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Probiotic treatment with Kefir reduces vascular oxidative stress while suppressing COX2 mediated relaxation in intestinal arteries of an animal model of menopause

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Functional foods such as probiotics are known to have benefits in various diseases including metabolic disorders and cardiovascular disease (CVD). In women, CVD has been shown to be linked with their gut microbiota and hormones. Here, we have evaluated the effects of chronic Kefir, a fermented milk beverage in Russia, Central Asia, Middle East and Eastern Europe, on mesenteric artery, using an animal model of menopause focusing on the superoxide anion and COX2 pathways. Two-month-old female Wistar rats were ovariectomized and treated by gavage with Kefir (5 % w/v, 3 mL/kg/day) or milk (Control) during two months. After this period, third-order mesenteric artery segments were isolated and mounted in a myograph system for evaluation of concentration-response curves to acetylcholine. We performed western blot analyses and measured oxidative stress through dihydroethidium (DHE) staining. Kefir reduced vascular oxidative stress, despite not changing SOD2 levels. COX2 levels were not changed by kefir, despite an apparent tendency towards reduction. However, in the functional experiments, under incubation with a COX2 inhibitor, a suppression of this pathway was observed in the kefir group, which suggests an interaction between inflammatory pathways and oxidative stress in this model. The effect of acute incubation with a superoxide anion scavenger on vascular responsiveness was equal in both groups. Kefir reduces vascular oxidative stress levels while suppressing COX2-mediated relaxation in mesenteric vessels in an animal model of menopause; which appears to involve an interplay between these two factors.

Keywords: COX2, Kefir, Menopause, Mesenteric vessels, Oxidative stress, Reactive oxygen species

Experimental and clinical studies have shown the benefits of functional foods in various conditions[1]. Currently, there is an important research focus on food products containing live microorganisms known as probiotics[2,3]. In particular, the fermented milk beverage known as Kefir — usually a homemade product — became a prominent probiotic originated from Russia and Central Asia, Middle East and other parts of Eastern Europe and Sweden. Kefir grains are composed of microbial species, such as lactic acid bacteria, acetic acid bacteria, yeasts and other fungi, which are incorporated in a matrix of viscous polysaccharides, called kefiran[4,5].

Emerging evidence point to the existence of an altered microbiota (dysbiosis) in some conditions, including cardiovascular (CVD) and metabolic diseases[6-9]. Effects of Kefir on CVD system has gained the attention of researchers during the latter half of last decade[10-15]. On the other hand, postmenopausal hormone deficiency is also associated with CVD impairment[16], and interestingly, there seems to be an interaction between the microbiota and female hormones[17]. Controversies regarding hormone replacement therapy[18,19] have generated interest in alternative non-hormonal treatments including probiotics, particularly the CD health of post-menopausal women[20,21]. Thus, we studied the effects of the probiotic Kefir in an experimental model of menopause by ovariectomy (OVX) in our lab. Initially, we observed that the participation of hydrogen peroxide in vascular function was lower in OVX rats treated with Kefir compared to those not treated, which was accompanied by an increase in tissue expression of catalase in the treated group[21]. In fact, estrogen probably exerts cardiovascular protective effects, with the development of vascular dysfunction after menopause being observed both in women and in animal models[20,22], which is possibly associated with oxidative stress and inflammatory markers[23-25].

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Therefore, in sequence to our previous studies, here, we hypothesize that the probiotic Kefir can induce positive vascular effects in a model of OVX rats, focusing on the superoxide anion and COX2 pathways.

