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Probiotic Kefir Prevents Renal Ischemia-Reperfusion Injury through Reduced Oxidative Stress and Apoptosis in Wistar Rats

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Abstract

Aim: Acute Kidney Injury (AKI) is an important healthcare issue with limited supportive care. Kefir is a probiotic agent that has been suggested to play a beneficial effect in kidney disease. The goal of the present study is to evaluate if the probiotic kefir may demonstrate beneficial effects in the treatment of AKI.

Methods: Male Wistar rats were treated with vehicle or Kefir (0.3mL/100g of body weight) for 14 or 60 days. Following treatment animals were submitted to sham or renal ischemia reperfusion surgery to induce AKI. Renal function was determined using inulin and para-aminohippurate clearance, and reactive oxygen species and apoptosis were quantified in kidney by flow cytometry.

Results: Our results demonstrate that 60-days kefir treatment was able to ameliorate AKI-induced renal injury, by reducing renal vascular resistance and increasing glomerular filtration rate. Animals receiving kefir during 60 days also presented attenuated superoxide production and apoptosis and rescued nitric oxide production within the kidney medulla.

Conclusion: Our data demonstrate a beneficial effect of kefir in AKI through reduction of oxidative stress and apoptosis.

Keywords: Acute kidney injury; Ischemia reperfusion; Kefir; Probiotics; Renal function

Introduction

Acute Kidney Injury (AKI) is an important healthcare issue worldwide. It is considered a clinical condition that occurs when Glomerular Filtration Rate (GFR) is acutely decreased leading to kidney failure [1]. Approximately 5% of hospitalized patients and 30% of critically ill patients present AKI [2,3], which has been associated with an increased risk of developing chronic kidney disease and end-stage renal disease [4,5]. Despite advances in treatment and in our understanding of the pathogenesis of AKI, the disease still remains subject to controversy, confusion and lack of consensus [6]. Due to the multiple causes leading to AKI, the current management is nonspecific and associated with limited supportive care. Thus, novel therapies aimed at preventing the development of AKI is in high demand [7]. The mechanisms underlying AKI are characterized by a complex interaction between predisposing chronic illnesses, hemodynamic disturbances, nephrotoxic insults and inflammatory responses leading to tubular cell injury and a decline in Glomerular Filtration Rate (GFR) [8]. Oxidative stress and inflammation are considered to play a central role in AKI [1-2,8] and previous investigations have demonstrated that the beneficial effects of probiotic use in the management of renal injury involves decreased reactive oxygen species and pro-inflammatory cytokines production [9]. Kefir, an acidic-alcoholic fermented milk product that presents a little acidic taste and creamy consistency [10] is a commonly used probiotic. In the kidney, studies showed that kefir administration reduces the progression of renal injury in

diabetic rats [11] and attenuates high-salt-induced kidney damage [12]. Therefore, the present study aimed to determine if Kefir can prevent the development of AKI.

Materials and Methods

Animals

Experiments were conducted in male Wistar rats (225-300g), maintained in the animal care facility at the Federal University of Espirito Santo, Brazil. The animals were housed in individual cages with a controlled temperature (22-23ºC) and humidity (60%) and were exposed to a 12:12-h light-dark cycle. All of the experimental procedures were performed in accordance with the National Institutes of Health (NIH) guidelines, and the experimental protocols were approved by the Institutional Animal Care and Use Committee (CEUA-UFES Protocol nº. 040-2013).

