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Protective effects of kefir in the angiotensin II-dependent hypertension

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ABSTRACT

Recently, we have reported cardiovascular protective effects of the probiotic kefir in a model of primary hypertension. Now, we evaluated the beneficial effects of kefir in a model of secondary hypertension under hyperactivation of the renin-angiotensin-system by partially clipping one kidney artery (2K1C) for 60 days and compared with Sham rats. Maximum levels of arterial pressure were reached 7–14 days post-clipping in both 2K1C and 2K1C-Kefir, but after that time the values were approximately 20% lower in 2K1C-Kefir rats. Also, kefir attenuated the angiotensin converting enzyme activity (intrarenal-40%/plasma-25%) preventing the increase of angiotensin II in both samples. Isolated aortic rings showed an impaired relaxation to acetylcholine in 2K1C (-38%) compared to the Sham group and this difference was attenuated in 2K1C-Kefir rats (~15%). Additional analysis revealed that kefir protected kidney and vascular endothelium against the synergistic oxidative stress/angiotensin II-axis. Thus, kefir is an effective nutraceutical therapy for prevention/treatment of hypertension.

1. Introduction

Hypertension is a common cardiovascular disease worldwide, characterized by persistent high arterial pressure, and is the main single contributor to all-cause disability and mortality (GBD, 2017; Kotchen, 2011; Oparil et al., 2018). Classically, it is known that endothelial dysfunction is a main marker of this disease, contributing directly to myriad pathophysiological outcomes (e.g., heart failure, stroke, chronic kidney disease, retinopathy) (Dias et al., 2018; Oparil et al., 2018; Vasquez, Pereira, Peotta, Baldo, & Campos-Toimil, 2019). While most patients develop hypertension due to an unknown cause (classified as essential or primary hypertension), the remaining patients (5–10%) have secondary hypertension, which is caused by other known pathophysiologic processes (Chokshi, Grossman, & Messerli, 2013; Mills, Stefanescu, & He, 2020).

Renovascular hypertension (RH) is one of the most common types of secondary hypertension with different pathophysiological triggers and mainly affects children and elderly people (Herrmann & Textor, 2019; Wells & Belsha, 1996). This disease is characterized by renal hypoperfusion and consequent hyperactivation of the renin-angiotensin system (RAS), culminating in an excessive production of reactive oxygen species (ROS), sympathoexcitation, and arterial and renal

Abbreviations: 2K1C, two-kidneys one-clip; ACE, angiotensin converting enzyme; Ach, acetylcholine; Ang II, angiotensin II; ANOVA, analysis of variance; AP, arterial pressure; AT1, angiotensin II receptor; Cox, cyclooxygenase; DCF, dichlorofluorescein; ΔAUC, area under the curves; DHE, dihydroethidium; H_2O_2, hydrogen peroxide; L-NAME, N(G)-nitro-l-arginine methyl ester; MFI, median fluorescence intensity; NO, nitric oxide; $\bullet O_2^-$, superoxide anion; pEC_{50}, sensitivity-used in dose-response curves; RH, renovascular hypertension; R_{max}, maximum response; ROS, reactive oxygen species; SHR, spontaneously hypertensive rat; SNP, sodium nitroprusside; TXA_2, thromboxane A2

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damage, leading to multidrug-resistant hypertension culminating in high morbimortality (Campos et al., 2011; Herrmann & Textor, 2019; Mehta & Fenves, 2010; Pereira et al., 2009). Thus, studies that investigate new therapeutic approaches to counteract the progression of RH are relevant, especially those addressing inexpensive approaches with minimal side effects.

Experimental RH was induced by Goldblatt in 1934 through unilateral partial stenosis of the renal artery in dogs, referred to as the two-kidney, one-clip (2K1C) model (Goldblatt, Lynch, Hanzal, & Summerville, 1934; Kotchen, 2011). In the last four decades, 2K1C has been an attractive model for studies in the hypertension field and has been used by our laboratory (Cabral & Vasquez, 1991; Dias et al., 2018; Moyses, Cabral, Marçal, & Vasquez, 1994; Pereira et al., 2009) and others (Campos et al., 2011; Campos, Oliveira-Sales, Nishi, Paton, & Bergamaschi, 2015; Navar et al., 1998; Oliveira-Sales et al., 2008) to investigate cardiac and vascular morphological changes, the interrelations among RAS, oxidative stress (generated by excessive ROS), endothelial dysfunction and sympathomodulation in addition to determining the possible preclinical efficacy of therapeutic candidates.

Among the varieties of functional foods available, kefir, a fermented milk containing lactic acid bacteria and yeasts, has been extensively studied for its beneficial effects, including its cardiovascular effects (Amorim et al., 2019; Friques et al., 2015; Pimenta et al., 2018; Rosa et al., 2017; Santanna et al., 2016). Recently, kefir was tested by our research group in spontaneously hypertensive rats (SHRs), a classical model for essential hypertension. We observed significant improvements in endothelial dysfunction and baroreflex control, in addition to the antihypertensive effects (de Almeida Silva, Mowry, Peaden, Andrade, & Biancardi, 2020; Friques et al., 2015; Klippel et al., 2016; Vasquez, Aires, Ton, & Amorim, 2020), by at least promoting inhibitory actions on the RAS and oxidative stress (Amorim et al., 2019; Friques et al., 2020). However, the detailed effects of kefir on endothelial dysfunction and related parameters in the 2K1C model have not yet been investigated. Therefore, this study was designed to test the hypothesis that kefir may have renal and vascular protective effects in 2K1C rats, antagonizing the synergistic oxidative stress/angiotensin II axis.

2. Materials and methods

2.1. Animals

The experiments were conducted in 138 male Wistar rats (140–160 g) provided by the Experimental Animal Care of Federal University of Espirito Santo, Brazil. All animals were kept in clean polypropylene cages in a constant light-dark cycle of 12 h, temperature from 20 to 25 °C, humidity of 70% prior to the start of experiments and during the 60 days of treatment. Animals were provided with *ad libitum* water and standard diet pellets until the treatment was completed. The experimental protocol was established according to the ethical principles of the research with animals by the National Technical Commission on Biosafety (CTNBio) and the Ethics Committee on the Use of Experimental Animals (CEUA-UFES, protocol # 489-2018).

2.2. Preparation and administration of kefir

Kefir was prepared as previously described (Friques et al., 2015). Briefly, the kefir grains, obtained from the University of Vila Velha since 2014 (Brazil), were added to whole milk, pasteurized, and cooled in a ratio of 4% (w/v). The milk inoculated with the grain was kept at room temperature for 24 h. After this period, the grain was filtered through a plastic sieve, and the resulting product was kept under refrigeration at between 5 and 15 °C for 24 h for the yeasts to multiply. At the end of this process, the kefir was aliquoted into sterile plastic tubes and stored at −20 °C until use.

The rats were randomly assigned to three experimental groups: sham-operated normotensive (Sham, n = 4–8/protocol), RH induced by clipping the left renal artery (2K1C, n = 4–8/protocol) and 2K1C rats administered kefir (2K1C-Kefir, n = 4–8/protocol). The dosing and administration of kefir were in accordance with our previous studies (Friques et al., 2015, 2020; Klippel et al., 2016). The 2K1C-Kefir group received kefir at a dose of 0.3 mL/100 g body weight/day (corresponding to human-equivalent doses of approximately 30 mL/70 kg/day, according to Reagan-Shaw, Nihal, and Ahmad (2008) and Nair and Jacob (2016), while the 2K1C animals received whole milk at an adjusted pH of 4.3. The doses were given by gavage daily for 60 days, starting after surgery on the same day.

2.3. Induction of 2K1C renovascular hypertension

2K1C renovascular hypertension was induced as previously described by our group (Cabral & Vasquez, 1991; Lincevicius et al., 2017; Shimoura et al., 2017). Briefly, under anesthesia with ketamine/xylazine (90 and 10 mg/kg, respectively, i.p.), the left renal artery was exposed through a retroperitoneal flank incision, and after careful isolation from the renal vein, nerves, and connective tissues, it was partially obstructed with a silver clip 0.2 mm wide placed around the renal artery near the abdominal aorta. Age-matched rats of the Sham group were subjected to the same procedure but without the placement of the clip. The incision was sutured, and the animals were housed in individual climatized cages (up to 20 days). After observation of a complete closure of the laparotomy incision, the rats were divided into cages containing 3 animals until the end of the study. The inclusion criterion in the 2K1C and 2K1C-Kefir groups was determined by animals that presented systolic arterial pressure measured by the tail-cuff method higher than 160 mmHg 7 days after surgery.

