Protective effect of bovine milk against HCl and ethanol-induced gastric ulcer in mice

Jeong-Hyun Yoo,^{*1} Jeong-Sang Lee,^{†1} You-Suk Lee,[‡] SaeKwang Ku,[§] and Hae-Jeung Lee^{*‡2}

^{*}Institute for Aging and Clinical Nutrition Research, Gachon University, Gyeonggi-do 13120, Republic of Korea

[†]Department of Functional Foods and Biotechnology, Jeonju University, Jeonlabuk-do 55069, Republic of Korea

[‡]Department of Food and Nutrition, Gachon University, Gyeonggi-do 13120, Republic of Korea

[§]Department of Korean Medicine, Daegu Haany University, Gyeongsanbuk-do 38610, Republic of Korea

ABSTRACT

The purpose of this study was to investigate the gastroprotective effects of bovine milk on an acidified ethanol (HCl-ethanol) mixture that induced gastric ulcers in a mouse model. Mice received different doses of commercial fresh bovine milk (5, 10, and 20 mL/kg of body weight) by oral gavage once a day for 14 d. One hour after the last oral administration of bovine milk, the HCl-ethanol mixture was orally intubated to provoke severe gastric damage. Our results showed that pretreatment with bovine milk significantly suppressed the formation of gastric mucosa lesions. Pretreatment lowered gastric myeloperoxidase and increased gastric mucus contents and antioxidant enzymes catalase and superoxide dismutase. Administration of bovine milk increased nitrate/nitrite levels and decreased the malondialdehyde levels and the expression of proinflammatory genes, including transcription factor nuclear factor- κ B, cyclooxygenase-2, and inducible nitric oxide synthase in the stomach of mice. These results suggest that bovine milk can prevent the development of gastric ulcer caused by acid and alcohol in mice.

Key words: bovine milk, gastric ulcer, gastroprotective effect, antioxidant enzyme activity, anti-inflammatory effect

INTRODUCTION

Gastric ulcer is one of the common diseases, affecting more than 10% of the world's population (Liu and Cho, 2000). Etiological factors of gastric ulcer include alcohol abuse, smoking, stress, drug overuse, and microorganism infection (Kim et al., 2014). Of these factors, alcohol can perturb the gastric mucosa and induce numerous metabolic changes, leading to mucosal dam-

ages and lesions in the stomach (Szabo et al., 1985). Alcohol also increases the generation of reactive oxygen species (ROS) and lipid peroxidation while suppressing the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase. These enzymes play important roles in protecting stomach against mucosa damages (Pan et al., 2008). Alcohol is an ulcerogenic substance that induces mucosal damage, which reduces intraluminal acid and promotes back diffusion of hydrogen ions. It also directly damages the oxyntic (parietal) cells and increases the gastric mucosal permeability by the increase in back diffusion and the transmucosal potential difference, which reflects surface cell layer exfoliation (MacMath, 1990; Keshavarzian et al., 1994). In addition, alcohol-induced mucosal damage can increase gut permeability, which allows the transport of bacterial endotoxins that are normally unable to cross the intestinal wall to enter the blood or lymph (Bode, 1980).

Milk contains various beneficial nutrients and, as such, it has been used as a valuable food source (Chung, 2010). Milk consumption is generally believed to be an important element in a healthy and balanced diet. Milk contains various bioactive peptides, including digestive health peptides (Mohanty et al., 2016); however, to the best of our knowledge, systemic assessment of a gastroprotective effect of bovine milk, especially against gastric damage induced by hydrochloride (HCl) and ethanol, has not been reported in an animal model such as the mouse. We hypothesized that bovine milk has protective effects against gastric mucosal damage via modulating antioxidant enzymes and anti-inflammatory activities. To test this hypothesis, we determined the gastroprotective effect of bovine milk against oxidative damages induced by HCl and ethanol in mice.

MATERIALS AND METHODS

Animals

Forty male ICR mice (6 wk old, 34.34 ± 1.60 g) were purchased from OrientBio (Gyeonggi-do, Korea). Mice

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¹These authors contributed equally to this paper.

²Corresponding author: skysea@gachon.ac.kr or skysea1010@gmail.com

Table 1. Experimental design, with milk administered as a pretreatment for 14 d

Group	Animals (n = 10)	Test substance ¹ (mg/kg)
Normal control	Untreated mice	Distilled water at 20 mL/kg of BW, oral administration
HE control	Gastric ulcer mice	Distilled water and HE administration
Experiment group	Gastric ulcer mice	Milk 5 mL/kg and HE administration
Experiment group	Gastric ulcer mice	Milk 10 mL/kg and HE administration
Experiment group	Gastric ulcer mice	Milk 20 mL/kg and HE administration

¹Milk = commercial fresh bovine milk (Na 100%, Seoul Dairy Corp., Seoul, Korea); HE = HCl-ethanol mixture (98% ethanol containing 150 mM HCl).

were housed in cages (20–25°C, 30–35% humidity, and a 12-h light/dark cycle). They were provided free access to water and standard rodent chow (38057, Purinafeed, Gyeonggi-do, Korea). After acclimation for 7 d, animals were randomly divided into 5 groups (n = 8 for each group, Table 1). All animal procedures were performed in accordance with guidelines issued by the Animal Care and Use Committee of Gachon University for the care and use of laboratory animals (approval number: GIACUC-R2016014).

