ORIGINAL RESEARCH

WHEY PRETREATMENT AMELIORATES GASTRIC AND HEPATIC OXIDATIVE DAMAGE IN ETHANOL-INDUCED GASTRIC ULCER VIA A NEUTROPHIL-DEPENDENT MECHANISM

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ABSTRACT

Objective: Whey is a dietary protein supplement with antioxidant effects. In this study, the protective effects of whey protein against ethanol-induced oxidative gastric injury and associated hepatic injury are evaluated.

Methods: Formation of reactive oxygen species in the gastric tissue was monitored, using chemiluminescence technique. Hepatic and gastric tissues were analyzed histologically, while malondialdehyde levels and myeloperoxidase activity of the gastric and hepatic tissues were measured to determine the degree of lipid peroxidation and tissue neutrophil infiltration, respectively.

Results: Whey treatment demonstrated protective effects against ethanol-induced gastric and hepatic injury, by inhibiting neutrophil infiltration and generation of cytotoxic free radicals.

Conclusion: A nutritional approach with a whey-based product might offer an attractive new choice to support the treatment of gastric lesions.

Keywords: Whey proteins, Ethanol, Gastric and hepatic oxidative damage

SÜT SERUMU PROTEİNLERİ UYGULAMASININ ETANOLLE OLUŞTURULAN MİDE ÜLSERİNDE MİDE VE KARACİĞER OKSİDATİF HASARI ÜZERİNE NÖTROFİL ARACILI ETKİSİ

ÖZET

Amaç: Süt serumu proteinlerinin antioksidan etkileri vardır. Bu çalışmada, süt serumu proteinlerinin etanolle oluşturulan oksidatif mide ve karaciğer hasarındaki koruyucu etkileri değerlendirildi.

Yöntem: Mide dokusunda reaktif oksijen türlerinin oluşumu kemilüminesans yöntemiyle takip edildi. Karaciğer ve mide dokuları histopatolojik olarak değerlendirilirken, lipit peroksidasyonu göstergesi olarak malondialdehit düzeyleri ve nötrofil infiltrasyonu için miyeloperoksidaz aktivitesi ölçüldü.

Bulgular: Süt serumu proteinleriyle ön beslenmenin etanolle oluşturulan mide ve karaciğer hasarında nötrofil infiltrasyonunu ve sitotoksik serbest radikal oluşumunu önleyerek koruyucu etki yaptığı gözlendi.

Sonuç: Peyniraltı suyu kaynaklı bir ürün ile beslenmenin gastrik lezyonların tedavisinde yararlı bir seçenek oluşturabileceği düşünülmektedir.

Anahtar Kelimeler: Süt serumu proteinleri, Etanol, Mide ve karaciğer oksidatif hasarı

INTRODUCTION

Gastric mucosal integrity is maintained by a balance between endogenous aggressive factors (hydrochloric acid, pepsin, bile and pancreatic enzymes) and a number of defense mechanisms, including gastric mucosal barrier, mucus secretion, gastric microcirculation, cellular regeneration and endogenous protective agents [1-3]. Ethanol, acting as an acute pro-inflammatory agent, is known to produce erosions, ulcerative lesions and petechial bleeding in the mucosa of the stomach in humans [4]. Ethanol rapidly penetrates the gastric mucosa, and causes membrane damage and erosions. Increased mucosal permeability together with the release of vasoactive substances from blood cells can lead to vascular injury, necrosis and ulcer formation [5]. Studies have shown alterations in the antioxidant status following ulceration, suggesting that free radicals play a crucial role in the development of

ethanol-induced ulceration in rats [6]. Moreover, the activities of enzymes such as superoxide dismutase, catalase and glutathione peroxidase were significantly inhibited by ethanol administration, indicating that the inhibition of these enzymatic activities was responsible for oxidative tissue damage to gastric mucosa after ethanol challenge [7].