Kefir Treatment and AKI induction

The kefir grains used in this study were generously donated by Ieda Carneiro Kalil, MSc, from the University of Vila Velha. The kefir beverage was prepared by adding kefir grains to pasteurized whole milk (4%) and allowing the solution to mix at room temperature for 24 hours. The mixture was then filtered using a plastic screen, and refrigerated for 24 hours to allow yeast growth. Kefir was aliquoted into sterile plastic tubes and stored at -20°C until use. Animals were administered with either vehicle (whole milk, adjusted pH 5.0, 0.3mL/100g of body weight) or kefir (0.3mL/100g of body weight) for 14 or 60 days, by gavage. After treatment, rats were submitted to renal ischemia reperfusion or sham surgery. Briefly, a midline abdominal incision was made, kidneys were exposed and within 5 minutes, the renal blood supply was interrupted for 45 min by clamping the renal pedicles of both kidneys with a suture line. Following the ischemic episode, kidneys were re-perfused for 24 hours. Sham surgery was performed following all the above-mentioned steps, except by the ligation of the renal artery. Postoperative dehydration was prevented by subcutaneous administration of 1.0 mL of 0.9% NaCl [13,14].

Renal Function Evaluation

Renal function was assessed using inulin clearance, which is considered the gold-standard method to determine GFR. The animals were anesthetized with sodium thiopental (50 mg/Kg ip.). The trachea was catheterized with a polyethylene tube (PE-90) to facilitate breathing, and a catheter (PE-240) was introduced into the bladder for urine sampling. The arterial catheter was connected to a pressure transducer (Cobe Laboratories, USA) plugged into a pressure-processor amplifier and data acquisition system (MP100, Biopac Systems, USA) for continuous monitoring of Mean Arterial Pressure (MAP) and Heart Rate (HR). The venous catheter was connected to an infusion pump (0.1 mL/min), which infused a saline solution (0.9%) containing 3% of mannitol over a period of 30 minutes. After this stabilization period, the animals received an intravenous injection of prime solution containing IN (300 mg/Kg) and and Para-Aminohippurate (PAH) (6.66 mg/kg) and were maintained on a continuous infusion of saline, inulin (15 mg/ml), PAH (4 mg/ml), and mannitol (3%) until the end of the experiment. At 30-minute intervals, urine and blood samples were taken, for a total of 4 samples. Haematocrit was measured using a heparinized capillary tube. Plasma and urinary IN and PAH concentrations were measured using a colorimetric assay. Inulin and PAH clearance were calculated using the standardized formula. Renal Blood Flow (RBF) and Renal Vascular Resistance (RVR) were calculated by the equations RBF = RPF / (1- haematocrit), and RVR= MAP / RBF, respectively.

Measurement of intracellular reactive oxygen species (ROS) production

ROS analysis was achieved by flow cytometry as previously described [15]. DHE (160 µM) and DAF (2mM) were added to the cell suspension (10^6 cells) and incubated at 37°C in the dark for 30 min and 180 min, respectively, to estimate intracellular $\bullet O_2^-$ or NO concentration [16,17]. For the positive control, samples were treated for 5 min with 10 µM doxorubicin or with 100µM sodium nitroprusside. Cells were then washed, resuspended in PBS, and kept on ice for an immediate detection by a flow cytometer (FACSCanto II,Becton Dickinson, San Juan, CA). Data were analyzed using the FACSDiva software (Becton Dickinson). For quantification of DHE and DAF fluorescence, samples were acquired in duplicate and 10,000 events were used for each measurement. Cells were excited at 488nm and DHE and DAF fluorescence were detected using, respectively, 585/42 and 530/30 bandpass filters. Data are expressed as geometric mean fluorescence intensity.

Apoptosis quantification

Apoptotic cells were quantified by Annexin V-FITC and Propidium iodide (PI) double staining, using an Annexin V-FITC apoptosis detection kit (Becton Dickinson, CA). In brief, after the separation of renal cortex and medulla, cells were washed twice with PBS, adjusted to 500 µl of the binding buffer ($5x10^5$ cells). Then, 2 µl of Annexin V-FITC and 2 µl of PI were added, and cells were gently vortexed. Cells were incubated for 15 min at room temperature (25°C) in the dark. Finally, cells were analyzed by flow cytometry (FACSCanto II, Becton Dickinson). Cells showing as Annexin V-/PI+ were recognized as necrotic, and those showing as Annexin V+/PI+ were interpreted as late apoptotic or secondary apoptotic, while Annexin V+/PI- cells were recognized as early or primary apoptotic cells.