Preparations and Administration of Test Materials

Commercial fresh bovine milk (Na 100%; Seoul Dairy Corp., Seoul, Korea) was purchased from a local market. Bovine milk was diluted with distilled water at a ratio of 1:4 and orally administered to mice once a day for 14 d. In our study, bovine milk was divided into 3 doses (5, 10, and 20 mL/kg of BW). The concentrations selected in the study were based on the daily milk intake of Koreans. The recommended intake of milk in South Korea is 400 mL, but, according to the Korean Nutrition Survey, daily milk intake is only 66.4 mL (Korea Health Industry Development Institute, 2014). The highest dose of milk in the current study was 20 mL/kg, which is equivalent to half a cup of milk; the middle dose (10 mL/kg) is equivalent to the average daily intake of Koreans. The human equivalent dose of the highest dose of milk would be an intake of 112 mL/d for an individual of 70 kg of BW [human equivalent dose (mg/kg) = mouse dose (mg/kg) × mouse Km (3)/human Km (37), where Km is the factor for converting mg/kg dose to mg/m² dose (BW to surface area)], as calculated by the body surface area normalization method (Reagan-Shaw et al., 2008). In addition, the highest dose corresponds to the limit highest oral dosage volume in mice (Flecknell, 1996; Korea Food and Drug Administration, 2015), and 10 and 5 mL/kg were selected as middle and lower dose groups using a common ratio of 2. Equal volumes of distilled water (20 mL/kg) were administered orally to mice in the normal control group and HE control group (Table 1).

Induction of Gastric Mucosa Damages

Mice were deprived of food for 24 h in a cage with wide-mesh wire bottoms to prevent coprophagia before conducting the experiment. One hour after the last (the 14th) administration of vehicle, an HCl and ethanol (HE) mixture (98% ethanol containing 150 mM HCl) was orally administered to mice at 5 mL/kg of BW according to a previous report (Oyagi et al., 2010). Untreated control mice were administered an equal volume of distilled water instead of HE solution.

Quantification of Gross Lesion

Animals were euthanized at 1 h after treatment with the HE mixture by cervical dislocation under inhalation anesthetized with 2 to 3% isoflurane (Hana Pharm. Co., Hwasung, Korea) in the mixture of 70% N₂O and 28.5% O₂, using rodent inhalation anesthesia apparatus (Surgivet, Waukesha, WI) and rodent ventilator (model 687, Harvard Apparatus, Cambridge, UK). The abdomen of each mouse was cut with a median incision and the stomach was removed. The excised stomach was opened along the greater curvature and rinsed with cold saline solution to remove gastric contents and blood clots. After examining the ulcer area on stomach, digital images of the ulcer were acquired and analyzed using an automated computer-based image analyzer (iSolution FL ver 9.1, IMT i-solution Inc., Vancouver, BC, Canada) according to methods of Suleyman et al. (2009), Morais et al. (2010), and Oyagi et al. (2010) with some modifications. Ulcer lesions were measured to calculate gastric damage score. For this purpose, total areas of ulcerous stomach regions were calculated as millimeters squared of gastric mucosa.

Determination of Gastric Myeloperoxidase Activity

Tissue samples (about 0.2 g) were homogenized in 2 mL of ice-cold potassium phosphate buffer (50 mM K₂HPO₄, pH 6.0; Sigma-Aldrich, St. Louis, MO) containing hexadecyltrimethylammonium bromide (0.5% wt/vol, Sigma-Aldrich; Morais et al., 2010). The ho-

mogenate was centrifuged at $12,000 \times g$ for 10 min at 4°C. After discarding the supernatant, the pellet was rehomogenized with an equivalent volume of 50 mM K_2HPO_4 containing 0.5% (wt/vol) hexadecyltrimethylammonium bromide and 10 mM EDTA (Sigma-Aldrich). Myeloperoxidase (MPO) activity was assessed by measuring H_2O_2 -dependent oxidation of *o*-dianisidine 2 HCl. One unit of enzyme activity was defined as the amount of MPO present in 1 g of tissue that caused a change in absorbance of 1.0/min. The absorbance was measured at wavelength of 460 nm and 37°C using an UV/Vis spectrometer (Optizen Pop, Mecasys, Daejeon, Korea; Bradley et al., 1982).

Measurement of Gastric Mucus Contents

Alcian blue (Sigma-Aldrich) binding assay was performed according to published method (Sun et al., 1991; Ribeiro et al., 2016). At euthanization, after removing gastric contents, some parts of the stomach were immersed in 10 mL of 0.02% Alcian blue and 0.16 M sucrose/0.05 M sodium acetate solution (pH 5.8) and incubated at 25°C for 24 h. The Alcian blue binding extract was centrifuged at $3,000 \times g$ for 10 min at 4°C. The absorbance of the supernatant was measured at 620 nm on a spectrophotometer. Free mucus in the gastric content was calculated based on the amount of Alcian blue binding to the gastric mucus (mg/g of tissue).