2.4. Hemodynamic parameters and measurement of cardiac and renal mass

Systolic arterial pressure was measured in conscious animals from day 0 (before surgery) to day 60 (end of treatment) by a noninvasive plethysmograph method following the protocol previously described (Friques et al., 2015). In addition, at the end of treatment with kefir, the animals were catheterized for direct measurements of AP. The rats were anesthetized with a combination of ketamine and xylazine (75 and 10 mg/kg, respectively, i.p.), and a catheter was inserted aseptically into the femoral artery to determine the direct hemodynamic parameters: systolic, mean and diastolic arterial pressure. One day after catheter placement, hemodynamic parameters were measured in conscious, freely moving rats in their cages using a disposable AP transducer (Cobe Laboratories, USA) connected to a pressure processor amplifier and data-acquisition system (MP100, Biopac Systems, USA). The data were analyzed on a beat-to-beat basis to quantify the baseline values of AP. Then, the rats were euthanized with a sodium thiopental injection (100 mg/kg, i.p.) Cardiac and renal hypertrophy were evaluated based on the left ventricle heart weight (mg) to tibia length (mm) ratio and the clipped to nonclipped weight ratio, as previously described (Klippel et al., 2016; Pereira et al., 2009).

2.5. Evaluation of angiotensin converting enzyme (ACE) activity in blood samples

After euthanasia of animals, kidneys were removed, immediately frozen in liquid nitrogen and subsequently stored in a freezer at −80 °C until sample preparation. In addition, blood was collected through the right ventricle using a syringe containing 100 μL of EDTA-free protease inhibitor cocktail (P2714, Sigma Aldrich, St. Louis, MO, US) and centrifuged, and the plasma was divided into aliquots and stored at −80 °C. For ACE activity determination, triplicate plasma samples (3 μL) were incubated for 15 min at 37 °C with 40 μL of solution containing the ACE substrate (Zphe-His Leu, 5 mM), sodium borate buffer (400 mM, pH 8.3) and NaCl (900 mM). For kidney analysis, each kidney

was homogenized in buffer (1:10 w/v, 100 mM Tris-HCl, 50 mM NaCl, pH 7.0). After homogenization, all samples were centrifuged (1000*g*) for 15 min at 4 °C. The product generated in the supernatant (His-Leu) was measured fluorimetrically (Synergy 2, Biotek, US) after 10 min of incubation with 17 μL of 2% o-phthaldialdehyde in methanol. The enzymatic reaction was stopped by the addition of 190 μL of 340 mM NaOH. Fluorescence measurements were performed at 37 °C on a 96-well black reading plate (black polystyrene, Corning, U.S.A.) with 365 nm excitation and 495 nm emission filters. The fluorescent reading plate was controlled by Gen5 software (BioTek, USA). A calibration curve with ECA substrate was included on each plate. The "blank" samples contained 40 μL of 'plasma buffer' (5 mM ECA substrate, 400 mM sodium borate buffer and 900 mM NaCl pH 8.3) or 'kidney buffer' (100 mM Tris-HCl, 50 mM NaCl, pH 7.0), plus 190 μL of 340 mM NaOH and 17 μL of o-phthaldialdehyde in methanol. The average values are shown as nmol/min/mL for serum and as nmol/min/mg protein for tissue homogenate. The protein measurement was performed by the Bradford method (Bradford, 1976).

2.6. Measurement of angiotensin II levels

The plasma and intrarenal levels (previously homogenized) of Ang II were measured via high-performance liquid chromatography (HPLC) at the Department of Nephrology at Unifesp as previously reported (Dias et al., 2018). Ang II was purified and extracted using an Oasis C18 column (Water Corporation, Milford, MA) that was previously activated with methanol (5 mL), tetrahydrofuran (5 mL), hexane (5 mL) and distilled water (10 mL). Subsequently, the samples were eluted in columns under a mixture of ethanol/acetic acid/water (at proportions 90% – 4% – 6%). The eluates were lyophilized and resuspended in 500 μL of mobile phase A (5% acetonitrile in 0.1% phosphoric acid) and then filtered with a 0.22 μm membrane for HPLC analysis. After the separation of peptides in each sample using a reversed-phase column Aquapore 300 ODS (250 × 4.6 mm, PerkinElmer's Brownlee Columns, Norwalk, USA) with 5 min of isocratic gradient followed by 20 min of linear gradient from 5% to 35% of mobile phase B (95% acetonitrile in 0.1% trifluoacetic acid) under a flow of 1.5 mL/min for 40 min by HPLC, Ang II was identified according to the retention period (< 6%) and height (< 5%) using a standard curve of known concentration, and the values of dosages in the kidney were normalized according to the kidney weight.

2.7. Scanning electron microscopy

Considering previous findings demonstrating vascular and renal structural changes in arterial hypertension models (Dias et al., 2018; Freitas et al., 2016; Leal et al., 2020), we analyzed the aorta microarchitecture and nephron apparatus integrity through scanning electron microscopy. Euthanized animals were perfused with a solution of paraformaldehyde (2%), glutaraldehyde (2.5%) and cacodylate buffer solution (0.1 mol/L, pH 7.2), and later, the kidneys were dissected and fixed in this solution for 24 h. The tissues were washed with 0.1 mol/L cacodylate buffer (pH 7.2–7.4). Subsequently, samples were postfixed in a solution of 2.0% osmium tetroxide (1.25% in water) of potassium ferrocyanide (2.5% + 0.2 M cacodylate buffer) for 1 h at room temperature, and a second wash with 0.1 mol/L cacodylate buffer (pH 7.2–7.4) was performed. Then, the samples were subjected to a cryofracture procedure, where a few drops of glycerol (30% in 0.1 mol/L cacodylate buffer) were added to the postfixed samples. The drops were added at 5 min intervals so that at the end of 30 min, the initial volume contained in the Eppendorf vials with the samples was folded. After 3 h, the liquid was discarded, and the samples were stored in a freezer at −80 °C until the day after, when the samples were dipped in liquid nitrogen for cryofractures. After fixation, the cuts were dehydrated in ascending grades of ethanol, critical-point dried and coated with gold. The preparations were processed for images using a scanning electron microscope (Jeol, JEM6610 LV, Jeol Inc. USA).

2.8. Vascular reactivity

After euthanasia, vascular reactivity was evaluated in isolated thoracic aortas as previously described in detail (Friques et al., 2015; Leal et al., 2020). After the equilibration period, a contraction was induced by the addition of phenylephrine (1 μM). Once the contraction stabilized, cumulative concentrations of acetylcholine (ACh, 10^{-11} to $10^{-4.5}$ log mol/L) were added to the bath to assess the endothelial integrity of the preparations. The vasorelaxation response to ACh was expressed as the percentage of vasodilation relative to the maximal phenylephrine-induced precontraction level, and for each curve, the maximum effect and the sensitivity were calculated using nonlinear regression analysis.

In some experiments, the rings were preincubated for 20 min with the following drugs: N(G)-nitro-l-arginine methyl ester (L-NAME, 100 μmol/L), a nonspecific NO synthase inhibitor; apocynin (100 μmol/L), a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor; or indomethacin (10 μmol/L), a nonspecific inhibitor of cyclooxygenase (Cox) 1 and 2, to evaluate their effects on ACh-induced relaxation. To verify whether the changes observed in ACh were in fact mediated by endothelium, sodium nitroprusside (SNP, 10^{-10} to 10^{-5} mol/L), an NO donor, was used to test the responsiveness of vascular smooth cells.

2.9. Determination of ROS in erythrocytes and aorta

Flow cytometry was performed using a FACS Canto II (Becton Dickinson - BD, CA, USA) instrument to quantify the endothelial cells and analyze the intracytoplasmic ROS content. For endothelial cell counting, the animals were anesthetized as described above, and the aortic arch was isolated, minced and digested using type II collagenase (1000 U/mL, at 37 °C for 60 min under constant shaking). The tissue fragments were removed by filtration using a sterile 70-μm nylon mesh. The free cells were immediately washed twice in PBS to remove excess collagenase, and the cell suspension was stored at −80 °C. The number of endothelial cells in the aortas was determined using an APC-conjugated monoclonal antibody against platelet endothelial cell adhesion molecule (CD31-APC). Briefly, 1×10^5 cells were resuspended in PBS and incubated with 5 μL of CD31-APC or the respective isotype control in the dark (20 min, RT). From each sample, 100,000 events were acquired and processed using FACS Diva software (Becton Dickinson - BD, CA, USA).