Materials and Methods

Animals and experimental groups

Female Wistar rats provided by the Federal University of Espirito Santo were ovariectomized at two months of age and arbitrarily split into two groups: treated by gavage once a day, for two months, with kefir beverage (Kefir group; 5% w/v, 3 mL/kg) or with the corresponding volume of milk without the kefir grain probiotics (control group). The kefir beverage was prepared by adding kefir grains to pasteurized whole cow's milk in a ratio of 5 % (w/v) and kept at room temperature (24ºC). After 24 h, this mixture was filtered through a plastic screen and the resultant product was refrigerated (averaging 10°C) to permit yeast growth for 24 h. At the end of this process, the kefir was aliquoted into sterile plastic tubes and stored at −20°C until use (Fig. 1). The microbiological analysis of random samples of the grains used showed that the dominant microflora of kefir included various beneficial bacteria (*Acetobacter aceti*, *Acetobacter* sp., *Lactobacillus delbrueckii*, *L. fermentum*, *L. fructivorans*, *Enterococcus faecium* and *Leuconostoc* spp.), in addition to *L. kefiranofaciens*, and yeasts (*Candida famata*, *Candida krusei*). Additionally, the microbiological analysis of kefir grains revealed that the global counting of microorganisms was 7.5×10^7 CFU/mL[10]. Treatment started on the day after surgery. The animals were kept in a 12-h light-dark cycle with free access to water and food (standard diet, Purina Labina, São Paulo, SP – Brazil).

Ethics approval

All procedures were approved by the Ethics Committee on Animal Use for Research of our University (nº 021/2018) and followed local laws and the recommendations of the National Institute of Health for animal use in laboratory experiments.

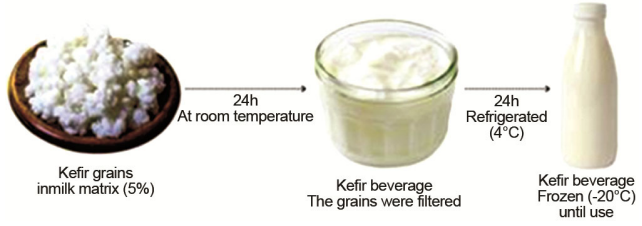

Fig 1 — Flow chart for kefir sample production.

Ovarian removal surgery

After intraperitoneal anesthesia with a ketamine/ xylazine mixture (70/10 mg/kg), the animals were subjected to a 1 to 1.5 cm incision in the skin, between the last rib and the thigh, 1 cm from the median line, followed by an incision in the muscle layer, opening the peritoneal cavity for later removal of the ovaries and ligation of the uterine tube. After removal of the ovaries, muscle and skin were sutured. The same process was carried out on the opposite side. At the end of the procedure, the animals were returned to individual cages.

Mesenteric vessels isolation and mounting in a myograph system

The rats were sacrificed by decapitation. The abdomen was upended and third-order mesenteric arteries were identified, isolated, dissected from the adjacent non-vascular tissue, and 2 mm rings were mounted in a myograph system (620 M; Danish Myo Technology, Aarhus, Denmark). Segments were maintained in chambers filled with Krebs solution at 37°C aired with carbogen (5% CO_2 and 95% O_2), as described by Mulvany & Halpern[26].

The internal circumference was standardized to 0.9·IC100, and the rings progressively stretched until their internal diameters corresponded to a transmural pressure of 100 mmHg. Rings had their endothelium rated as intact when the relaxation observed in response to ACh was ≥70%. LabChart 8 software was used for data acquisition (AD Instruments Pty Ltd., New South Wales, Australia).

Concentration-response curve of relaxation to ACh

After contraction with 3 μM of phenylephrine (Phe), concentration-response curves were built for the cumulative addition of acetylcholine (ACh). ACh response was investigated after incubation for 30 min with the following inhibitors: Tiron (superoxide anion scavenger, 1 μM) or NS398 (COX-2 specific inhibitor, 1 μM). All inhibitors were obtained from Sigma-Aldrich (St. Louis, MO - USA).

