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Original article

Immunomodulation and nitric oxide restoration by a probiotic and its activity in gut and peritoneal macrophages in diabetic rats

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SUMMARY

Background: The hyperglycemia leads to increased oxidative stress, causing lipid peroxidation and imbalance in the immune system.
Aims: To investigate the effect of Kefir – a probiotic containing different strains – on metabolic parameters, cytokines, nitric oxide (NO) production, phagocytic activity of peritoneal macrophages and intestinal modulation in diabetes.
Methods: Wistar rats received injection of streptozotocin (45 mg/kg, intravenously) and diabetes was defined as glycemia ≥200 mg/dL. The animals were distributed in four groups: control (CTL); control Kefir (CTLK); diabetic (DM); diabetic Kefir (DMK). Kefir was given at 1.8 mL/day by gavage, started on the 5th day of diabetes, during 8 weeks. The animals were allocated in metabolic cages, pre and post treatment with Kefir, for measurement of the metabolic parameters, such as water intake, food intake, diuresis, glycemia, body mass, insulin and lipid profile, these last two were only measured at the end of Kefir protocol. After treatment, the animals were euthanized and the peritoneal cavity was prepared, resident macrophages were collected and cultured for analysis of the phagocytic activity, cytokines (IL-10, TNF-α, IL-17, IL-1β) and NO. The intestinal modulation was performed by the quantification of Peyer's patches (PP) in the small intestine. The data were presented as mean ± SEM, with significance of $p < 0.05$.
Results: DM when compared to CTL showed increase in water intake (133 ± 7 *vs.* 28 ± 1 mL, $p < 0.0001$), food intake (40 ± 2 *vs.* 16 ± 1 g, $p < 0.0001$), diuresis (102 ± 5 *vs.* 13 ± 1 mL, $p < 0.0001$) and glycemia (567 ± 12 *vs.* 84 ± 3 mg/dL, $p < 0.0001$), while in DMK group all these metabolic parameters were decreased (96 ± 14; 36 ± 1; 86 ± 7 and 407 ± 19, respectively, $p < 0.0001$), presenting increase of body mass (42 ± 5 *vs.* 16 ± 4Δ, $p < 0.0001$) and insulin levels (0.3 ± 0.8 *vs.* 0.1 ± 0.04 ng/mL, $p < 0.0001$) compared to DM. The lipid profile of the diabetic groups showed tendency to increase compared to the respective controls. In relation to function of peritoneal macrophages, DMK group *vs.* DM showed improvement in phagocytic capacity (70 ± 5 *vs.* 51 ± 7%, $p = 0{,}0023$) and increased concentration of all the cytokines analyzed (pg/mL), as IL-10 (926 ± 69 *vs.* 556 ± 92, $p = 0.0004$), TNF-α (178 ± 20 *vs.* 109 ± 20, $p = 0.005$), IL-17 (33 ± 1 *vs.* 9 ± 1, $p = 0.0001$) and IL-1β (102 ± 14 *vs.* 70 ± 5, $p = 0.0129$), after 24 h of LPS stimulation; including NO bioavailability after 24 h (102 ± 9 *vs.* 66 ± 5 μM/mL, $p = 0.0029$) or 48 h (143 ± 8 *vs.* 119 ± 4 μM/mL, $p = 0.0102$) of LPS stimulation. Moreover, the number of PP in the whole small intestine of DMK group was also increased as compared to DM (22 ± 1 vs. 18 ± 1, $p = 0.0292$).
Conclusion: These results show that Kefir has a potential to modulate the immune response and activate peritoneal macrophages in diabetic animals, which suggests that it could enhance the immunocompetence of patients affected by diabetes mellitus. The hypoglycemic effect of this probiotic could be used as a tool to control glycemia, reducing or delaying the onset of complications associated with this disease.

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1. Introduction

Diabetes causes several complications like atherosclerosis, neuropathy, nephropathy and retinopathy and it is characterized by hyperglycemia, which results from impaired action or insulin secretion. Chronic hyperglycemia induces the production of reactive oxygen species that cause lipid peroxidation of membranes, which can influence the immune response. Furthermore, diabetic patients have increased susceptibility to fungal and bacterial infections; this happens because there is a reduced microbicidal activity of the macrophages, which can be restored by insulin [1].

In diabetic patients and rats, macrophages are deficient, affecting the production of NO, which mediates immune and inflammatory processes, with important role in the physiological and pathological signaling, contributing to liberation of cytokines [2]. The production of cytokines by phagocytic cells in response to agents and/or pathogenic bacteria is important to maintain the state of controlled inflammation that occurs in the intestinal mucosa under physiological conditions [3].

The intestine has a natural defense system, with a variety of mononuclear phagocytes acting as antigen-presenting cells (macrophages and dendritic cells), that resides in the gut-associated lymphoid tissue (GALT), composed of mesenteric lymph nodes (MLNs), Peyer's patches (PP) and the lamina *propria* tissue. Increasingly, studies have shown that regular intake of probiotics can change the intestinal microflora and contribute to the overall health [4,5].