The intracytoplasmic ROS content was determined in isolated endothelial cells, as previously described (Friques et al., 2015). Intracellular superoxide anion ($^{\cdot}O_2^-$) and hydrogen peroxide (H_2O_2) were monitored separately by measuring changes in the median fluorescence intensity (MFI) emitted by dihydroethidium (DHE) and dichlorofluorescein (DCF), respectively. Briefly, 10^6 cells were incubated with 160 mmol/L DHE or 20 mmol/L DCF at 37 °C for 30 min (DHE, DCF) in the dark. The samples were then washed, resuspended in PBS and kept on ice until the acquisition of 10,000 events by flow cytometry, which were subsequently analyzed using FCS Express software (De novo, USA).

2.10. Data and biostatistical analysis

The values are expressed as the mean ± SEM. For each concentration-response curve, the maximum effect (R_{max}) and the molar concentration of compound required to elicit 50% of maximum relaxation (EC_{50}) were calculated by nonlinear regression analysis using the nonlinear fitting function of Prism software (Prism 8, GraphPad Software, Inc., San Diego, CA, USA). The sensitivities of phenylephrine precontracted rings to ACh or SNP were calculated using the pEC_{50} ($-\log EC_{50}$). The differences in the area under the curves (ΔAUC) were

calculated using the responses of the aortic rings before and after the presence of the inhibitor, and these results were expressed in arbitrary units (a.u.).

Considering that our samples had a normal distribution, the statistical comparisons between the different groups were performed by either one-way or two-way analysis of variance (ANOVA) for repeated measures or completely randomized, followed by Bonferroni's *post hoc* test. The statistical analyses were performed using Prism software. A value of $p < 0.05$ was considered statistically significant.

2.11. Drugs and chemicals

Acetonitrile, apocynin, ACh, cacodylate, DHE, DCF, indomethacin, L-NAME, o-phthaldialdehyde, osmium tetroxide, paraformaldehyde, glutaraldehyde, phenylephrine, potassium ferrocyanide, SNP, sodium cacodylate, tetrahydrofuran and Tris-HCl were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sodium thiopental (Thipentax®) was obtained from Bayer (Germany) and Cristalia (São Paulo, Brazil). All other chemical solvents used were of analytical grade.

3. Results

3.1. Kefir reduces blood pressure in renovascular hypertensive rats

Fig. 1A shows the average values of the resting systolic, mean and diastolic arterial pressure measurements in conscious animals 60 days after renal artery clipping. As expected, 2K1C rats developed hypertension compared to the Sham group (systolic: 205 ± 7 vs. 139 ± 6 mmHg; mean: 171 ± 6 vs. 95 ± 2 mmHg; diastolic: 130 ± 4 vs. 69 ± 5 mmHg, $p < 0.05$). The novel result was that kefir supplementation demonstrated antihypertensive effects in 2K1C rats, as observed through systolic, mean and diastolic arterial pressure (reduction by 13%, 23% and 30%, respectively, $p < 0.05$). Interestingly, periodic noninvasive systolic arterial pressure revealed that the prevention of hypertension using kefir was consolidated from the 28th day of supplementation with a reduction of 13% (2K1C: 227 ± 9 vs. 2K1C-kefir: 198 ± 9 mmHg, $p < 0.05$, Fig. 1B), extending to 45th and 60th days (reduction by 13% and 16%, respectively $p < 0.05$).

3.2. Determination of ACE activity and angiotensin II (plasma and intrarenal)

The average values in Fig. 2 show that after 60 days of RH, ACE activity was increased in both plasma (~20%) and intrarenal samples (nonclipped kidney: 24% and clipped kidney: 43%) of 2K1C rats compared to the normotensive Sham group ($p < 0.05$). The novel result was that treatment with kefir was able to significantly reduce ACE activity in the three target sites (plasma: 24%; nonclipped and clipped kidney: 40%, $p < 0.05$), suggesting that this probiotic may also reduce the production of Ang II. Confirming our hypothesis, we observed that Ang II levels were significantly reduced in both plasma and tissues from nonclipped and clipped kidneys in the 2K1C rats treated with kefir (54%, 50% and 35%, respectively) compared with the nontreated 2K1C group ($p < 0.05$).

3.3. Effects of kefir on the morphological alterations induced by renal clipping in the left ventricle and kidney

The classical changes in the mass of cardiac ventricles (hypertrophy) and clipped (atrophy) and nonclipped (hypertrophy) kidneys

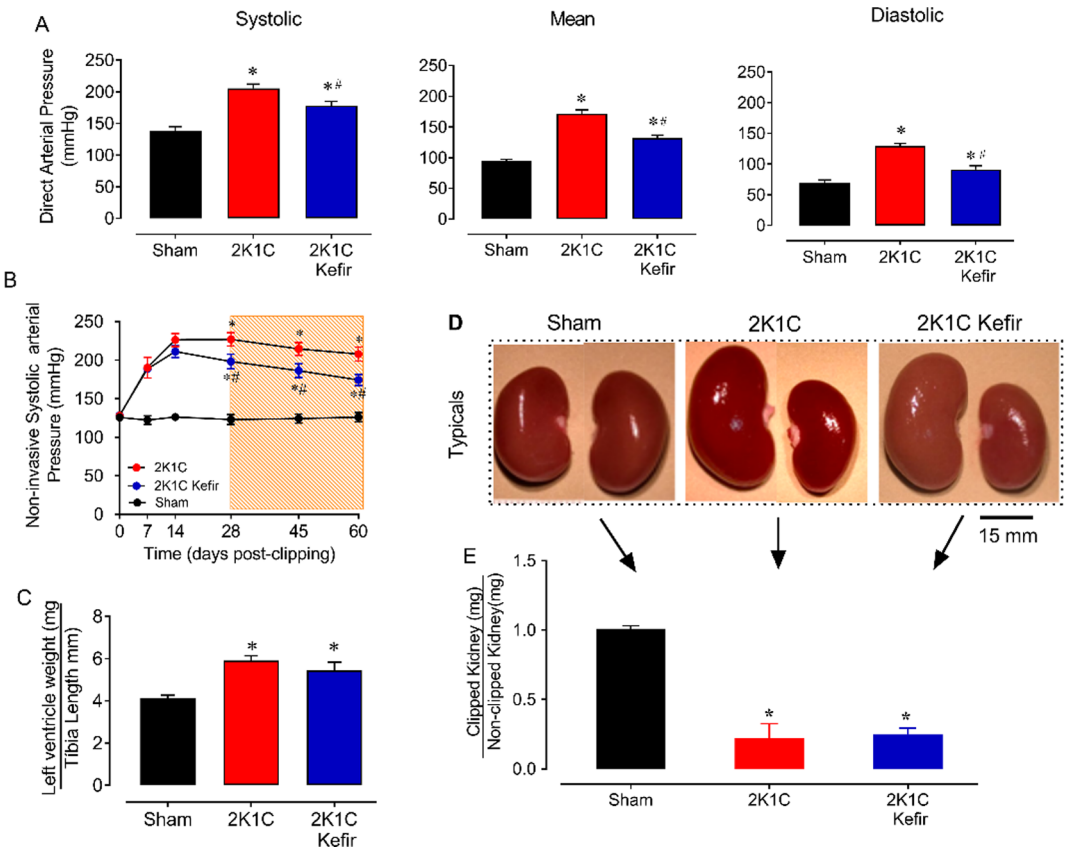

Fig. 1. Kefir reduced renovascular hypertension in 2K1C rats. Invasive measurements of arterial pressure (systolic, mean and diastolic, N = 8/group) (A) and noninvasive systolic values in conscious Sham, 2K1C and 2K1C-Kefir rats (N = 8/group) (B). Bar graphs show that kefir did not attenuate left ventricular hypertrophy (N = 6/group) (C) and did not prevent renal atrophy, as observed in typical macroimages (D) or bar graphs (N = 6/group) (E). Values are the mean ± SEM. $^{*}p < 0.05$ vs. Sham group and $^{\#}p < 0.05$ vs. 2K1C group; one-way (bar graphs) or two-way (line graph) ANOVA.