Tissue SOD Activity

Gastric SOD activity was determined by the method of Minami and Yoshikawa (1979), with slight modifications. Briefly, 15 μ L of gastric homogenate was mixed with 450 μ L of cold deionized water, 125 μ L of chloroform, and 250 μ L of ethanol. The mixture was then centrifuged at $8,000 \times g$ for 2 min at 4°C. The extract (500 μ L) was added to the reaction mixture containing 500 μ L of 72.4 mM Tris-cacodylate buffer, 3.5 mM diethylene pentaacetic acid (pH 8.2), 100 μ L of 16% Triton X-100, and 250 μ L of 0.9 mM nitro blue tetrazolium (all from Sigma-Aldrich). The reaction mixture was incubated at 37°C for 5 min before adding 10 μ L of 9 mM pyrogallol (Sigma-Aldrich) dissolved in 10 mM HCl. After incubating at 37°C for exactly 5 min, the reaction was stopped by adding 300 μ L of 2 M formic buffer (pH 3.5) containing 16% Triton X-100. The absorbance of the mixture was then measured at 540 nm on a spectrophotometer. One unit of SOD enzymatic activity was equal to the amount of enzyme that diminished the initial absorbance of nitro blue tetrazolium by 50% (mmol/min per milligram of tissue).

Tissue CAT Activity

Catalase activity was determined according to the method of Rice-Evans and Diplock (1991). Homogenate of mouse gastric mucosa was diluted with buffer as described earlier to obtain adequate dilution of the enzyme. Two milliliters of the enzyme dilution was then added to a cuvette and mixed with 1 mL of 30 mM H_2O_2 . The absorbance was measured at 240 nm for 100 s using a UV/Vis spectrometer. The initial absorbance of the reaction mixture was around 0.5. Enzyme activity was expressed as the first-order constant that described the decomposition of H_2O_2 at room temperature in millimoles per minute per milligram of tissue.

Determination of Lipid Peroxidation or Malondialdehyde Formation

Determination of malondialdehyde (MDA) formation by thiobarbituric acid was used as an index for the extent of lipid peroxidation using published method (Ohkawa et al., 1979). Briefly, mouse stomach was promptly excised and rinsed with cold saline. To minimize the possibility of hemoglobin interfering with free radicals, any blood adhering to the mucosa was carefully removed. The corpus mucosa was scraped, weighed, and homogenized in 10 mL of 100 g/L KCl (Sigma-Aldrich). The homogenate (0.5 mL) was added to a solution containing 0.2 mL of 80 g/L SLS (Sigma-Aldrich), 1.5 mL of 200 g/L acetic acid (Merck, Darmstadt, Germany), 1.5 mL of 8 g/L 2-thiobarbiturate (Sigma-Aldrich), and 0.3 mL of distilled water. This mixture was then heated at 98°C for 1 h. After cooling to room temperature, 5 mL of n-butanol:pyridine (15:1; Sigma-Aldrich) was added. The reaction mixture was vortexed for 1 min and centrifuged at $2,000 \times g$ for 30 min at 4°C. The supernatant was collected and its absorbance was measured at the wavelength of 532 nm using a UV/Vis spectrometer (Optizen Pop, Mecasys). A standard curve was obtained by using 1,1,3,3-tetramethoxypropane (Sigma-Aldrich). Results are expressed as nanomoles of MDA per gram of wet tissue.

Gastric Nitrate/Nitrite Contents

Gastric nitric oxide (NO) levels were measured as both total nitrate and nitrite levels using Griess reagent (Green et al., 1982). Briefly, mouse stomach was homogenized in 50 mM potassium phosphate buffer (pH 7.8; Sigma-Aldrich) and centrifuged at $11,000 \times g$ for 15 min at 4°C. Supernatant (100 μ L) was then mixed with 100 μ L of Griess reagent [0.1% *N*-(1-naphthyl) ethylenediamide dihydrochloride, 1% sulfanilamide in 5% phosphoric acid; all obtained from Sigma-Aldrich].

After 10 min of reaction at room temperature, absorbance of the mixture was measured at wavelength of 540 nm using a microplate reader (Tecan, Männedorf, Switzerland). A standard curve was obtained by using sodium nitrite as reference. Results are expressed as micromoles of nitrate or nitrite per gram of protein. Protein concentration of the sample was determined by Bradford assay (Bradford, 1976).

Real-Time Reverse Transcription-PCR for Gastric mRNA Expression

Expression levels of nuclear factor (NF)- κ B (*Nfkb1*), cyclooxygenase-2 (*Cox2*), and inducible nitric oxide synthase (*inos*) mRNA inside the prepared gastric tissues were determined by real-time PCR. Briefly, RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA). Purification was carried out with a commercial RNA extraction kit (Qiagen, Valencia, CA); RNA concentrations and quality were determined by CFX96 Real-Time System (Bio-Rad, Hercules, CA). To remove contaminated DNA, samples were treated with recombinant DNase I (DNA-free; Ambion, Austin, TX). The RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions (<https://www.thermofisher.com/order/catalog/product/4368814>). Real-time PCR was then carried out using ABI Step One Plus Sequence Detection System (Applied Biosystems). Expression levels were calculated relative to vehicle control. Expression level of *Gapdh* mRNA was used as an internal control for all samples. Gene-specific primers used for real-time PCR are listed in Table 2.