Statistical analysis

Values are expressed as means ± S.E.M. Statistical comparisons between the different groups were performed by one-way Analysis of Variance (ANOVA) followed by Tukey´s post hoc test.

The statistical analyses were performed using Prism software (Prism 6, GraphPad Software, Inc, San Diego, CA, USA). A value of $p<0.05$ was regarded as statistically significant.

Results

Figure 1 demonstrates the results of GFR (A) and RPF (B), as assessed by inulin and PAH clearance; as well as renal RBF (C) and RVR (D) determination. Treatment with kefir did not change GFR (mL/min/Kg) in sham group (vehicle: 6.2 ± 0.3; kefir: 5.2 ± 0.4). As expected, ischemia reperfusion resulted in a decreased inulin clearance (3.0 ± 0.3 mL/min/Kg, $p<0.05$ vs. sham) and the treatment with kefir during 14 days did not ameliorate IR-induced renal dysfunction (2.9 ± 0.2 mL/min/Kg, $p<0.05$ vs. sham). However, after 60-days of kefir administration the decline of GFR was ameliorated (4.8 ± 0.7 mL/min/Kg, $p<0.05$ vs. IR vehicle and IR kefir 14d). Induction of AKI resulted in decreased RPF (7.9 ± 1.6 mL/min/Kg, $p<0.05$ vs. sham) and RBF (12.7 ± 2.8 mL/min/Kg, $p<0.05$ vs. sham) in animals receiving vehicle when compared to sham vehicle (RPF: 17.9 ± 1.9; RBF: 29.9 ± 3.3 mL/min/Kg) and sham kefir (RPF: 14.8 ± 2.5; RBF: 25.1 ± 4.5 mL/min/Kg) group. The administration of kefir in I/R rats during 14 or 60 days did not affect RPF (14d: 11.2 ± 1.6; 60d: 10.3 ± 1.2 mL/min/Kg) or RBF (14d: 18.9 ± 3.0; 60d: 17.6 ± 2.3 mL/min/Kg). Although no statistical differences were reached, the treatment with kefir in I/R rats appears to elevate RPF and RBF. RVR was not changed by kefir treatment in sham groups (vehicle: 3.6± 0.5; kefir: 4.6± 0.5 a.u.), however I/R vehicle-treated animals exhibited a striking increase in RVR (10.32 ± 1.0, $p<0.05$ vs. sham). The elevated RVR in I/R animals was ameliorated by kefir administration for 14 (5.5± 0.7, $p<0.05$ vs. IR vehicle) and 60 days (7.0± 0.8, $p<0.05$ vs. IR vehicle).