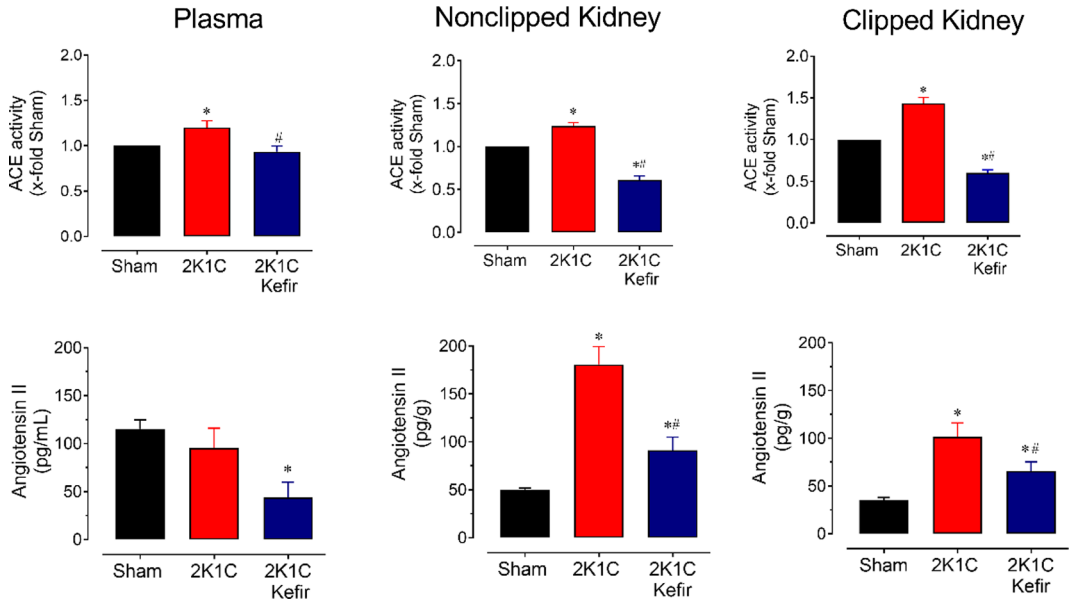

Fig. 2. Values of plasma and intrarenal (nonclipped and clipped kidney) angiotensin II in Sham, 2K1C renovascular hypertensive rats, and 2K1C rats treated with kefir. Each value represents the mean ± SEM (N = 6–8). *p < 0.05 vs. Sham group; #p < 0.05 vs. 2K1C group (one-way ANOVA).

that occur in the rat model of RH were confirmed in the present study (Fig. 1C–E). Despite the antihypertensive effect exhibited by kefir in the renovascular hypertensive group, kefir was not effective in mitigating left ventricular hypertrophy (2K1C: 5.9 ± 0.2 vs 2K1C-kefir: 5.5 ± 0.5 mg/mm, p > 0.05) compared to the values in the Sham (Sham: 4.3 ± 0.2 mg/mm, p < 0.05, Fig. 1C) or renal atrophy (2K1C: 0.22 ± 0.1 vs 2K1C-kefir: 0.24 ± 0.04 mg/nonclipped kidney, p > 0.05, Fig. 1D and E) groups.

The morphological analysis of the ultrastructure of the kidneys performed by scanning electron microscopy is shown in Fig. 3. In the nontreated 2K1C group, some typical damage in the renal glomerulus, podocytes and tubules was noted compared to the Sham group. Kefir treatment apparently reduced kidney damage, maintaining morphological characteristics similar to those in the Sham group.

3.4. Kefir reduces aortic endothelial dysfunction in 2K1C hypertensive rats

Due to the magnitude of endothelial dysfunction in the 2K1C group, we decided to use some animals from each group (n = 4 per group) to evaluate the typical damage to the architecture of the endothelial surface. As expected, scanning electron micrographs (Fig. 4) of the thoracic aortas from normotensive animals showed a confluent endothelial cell layer. In contrast, the 2K1C group exhibited gaps between endothelial cells, with clear damage to the luminal surface layer exposing the internal elastic membrane. Interestingly, the 2K1C animals treated with kefir (60 days) exhibited a better-preserved endothelial surface than non-treated 2K1C and showed no gaps.

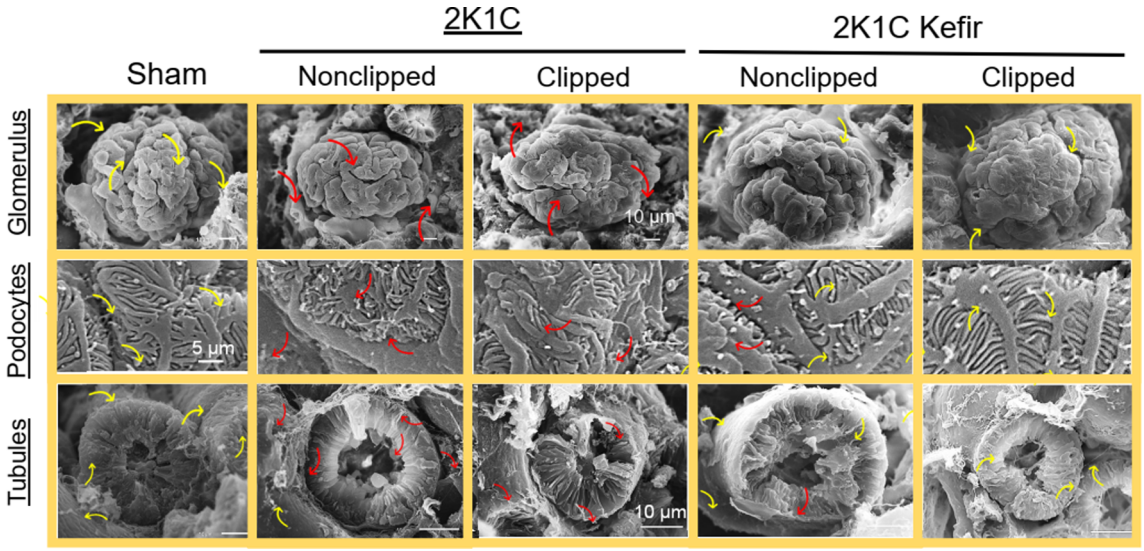

Fig. 3. Effects of kefir on kidney microstructures of renovascular hypertensive rats. Typical photomicrographs of the left kidney obtained by scanning electron microscopy showing glomeruli, podocytes, and renal tubules. Red arrows show sites of damage in architecture of the kidney, mainly observed in the clipped and nonclipped kidneys of 2K1C hypertensive rats. Yellow arrows suggest well-preserved structures in the kidney of Sham rats and in hypertensive rats treated with kefir (N = 5–6). Horizontal bars indicate the relative magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

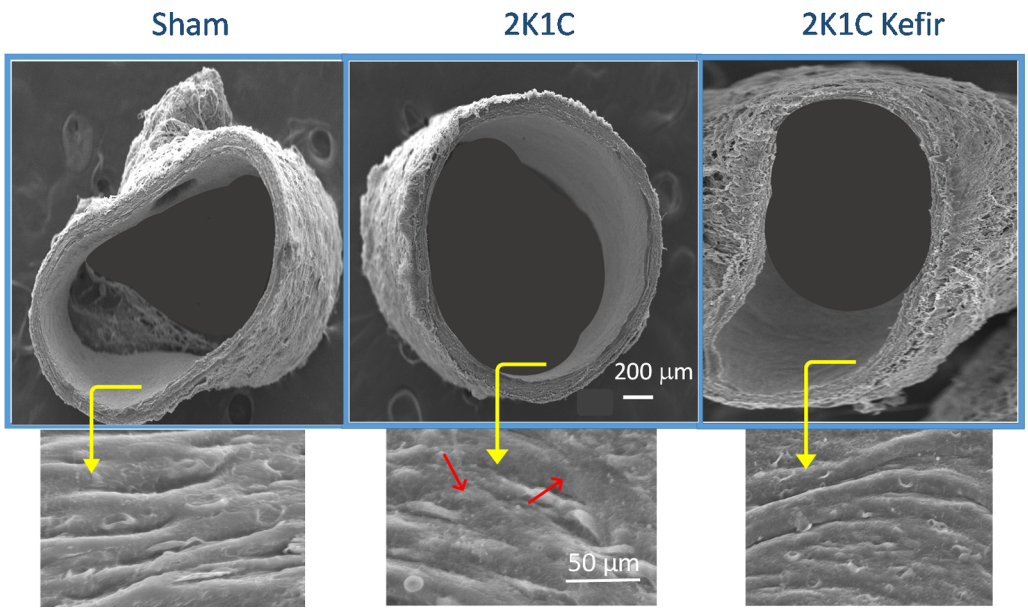

Fig. 4. Effects of kefir on the morphology of aortic vessels in renovascular hypertensive rats. Typical cross-section photomicrographs of the aorta obtained by scanning electron microscopy. Bottom images indicated by yellow arrows are magnifications of the endothelial surface. Suggestive damage to the endothelial surface in the 2K1C rat is indicated by red arrows lacking cellularity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Kefir reduces aortic endothelial dysfunction in 2K1C hypertensive rats

To evaluate the physiological conditions of endothelial function in the aortic rings, we first evaluated vascular responsiveness to 3 classical agents: ACh (to test endothelium-dependent relaxation in phenylephrine-precontracted vessels), the direct NO donor SNP (to test endothelium-independent relaxation) and phenylephrine (to investigate the contractile response).