Histopathology

Approximated regions of individual stomach (between cardiac and pylorus, the fundus) were sampled and cross-trimmed based on the lumen. All trimmed fundi were fixed in 10% neutral buffered formalin for 24 h. After paraffin embedding, sections (3–4 μ m in thickness) were prepared. Representative sections were stained with hematoxylin and eosin for light microscopy. Next, the histological profile of individual cross-trimmed fundus was observed. To obtain detailed

changes, total thickness of fundic mucosa from luminal mucosal surface to muscularis mucosa on peri-ulcerative regions of cross-trimmed histological specimens were measured using an automated computer-based image analyzer under microscopy (model Eclipse 80i, Nikon, Tokyo, Japan). In addition, percentage of invaded lesion in fundus was calculated by using equation 1 according to the method of Ku et al. (2009):

$$\text{Percentage of invaded lesion (\%)} = \left(\frac{\text{length of lesion on cross-trimmed fundic wall}}{\text{total thickness of cross-trimmed fundic wall}} \right) \times 100. \quad [1]$$

Based on general and histomorphometrical analysis results, semiquantitative scoring was performed using the following 4 degrees: 0, normal intact mucosa; 1, slight surface erosive damages; 2, moderate mucosa damages; and 3, severe total mucosa damages. The histopathologist was blinded to group distribution when this analysis was made.

Statistical Analyses

All statistical analyses were conducted using software SPSS version 23 (IBM Corp., Armonk, NY). Experimental data are expressed as mean $\pm$ standard deviation ($n = 8$ per group). Differences between mean values for individual groups were assessed by 1-way ANOVA with Duncan's multiple range test. Statistically significant differences were considered at $P < 0.05$.

RESULTS

Changes in BW of Mice

No significant difference in BW change was found between HE control mice and normal control mice. We observed no significant differences in BW changes between mice administered 3 doses of bovine milk (5, 10, and 20 mL/kg) and HE control mice during the whole experimental period (Table 3).

Changes in Gastric Mucosa Gross Lesions

In mice treated with HCl/ethanol, focal hemorrhagic ulcerative lesions were observed throughout the whole

Table 2. List of primers used for real-time PCR

Gene	NCBI accession No.	Forward sequence	Reverse sequence
<i>Nfkb1</i>	NM_008689	CAATGGCTACACAGGACCA	CACTGTCACTTGGGAACCAGA
<i>Cox2</i>	NM_011198	CACTACATCCTGACCCACTT	ATGCTCCTGCTTGAGTATGT
<i>inos</i>	NM_001313922	CCCTTCCGAAGTTTCTGGCAGCAG	GGCTGTCAGAGCCTCGTGGCTTTGG
<i>Gapdh</i>	NM_008084	CATCTTCCAGGAGCGAGACC	TCCACCACCCTGTTGCTGTA

Table 3. Body weight gains of control and milk group treated for 14 d

Item ¹	BW by time		BW gain (terminal – initial)
	Initial (g)	Terminal (g)	
Control			
Normal	30.45 ± 1.39	31.93 ± 1.96	1.48 ± 0.88
HE	30.23 ± 1.31	32.01 ± 1.80	1.79 ± 0.75
Milk			
5 mL/kg	30.31 ± 1.41	32.31 ± 2.07	2.00 ± 1.05
10 mL/kg	30.33 ± 1.66	32.21 ± 1.84	1.89 ± 0.71
20 mL/kg	30.19 ± 2.52	32.30 ± 3.14	2.11 ± 0.74

¹Values are expressed as mean ± SD (n = 8 per group). HE = HCl-ethanol mixture (98% ethanol containing 150 mM HCl).

gastric mucosa; however, mice in the control groups did not show any macroscopic changes. Gross gastric damages in mice pretreated with 3 different doses of bovine milk (5, 10, and 20 mL/kg) were significantly ($P < 0.05$) inhibited compared with those in the HE control group. Gastric mucosa gross lesion area in the HE control group was increased 3,009.23% compared with that in the normal control group; however, oral administration of bovine milk at 5, 10, and 20 mL/kg ameliorated damage to the gastric mucosa by 31.14, 48.13, and 74.66%, respectively, compared with HE control [all $P < 0.05$, for HE-treated group vs. normal control % change = [(HE-treated – normal control)/normal control] × 100, for milk-treated group % change = [(milk-treated – HE-treated)/HE-treated] × 100; Figures 1 and 2].