Kefir is a fermented milk, manufactured from Kefir grains, which contain a complex mixture of lactic acid bacteria, including various species of *lactobacilli* and yeasts. In recent years, lactic acid bacteria has received increased research attention by its capacity of tumor growth inhibition, induction of apoptosis in tumor cells, modulation of gut microbiota and anti-allergic and anti-inflammatory action [5].

Prior study in our laboratory showed that Kefir reduced oxidative stress and hyperglycemia in diabetic rats [6], however, the immune response and the local immune system (small intestine) had not been assessed. Besides, there are many researches that analyze Kefir and its fractions in type 2 diabetes but not on type 1 diabetes [7,8].

The aim of this study is to assess the immunomodulation and nitric oxide by Kefir, as well as its activity in gut and peritoneal macrophages, in a rat model of type1 diabetes.

2. Materials and methods

2.1. Animals

The male Wistar rats, weighing ±250 g and with 8 weeks of age were obtained from Central Animal Housing, Sao Paulo, Brazil. The protocol was approved by the Ethics Committee of Universidade Federal de Sao Paulo (Protocol #0278/11). The animals were kept in collective boxes in the Animal Housing of the Nephrology Division, at controlled temperature of 22 ± 2 °C, in an environment with photoperiodic cycles of 12/12 h beginning at 6 am, receiving chow (Nuvital, Nuvilab, PR, Brazil) and water *ad libitum*.

2.1.1. Type 1 diabetes

After a period of 7 days of adaptation, it was performed the induction of diabetes in the animals with an intravenous injection of streptozotocin (STZ) 45 mg/kg/BW (Sigma Chemical Co., USA), diluted in 1 mL/kg/BW of citrate buffer (0.1 M, pH 4.5); control group animals received drug vehicle. Diabetes was confirmed after 72 h of STZ induction; glycemia was measured by glucometer (Accu-Chek Advantage II, Roche, USA) in blood aliquot from the tail vein; rats were considered diabetic when the fasting blood glucose was ≥200 mg/dL, and animals outside this criterion were excluded.

2.1.2. Treatment of the animals

After confirmation of diabetes, the animals were distributed into 4 groups of 5–10 animals each:

CTL: normoglycemic animals that received water
CTLK: normoglycemic animals that received Kefir
DM: diabetic animals that received water
DMK: diabetic animals that received Kefir

The DMK and CTLK groups received 1.8 mL/day of Kefir (Danisco Biolacta, Olstyn, Poland) by gavage for 8 weeks and the other groups received water in the same volume and route mentioned above.

The Kefir bacteria dose was based on human healthy and safe intake and its preparation was carried out according to manufacturer's instructions. Briefly, once a week, 20 mg freeze-dried Kefir culture, with 10^{10} colony-forming units per gram (CFU/g) of lactic acid bacteria and 10^4–10^7 CFU/g of yeast, containing *Lactobacillus* sp., *Lactococcus lactis subsp*., *Streptococcus termophilus*, *Leuconostoc sp*. grains and yeast of Kefir were diluted in 100 mL of reconstituted milk; the fermentation was performed for 16 h at 23 °C, monitored by the Cinac System (Cynetiqued' acidification, Ysebaert, Frépillon, France) until reach pH 4.6, when the fermentation was stopped and Kefir was stored under refrigeration until its use. All these processes were developed at the Laboratory of Food Technology, Faculty of Pharmaceutical Sciences, Universidade de Sao Paulo, Sao Paulo, SP, Brazil.

2.1.3. Metabolic parameters of the animals

The animals were allocated in metabolic cages for 24 h, before and after Kefir treatment, with chow and water *ad libitum*, for quantification of water intake, food intake and diuresis. The glycemia was checked every two weeks after starting treatment with Kefir (5th day of diabetes), in blood aliquot from the tail vein, determined by glucometer after 3 h fasting, without anesthetic; the body mass was checked weekly and the insulin was measured only post Kefir treatment, using the Rat/Mouse Insulin ELISA Kit (EMD Millipore, Missouri, USA), according to manufacturer's instructions. The lipid profile was measured in the plasma of rats after 12 h of overnight fasting, by colorimetric assay of Colesterol Liquiform, Triglicerides Liquiform and LDL Liquiform kits (Labtest, Centerlab, MG, Brazil).

2.2. Obtaining cells from the peritoneal cavity

After 12 h of overnight fasting, the animals were sacrificed in a CO_2 chamber, exsanguinated, dipped in 70% alcohol and taken to the laminar flow. Previous to the opening of the abdomen skin, we injected 60 mL of sterile PBS, pH 7.4 into the peritoneal cavity of animals. After abdominal massage to facilitate the detachment of the macrophages, the contents were aspirated using a sterile and apyrogenic Pasteur pipette, and the fluid was maintained in sterile polypropylene tubes, in ice bath. The cell suspension was centrifuged for 10 min at 1500 rpm and washed 3 times with sterile PBS. After the last wash, the cell pellet was resuspended in 3 mL of McCoy's medium (Vitrocell, Sao Paulo, Brazil), pH 7.4, sterile, containing 10% fetal calf serum.

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