In recent years, milk and its constituents have been recognized as functional foods [8,9]. Whey is a natural by-product of the cheese making process and is made up of different protein fractions, each having a critical role in the support of healthy metabolism [8]. Recent studies confirm a number of whey-linked health benefits including anticancer, antihypertensive and antioxidant effects as well as protection against viral and bacterial infections, and immunomodulation [9,10]. It has been shown that whey proteins boost immune status by increasing glutathione synthesis [11,12]. Gastrointestinal mucosal defense has been our major focus among other biological effects of whey proteins, since once ingested, they would first come into contact with the mucosa of the digestive tract. Whey protein, owing to the sulfhydryl compounds present in its composition, was shown to protect the gastric mucosa from ethanol damage by its capacity to stimulate glutathione synthesis [13].

It is well known that gastroprotective mechanisms act mainly by stimulating the defensive factors in the gastric mucosa rather than inhibiting the aggressive factors [14]. The reinforcement of the defensive factors may protect against damage induced by free radicals. In an attempt to explore the possible protective effect of whey-protein supplementation on oxidative tissue injury, we investigated the impact of orally administered whey solution on ethanol-induced gastric ulcer and associated hepatic injury by measuring lipid peroxidation and by histopathological analysis. We also assessed whether the mechanisms of possible gastroprotection of whey proteins involve the activation of neutrophils and generation of reactive oxygen species.

METHODS
Animals
All experimental protocols were approved by the Marmara University School of Medicine Animal Care and Use Committee. Both sexes of Wistar albino rats (200-250 g) were kept at a constant temperature (22 ± 1 °C) with 12 h light and dark cycles in individual wire-bottomed cages.

Animals were fed a standard rat chow and were weighed daily to follow body weight changes.

Experimental protocol

Whey powder was obtained from Bahçıvan Milk Products (İstanbul, Turkey) and the whey solution was freshly prepared by dissolving 16 g of whey powder in 40 ml of saline. Rats were administered 2.5 ml of isotonic saline or whey solution by gastric gavage every second days for 2 weeks.

Following a 24-h of fasting period, on the 15th day of the treatment, gastric ulcer was induced two hours after whey or saline administration. The whey-treated rats (n=6) and half of the saline-treated rats (n=6) were anesthetized with ether and were administered ethanol (99%; 5 ml/kg) orogastrically. The rest of the saline-treated rats (n=6) were subjected to the same procedure with the exception that isotonic saline was substituted for ethanol.

Animals were decapitated 30 minutes after orogastric administration of ethanol or saline. Stomachs were rapidly removed, washed and opened along the greater curvature to examine macroscopically for the evaluation of gastric damage. Gastric lesions were examined macroscopically to measure the length of ulcer (ulcer index; mm) along their greatest diameters. Macroscopic score assessment was made as follows: normal mucosa: 0; 1-4 petechie: 1; >5 petechie or <4 mm bleeding areas: 2; >5 mm bleeding or erosion areas: 3. Stomach and liver were excised and stored at -80 °C for the measurement of myeloperoxidase activity and malondialdehyde levels. A small part of the stomach was reserved for chemiluminescence measurements which were made on fresh gastric tissue.

Malondialdehyde (MDA) assay

Hepatic and gastric tissue samples were homogenized with ice-cold 150 mM KCl. The MDA levels were assayed as products of lipid peroxidation by monitoring thiobarbituric acid reactive substance formation as described previously [15]. Lipid peroxidation was expressed in terms of MDA equivalents using an extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$ and results were expressed as nmol/g tissue.

Myeloperoxidase (MPO) activity

MPO, an enzyme of activated polymorphonuclear leukocytes, is used as an indication of tissue neutrophil accumulation. MPO activity was measured using a procedure similar to that

documented previously [16]. Hepatic and gastric tissue samples were homogenized in 50 mM potassium phosphate buffer (PB, pH 6.0), and centrifuged at 41,400 x g (10 min); pellets were suspended in 50 mM PB containing 0.5 % hexadecyltrimethylammoniumbromide (HETAB). After three freeze and thaw cycles, with sonication between cycles, the samples were centrifuged at 41,400 x g for 10 min. Aliquots (0.3 ml) were added to 2.3 ml of reaction mixture containing 50 mM PB, o-dianisidine, and 20 mM H_2O_2 solution. One unit of enzyme activity was defined as the amount of the MPO present that caused a change in absorbance measured at 460 nm for 3 minutes MPO activity was expressed as U/g tissue.