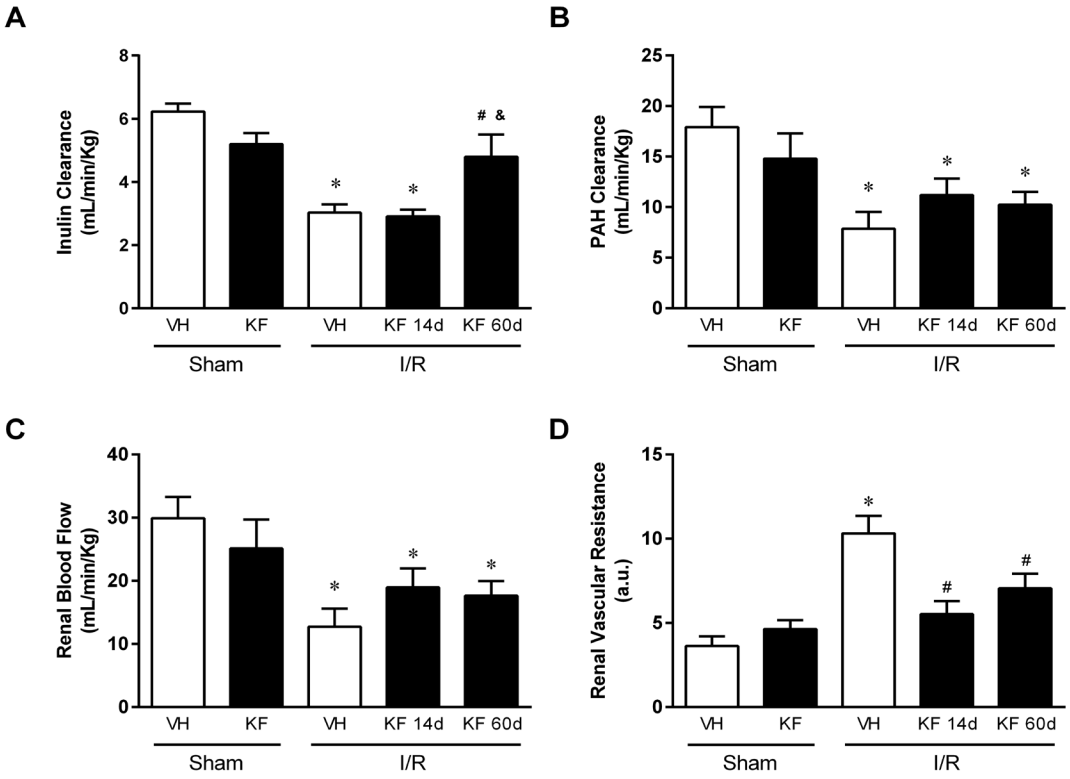

Figure 1: Effects of kefir treatment on renal hemodynamics were evaluated through determination of glomerular filtration rate (GFR) (using inulin clearance) **(A),** Renal Plasma Flow (RPF) (using PAH clearance) **(B),** renal blood flow (RBF) **(C),** and renal vascular resistance **(D)** in animals receiving vehicle (white bars) and kefir (black bars). GFR was significantly decreased in I/R vehicle. Kefir treatment prior to AKI induction resulted in an amelioration of GFR. Renal vascular resistance (RVR) was increased in I/R animals and kefir treatment ameliorated the rise in RVR. N=6-7. Values are means ± SEMs. *$p<0.05$ *vs*. sham; #$p<0.05$ *vs*. I/R vehicle, &$p<0.05$ vs. I/R kefir 14 days. One-way ANOVA followed by Tukey's post hoc test.

Renal production of $\bullet O_2^-$ and NO was assessed through DHE (left panel) and DAF (right panel), as displayed in Figure 2. The analysis of the cortical DHE and DAF fluorescence showed that superoxide anions (2.0 ± 0.1 a.u.) and NO (2.2 ± 0.1 a.u.) production was not changed by kefir treatment in sham animals ($\bullet O_2^-$: 1.8 ± 0.1; NO: 2.3 ± 0.1 a.u.). As expected, we detected an increased $\bullet O_2^-$ (2.4± 0.1 a.u, $p<0.05$ vs. sham) and decreased NO (1.7± 0.1 a.u, $p<0.05$ vs. sham) generation in I/R animals treated with vehicle. After 14 (1.9± 0.1 a.u. $p<0.05$ vs. IR vehicle) and 60 (1.7± 0.1 a.u, $p<0.05$ vs. IR vehicle) days of kefir administration $\bullet O_2^-$ production was reduced in I/R group. NO production was rescued in the 60 days' group (2.7± 0.1 a.u, $p<0.05$ vs. IR vehicle) but not in the 14 days' group (2.1± 0.1 a.u.). Medullary analysis of ROS production showed similar results. Treatment with kefir did not change $\bullet O_2^-$ (2.0 ± 0.1 a.u.) or NO (2.4 ± 0.1 a.u.) generation in sham animals ($\bullet O_2^-$: 1.9± 0.1; NO: 2.4 ± 0.1 a.u.). I/R resulted in increased $\bullet O_2^-$ (2.8± 0.1 a.u, $p<0.05$ vs. sham) and decreased NO (1.9± 0.1 a.u, $p<0.05$ vs. sham) synthesis in the vehicle-treated group. Treatment with kefir for 14 days normalized $\bullet O_2^-$ (2.1± 0.1 a.u, $p<0.05$ vs. IR vehicle) and NO (2.7± 0.1 a.u, $p<0.05$ vs. IR vehicle) production. The same effect was seen with treatment with kefir for 60 days ($\bullet O_2^-$: 1.7± 0.1; NO: 2.7± 0.1 a.u, $p<0.05$ vs. IR vehicle).