Effects on Gastric Mucus Contents

A significant decrease in gastric mucus content was observed in HE control mice compared with that in normal control mice based on Alcian blue binding assay; however, gastric mucus contents in bovine milk treatment groups (5, 10, and 20 mL/kg) were significantly ($P < 0.05$) higher than those of the HE control group. Gastric mucus contents in HE control group were decreased by 74.47% compared with those in the normal control group; however, gastric mucus contents in groups pretreated with bovine milk at 5, 10, and 20 mL/kg were increased 68.83, 89.63, and 185.90% ({[M20(mean) – HE(mean)]/HE(mean)} × 100), respectively, compared with that in the HE control group ($P < 0.05$; Figure 2).

Changes in Gastric MPO Activities

As shown in Figure 3, administration of HE resulted in a significant ($P < 0.05$) increase in gastric MPO activities compared with the normal control group. Compared with the HE control group, bovine milk groups (5, 10, and 20 mL/kg) showed significant ($P <$

0.05) attenuation in ethanol-induced increase of MPO activity. Gastric MPO activity in HE control group was increased by 1,453.85% ($P < 0.05$) compared with that in the normal control group. In groups of mice pretreated with bovine milk at 5, 10, and 20 mL/kg, gastric MPO activities were decreased 44.61, 69.64, and 82.14%, respectively, compared with those in the HE control group ($P < 0.05$).

Effects on the Antioxidant Defense System

To evaluate antioxidant defense properties, we determined SOD activity, CAT activity, MDA content, and nitrate/nitrite content in gastric tissues; results are shown in Figure 4. Gastric SOD and CAT activities decreased 62.45 and 70.54% ($P < 0.05$) in the HE control group, respectively, compared with those in the normal control group. However, in groups pretreated with bovine milk at 5, 10, and 20 mL/kg, significant ($P < 0.05$) increases in SOD (38.53, 58.75, and 102.55%, respectively) and CAT (47.21, 85.28, and 157.29%, respectively) activities were observed compared with the HE control group. The content of gastric MDA (225.74%) was significantly ($P < 0.05$) higher in the HE control group compared with that in the normal control group. In groups pretreated with bovine milk at 5, 10, and 20 mL/kg, gastric MDA contents were 28.56, 38.88, and 58.70% lower ($P < 0.05$), respectively, compared with those in the HE control group. Gastric nitrate/nitrite content in the HE control group decreased by 60.35% compared with that in the normal control group; however, gastric nitrate/nitrite contents in groups pretreated with bovine milk at 5, 10, and 20 mL/kg were increased ($P < 0.05$) 29.85, 52.62, and 90.70%, respectively, compared with those in the HE control group.

Effects on Gastric *Nfkb1*, *Cox2*, and *inos* mRNA Expression

Results of gastric mRNA expression are shown in Figure 5. Expression levels of *Nfkb1* (516.25%), *Cox2*

(1,170.83%), and *inos* (996.21%) mRNA were significantly higher in the HE control group compared with those in the normal control group (all $P < 0.05$). However, groups of mice pretreated with bovine milk at 5, 10, and 20 mL/kg exhibited significant ($P < 0.05$) decreases in the expression of *Nfkb1* (27.31, 48.17, and 64.85%, respectively), *Cox2* (39.47, 56.45, and 79.54%, respectively), and *inos* (34.45, 57.16, and 77%, respectively) compared with the HE control group.

Changes in Gastric Mucosa Histopathology

As shown in Figures 6 and 7, severe focal extensive superficial epithelial damage, desquamation of focal epithelium, congestion/hemorrhages, inflammatory cell infiltration and necrosis of gastric glands, submucosa edematous changes, and ulcerative lesions were detected on fundi after treatment with HCl/ethanol. However, these microscopic ulcerative lesions were markedly

Figure 1. Effect of bovine milk on stomach gross lesion. (a) Representative gross stomach images, where CA = cardiac region; FU = fundus region; PY = pylorus region; (b) gross lesion area of gastric mucosa. Normal = untreated; HE = treatment with acidified ethanol (98% ethanol containing 150 mM HCl); M5, M10, and M20 = pretreatment with commercial fresh bovine milk at 5, 10, and 20 mL/kg of BW before HE treatment. Values are expressed as mean $\pm$ SD ($n = 8$ per group). Different letters (a–e) indicate significant differences ($P < 0.05$) between treatments determined by Duncan's multiple range test. Color version available online.

Figure 2. Effect of bovine milk on gastric mucus content. Normal = untreated; HE = treatment with acidified ethanol (98% ethanol containing 150 mM HCl); M5, M10, and M20 = pretreatment with commercial fresh bovine milk at 5, 10, and 20 mL/kg of BW before HE treatment. Values are expressed as mean $\pm$ SD ($n = 8$ per group). Different letters (a–d) indicate significant differences ($P < 0.05$) between treatments determined by Duncan's multiple range test.

diminished by pretreatment with 3 doses of bovine milk. Based on histopathological and semiquantitative analysis, significant ($P < 0.05$) increases in invaded lesions and histological scores with significant decreases in peri-ulcerative mucosa thicknesses were observed in HE control mice compared with normal control mice; however, they were significantly ($P < 0.05$) ameliorated by pretreatment with bovine milk at 5, 10, and 20 mL/kg. Invaded lesions in the HE control group were larger (at 5,027.29%) compared with those in the normal control group. However, they were significantly