Chemiluminescence (CL) assay
Reactive oxygen species were determined in fresh gastric tissues using luminol (detects a group of reactive species including .OH, H_2O_2, HOCl) and lucigenin (selective for O_2-) as probes for chemiluminescence (CL) [17-19]. Lucigenin (bis-N-methylacridiniumnitrate) and luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) were obtained from Sigma (St. Louis, MO, USA). Measurements were made at room temperature using Junior LB 9509 luminometer (EG&G Berthold, Germany). Specimens were put into vials containing PBS-HEPES buffer (0.5 M PBS containing 20 mM HEPES, pH 7.2). ROS were quantitated after the addition of enhancers (lucigenin or luminol) at a final concentration of 0.2 mM. Counts were obtained at 1 minute intervals and the results were given as the area under curve (AUC) for a counting period of 5 minutes [19]. The counts were corrected for wet tissue weights and expressed as relative light units (rlu).

Histological assessment of stomach injury
Small pieces of stomach were placed in 10% (v/v) formalin solution and processed routinely by embedding in paraffin. Samples from the fundus of the stomach were placed in 10% formaldehyde and processed routinely for paraffin embedding. Tissue sections (5 μm) were stained with hematoxylin and eosin for general morphology. Some of the sections were stained with 1% acidified toluidine blue (TB) for mast cell investigation. Stained sections were examined under a photomicroscope (Olympus BX50, Tokyo, Japan) by an experienced histologist who was unaware of the treatment conditions. Gastric

damage was evaluated as follows: degeneration of surface mucous cells (0: none; 1: mild; 2: moderate; 3: severe); hemorrhage, focal necrosis and mucosal congestion (0: none; 1: mild; 2: moderate; 3: severe); degeneration of glandular cells (0: none; 1: mild; 2: moderate; 3: severe); inflammatory cell infiltration (0: none; 1: mild; 2: moderate; 3: severe).

For scanning electron microscopic investigations, tissue samples were fixed for 2 hours in 2.5% phosphate buffered glutaraldehyde solution (0.1 M, pH 7.4), postfixed in 1% phosphate buffered osmium tetroxide solution, and passed through an increasing alcohol and amylacetate series. After drying with a critical point dryer (Bio-Rad E 3000, Hertfordshire, UK) and coating with gold (Bio-Rad SC 502, Hertfordshire, UK) tissue samples were examined under a scanning electron microscope (Jeol 5200 JSM, Tokyo, Japan).

Statistical analysis
All data are expressed as means ± SEM. Statistically significant differences among groups were identified using analysis of variance (ANOVA) followed by Tukey-Kramer test. Differences were considered to be significant at p< 0.05.

RESULTS

Body weight increase during the 15-days follow-up period was not significantly different among experimental groups (approximately 12.5% increase in the basal weights; p>0.05). Gastric ulcer index and macroscopic ulcer score were not changed by whey pretreatment (Table I). However, in the saline-treated ulcer group, stomach wet weight was elevated, while whey pretreatment abolished this increase (p<0.05). When compared to the non-ulcer group, gastric and hepatic MDA levels were increased in the saline-treated ulcer group approximately 5 and 2-fold, respectively (p<0.01 and p<0.05), indicating ulcer-induced increase in lipid peroxidation. However, whey pretreatment prevented ethanol-induced increase in lipid peroxide levels of both tissues. MPO activities in the stomach and liver of the saline-treated ulcer group were significantly increased (p<0.05), indicating the enhanced neutrophil recruitment in the inflamed tissues as compared to control group. These increases in MPO activities were found to be significantly lower in the whey-treated ulcer group (p<0.05).

