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Article

Pretreatment with water kefir reduces the development of acidified ethanol-induced gastric ulcers

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To evaluate the gastroprotective and antioxidant effects of pretreatment with water kefir on ulcers induced with HCl/ethanol. All pretreatments lasted 14 days. Male mice were separated into five groups: the control (C) group received vehicle without ulcer induction; the ulcerated (U) group received vehicle; the lansoprazole (L) group received 30 mg/kg/day lansoprazole; the water kefir (WK15 and WK30) groups received WK at a dose of 0.15 or 0.30 ml/kg/day, respectively. Gastroprotection was measured by ulcer area, ulcer index and ulcer reduction percentage. Antioxidant effects were quantified by measuring advanced oxidized protein products (AOPPs), superoxide dismutase (SOD), and catalase activity in the stomach. Pretreatment with WK at both doses promoted gastroprotection against HCl/ethanol-induced ulcers much like the pretreatment with lansoprazole. In addition, WK decreased protein oxidation while increasing SOD and catalase activity. We concluded that pretreatment with water kefir increases the activity of antioxidant enzymes, preventing gastric lesions induced by HCl/ethanol by maintaining the antioxidant performance in gastric tissue.

Keywords: Ulcer lesion. Antioxidant activity. Natural products. Probiotic.

INTRODUCTION

Gastric ulcers are lesions developed in the gastric mucosa that can extend from the mucosa layer to the submucosa, and even to deep layers of the stomach (Uyanikoğlu *et al*., 2012). These lesions are known to develop from an imbalance between gastroprotective factors such as mucus, bicarbonate, prostaglandins, nitric oxide (NO) and stomach stressors, such as HCl and pepsin (Magierowski *et al*., 2015). In addition to these endogenous factors, there are exogenous factors that can influence the development of gastric ulcers, such as *Helicobacter pylori* infection, abuse of nonsteroidal anti-inflammatory drugs, alcohol abuse, stress and diet (de Araújo *et al*., 2018; Tulassay, Herszényi, 2010).

Among the factors described above, alcohol is an important factor contributing to the development of gastric ulcers, as it is considered an acceptable drug and its abuse is widespread in the world (Trinovita, Chany, Mun'im, 2018). In agreement with the World Health Organization (WHO), alcohol consume is considered a health problem, having a great impact on public health (Glantz et al., 2019; WHO - World Health Organization, 2018). The consumption of alcohol by the world population was up to 6.4 L/person in 2018, with such abuse predisposing people to develop many health problems, including gastric disease (Na, Lee, 2017).

Ethanol can attack the gastric mucosa, inducing lesions through mucus destruction, bicarbonate depletion, increased H^+ concentration, histamine delivery and cellular necrosis (de Araújo *et al*., 2018; Monforte *et*

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al., 2012). Cellular necrosis is the result of a sequence of events that involve the infiltration of inflammatory cells and the release of reactive oxygen species (ROS). As a consequence, an increase in lipid peroxidation and other oxidative stress biomarkers can be observed, demonstrating the importance of oxidative stress in lesion development (Monforte *et al*., 2012).

Gastric ulcers are estimated to affect approximately 4 million people worldwide per year (Chung, Shelat, 2017). Many drugs have been developed to treat gastric ulcers, such as proton pump inhibitors, anticholinergics and antihistamines; however, these drugs can produce considerable side effects (Farzaei, Abdollahi, Rahimi, 2015; Shi, Klotz, 2008), what calls for alternative treatments. Probiotics and natural plant-based products are promising new therapeutic resources, for they produce fewer side effects and have a high tolerability profile by users (Rodrigues *et al*., 2016).

Currently, there is a demand for foods containing probiotic and functional substances that can contribute to health. Thus, interest in kefir consumption is growing (Prado *et al*., 2015). Kefir grains are a symbiosis of lactic acid bacteria and yeasts adhered to a polysaccharide matrix (Chen *et al*., 2015; Prado *et al*., 2015). These grains can be fermented using milk or sugar-water as a culture medium; the latter produces water kefir (WK) or sugary kefir grains, which have similar bacterial and yeast profiles as milk kefir (Fiorda *et al*., 2016; Prado *et al*., 2015).