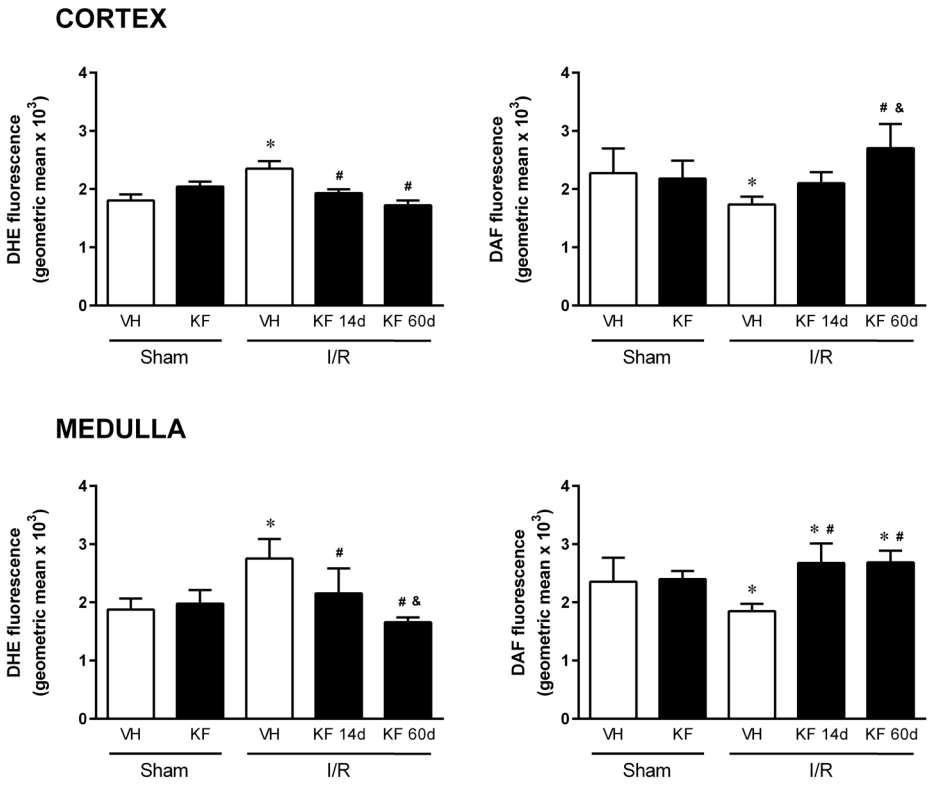

Figure 2: Effects of kefir treatment on anion superoxide (left) and nitric oxide (right) production in the renal cortex (upper) and medulla (bottom). Renal cells from vehicle (white bars) and kefir (black bars) treated animals were evaluated using flow cytometry. In the renal cortex and medulla, I/R led to increased superoxide anion and decreased nitric oxide production. Kefir treatment normalized the production of reactive oxygen species; however, it did not affect the bioavailability of nitric oxide in the renal cortex. N=5-13. Values are means ± SEMs. *$p<0.05$ *vs*. sham; #$p<0.05$ *vs*. I/R vehicle; &$p<0.05$ vs. I/R kefir 14 days. One-way ANOVA followed by Tukey's post hoc test.