Western blot analysis

Mesenteric artery segments were homogenized in lysis buffer [Tris-HCL pH 7.4 (10 mM), PMSF (1 mM), NaVO3 (1 mM), SDS (1%), DTT (0.5 mM) and EDTA (5 mM)] containing protease inhibitor cocktail (1:100 dilution) and submitted to electrophoresis in SDS-PAGE at 10%. After blockade with 5% non-fat

dry milk, the membranes were exposed to primary antibodies for the following proteins: SOD2 ([1:1000], Sigma-Aldrich, St. Louis, MO, USA), COX2 ([1:200], Cayman Chemical, USA), and β-actin ([1:1500], Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were then incubated with the secondary antibody ([1:5000] Sigma-Aldrich, St. Louis, MO, USA). Bands were detected through chemiluminescence (ECL) using the Chemi-Doc XRS+ system (Bio-Rad, Inc.). Protein expression levels of each protein were normalized to β-actin and quantified by band density using the Bio-Rad Image Lab 6.0 software. Total protein content was determined through the Bradford method and all samples were analyzed in duplicate.

Detection of Reactive oxygen species (ROS)

ROS production was detected using DHE (dihydroethidium) dye. Mesenteric arteries were immersed in Tissue-Tek OCT compound (Sakura Finetek, USA) medium and stored at -80° C. Cryostat transverse cuts (10 μm) were topically incubated for 30 min at 37 °C in 1 mL of Krebs/HEPES buffer containing DHE (2 μM). Images were acquired using an optical microscope DM-2500 (Leica Microsystems, Germany) with a 40X objective connected to a camera system. Fluorescence intensity was analyzed (Image J software; Wayne Rasband, National Institute of Health - NHI, USA) and values expressed as percentage of control group.

Statistical analysis

The results were expressed as mean ± standard error of the mean (SEM) and analyzed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Comparisons between two means were performed using Student's t-test or Mann Whitney test. Concentration-response curves underwent two-way analysis of variance (ANOVA) followed by Fisher's LSD post hoc test. For all the statistical analysis, the value of P <0.05 was considered statistically significant.

Results

The chronic treatment of OVX rats with Kefir had an interesting outcome regarding oxidative stress and inflammatory pathways. Kefir attenuated the DHE signaling in mesenteric vessels, a marker of reduced oxidative stress production (Fig. 2A). However, no changes were observed in the tissue expression of SOD2 (Fig. 2B). Regarding pro-inflammatory pathways, Kefir did not change significantly the protein expression of COX2 in the mesenteric vascular tissue, in spite of an apparent trend towards reduction (Fig. 2C).

We then conducted functional experiments to investigate the impact of these findings in the physiology of the mesenteric vessels. Concentration-response curves in response to acetylcholine, before and after incubation with Tiron, revealed that this superoxide anion scavenger reduced the vascular responsiveness in control animals only slightly (Fig. 3 A and B). However, a similar outcome was observed in the Kefir-treated group (Fig. 3 C and D), showing that treatment with kefir

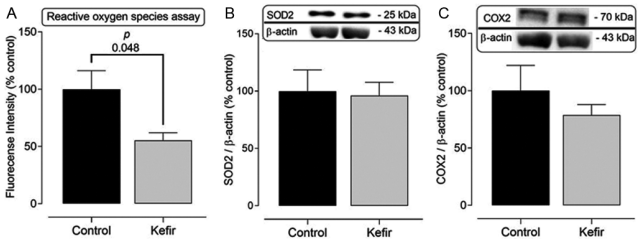

Fig. 2 — Investigation of oxidative stress and inflammatory pathway machineries. Evaluation of mesenteric vessels (n = 5-9). (A) ROS levels measured by DHE; (B and C) Western blot analysis of the protein expression levels of SOD2 and COX2. [P <0.05 was considered statistically significant]