Previous studies have shown the gastroprotective effect of milk kefir in different animal models, suggesting that this probiotic drink is efficient in combating ROS (Barboza *et al*., 2018; Fahmy, Ismail, 2015). Rodrigues *et al*., (2016) showed that beer fermented with kefir was capable of decreasing the damage caused by ethanol when compared to control groups. Their results suggested that kefir had probiotic and prebiotic properties; however, the hypothesis that the anti-ulcer effects could be attributed to other constituents of the handmade beer, not only to kefir, could not be disproved.

Therefore, the effects of WK on gastric ulcer induction and its mechanism of protection remain to be determined. Thus, the aim of the present study was to investigate the effects of pretreatment with water kefir in an experimental model of gastric ulcers induced by acidified ethanol.

MATERIAL AND METHODS

Animals

Male mice (C57BL/J6) aged between 2-3 months and weighing 20-30 g were separated into five groups (n=7 each). The animals were provided by the Animal Care Facility at the Experimental Monitoring Laboratory of University Vila Velha (UVV). All experimental protocols were performed in agreement with the guidelines for the care and handling of laboratory animals recommended by the National Institutes of Health (NIH) and were approved by the Institutional Animal Care Committee (Protocol nº 434-2017). The animals were maintained in Alesco© mini-insulator IVC (Individually Ventilated Caging) Racks at controlled temperature (~23°C) and humidity and were exposed to a 12/12-h light–dark cycle with *ad libitum* access to food and water.

Water kefir preparation

The water kefir beverage was prepared by adding kefir grains, obtained by household preparations (Gulitz *et al*., 2011), at a ratio of 4% (w/v) to water mixed with brown sugar (7 g/100 mL) at room temperature. This preparation is commonly consumed by Brazilian people. After 24 h, this mixture was filtered and the resulting product refrigerated (~8°C) to allow yeast growth for 24 hours (Gulitz *et al*., 2011). The pH of the final product, determined using a pHmeter (K-39-1014B, Kasvi®, China), was found to be 3.99. After this period, the kefir beverage was administered to the animals. Two different doses – 0.30 and 0.15 mL/100g of body weight – were used here, having been adapted from a previous study by Friques *et al*., (2015).

Experimental protocol

The animals were separated into groups and treated for 14 days prior to ulcer induction (Xie *et al*., 2017). The animals were separated as follows: the control (C) and ulcerated (U) groups received tap water – the vehicle of water kefir – daily (0.1 mL); the lansoprazole group (L) received lansoprazole (30 mg/kg/day) daily by gavage; The water kefir 15 (WK15) and 30 (WK30) groups received

water kefir (0.15 and 0.30 mL/kg) daily. All treatment groups, except for the control group, completed the ulcer induction protocol at the end of the treatment period.

For ulcer induction, the animals were submitted to fasting for at least 24 h with free access to glucose solution (10%), after which they received 0.2 mL of HCl/ethanol solution (60%, 0.3 μM HCl) by gavage (Silva-Junior *et al*., 2016). One hour later, the mice were euthanized with a mixture of ketamine and xylazine (75/7.5 mg/kg) and the stomachs removed.

Evaluation of macroscopic gastric lesions

After removal, the gastric contents were separated and the pH determined by titration. Next, the stomach was cleaned with cold saline and opened along the great curvature to expose the lumen. The stomach was mounted between two glass plates to guarantee the visualization of lesions and then photographed using a digital camera for the evaluation of macroscopic lesions (Guzmán-Gómez *et al*., 2018). The stomach was then separated into two pieces by its lesser curvature, with both pieces maintaining all parts of the organ: one was kept at -80ºC for oxidative stress evaluation, and the other piece was stored in formalin buffer for the evaluation of macroscopic lesions.

For macroscopic evaluation, the images were analyzed using a free imaging software (ImageJ 1.35 d, NIH, Bethesda, MD, USA), and the results expressed as mm^2 of total stomach area. The ulcer index (UI) was calculated for each animal as follows: [(lesion area x 100)/ total stomach area]. The percentage of ulcer reduction (PR) was determined as follows: [(UI of the ulcerated group – UI of the treated group)/UI of the ulcerated group] x 100 (Guzmán-Gómez *et al*., 2018).