Cortical (A) and medullary (B) apoptosis were also determined, and the results are displayed in Figure 3. The total number of apoptotic cells was increased by I/R in the cortex (17.9 ± 3.1 %, $p<0.05$ vs. sham) and medulla (20.1 ± 2.4 %, $p<0.05$ vs. sham) in vehicle-treated animals when compared to sham vehicle group (cortex: 1.3 ± 0.1; medulla: 1.3 ± 0.3 %). Kefir treatment did not change apoptosis in the sham group (cortex: 4.7± 1.0; medulla: 4.0 ± 1.3 %). After 14 days' treatment, kefir decreased the number of apoptotic cells in the renal cortex (6.9 ± 1.5 %, $p<0.05$ vs. IR vehicle), but not in the medulla (23.4 ± 1.2 %). Sixty days of treatment with kefir ameliorated cortical (8.6 ± 0.7 %, $p<0.05$ vs. sham and IR vehicle) and medullary (5.5 ± 0.3 %, $p<0.05$ vs. IR vehicle) I/R-induced apoptosis.

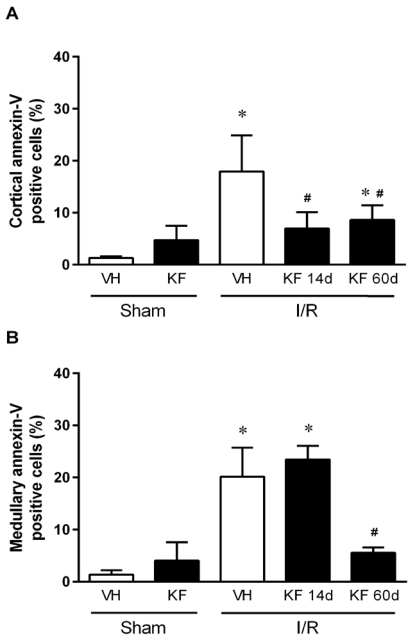

Figure 3: Effect of kefir treatment on apoptosis in the renal cortex and medulla. Apoptosis was determined in the renal cortex **(A)** and medulla **(B)** using Annexin V-FITC and Propidium Iodide (PI) double staining. Renal cells from vehicle (white bars) and kefir (black bars) treated animals were evaluated using flow cytometry. The number of apoptotic cells was substantially increased in the renal cortex and medulla of animals with AKI. Treatment with kefir for 60 days led to a marked reduction in apoptosis, while treatment for 14 days reduced the number of apoptotic cells in the renal cortex alone. N=5-13. Values are means ± SEMs. *p<0.05 *vs*. sham; $^{\#}$p<0.05 *vs*. I/R vehicle, $^{\&}$p<0.05 vs. I/R kefir 14 days. One-way ANOVA followed by Tukey's post hoc test.

Discussion

The present study assessed the effects of the probiotic agent kefir on the development of AKI. Using the renal ischemia-reperfusion model, we demonstrate that chronic treatment (60 days) with kefir was able to prevent renal dysfunction induced by I/R, by reducing superoxide anion, increasing nitric oxide production and preventing apoptosis. Although the present study did not evaluate the composition of kefir grains, a previous study had characterized Brazilian kefir grains to consist of a matrix of polysaccharides and protein containing acidic bacteria belonging to the genuses *Leuconostoc* spp, *Lactococcus* spp, *Lactobacillus* spp, as well as yeast [18]. A recently published study from our laboratory has demonstrated that the biofilm surrounding the kefir grain was composed of short and long curved bacilli or ovoid-shaped yeast through scanning electron microscopy. The microbiological analysis of kefir samples also demonstrated the presence of the *Lactobacillus kefiranofaciens* species [19], which produces kefiran, the main functional component of the beverage [20].