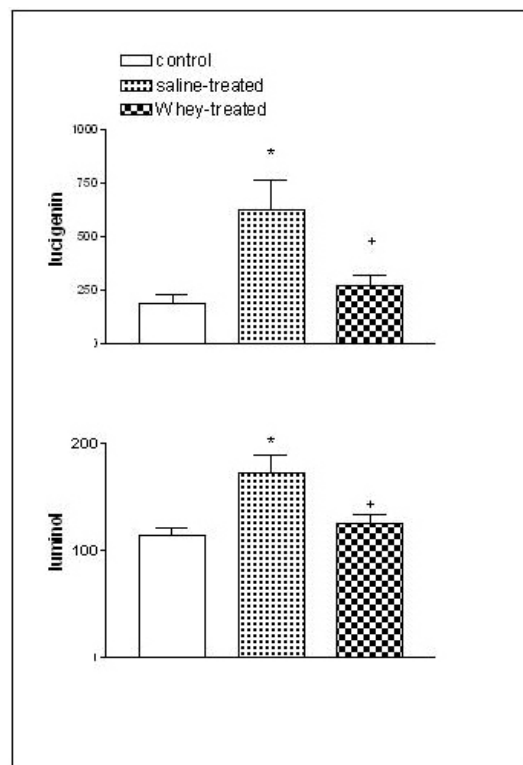Luminol and lucigenin CL levels in the gastric tissues of the saline-treated ulcer group were significantly increased (Fig. 1). On the other hand, whey pretreatment attenuated the generation of free radicals without any selectivity as assessed by the two different probes.

Table I: The effect of whey pretreatment on gastric injury as assessed by stomach wet weight, ulcer index, macroscopic score, gastric myeloperoxidase activity (MPO) and malondialdehyde (MDA). Hepatic injury due to gastric ulcer was also determined by MDA and MPO.

	Control group (n=6)	Saline-treated ulcer group (n=6)	Whey-treated ulcer group (n=6)
Wet tissue weight (g)	0.44 ± 0.01	0.60 ± 0.03 *	0.46 ± 0.03 [+]
Ulcer index (mm)	0	26.4 ± 0.7	24.7 ± 0.9
Macroscopic score	0	2.7 ± 0.2	2.1 ± 0.4
MPO (U /g tissue)			
gastric	17.8 ± 4.0	29.7 ± 3.9 *	19.8 ± 1.3 [+]
hepatic	2.05 ± 0.40	8.07 ± 1.10 *	1.81 ± 0.37 [+++]
MDA (nmol / g tissue)			
gastric	2.78 ± 0.58	13.29 ± 2.95 **	6.18 ± 0.70 [+]
hepatic	7.23 ± 0.82	14.33 ± 1.18 *	10.79 ± 1.05 * [+]

* $p < 0.05$; compared to control group.
+ $p < 0.05$ and [+++]$p < 0.001$; compared to saline-treated ulcer group.