Evaluation of antioxidant activity

Tissue preparation

Stomach samples were homogenized in cold phosphate-buffered saline (PBS), after which the homogenate was centrifuged at 3500 rpm for 10 minutes at 4ºC. The supernatant was aliquoted and kept at -80ºC until use. Protein concentration was determined by the Bradford protein assay and the results normalized by protein content (Bradford, 1976).

Advanced oxidized protein product (AOPP) determination

Samples of homogenized tissue were used for AOPP determination according to the protocol described by Witko-Sarsat *et al*. (1996), with few modifications. The samples were mixed with potassium iodide (1.16 μM) and acetic acid in a 96-well plate. After 6 minutes of incubation, the plate was read at 340 nm using a microplate reader (FilterMax F3/F5 Multi-Mode Microplate Readers, Molecular Devices, USA). The quantification of oxidized products was performed using a standard curve of Chloramine T (0–100 μmol). The results were expressed as μmol of Chloramine T equivalent/mg of protein (μmol/mg).

Determination of antioxidant enzymes activity (SOD and catalase)

Superoxide dismutase (SOD) activity was determined by the ability of SOD in preventing the autoxidation of epinephrine (Misra, Fridovich, 1972). For that, the samples were mixed with epinephrine (0.025 M) and sodium phosphate buffer (pH = 7.2) containing KCl (0.015 M). The absorbance was read using a spectrophotometer at 480 nm at 15-second intervals for one minute. One SOD unit is considered to be the amount of enzyme capable of inhibiting the autoxidation of epinephrine by 50%. The results were expressed as units of SOD/mg of protein (USOD/mg protein).

For catalase activity evaluation, the sample was mixed with phosphate buffer (0.2 M) and hydrogen peroxide (0.3 M) was added to the mixture. The absorbance was read at 240 nm using a spectrophotometer at 15-second intervals for one minute. The results were expressed as peroxide extinction coefficient/minute/mg of protein (ΔE/min/mg of protein) (Aebi, 1984).

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM), having been submitted to the Kolmogorov-

smirnov normality test. Differences between groups were determined by ANOVA followed by Tukey's post hoc test.

RESULTS

Our results revealed that pretreatment with WK was capable of promoting protection against gastric ulcers, as shown in Figure 1. Panels A-E display representative images of stomachs after ulcer induction, with the results of macroscopic evaluation having been shown in Panel F. As can be seen in Figure 1 (Panel F), the 14-day treatment with water kefir at both doses used here led to a decrease in gastric lesion area (C: 14477±6204; U: 127578±33260; L: 39958±13921; K15: 79357±23814; K30: 63252±22435 μm^2. $p<0.05$ compared to the U group), which indicates gastroprotection.

The gastroprotection induced by water kefir was not different from that promoted by lansoprazole, which is the drug of reference for gastric ulcer. This is a very interesting result, for it could lead to water kefir being used as an adjuvant to prevent ulcer in risk groups. In order to better investigate water kefir's gastroprotective ability, we calculated the ulcer index (UI) and protection percentage. Table I summarizes those results and highlights the gastroprotective effect of water kefir, as the K30 group was found to promote 59.5% of gastroprotection.

Oxidative stress evaluation was performed through the quantification of antioxidant enzymes activity (SOD and Catalase) and by determining the products of reactive oxygen species (ROS), such as AOPP. Table I shows the results regarding SOD and catalase activity. A strong decrease in the activity of both enzymes can be observed in the ulcerated group (U), what was completely reversed by lansoprazole and WK treatment at both doses. It is worthy of note that the activity of the antioxidant enzymes evaluated here was restored in a similar way following water kefir and lansoprazole treatment.

The analysis of protein oxidation products revealed increased AOPP values in the ulcerated group (Figure 2: 0.018±0.004; U: 0.036±0.004; L: 0.021±0.004; WK15: 0.014±0.006; WK30: 0.023±0.003 μM of chloramine T/mg of protein). Much like what was observed regarding antioxidant enzymes, treatment with WK decreased AOPP values at both doses employed here, with this decrease being, again, similar to that induced by lansoprazole treatment. Taken together, these results demonstrate that water kefir promotes gastroprotection by decreasing the oxidative stress induced by ethanol, which leads to an improvement in the redox status of treated animals.