The effects of kefir on renal damage have not been extensively studied; however, the published results are promising. Acting as an ACE inhibitor, kefir treatment for 30 days decreased high-salt-induced renal damage [12]. A study in diabetic rats receiving kefir for 8 weeks demonstrated an amelioration of renal function due to a reduction of hyperglycemia and oxidative stress [11]). The beneficial effects of kefir treatment in kidney injury is expected to also extended to its action as an inducer of PPARα and PPAR-β/δ expression in the kidney [21] which is expected to ameliorate in plasma lipoprotein levels profile, inflammation, and insulin resistance, and contribute to delay renal dysfunction when these factors participate in the progression of renal disease. We have demonstrated that the protective effects of kefir in treatment of AKI involve changes in oxidative stress status. We have shown that kefir treatment reduced $\bullet O_2^-$ and increased NO production, indicating that these effects may be related to the amelioration of renal function. It is well established that NO in an important molecule regulating renal hemodynamics and function [22]. NO bioavailability can be reduced by reaction with superoxide anions, resulting in peroxynitrite [23] and worsening renal function. This scenario is observed in different renal diseases, such as diabetic nephropathy [24], chronic kidney disease [25] and also in AKI [26]. The amelioration of NO/peroxynitrite balance seems to have a beneficial effect [27]. Similar to other probiotics, Kefir contains high levels of lactic acidic bacteria, providing kefir with strong antioxidant properties [28]. Previous studies have demonstrated the potential mechanisms by which kefir may improve oxidative profile, such as reduction of iNOS expression [11] and increased activity of the antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase [9]. That antioxidant action of kefir may also be related to the release of bioactive peptides during milk fermentation by proteolytic lactic acid bacteria which have been shown to scavenge ROS [29] and ameliorate oxidative stress.

AKI is pathologically characterized by sublethal and lethal damage of renal tubules [30]. Studies from our lab have recently demonstrated that reduced tubular apoptosis was correlated with improved renal function in rats submitted to ischemia reperfusion [31]. Furthermore, specific deletion of Bax and Bak, two pro-apoptotic Bcl-2 family proteins, in proximal tubules resulted in a protective effect in mouse submitted to ischemic AKI [32]. These studies highlight the importance of apoptosis in ischemia-reperfusion-induced renal dysfunction. Our current study has shown that kefir administration resulted in reduced apoptosis in both renal cortex and medulla. The effects of fermented milk on apoptosis are still controversial; results have shown that kefir may either stimulate [33] or inhibit [34] apoptosis. Caspases activation is a well-established pathway that leads to apoptosis in multiple cell types, including tubular cells [30]; however, in high salt-induced hyper-

tensive rats, kefir treatment did not modify caspase-3 like enzyme activity [12]. However non-caspases proteases such as cathepsins have been reported as essential downstream effectors of caspases in TNF-mediated apoptosis [35]. Studies have demonstrated that kefir is able to reduce TNF-α [9] and cathepsin B [12] expression, indicating that the protective effect of kefir in apoptosis may involve these proteins.

The effects of kefir treatment on ischemia reperfusion have also been recently elucidated by Yener, et al. [9]. Similar to our results, the authors also observed that kefir treatment lead to the amelioration of renal dysfunction and reduced oxidative stress. However, several differences between the studies must be highlighted: 1) the authors performed an aortic ischemia reperfusion, which also leads to significant alterations in lung physiology; 2) renal function was evaluated using plasma creatinine and urea; and 3) oxidative stress was evaluated using lipid peroxidation quantification. In our study we performed ischemia from renal artery for 45 min followed by 24-hour reperfusion, which is considered the most appropriate animal model to mimic the hemodynamic changes that happen in renal function in humans with AKI [36]. We also determined renal function using inulin clearance, which is considered the gold standard to GFR determination. The present investigation also shows a complete evaluation of renal hemodynamics, including RPF, RBF and RVR. Additionally, in our study we were able to quantify different ROS (superoxide anion and nitric oxide) production using flow cytometry. However, both studies showed a beneficial effect of kefir into the kidney, demonstrating a potential therapeutic use of this substance in preventing renal diseases.

Conclusion

Our results indicate the renal protective role of kefir in ischemia-reperfusion acute kidney injury, through reduction of oxidative stress and apoptosis. Chronic use of kefir as functional food may be considered a promising therapeutic agent to prevent of renal dysfunction.

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