but reduced in DMK rats compared to DM rats (5 ± 2, p < 0.05). This is shown in Fig. 3B. The Western Blot analysis of the renal cortex showed that there was no difference in eNOS expression in the DM and DMK rats. However, there was a significant reduction in the expression of iNOS in the DMK group when compared to the DM group (101 ± 2 vs 113 ± 5, p < 0.05), as shown in Figs. 4A and B.

Likewise, a similar increase in superoxide anion (OD) was observed in the renal cortex of DM rats when compared to CTL rats (0.107 ± 0.012 vs 0.023 ± 0.007, p < 0.001). However, DMK rats presented with lower values in this parameter than DM rats (0.047 ± 0.019, p < 0.01), and in the DMK rats, this value approached that of the CTLK rats (0.021 ± 0.002, NS), as seen in Fig. 5A.

In Table 2, we observed that plasmatic TBARS was higher in the DM group however, not demonstrated difference between DMK and CTLK; otherwise, the excretion of TBARS at the end of Kefir treatment was different among the groups, and the main differences were observed between the control and diabetic animals. The excretion of TBARS was three times higher in the diabetic group when compared to DM and these values approached of the CTLK group. However, as shown in Fig. 5B, this elevation was more pronounced in the DM group compared to the CTL group (0.65 ± 0.06 vs 0.22 ± 0.04, p < 0.001).

The CRP concentration in the DM group was significantly increased when compared to the CTL group (0.28 ± 0.01 vs 0.18 ± 0.04, p < 0.05). The DMK group showed a reduction of this parameter compared to the DMK group (NS) and an increase when compared to the CTLK group (0.23 ± 0.03 vs 0.16 ± 0.01, NS) (Fig. 5C).

Discussion

STZ-induced hyperglycemia is a recognized experimental model that is used to study both type 1 and type 2 diabetes [14]. In this study, a lower dose of STZ (45 mg/kg) was used and the rats became permanently diabetic. This allowed the production of a diabetic state with moderate to severe hyperglycemia throughout the protocol. Among the most important findings in this study, Kefir treatment in diabetic rats resulted in better glycemic control with reduced polyuria, polydipsia and polyphagia, a partial improvement in renal function, increased NO excretion and reduction of superoxide anion, LPO and inflammation.

The mechanisms by which probiotic bacteria modulate hyperglycemia are not fully understood, although previous studies have reported that supplementation with a strain of *Lactobacillus* significantly delayed the onset of glucose intolerance, hyperglycemia, hyperinsulinemia, and oxidative stress in rats with type 2 diabetes and also reduced the risk of the development of associated complications [15,16].

Additionally, studies that utilized animals fed with Kefiran, an exopolysaccharide isolated from Kefir grains, demonstrated that it reduced blood pressure and serum cholesterol in stroke-prone spontaneously hypertensive rats (SHR-SP) [17] and reduced atherosclerosis in rabbits with type 2 diabetes [18]. It also decreased the blood glucose levels in a genetic diabetic animal model (KKAy) that showed impaired glucose tolerance to an intraperitoneal glucose administration [19].
It is known that probiotics can modulate the microbiota, and/or the intestinal immune system and influence intestinal physiology. This was observed in an in vivo study using healthy female rats that consumed a Kefir-supplemented diet. Kefir supplementation was shown to have an effect on enzymes and proteins, increase the activity of the intestinal dipeptidase and decrease the Na⁺-dependent uptake of intestinal sugar, which contributes to protein digestion and glycemia reduction, respectively [20].

Hyperglycemia is the main factor in the progression of DN, leading to the dysfunction of multiple organs and increasing the morbidity and mortality in patients with diabetes [21]. In our study, the kidneys of the diabetic animals were injured when compared to control animals, but after Kefir administration, this was partially improved. This is in agreement with the literature, where it has been previously reported that probiotic bacteria benefited both human and animals with kidney failure by increasing gut bacterial metabolism for the excretion of ammonia, thus reducing blood urea-nitrogen levels [22,23].

In the present investigation, we observed significant proteinuria in diabetic rats, suggesting increased permeability of the glomerular basement membrane [24], but this was not significantly reduced in the Kefir-supplemented group. Nevertheless, we observed a strong and inverse correlation between proteinuria and NO among control and diabetic animals. This shows the importance of NO on this parameter, a matter which is still controversial [3] but has been reproduced in our previous study [9]. Moreover, glycogen accumulation was observed in the kidney tubules of DM rats and this alteration was significantly reduced after Kefir treatment, most likely as a consequence of better glycemic control in these animals.