Fig. 5 shows that ACh-induced relaxation was significantly impaired in the 2K1C group compared to the Sham group (Rmax: 50 ± 3% and 81 ± 2%, respectively, $p < 0.05$). Treatment with kefir for 60 days caused a marked reduction in that difference (Rmax for the 2K1C-kefir group: 68 ± 3%, $p < 0.05$ vs. 2K1C group), although this value was still statistically reduced with respect to the Sham group ($p < 0.05$). The 2K1C group also exhibited a significant decrease in the sensitivity to ACh compared to that in the Sham group (pEC50: 6.8 ± 0.2 vs. 7.5 ± 0.1, respectively, $p < 0.05$), and kefir was able to abolish that difference (pEC50 for the 2K1C-Kefir group: 7.5 ± 0.1, $p > 0.05$ with respect to the Sham group).

The endothelium-independent relaxation was tested using SNP (10^{-10} to 10^{-5} mol/L). As shown in the middle graph of Fig. 5, the relaxant effect of this direct NO donor was significantly reduced in the 2K1C group compared to the Sham group (Rmax: 86 ± 3 and 95 ± 1%, respectively, $p < 0.05$), although this reduction was of a lower magnitude than that observed with ACh. Kefir treatment completely prevented that alteration (Rmax for the 2K1C-Kefir group: 90 ± 2%, $p < 0.05$ vs. 2K1C). There was also a significant reduction in the sensitivity to ACh in the 2K1C hypertensive animals in comparison to the Sham group (pEC50: 7.1 ± 0.1 and 8.0 ± 0.1, respectively, $p < 0.05$). However, 2K1C-Kefir showed no difference in sensitivity to ACh when compared to the 2K1C group (pEC50: 7.7 ± 0.1, $p > 0.05$). Therefore, treatment with kefir did not change muscle dysfunction in 2K1C animals.

As seen in the right graph in Fig. 5, the aortic rings of the 2K1C group were hyperreactive to phenylephrine (Rmax: 85 ± 1%) compared to those of the Sham group (Rmax: 74 ± 2%, $p < 0.05$). Additionally, kefir treatment reduced vascular hyperreactivity (Rmax: 78 ± 2%). On the other hand, 2K1C-Kefir group showed no difference in the sensitivity to phenylephrine compared to the 2K1C or Sham groups (6.2 ± 0.1%; 6.2 ± 0.1%; 6.1 ± 0.15, respectively, $p > 0.05$).

3.6. Kefir restores NO bioavailability in 2K1C hypertensive rat aorta

The contribution of NO bioavailability to endothelial dysfunction in 2K1C cells and to the effect of kefir was evaluated using L-NAME (Fig. 6, Table 1). To achieve this goal, we calculated the average values of the maximum response, pEC50 (negative logarithm of required concentration to produce 50% of the maximum response – 'sensitivity') and the difference in the area under the curve (ΔAUC) from the concentration-response curve obtained during the blockade of the basal NO/cGMP molecular pathway with L-NAME. Interestingly, preincubation with this NOS inhibitor abolished the vasorelaxation induced by ACh in all 3 experimental groups (Fig. 6).

NOS blockade with L-NAME caused intense changes in the Rmax of concentration–response curves to ACh in the Sham (from 81 ± 8% to −1.1 ± 0.3%) and 2K1C (from 50 ± 4 to 5.4 ± 0.4%) groups and in the hypertensive rats treated with kefir (from 62 ± 7% to

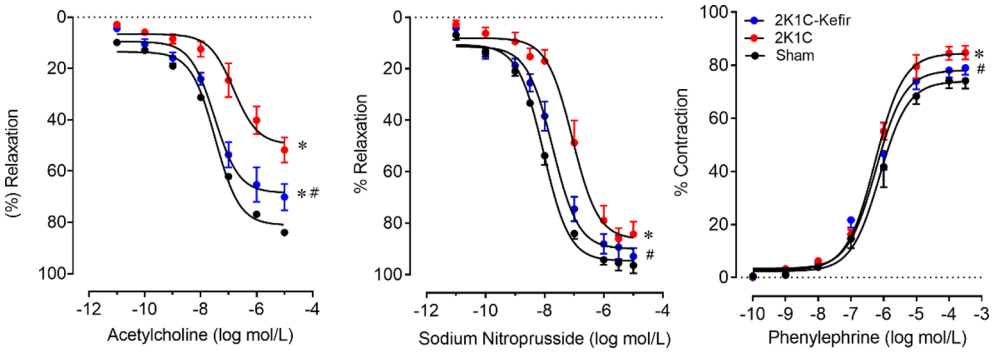

Fig. 5. Effects of kefir on vascular reactivity in renovascular hypertensive rats. Relaxation induced by cumulative concentrations of ACh (A) and SNP (B) in phenylephrine (1 μM) precontracted rings and contraction induced by cumulative concentrations of phenylephrine (C). Sham: control normotensive rats; 2K1C: renovascular hypertensive rats; 2K1C-Kefir: renovascular hypertensive rats treated with kefir. Each point represents the mean ± SEM of 8 experiments. Statistical significance: *$p < 0.05$ vs. Sham group; #$p < 0.05$ vs. 2K1C group (two-way repeated measures ANOVA).

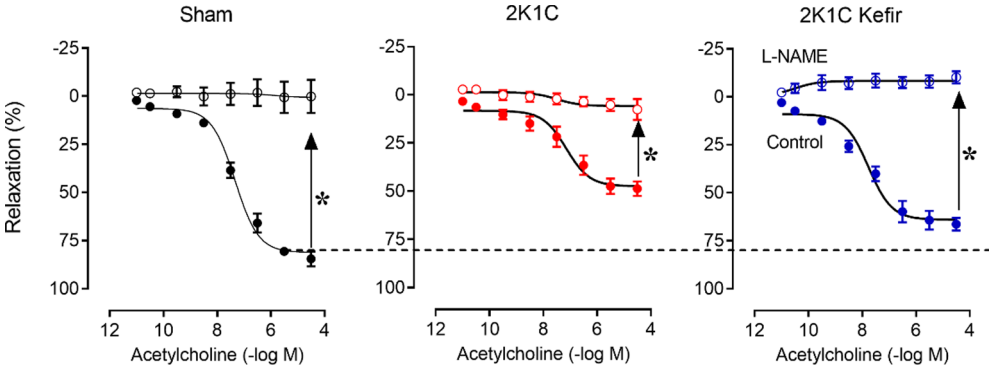

Fig. 6. Contribution of NO bioavailability to endothelial dysfunction in 2K1C cells and the effect of kefir treatment. The graphic shows the changes in the dose–response to Ach (●) following endothelial NO synthase blockade (o) with L-NAME (100 μmol/L). Each point represents the mean ± SEM of 8 experiments. Statistical significance: *p < 0.05 vs. Sham group (two-way repeated measures ANOVA).

7.2 ± 0.7%). The ΔAUC was smaller in the 2K1C group (ΔAUC: 155 ± 9 a.u.), in comparison with Sham animals (AUC: 255 ± 11 a.u., p < 0.05), and this value was restored by treatment with kefir (ΔAUC for the 2K1C-Kefir group: 247 ± 8 a.u., p < 0.05. The sensitivity was significantly altered only in the Sham (7.3 ± 0.4 to 5.5 ± 0.4) and 2K1C-Kefir (from 7.8 ± 0.5 to 10 ± 0.3) groups but not in the 2K1C group (Table 1).

3.7. Kefir reduces oxidative stress in 2K1C hypertensive rat aorta

The relative contribution of the ROS derived from NADPH oxidase in the ACh-induced relaxation was evaluated using apocynin. Regarding the maximum response, as shown in Fig. 7 (left panel), incubation with apocynin did not alter the relaxation (Rmax) in the aortic rings from the Sham group (before blockade: 81 ± 8 vs. after: 81 ± 7%) but significantly increased relaxation in t the 2K1C group (from 42 ± 4 to 58 ± 3%, p < 0.05, middle panel). Interestingly, treatment with kefir restored this abnormality (before blockade: 78 ± 8% vs. after: 78 ± 8%, Fig. 7-right panel), suggesting a vascular antioxidant effect. The ΔAUC analysis of the effects of ROS blockade showed an increased value in the 2K1C group (ΔAUC: 85 ± 9 a.u.), compared with Sham animals (AUC: 26 ± 4 a.u., p < 0.05), and the treatment of hypertensive animals with kefir restored the values (32 ± 3 a.u., p < 0.05) close to the values observed in Sham animals (Fig. 7, Table 1). The sensitivity parameter was significantly augmented by ROS blockade only in the 2K1C group, and it was restored by kefir treatment (before blockade: 7.8 ± 0.3 vs. after: 8.4 ± 0.2, p < 0.05).