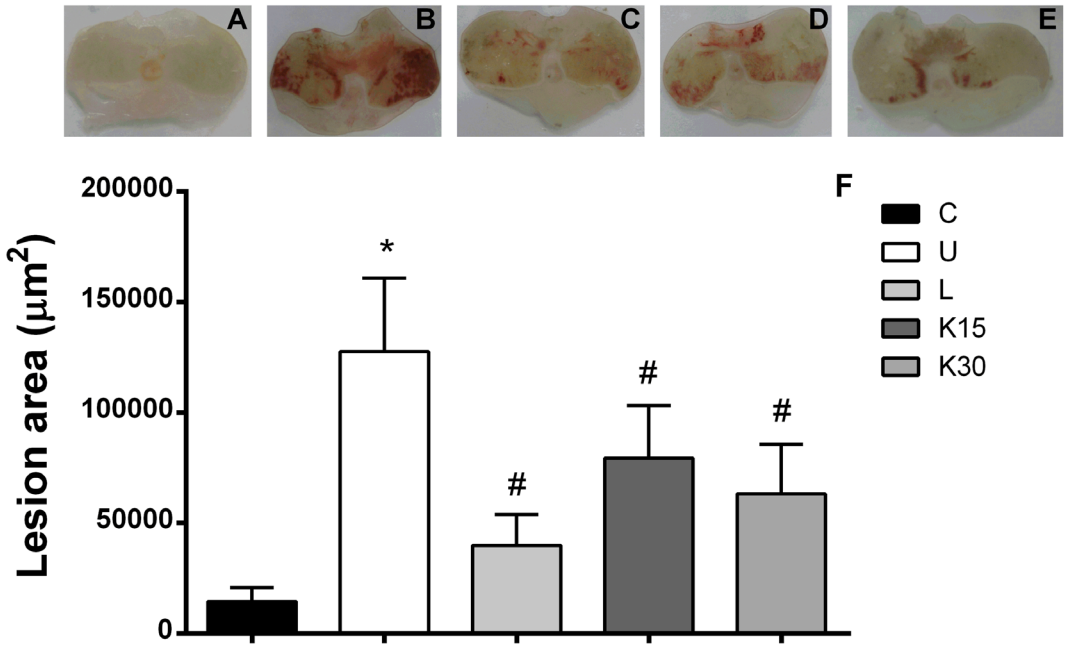

FIGURE 1 - Results of macroscopic lesion evaluation after pretreatment with lansoprazole or water kefir and ulcer induction with acidic ethanol. **Panels A – E** depict representative images of the animals' stomachs. A: Control; B: Ulcerated; C: Lansoprazole; D: K15; E: K30. **Panel F**: Bar chart showing quantitative analysis of lesion area. *p<0.05 compared to the control group; #p<0.05 compared to the ulcer group. Data are presented as mean ± standard error of the mean (SEM).