Figure 3. Effect of bovine milk on gastric myeloperoxidase (MPO) activities. Normal = untreated; HE = treatment with acidified ethanol (98% ethanol containing 150 mM HCl); M5, M10, and M20 = pretreatment with commercial fresh bovine milk at 5, 10, and 20 mL/kg of BW before HE treatment. Values are expressed as mean $\pm$ SD ($n = 8$ per group). Different letters (a–d) indicate significant differences ($P < 0.05$) between treatments determined by Duncan's multiple range test.

decreased by pretreatment with bovine milk at 5, 10, and 20 mL/kg (46.09, 62.42, and 75.49%, respectively) compared with those in the HE control group ($P < 0.05$). Peri-ulcerative mucosa thickness was decreased in the HE control group (59.62%) compared with those in the normal control group but increased in groups of mice pretreated with bovine milk at 5, 10, and 20 mL/kg (52.30, 94.79, and 117.53%, respectively) compared with those in the HE control group ($P < 0.05$). Semiquantitative histological scores in the HE control group were increased (633.33%) compared with those in the normal control group; however, they were significantly decreased in groups pretreated with bovine milk at 5, 10, and 20 mL/kg (31.82, 45.45, and 59.09%, respectively) compared with those in the HE control group ($P < 0.05$).

DISCUSSION

We evaluated a possible protective effect of fresh bovine milk against acidified ethanol-induced gastric ulcer in mice. Alcohol-induced gastric ulcer models have been commonly used to study both pathogenesis and therapies for human ulcerative disease (Szabo and Brown, 1987). In the present study, 3 doses of fresh commercial bovine milk (5, 10, and 20 mL/kg) were orally administered to mice once daily for 14 d. One hour after the last (14th) milk treatment, a single dose of HCl/ethanol mixture (5 mL/kg of 98% ethanol containing 150 mM HCl) was orally administered to induce severe gastric damages.

Milk and dairy products are nutritious food containing various essential nutrients. Biological studies have reported that dairy products or components of milk have gastroprotective properties. Yogurt containing *Lactobacillus gasseri* OLL2716 inhibited the formation of acute gastric lesions and significantly increased prostaglandin E₂ (PGE₂) in the gastric mucosa and inhibit the formation of antral ulcer (Uchida and Kurakazu, 2004). Fermented milk that contains *Bifidobacterium bifidum* BF-1 was reported to exhibit gastroprotective effects by increasing mucin 5ac (*MUC5AC*) gene expression level and gastric mucin production in an acute gastric injury rat model (Gomi et al., 2013). In various studies, it has been shown that whey protein in milk protects the gastric mucosa (Marshall, 2004; Rosaneli et al., 2004). Bovine whey protein reduced ulcerative lesions by 50.1 and 44% in a rat model of indomethacin-induced gastric ulcer (Rosaneli et al., 2004). In addition, α -LA, one of the major proteins in milk, has been reported to inhibit ulcers by lowering gastric damage, increasing prostaglandin synthesis (including elevation of gastric luminal pH and gastric fluid volume), and increasing mucin contents of both the gastric fluid and

adherent mucus gel layer in an ethanol- and stress-induced gastric mucosal injury model (Matsumoto et al., 2001) and a naïve model (Ushida et al., 2003).

Our results showed no significant changes in BW or BW gains of HE control mice compared with normal control mice. No significant changes in BW or BW gains were observed in groups pretreated with bovine milk at different doses (5, 10, and 20 mL/kg) compared with those of HE control mice throughout the experimental period.

Our results confirmed that administration of acidified ethanol caused severe gastric damage. Similar results have been reported, showing that gastric ulcer model is accompanied by significant increases in hemorrhagic gross lesion and MPO activities but a significant decrease in mucus (mucin) content (Fahmy and Ismail, 2015; Carvalho et al., 2016). Gastric mucosa gross lesions area was markedly increased in the HE control group compared with that in the normal control group. However, pretreatment with bovine milk significantly

improved gastric damages induced by the acidified ethanol mixture. Fahmy and Ismail (2015) reported that kefir milk pretreatment before exposure to γ -radiation or ethanol can alleviate gastric ulcers, resulting in a significant reduction in ulcer number, volume of gastric juice, and total acidity. Hemorrhagic lesions are associated with changes in gastric mucosal permeability, resulting in back-diffusion of acid across damaged gastric mucosa (Skillman et al., 1970). Increased mucosal permeability can allow the transport of large molecules, consequently promoting the development of gastric ulcer. Therefore, decrease or inhibition of gross hemorrhagic lesion area has been regarded as a valuable indication that the test substance has a favorable gastric mucosa protective effect.