3.8. Determination of $^{\cdot}O_2^-$ and H_2O_2 levels in blood cells and endothelial aortic cells

To quantify the bioavailability of $^{\cdot}O_2^-$ and H_2O_2 in whole blood cells and endothelial aortic cells, we used flow cytometry with DHE and DCF indicators (respectively) in all groups. As summarized in Fig. 8 (left panel), we observed an increase in ROS blood production in 2K1C animals (DHE: 929 ± 86, DCF: 551 ± 42 a.u.) compared to the Sham group (DHE: 516 ± 60, DCF: 252 ± 5 a.u., p < 0.05). According to the left panel of Fig. 8, ROS upregulation was also detected in endothelial cells of the aorta in the same hypertensive animals (DHE: 1771 ± 32, DCF: 562 ± 41 a.u., respectively) compared to the sham group (DHE: 1129 ± 85, DCF: 191 ± 2, a.u., p < 0.05). Interestingly, kefir supplementation prevented the ROS increase both in the blood (reduction of DHE: 60% and DCF: 53%, p < 0.05) and in the aortic cells (reduction of DHE: 61% and DCF: 65%, p < 0.05).

3.9. Kefir improves the balance vasodilation and vasoconstriction in the prostanoid pathway in 2K1C hypertensive rat aorta

To evaluate the arachidonic acid-Cox pathway and the influence of prostanoids on vascular responsiveness, some aortic rings were pre-incubated with indomethacin, followed by the construction of concentration-response curves to ACh (Fig. 9, Table 1). In the Sham group, no difference was found between pre- and post-blockade, since indomethacin did not modify the relaxation of the rings (Fig. 9, Table 1). On the other hand, the 2K1C group showed a greater relaxation in the presence of the Cox blocker, as indicated by the Rmax (48 ± 5% before blockade vs. 66 ± 4% after, p < 0.05) and the ΔAUC (87 ± 6 a.u.), in comparison to the Sham group (2.9 ± 0.2 a.u., p < 0.05). This suggests an increase in vasoconstrictor prostanoids in the 2K1C

Table 1
Average values of vascular parameters derived from the concentration–response curves to ACh: Maximum response (Rmax), sensitivity (pEC50) and area under the curve (AUC) aiming to investigate the molecular pathways involved in the impaired vasorelaxation in this model of experimental hypertension and the therapeutic effectiveness of kefir.

Parameters	Sham		2K1C		2K1C Kefir	
	Control	Blockade	Control	Blockade	Control	Blockade
NOS blockade effect						
Rmax (%)	81 ± 8	−1.1 ± 0.3[a]	50 ± 4	5.4 ± 0.4[a]	62 ± 7	7.2 ± 0.7[a]
ΔAUC (a.u.)	–	255 ± 11	–	155 ± 9[a]	–	247 ± 8
pEC50	7.3 ± 0.4	5.5 ± 0.4[a]	7.0 ± 0.2	7.0 ± 0.3[b]	7.8 ± 0.5	10 ± 0.3[ac]
ROS blockade effect						
Rmax (%)	81 ± 8	81 ± 7	42 ± 4	58 ± 3[a]	81 ± 9	78 ± 8[c]
ΔAUC (a.u.)	–	26 ± 4	–	85 ± 9[b]	–	32 ± 3[bc]
pEC50	7.5 ± 0.4	7.6 ± 0.6	7.8 ± 0.3	8.4 ± 0.2[a]	7.1 ± 0.4	6.8 ± 0.4
COX blockade effect						
Rmax (%)	100 ± 10	99 ± 9	48 ± 5	66 ± 4[ab]	83 ± 7	91 ± 8[c]
ΔAUC (a.u.)	–	2.9 ± 0.2	–	87 ± 6[b]	–	5.4 ± 2[bc]
pEC50	7.8 ± 0.6	8.2 ± 0.8	7.1 ± 0.3	8.3 ± 0.3[a]	7.1 ± 0.5	7.4 ± 0.3[c]

Values are mean ± SEM. [a]p < 0.5 vs. control value into the same group; [b]p < 0.05 vs. Sham group; [c]p < 0.05 vs. 2K1C group.

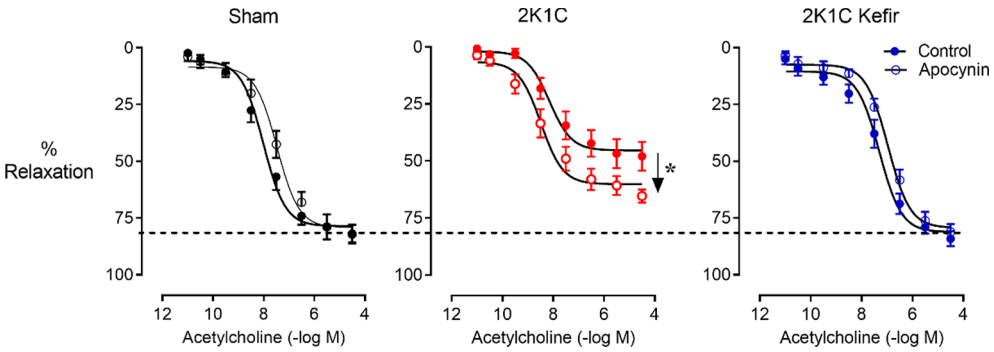

Fig. 7. Contribution of NADPH oxidase-generated ROS to endothelial dysfunction in 2K1C cells and the effect of kefir treatment. Concentration-response curves induced by Ach in rat aortic rings in the absence (●) or in the presence (o) of apocynin (100 μmol/L). Each point represents the mean ± SEM of 8 experiments. Statistical significance: *p < 0.05 vs. Sham group (two-way repeated measures ANOVA).

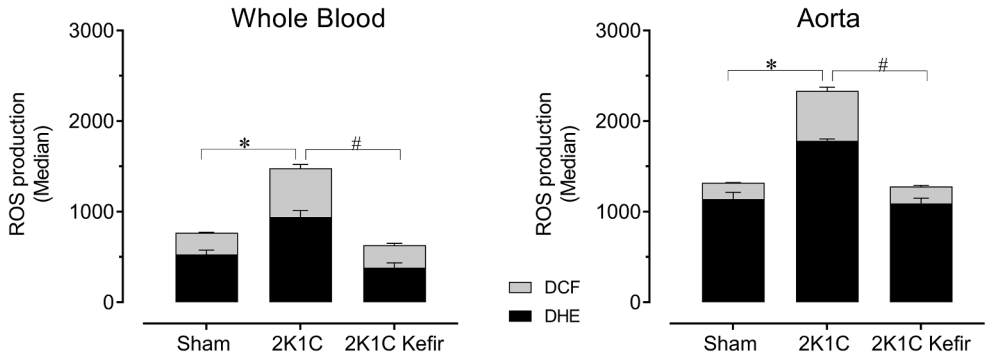

Fig. 8. Kefir prevents ROS production in blood and endothelial aortic cells in rats with renovascular hypertension. Graphical representation of flow cytometry showing the fluorescence intensity of DHE and DCF with severe overproduction of $^{\cdot}O_2^-$ and H_2O_2 in the 2K1C groups, being which was completely reversed by kefir supplementation in both samples. Values are presented as the mean ± SEM for n = 4–6 animals per group. *p < 0.05 vs. control group and #p < 0.05 vs. 2K1C group (one-way ANOVA).

pathway, but not in 2K1C treated with kefir, which demonstrated discreet participation (Rmax after blockade: 91 ± 8% and ΔAUC: 5.4 ± 2 a. u, p < 0.05). Following the same protocol, Cox pathway blockade caused a significant increase in the sensitivity in the 2K1C group (from 7.1 ± 0.3% to 8.3 ± 0.3%) but not in the Sham and 2K1C-Kefir groups (Fig. 9, Table 1).