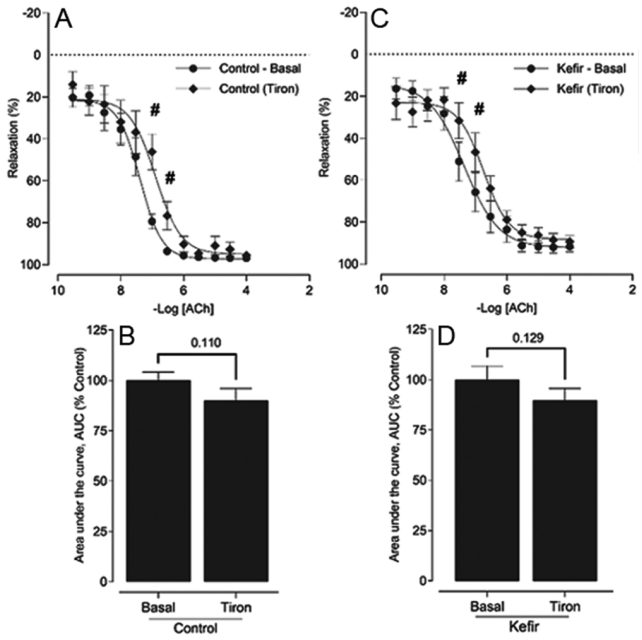

Fig. 3 — ROS pathway in endothelium-dependent response to acetylcholine. Evaluation of third-order mesenteric arterial segments. Concentration-response curves and the associated area under the curve (AUC) before and after superoxide anion scavenging using (A and B) Tiron: control; and (C and D) Kefir-treated groups. [Data are presented as mean ± SEM (n = 7-9). #Significant difference before and after Tiron]

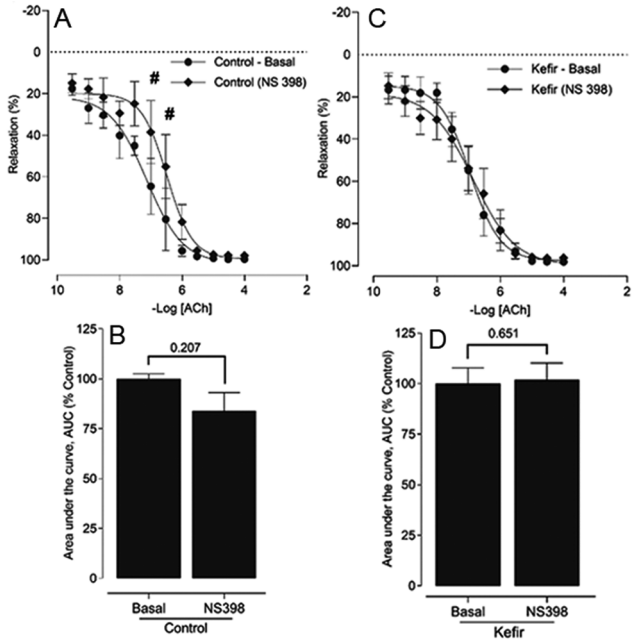

Fig. 4 — COX2 pathway in endothelium-dependent response to acetylcholine. Evaluation of third-order mesenteric arterial segments. Concentration-response curves and the associated area under the curve (AUC) before and after COX2 inhibition using (A and B) NS398: control; (C and D) and Kefir-treated groups. [Data are presented as mean ± SEM (n = 5-8). #Significant difference before and after NS398]

did not change the response pattern compared to the control group.

Next, we assessed the role played by the pro-inflammatory pathway linked to COX2. Upon incubation with NS398 – a specific COX2 inhibitor, control animals showed partially reduced vascular responsiveness to acetylcholine (Fig. 4 A and B). Interestingly, the Kefir-treated group remained wholly unaffected by COX2 inhibition with NS398, displaying a pattern of responsiveness close to that observed in basal condition (Fig. 4 C and D). Taken together, these data showed a reduction in vascular oxidative stress levels due to Kefir treatment without changes in the antioxidant machinery of SOD2, which may have indirectly suppressed the regulation of the vascular function by the COX2 pathway in the kefir-treated group.

Discussion

Despite being a well-known probiotic food, little is known about the effects of Kefir on vascular function in ovarian hormones deficient states, as in post-menopausal women and ovariectomized animals. In this study, Kefir reduced vascular oxidative stress levels while it suppressed the participation of COX2 in the regulation of vascular function in an animal model of menopause.