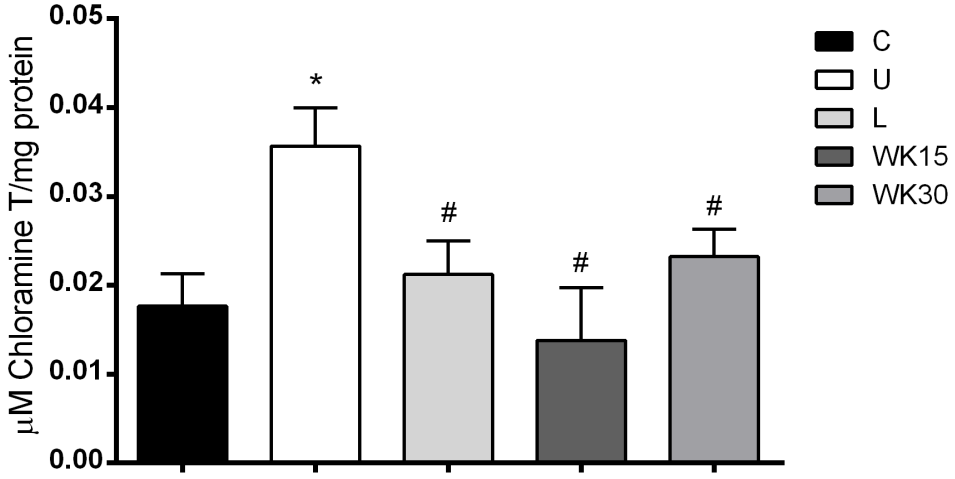

FIGURE 2 - Evaluation of advanced oxidized protein products (AOPPs) in stomach tissue. Both water kefir and lansoprazole promoted a decrease in AOPP values after 14 days of treatment. *p<0.05 compared to C; #p<0.05 compared to U. Data are presented as mean ± standard error of the mean (SEM).

TABLE I - Macroscopic evaluation of ulcer lesion and antioxidant enzyme activity

	Groups				
	C	**U**	**L**	**K15**	**K30**
Ulcer Index (UI)	-	12.67±3.16	1.42±0.52	5.33±1.51	3.20±1.59
% of protection	-	-	70.9	19.1	59.5
SOD (USOD/ mg protein)	21.75±8.16	2.91±1.63*	27.27±2.97#	28.79±4.84#	28.25±8.69 #
Catalase (ΔE/ min/mg protein)	5.73±1.63	0.78±0.23*	4.29±1.21 #	4.28±0.74 #	3.56±1.23 #

Data are presented as the mean ± standard error of mean. Data were analyzed by ANOVA followed by Tukey's post hoc test. *$p<0.05$ compared to the control group; #$p<0.05$ compared to the ulcer group.

DISCUSSION

The main result of the present study was that pretreatment with different doses of WK (0.15 and 0.30 mL/kg), administered for 14 days, promotes gastroprotection in a similar way to lansoprazole, the standard drug used for that purpose. In addition, gastroprotection was accompanied by a decrease in oxidative stress, as observed by an increase in SOD and catalase activity and a decrease in AOPP values.

Macroscopic analysis of the gastric mucosa showed that ethanol induced the appearance of lesions, with areas presenting hemorrhagic ulcers and an increased ulceration index. These findings were similar to previously reported ones (Lebda *et al*., 2018), which showed that exposure to short-term ethanol caused gastric damage.

Currently, the main drugs used for the treatment of gastric ulcers are proton pump inhibitors (PPIs), such as lansoprazole (Ohkuma *et al*., 2018). Lansoprazole inhibits the activity of the H^+/K^+-ATPase protein located in the gastric parietal cell membrane, raising the pH of the gastric lumen by reducing the concentration of H^+ (Shi, Klotz, 2008). Although this mechanism of action does give lansoprazole a good efficacy for the treatment and prevention of gastric lesions, the use of PPIs has been reported to produce undesirable effects, such as pancreatic cancer (Hwang, Chang Park, 2018), making the search for alternative treatments a rather pressing issue.

Accordingly, the interest in probiotics to treat diseases is growing, for these compounds promote many health benefits, not only through gut modulation but also by producing beneficial substances that can affect pathophysiology (Sah *et al*., 2014). Foods containing microorganisms, such as bacteria and yeasts in amounts that may promote health benefits, are considered probiotic foods (Nielsen, Gurakan, Unlu, 2014). Among the probiotic foods, kefir has gained attention as it is formed by a symbiotic mixture of bacteria and yeasts that promote many health benefits (Bourrie, Willing, Cotter, 2016; Brasil *et al*., 2018).

A systematic review revealed that probiotic treatments usually range from 6 to 77 days, with the most frequent period being 14 days (Wang *et al*. 2016). A meta-analysis found beneficial effects in patients with *H. pylori* infection who underwent a 14-day treatment, which improved eradication rates and reduced antibiotic-associated side effects (Wen *et al*. 2017). A double-blind, randomized, placebo-controlled study showed that the efficacy of antibiotic therapy plus *Saccharomyces boulardii* resulted in a reliable cure rate of infection in dyspeptic patients, besides decreasing side effects (Chotivitayatarakorn, Mahachai, Vilaichone, 2017). Our data corroborate those found in the literature, suggesting that 14 days of treatment with water kefir can promote gastroprotection.