4. Discussion

In the last two decades, the abrupt jump in the number of scientific publications (i.e., from 13 in 2001 to 7725 papers in 2019) has generated extraordinary new knowledge and simultaneously clarified many unanswered questions about the interaction between the gut microbiota and the function of the organs and systems in health and diseases (Companys et al., 2020; Khalesi et al., 2019). Moreover, there is a consensual opinion that probiotic supplementation contributes to preventing chronic disorders, including cardiovascular diseases (Pimenta et al., 2018). On the other hand, even with important advances, there is still a large step needed to further understand the contribution of probiotic consumption in the potential protective effectiveness in cardiovascular diseases (Peng, Xiao, Hu, & Zhang, 2018). In parallel, a growing number of studies are designed to study the beneficial effects of prebiotics and probiotics targeting disorders in the cardiovascular system, such as cardiac dysautonomia and abnormal vascular responsiveness (Friques et al., 2020; Klippel et al., 2016). On the other hand, there are many open questions that need to be clarified, as recently stated by Sanders, Merenstein, Reid, Gibson, and Rastall (2019); despite important advances, more research and translational efforts on prebiotics and probiotics are still needed (Khalesi et al., 2019; Sanders et al., 2019).

This study was designed to investigate the possible protective effects of chronic supplementation with the probiotic kefir on the cardiovascular-renal system in the 2K1C model. The main beneficial effects observed in this study and discussed below are (a) a moderate decrease in high blood pressure, (b) reduction of Ang II levels in both plasma and kidney tissue, (c) improvement of vascular endothelial responsiveness to ACh and (d) protective role in the architecture of nephron segments and aorta endothelial surface.

Our experimental design was inspired by previous data from our laboratory and from other research groups. First, several studies have shown that changes in the composition of the gut microbiota in murine models and humans may contribute to the development of arterial hypertension (Bellikci-Koyu et al., 2019; Honour, 1982; Jama, Kaye, & Marques, 2019; Tang, Kitai, & Hazen, 2017; Venkatakrishnan, Chiu, & Wang, 2020). This is in agreement with recent data demonstrating that the oral administration of kefir reduces arterial pressure in both SHR

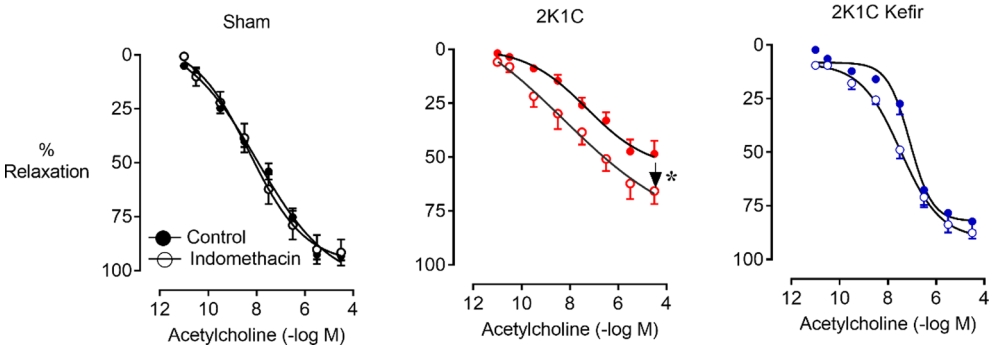

Fig. 9. Contributions of prostanoids to the effect of kefir on endothelial dysfunction in renovascular hypertensive rats. Concentration-response curves induced by Ach in rat aortic rings in the absence (●) or in the presence (o) of indomethacin (10 μmol/L). Each point represents the mean ± SEM of 8 experiments. Statistical significance: *p < 0.05 vs. Sham group (two-way repeated measures ANOVA).

(model of primary hypertension) (de Almeida Silva et al., 2020; Friques et al., 2015; Tang et al., 2017) and 2K1C rats (a model of secondary hypertension) (Amorim et al., 2019). Moreover, recent studies have shown that kefir administration results in attenuated endothelial dysfunction and improves baroreflex control of arterial pressure, probably by antioxidative and anti-inflammatory actions (Brasil et al., 2018; de Almeida Silva et al., 2020; Friques et al., 2015). However, it is difficult to accurately determine a major mechanism of action of a probiotic that has beneficial effects in diverse diseases. Our alternative strategy was to study the impact of this probiotic in the 2K1C rat, an experimental model mostly dependent on Ang II (Campos et al., 2015; Herrmann & Textor, 2019; Navar et al., 1998; Pereira et al., 2009).

Interestingly, while kefir lowered blood pressure only after 60 days of treatment (Friques et al., 2015), Our current investigation demonstrated that 2K1C rats treated with the same low dose of kefir achieved early antihypertensive efficacy (from 28 days), providing further insight into the mechanisms of its antihypertensive properties. Interesting that 1) RAS plays an important role in the maintenance of 2K1C hypertension (Campos et al., 2015; Herrmann & Textor, 2019; Navar et al., 1998; Pereira et al., 2009), 2) ACE activity in serum and tissues significantly increases during the development of RH (Okamura, Miyazaki, Inagami, & Toda, 1986; Sharifi, Akbarloo, Heshmatian, & Ziai, 2003); and 3) kefir peptides are capable of inhibiting ACE activity *in silico* and *in vivo* using proteomic and pharmacological approaches, respectively (Amorim et al., 2019). We suggest that kefir may exhibit characteristics of an effective 'captopril-like' nutraceutical, justifying our investigation in this chronic hypertensive Ang II-dependent model.

ACE is a decapeptidyl peptidase that catalyzes the degradation of bradykinin to inactive fragments (reducing the serum levels of endogenous vasodilators) (Ferreira, 2000; Ferreira, Greene, Alabaster, Bakhle, & Vane, 1970). At the same time, it catalyzes Ang II production, contributing directly to the increase in arterial pressure via complementary mechanisms (through AT1 receptors), including vasoconstriction, sympathoexcitation coupled with aldosterone and vasopressin release under the influence of oxidative stress (Campos et al., 2015; Lavoie & Sigmund, 2003; Peotta, Gava, Vasquez, & Meyrelles, 2007). Confirming our hypothesis, we found that both ACE activity and Ang II bioavailability (in both blood and contralateral/clipped kidneys) were decreased in the 2K1C group treated with kefir. These data corroborate recent findings by Amorim et al. (2019) from our laboratory, suggesting that several peptide candidates found in kefir could act as ACE inhibitors, justifying the impact of this 'captopril-like' probiotic in target organs, such as kidneys and systemic arteries, as discussed below.

Regarding the renal parameters, previous studies have already demonstrated some nephroprotective properties of kefir in different experimental models (diabetes, low-dose aspirin, ischemia-reperfusion injury), observing improvement in renal function, probably due to antioxidative effects exhibited by this probiotic, although by unknown mechanisms (Kanbak et al., 2014; Punaro et al., 2014; Yener et al., 2015). In the present study, we performed morphological analysis of clipped and nonclipped kidneys through scanning electron microscopy. The typical images (Fig. 3) revealed a clear integrity of glomerulus, podocytes and kidney tubules in the 2K1C hypertensive rats treated with kefir when compared with the structural damage observed in some nontreated 2K1C rats exposed to high levels of plasma and intrarenal Ang II, corroborating other studies (Dias et al., 2014; Pereira et al., 2009; Satou, Penrose, & Navar, 2018). Therefore, we suggest that due to the 'captopril like' effect, the pro-oxidative and pro-inflammatory effects of Ang II might be partially antagonized by kefir, reflecting a diminished glomerular and tubular injury in RH, despite the maintenance of mass of clipped and nonclipped kidneys. Although morphological analysis of the kidneys was not the main focus of this study, these exciting findings open new avenues for investigation of renal function. In this regard, further protocols such as specific staining tools, renal address biomarkers (urea, creatinine and cystatin C), glomerular filtration rate and renal plasma flow need to be designed to clearly detail these questions.