We have recently reported that Kefir reduces the participation of hydrogen peroxide in vascular function in OVX rats, which was accompanied by an increase in tissue expression of catalase[21]. Interestingly, a previous report showed that OVX rats have a reduction in catalase expression[27] and activity levels[28], an outcome that Kefir seems to be able to counteract[21]. These data are in agreement with the reduced levels of tissue oxidative stress found in kefir-treated rats in the present study. Yet another study showed that a Kefir treatment with a duration similar to our own reduced aortic cell intracytoplasmic ROS production in hypertensive male rats[10].

It is worthy of note that vascular oxidative stress and associated machinery are upregulated in OVX rats[23,25]. On the other hand, studies on other probiotics also found improvements in antioxidant defenses along with reduced oxidative stress, both in humans and in animal models[29-31]. In particular, a randomized study showed that “Lactobacillus casei” increased the activity of plasma catalase and the antioxidant capacity in women[31].

It has been also shown that Kefir attenuates oxidative stress in a model of gastric lesions in mice[32]. Meanwhile, our colleagues found that kefir reduced superoxide anion levels in a model of health impairment by bisphenol A[33]. In this context, our results support an antioxidant effect of Kefir that could be protective in estrogen-deficient states such as menopause, despite SOD2 levels remained unaffected.

Another important issue regarding OVX rats is the increased levels of inflammatory markers, such as TNFα[24], while presenting increased prostacyclin (PGI_2) release induced by ACh[34]. Therefore, we evaluated the expression of COX2, as well as the role of COX2 in vascular function in OVX rats. Interestingly, Kefir attenuated the participation of COX2 in vascular function, which was accompanied by an apparent tendency towards reduced COX2 expression.

The results of the COX2 in contrast to the other results allow us to suggest an interaction of COX2 and oxidative stress. In fact, it is known that hydrogen peroxide can activate COX[35], while COX activation can generate oxidative stress[36]. Considering our previous results of reduction of the hydrogen peroxide pathway in OVX rats treated with kefir[21]; we suggest that there is a two-way interplay between these factors in our model, which was reduced by Kefir.

Interestingly, Brasil *et al*.[13] found that the non-bacterial fraction of kefir improved inflammatory parameters in hypertensive rats, while Friques *et al*.[33] found that kefir decreased vasoconstriction to prostanoids in aortic rings of animals under the deleterious effects of bisphenol A[33]. In addition, other studies reported anti-inflammatory effects of Kefir[12,37,38], including protection against colitis in animal models, although kefir worsened colitis when used in very high doses[37,38].

The beneficial effects of kefir can be explained by the presence of bacteria and yeast that share a close symbiotic relationship in the kefir grains, resulting in the production of metabolites from fermented milk in the beverage[39]. Previous data show that kefir inhibits the angiotensin converting enzyme (ACE), which lead to a decrease in blood pressure in hypertensive animals [10,13.] Brasil *et al*.[13] used the soluble non-bacterial fraction of kefir beverage, confirming the presence of ACE inhibitor peptides in the Kefir and supporting the hypothesis that they are involved in the antihypertensive effects of this dairy beverage. A list of 35 peptides with potential anti-hypertensive activity due to ACE inhibition were identified[39]. The main polysaccharide in kefir grains is kefiran, a heteropolysaccharide composed of equal proportions of glucose and galactose that is produced mainly by *Lactobacillus kefiranofaciens*. This polysaccharide has many biological properties such as antioxidant, anti-inflammatory, and antihypertensive, which further enhance kefir properties[40,41].

Conclusion

The above results have demonstrated that Kefir beverage promotes beneficial vascular adaptations, reducing oxidative stress levels while suppressing COX2-mediated relaxation in mesenteric vessels in an animal model of menopause. This effect possibly involves an interplay between oxidative stress and COX2. Further studies are needed to verify the translational potential of these findings.

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Conflict of interest

Authors declare no competing interests.

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