Gastric mucus is secreted to form a gel layer on the gastric mucosa. This gel layer covers the gastric mucosal surface and sustains basophilic pH of the mucosal surface. Gastric mucus, which is produced in mucus-producing cells, plays an important role in preventing

Figure 4. Effect of bovine milk on gastric antioxidant defense system; (a) superoxide dismutase (SOD) activity; (b) catalase (CAT) activity; (c) malondialdehyde (MDA) content; (d) nitrate/nitrite level. Normal = untreated; HE = treatment with acidified ethanol (98% ethanol containing 150 mM HCl); M5, M10, and M20 = pretreatment with commercial fresh bovine milk at 5, 10, and 20 mL/kg of BW before HE treatment. Values are expressed as mean $\pm$ SD ($n = 8$ per group). Different letters (a–d) indicate significant differences ($P < 0.05$) between treatments determined by Duncan's multiple range test.

Figure 5. Effect of bovine milk on mRNA levels of (a) *Nfκb1*, (b) *Cox2*, and (c) *inos*. Normal = untreated; HE = treatment with acidified ethanol (98% ethanol containing 150 mM HCl); M5, M10, and M20 = pretreatment with commercial fresh bovine milk at 5, 10, and 20 mL/kg of BW before HE treatment. Values are expressed as mean $\pm$ SD (n = 8 per group). Different letters (a–e) indicate significant differences ($P < 0.05$) between treatments determined by Duncan's multiple range test.

gastric mucosa against stimulating substances such as acidified ethanol (Ichikawa and Ishihara, 2011). Our results showed that gastric mucus contents were markedly decreased in the HE control group compared with those in the normal control group. However, pretreatment with milk reversed this decrease. Ushida et al. (2003) reported that oral administration of bovine α -LA, a major milk protein, could elevate gastric PGE₂ levels, gastric mucin contents, gastric luminal pH, gastric fluid volume, and gastric mucus gel layer in naïve rats. In addition, α -LA treatment can increase the synthesis and secretion of mucin and increase PGE₂ synthesis in RGM1 cells (Ushida et al., 2007). In an in vivo study, oral administration of α -LA increased the thickness of the mucus gel layer by stimulating either mucin production or gastric mucus-producing cells (Ushida et al., 2007).

Myeloperoxidase, a heme enzyme mainly secreted in neutrophils, indicates gastric mucosal neutrophil infiltration and can be used as a gastric mucosal injury indicator. Neutrophil infiltration in inflamed mucosa has been observed in ulcerative colitis (Masoodi et al., 2011). Neutrophils are associated with upregulated inflammatory response with increased gastric expression of NF- κ B, leading to increased production of proinflammatory cytokines, including tumor necrosis factor- α (Arab et al., 2015). Our results showed that MPO activity in the stomach was significantly increased in the HE control group compared with those in the normal control group; however, pretreatment with bovine milk ameliorated the HE-induced increase in MPO activity. These results suggest that bovine milk has gastroprotective effects against gastric ulcer in the mouse model by protecting gastric mucosa and improving gastric mucus contents and MPO activity.

It is well known that ethanol induces ROS, which stimulates inflammatory processes, including the secretion of proteolytic enzymes, lipoxygenases, and cyclooxygenase and the release of signaling proteins (Leonard et al., 2004). Ethanol administration may induce variations in antioxidant/oxidant status that can be detected in mice. In tissues where ROS is produced, antioxidants such as SOD, CAT, and glutathione peroxidase are known to protect against oxidative stress-induced damages (Alirezai et al., 2012). In our study, acidified ethanol inhibited SOD and CAT activities in the HE control group; however, all doses of bovine milk increased SOD and CAT activities. These results indicate that antioxidant enzymes play important roles in eliminating gastric damage by partially preventing oxidative damage.

Increasing lipid peroxidation products, such as MDA, is often used to indicate the extent of oxidative stress (Harrison-Findik, 2007). As a result of lipid peroxida-

Figure 6. Effect of bovine milk on histopathology image features, where the difference in the thickness of mucosa and edema between the HE group and the M5, M10, and M20 groups can be confirmed. Normal = untreated; HE = treatment with acidified ethanol (98% ethanol containing 150 mM HCl); M5, M10, and M20 = pretreatment with commercial fresh bovine milk at 5, 10, and 20 mL/kg of BW before HE treatment. Scale bars = 180 μ m. LU = lumen; MU = mucosa layer; MM = muscularis mucosa; SM = submucosa; ML = muscular layer. Color version available online.

tion, toxic products such as lipid peroxide, alcohol, and aldehyde can damage tissues (Freeman and Crapo, 1982; Comporti, 1985). Our results showed that gastric MDA content was increased gradually after treatment

with acidified ethanol in the HE control group. However, MDA levels in groups pretreated with bovine milk were significantly reduced compared with those in the HE control group.

Figure 7. Effect of bovine milk on histopathology images with quantitative analysis. (a) Percentage of invaded lesions; (b) mean peri-ulcerative mucosa thickness; and (c) semi-quantitative score. Normal = untreated; HE = treatment with acidified ethanol (98% ethanol containing 150 mM HCl); M5, M10, and M20 = pretreatment with commercial fresh bovine milk at 5, 10, and 20 mL/kg of BW before HE treatment. Values are expressed as mean $\pm$ SD ($n = 8$ per group). Different letters (a–e) indicate significant differences ($P < 0.05$) between treatments determined by Duncan's multiple range test.