Endothelial dysfunction is an important biomarker of vascular damage commonly observed in high blood pressure and neuro-humoral factors (Choi et al., 2014; Dias et al., 2014; Fahning et al., 2015; Konukoglu & Uzun, 2017; Schäfer et al., 2012; Vanhoutte, Shimokawa, Tang, & Feletou, 2009; Vasquez et al., 2016, 2020), such as in the present model. In this context, previous data have shown that upregulation of the ACE/Ang axis culminates with the impairment of endothelium-dependent relaxation to ACh and hyperresponsiveness to norepinephrine. These abnormalities are accompanied by a marked decrease in NO bioavailability (triggered by ROS generation) and improved vasoconstriction to prostanoids (Choi et al., 2014; Dias et al., 2014; Schäfer et al., 2012). In the current study, we evaluated the effects of kefir treatment on endothelial dysfunction in hypertensive 2K1C rats. First, endothelium-dependent (to ACh) and endothelium-independent relaxation (to SNP) were diminished in 2K1C rats, indicating that vascular damage may be due to impaired function linked to molecular signaling in both endothelial cells (frequently observed in this model) and vascular smooth muscle cells (rarely observed in this model) (Choi et al., 2016; Lee et al., 1995). The novel finding is that kefir helped to protect these two layers of the aorta, as indicated by the parameters of the dose–response curve to ACh and SNP (Fig. 5). This differs from what we have observed in SHR rats treated with kefir demonstrating a protective effect only in endothelial surface cells (Friques et al., 2015). In addition, we found hypercontractility in aortic rings from 2K1C, which was attenuated by kefir (Fig. 5). To investigate possible mechanisms that may explain the vascular beneficial effects of kefir and the partial recovery of the architecture of the endothelial surface, we designed standard protocols using pharmacological inhibitors to assess the relative contribution of each ACh-dependent molecular pathway.

It is well known that along artery segments, there is a varying participation of vasoactive substances (e.g., NO, ROS, prostanoids, endothelium-derived hyperpolarizing factor) (Bolz, de Wit, & Pohl, 1999; Dias et al., 2014; Goto & Kitazono, 2019; Meyrelles, Peotta, Pereira, & Vasquez, 2011). Moreover, studies have demonstrated that NO is the main contributor to vasodilation in the aorta of rats, justifying our investigation of downstream-related pathways. Corroborating these previous studies, our protocol using L-NAME emphasizes that the impairment of NO/cGMP signaling is the primary contributor to vascular damage (observed by abolition of vasodilator response), and interestingly, the vascular protection generated by kefir is highly NO-dependent. In addition, since previous studies have shown the involvement of NO physiological antagonists such as ROS and vasoconstrictor prostanoids in Ang II-dependent hypertensive animals (Choi et al., 2016; Dias et al., 2018; Leal et al., 2017; Virdis, Duranti, & Taddei, 2011), we performed complementary procedures in our study. After blockade of the main source of ROS with apocynin (an NADPH oxidase inhibitor), we noted a significant improvement in the partial endothelium-dependent relaxation induced by ACh in 2K1C aortic rings. Thus, our results are in agreement with others that demonstrated a relevant participation of the Ang II/AT1/NADPH oxidase/ROS axis in RH (Campos et al., 2011; Choi et al., 2014; Dias et al., 2014; Virdis et al., 2011). Despite the relevant results observed in the present data, further studies are needed to evaluate whether the above results can be reproduced in resistance arteries and to determine the possible contribution of the EDH/EDHF pathways in this process. In agreement with the vascular reactivity, our findings using flow cytometry also supported the antioxidant properties of chronic kefir supplementation, both at systemic and local levels, as we recently reported (Friques et al., 2015, 2020; Ton et al., 2020) and others (Fahmy & Ismail, 2015). Although recent data have shown that kefir grain fermentation has intrinsic antioxidant activities (justified by chelation of transition metals by lactoferrin/albumin or free radical scavenging by tyrosine/cysteine) (Hernández-Ledesma, Amigo, Ramos, & Recio, 2004; Sabokbar & Khodaiyan, 2016), we hypothesized that the 'captopril-like' effect

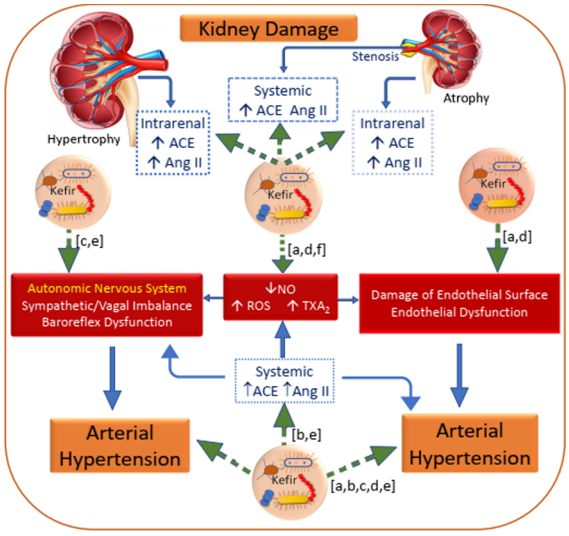

Fig. 10. Summary of the beneficial effects of the probiotic kefir on the morphological and functional changes occurring in the blood vessels and kidney that are observed in Goldblatt renovascular hypertension. The scheme is based on the findings from our laboratory and from others. ACE: angiotensin converter enzyme; Ang II: angiotensin II; NO: nitric oxide; ROS: reactive oxygen species; TXA2: thromboxane. The letters in square brackets correspond to the following references: [a] Friques et al. (2015); [b] Amorim et al. (2019); [c] Klippel et al. (2016); [d] Friques et al. (2020); [e] Brasil et al. (2018) and [f] Ton et al. (2020).

promoted by kefir might be another important factor to antagonize the prooxidant and harmful influences of Ang II in hypertension. Moreover, we consider that in addition to the AT1/NADPH pathway, complementary mechanisms have been recently proposed, such as the direct generation of mitochondrial ROS via mitochondrial Ang II internalized via AT1 receptors (Vajapey, Rini, Walston, & Abadir, 2014; Valenzuela et al., 2016). However, regardless of the activation mechanism, the antioxidant effect provided by this nutraceutical seems extremely attractive for hypertension control, mainly in patients refractory to antihypertensive medications.

In addition, since previous studies suggested that Ang II mediated by ROS production could stimulate COX-1 activity and consequently lead to an increase in vasoconstrictor prostanoids (e.g., PGH2, TXA_2) (Leal et al., 2017; Schäfer et al., 2012; Virdis et al., 2007, 2011), we explored this pathway using a Cox inhibitor and indirectly observed their contributions to the relaxation response induced by ACh in all groups. Interestingly, indomethacin partially ameliorated the Ang II-induced endothelial dysfunction only in 2K1C animals, corroborating previous data (Schäfer et al., 2012; Virdis et al., 2007, 2011). Fortunately, 2K1C treated with kefir was able to abolish this difference, contributing even more to its vascular protective effects against hypertension-associated complications (Vasquez et al., 2019).

A further design should include an analysis of the physiological parameters of the kidneys (blood flow and glomerular filtration) and potential biomarkers of glomerular and tubular damage. In addition, due to the important 'captopril-like' effect of this probiotic, the bradykinin pathway needs to be investigated in this experimental model. The hypothesis to be tested is that, at least in part, the cardiovascular protective effects could be triggered by the accumulation of bradykinin and not only by reductions in the harmful effects of Ang II.

5. Conclusion

This study is the first to demonstrate that probiotic supplementation with kefir, even at a low dose, was able to decrease the Ang II-dependent high blood pressure in 2K1C rats early. The data show that the hypotensive effects of kefir seem to occur, at least in part, via ACE-Ang II inhibition. As summarized in Fig. 10, other remarkable beneficial effects included the improvement of nephron structure, endothelial dysfunction, attenuation of the high levels of ROS in the plasma and kidney tissues and attenuation of damage to the architecture of the aortic endothelial surface. These findings suggest that kefir (and probably other probiotics) is a promising coadjuvant nutraceutical against the progression of hypertension.

6. Ethics statement

The experiments were conducted in 138 male Wistar rats (140–160 g) provided by the Experimental Animal Care of Federal University of Espirito Santo, Brazil. All animals were kept in clean polypropylene cages in a constant light-dark cycle of 12 hours, temperature from 20 to 25 °C, humidity of 70% prior to the start of experiments and during the 60 days of treatment. Animals were provided with *ad libitum* water and standard diet pellet until the treatment was completed. The experimental protocol was established according to the ethical principles of the research with animals, established by the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978), National Technical Commission on Biosafety (CTNBio) and the Ethics Committee on the Use of Experimental Animals (CEUA-UFES, protocol # 489-2018).

Author contributions

BLM, SCW and **MASL** carried out the experiments and provided data acquisition, analysis and interpretation of the main results. **RY, ATD** and **DEC** and carried out the protocol of Angiotensin peptides at the Federal University of Sao Paulo. **MCT** and **RRC** participated in study's design and in the critical revision of the manuscript. **BPC** and **SSM** conducted the cytometric analysis in this study. **BLM, SCW and BVN** performed the scanning electron microscopic analysis. **TMCP** and **ECV** contributed to the conception, design and supervision of the study and interpretation of all data. All authors read and approved the final version of the manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2020.104260.

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