The bioavailability of vascular NO is decreased in diabetes, both in human and animals, possibly due to elevated oxidative stress stimulated by the chronic hyperglycemic state [25]. This is because NO can be inactivated by reaction with superoxide, resulting in peroxynitrite, or may be transformed into the more stable hydrogen peroxide radical [3,14]. In this study, superoxide anion, one of the main types of ROS, was higher in the renal tissue of the DM group and was significantly reduced in the DMK group. These findings agree with that reported by Ishii et al., who showed that NO activity is augmented in the kidney cortex of diabetic rats and as such, larger quantities of superoxide are produced [26].

In the present investigation, NO excretion was reduced in diabetic animals in comparison to their respective control and was increased after Kefir treatment to control levels. Another study showed the role of some probiotic bacteria in the generation of local NO in the intestinal lumen by nitrate reduction or acid dependent mechanisms, which may be counteracted by rapid NO consumption by other strains or diffused into the surrounding tissues. This may explain some of the health promoting effects of these bacteria, for example, by potentiating the antimicrobial property of NO [27,28].

Moreover, the increase in blood glucose concentration activates high expression of iNOS through NF-κB. This is followed by the increased production of NO, which influences several signal pathways [29]. This up regulation of iNOS under conditions of oxidative stress may be a compensatory mechanism for reduced NO availability, indicating roles for NO, ROS, and peroxynitrite in the development of early diabetic damage prior to the development of complications [30].

Furthermore, researchers have shown that the uncoupling of eNOS by peroxynitrite may be a secondary mechanism by which hyperglycemia can quench and/or inactivate NO and consequently, lead to endothelial dysfunction [30,31]. However, in this study, there was a reduction of eNOS expression in both groups, DM and DMK, when compared to CTL and CTLK, although there was no difference between them. Nevertheless, in DM animals, higher iNOS expression was observed, and this was accompanied by elevated oxidative stress in the renal cortex, as reflected by an increase in NO-scavenging superoxide anions and MDA. This suggests that in the diabetic kidney, NO is deficient and consequently, its bioavailability is reduced [3,32]. This status was reversed in DMK animals, which showed reduced oxidative stress and iNOS and the restoration of NO excretion.

The measurement of LPO was based on the extinction coefficient of MDA, which is the end product of fatty acid peroxidation that reacts with thiobarbituric acid (TBA). This is accepted as an index of LPO and is widely utilized in studies relating to diabetes [4]. It has also been demonstrated that MDA in the urine is a more sensitive measure than that in the plasma [33], a finding that supports previous published results of our research group [9].

In the present study, there was a significant increase of LPO in diabetic rats, most likely due to an increase in blood glucose levels that generate ROS upon auto oxidation [2]. This indicates that peroxidative injury may be involved in the reduction...
of antioxidant defense mechanisms and the development of diabetic complications. In our study, Kefir treatment significantly decreased LPO, and this was accompanied by a reduction in hyperglycaemia, perhaps by the preservation of pancreatic β-cells, which are vulnerable to oxidative damage [14], or by the antioxidant effect of fermented milk. It is known that the bioactive peptides released during fermentation by proteolytic lactic acid bacteria can scavenge the ROS and inhibit LPO [34].

In contrast, CRP, a recognized marker of inflammation associated with endothelial dysfunction, is elevated in the blood of patients with both type 1 [35] and type 2 diabetes [36]. Researchers have reported no change [37] or an increase [21] in plasmatic CRP levels in STZ-treated diabetic rats. In this study, a significant increase in plasmatic CRP was observed in diabetic rats; however, this parameter was decreased after Kefir treatment, perhaps due to the better control of hyperglycaemia in these animals, which could modulate the activation of NF-κB and reduce the iNOS expression. It was demonstrated that Kefir could inhibit the innate response of intestinal epithelial cells, suggesting the modulation of the NF-κB pathway [38].

Further, other studies have shown that altered bowel function contributes to the development of diabetes mellitus. In particular, the intestinal barrier has become a focus of much research because increased intestinal permeability has been associated with type 1 and type 2 diabetes; suggesting that this would precede the onset of clinical diabetes in humans and rats and that enhancing intestinal barrier function may be important for preventing pathophysiological mechanisms in diabetic patients [39, 40].

Studies with probiotics could be used to design clinical plans for prevention-based treatment in which microbiota could be beneficial in the attenuation or delay of disease progression [41]. While the effects and the precise mechanisms of probiotics involved in the diabetes remain unknown, they are likely related to a decrease in oxidative stress that is associated with the immune-modulatory and anti-inflammatory properties of these bacteria, the modification of intestinal microbiota and maintenance of gut integrity. Further studies are necessary to provide new insights into the effective therapeutic approaches for type 1 diabetes mellitus.

Conclusion

The results obtained in this study show that Kefir treatment significantly reduced the progression of STZ-induced hyperglycaemia and oxidative stress in rats. Thus, Kefir may play a role in slowing the metabolic changes that contribute to DN.

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