A comparison of the microbial diversity between water and milk kefir revealed that the main difference is found in the yeast group (Gulitz *et al*., 2013). The higher concentration of sucrose present in the sugar-water matrix may stimulate the growth of *Saccharomyces*

species. In this context, Girard *et al*., (2010) demonstrated a dose-dependent gastroprotective effect of *Saccharomyces boulardii* in a rat model of ibuprofen-induced gastric ulcers, by reducing the number of gastric ulcers and the ulcerated surface of the gastric mucosa. Taketani *et al*., (2014) showed a gastroprotective effect of orally administered thioredoxin derived from the edible yeast *Saccharomyces cerevisiae*, in an HCl/ethanol-induced model. Their results brought forward an interesting possibility of promoting wound-healing responses through the administration of this compound. Besides, these results suggest that the *Saccharomyces species* and metabolites may be responsible for the gastroprotective effects of water kefir observed in the present study.

Evidence shows that beverages produced by fermentation of kefir in a sugar matrix have beneficial properties to health, which can be attributed to both the microorganisms and the metabolites formed during fermentation (Moreira *et al*., 2008; Muneer *et al*., 2013). Moreira *et al*., (2008) demonstrated that the use of a cell-free fraction isolated from kefir promoted anti-inflammatory and healing activity in experimental animals. Yet another study addressing the benefits of water kefir consumption reported that this beverage can be an interesting source of natural antioxidants with good potential for health improvement, which may be related to the presence of lactic and acetic acid bacteria, yeasts and their metabolites (Muneer *et al*., 2013).

It has been suggested that the health benefits of kefir are related to its antioxidant proprieties (Cenesiz *et al*., 2008; Fahmy, Ismail, 2015). In the present study, treatment with water kefir was found to improve the levels of oxidative stress markers. These data are further supported by studies in which the antioxidant activity of milk-fermented kefir on experimental models had been evaluated; the antioxidant capacity of kefir was found to be involved with insulin resistance improvement (Rosa *et al*., 2016), and oxidative stress markers were reported to be reduced in mice with marked intestinal crypts induced by azoxymethane (Cenesiz *et al*., 2008). The antioxidant effect of the water kefir pretreatment can be explained by the presence of beneficial bacteria in its grains (Friques *et al*., 2015), with evidence indicating that probiotic bacteria have high antioxidant capacity (Shen, Shang, Li, 2011; Wang *et al*., 2017a, 2017b).

The use of kefir as a functional food was also verified by Rodrigues *et al*., (2016), who carried out a study with the objective of developing a kefir-based beer with anti-inflammatory and antiulcerogenic activities. The resulting beverage was reported to be able to improve inflammation and gastric lesions in experimental animals, what appeared to be due to the synergistic interaction between the polyphenols present in barley malt and the probiotic properties of kefir. In this way, beer can be characterized as a functional food capable of producing beneficial effects when consumed.

Therefore, the prevention of gastric lesion development described in the present study may be directly related to the antioxidant activity of water kefir, which acted by restoring the balance between the oxidation of protein products and improving the activity of antioxidant enzymes. These findings were corroborated by other studies addressing the beneficial activities of water kefir (Fiorda *et al*., 2017; Moreira *et al*., 2008; Muneer *et al*., 2013; Rodrigues *et al*., 2016).

CONCLUSION

Water kefir was able to prevent the gastric mucosal damage induced by the use of alcohol in experimental animals. This protection may be established, at least in part, by the antioxidant potential of the beverage prepared with kefir fermented in sugar-water. However, further research is needed to better elucidate the pathways and effects of water kefir on the body.

LIMITATION OF THE STUDY

A limitation of this study is that microbiological analyses of the grains used here and the fermented products of water kefir were not performed. Although the literature indicates the presence of microorganisms in water kefir with gastroprotective activity and potential antioxidant effects, which support our results, further studies are necessary to identify the probiotic action of microorganisms, in order to better relate it with their biological function.

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