Nitric oxide is a crucial mediator of gastrointestinal mucosal defense (Wallace and Miller, 2000). It has been reported that gastric NO can maintain the viscoelastic layer of mucus by boosting mucus and bicarbonate secretion, regulating gastric blood flow and microcirculation, and downregulating neutrophil aggregation and secretion (Wallace and Miller, 2000; Wallace, 2006). Our findings on nitrate/nitrite levels in gastric tissues further confirmed this view. Total nitrate/nitrite level, a marker of endogenous NO (Morais et al., 2010), was markedly reduced by HE treatment; however, its level was significantly elevated in mice pretreated with bovine milk at 3 doses. These results suggest that administration of bovine milk can improve antioxidant defense system and decrease lipid peroxidation in the stomach of mice treated with HE mixture.

It is known that ethanol administration activates immune response and upregulates levels of inflammatory cytokines (Kawaratani et al., 2013). Nuclear factor- κ B is a transcription factor that can promote the expression of over 100 target genes that have NF- κ B binding sites in their promoters. Nuclear factor- κ B signaling pathway plays an important role in the immune system through regulating the activation of inflammation-related genes such as *TNFA*, *IL6*, *PTGS2*, and *INOS* (Lawrence, 2009). Mucosal inflammation is closely associated with NF- κ B activation (Sokolova and Naumann, 2017); therefore, inhibiting NF- κ B is a key to attenuating gastric ulcer formation. Cyclooxygenase, the key enzyme for synthesis of prostaglandins, has 2 isoforms (COX-1 and COX-2); COX-1 is generally constitutive in most tissues and COX-2 is an enzyme that is induced by inflammatory stimuli, including cytokines, endotoxin, growth factor, and oncogenes (Smith et al., 1996). Prostaglandins produced by COX-1 are necessary for maintaining homeostasis of gastric mucosal tissues (Dubois et al., 1998); however, *Cox2*, one of the NF- κ B target genes induced by inflammatory stimuli, can mediate the synthesis of prostaglandins that promote cell proliferation and macrophage infiltration in stomach tissues (Shao et al., 2014). Therefore, inhibition of COX-2 mRNA expression is a major factor for the treatment of gastric ulcers (Wang et al., 2014). Inducible NO synthase (iNOS) is normally not expressed in normal conditions; however, it is expressed after specific stimuli, such as stomach injury. Inducible NO synthase is regulated by inflammation-related cytokines via transcriptional factor NF- κ B. Significant increases in iNOS expression and activity have been reported in severely inflamed ulcer tissues during ulcer formation (Guo et al., 2003). In our study, exposure to HE significantly increased gastric *Nfkb1*, *Cox2*, and *inos* mRNA expression levels. However, significant

downregulation of gastric *Nfkb1*, *Cox2*, and *inos* mRNA expression levels were detected in all groups pretreated with bovine milk compared with the HE control group. Our results indicate that bovine milk administration might have an anti-inflammatory effect via modulating NF- κ B signaling pathway.

Histopathologically, severe focal extensive superficial epithelial damage, desquamation of focal epithelium, focal hemorrhages/congestions, inflammatory cell infiltrations and necrosis of gastric glands, submucosa edematous changes, and ulcerative lesions were detected on fundi after treatment with the acidified ethanol mixture in the current and previous studies (Yesilada and Gurbuz, 2003; Oyagi et al., 2010). Histopathological images have been used to confirm gastric protective effects in many studies (Yesilada and Gurbuz, 2003; Graziani et al., 2005; Oyagi et al., 2010). In our results, acidified ethanol-induced microscopic ulcerative lesions were markedly inhibited by pretreatment with bovine milk. Such results corresponded to others based on gross observation, gastric MPO and nitrate/nitrite levels, *Nfkb1*, *Cox2*, and *inos* mRNA expression levels, and antioxidant defense systems. These effective changes were also reconfirmed by histomorphometrical analysis based on percentages of invaded lesions and peri-ulcerative mucosa thicknesses as well as semiquantitative histological scores. Peri-ulcerative mucosa thicknesses were significantly decreased and percentages of invaded lesions and semiquantitative histological scores were markedly increased by treatment with acidified ethanol. However, they were significantly ameliorated by 14 d of continuous oral pretreatment with 3 doses of bovine milk. Taken together, results of the present study demonstrate that bovine milk has a gastroprotective effect against acidified ethanol-induced gastric ulcer in a mouse model.

One limitation of our study might be the lack of an isocaloric group. The main reason we did not add the isocaloric group was that our focus was on investigating the effects of bovine whole milk rather than a specific component. However, the caloric value of bovine milk cannot be ruled out for the observed mitigation of the gastric ulcer. Further studies with isocaloric groups are needed to evaluate bovine milk in itself.

In summary, pretreatment with bovine milk can elevate the antioxidant defense system and ameliorate expression of inflammation-related genes by modulating the NF- κ B pathway in mice with acidified ethanol-induced gastric mucosal injury. These findings suggest that pretreatment with bovine milk before gastric damage may prevent gastric ulcer development triggered by alcohol and acid.

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