Fig. 1: Luminol and lucigenin chemiluminescence levels in the gastric tissues of the saline- or whey protein-treated rats with ethanol-induced ulcer compared to control rats without ulcer.
$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the control group, [+]
$p < 0.05$, [++] $p < 0.01$ compared with the saline-treated group.
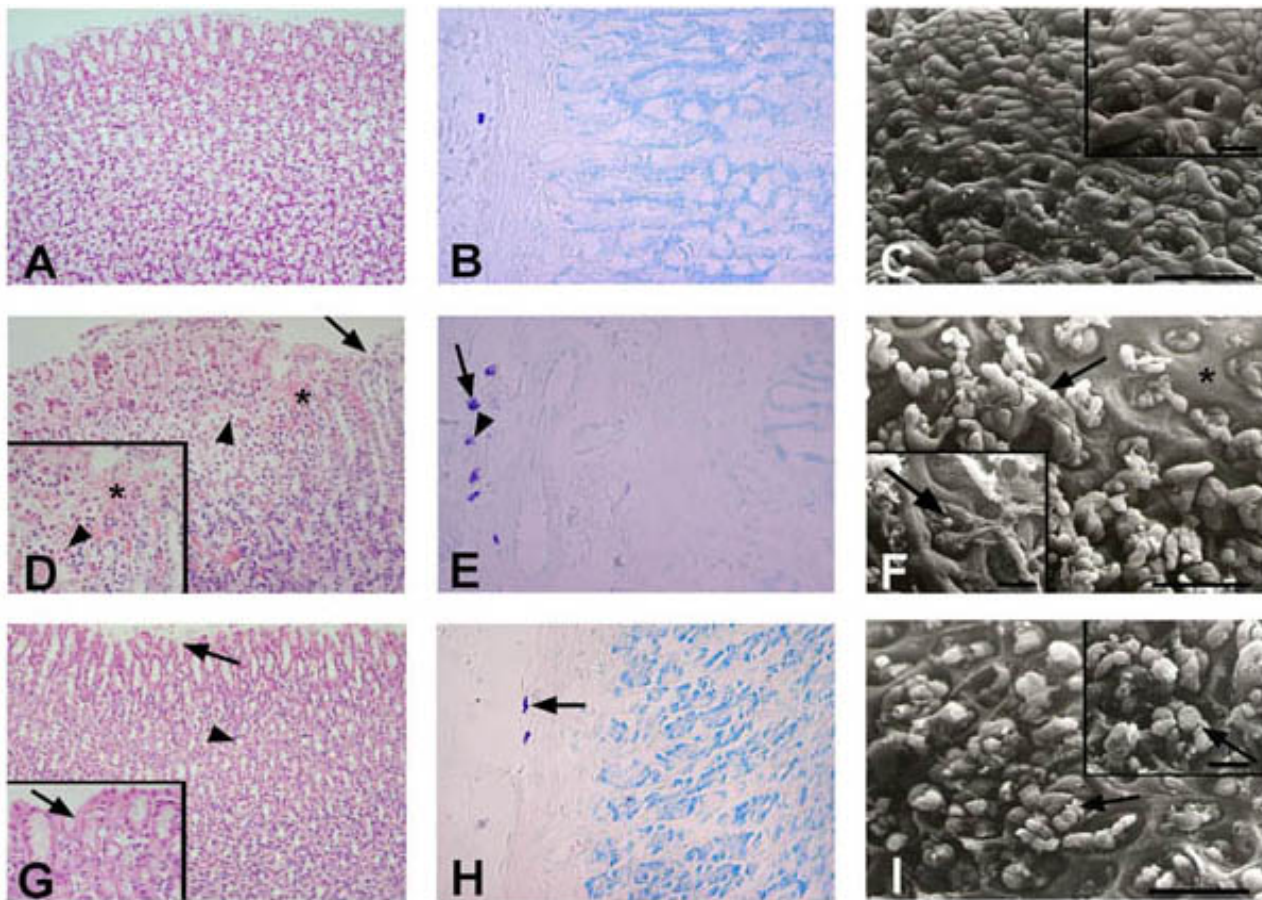
Fig. 2: Photomicrographs show the light microscopic sections with hematoxylin-eosin (left panel) or toluidine blue (middle panel) staining and the scanning electron microscopic (right panel) views.
Control group: A) Regular gastric surface epithelium and glandular cells. B) Granulated mast cell in the mucosa. C) Regular gastric surface epithelium with gastric pits.
Saline-treated ulcer group: D) Severe damage of surface mucus cells with necrosis (¢) and degenerated glandular cells (►), severe inflammatory cell infiltration and hemorrhage (*) in mucosa. E) Granulated (¢) and degranulated (►) mast cells in the mucosa.
F) Severe damage of surface mucus cells (¢) with degeneration of gastric pits and denuded lamina propria (*).
Whey protein-treated ulcer group: G) Regular surface mucus cells with gastric pits (¢), mild degeneration of glandular cells (►) in mucosa. H) Granulated mast cells (¢) in the mucosa. I) Focal damage of surface mucus cells (¢), regenerated gastric pits.
A, D, G: H & E staining, x 200, insert: x 400.
B, E, H: toluidine blue staining, x 400.
C, F, I: Scale bars: 50 μm, insert: 10 μm.

Light and scanning electron microscopic evaluation of the gastric tissue in the control group revealed regular stomach mucosa with surface mucus cells and glandular cells, and a few granulated mast cells in the mucosa (Fig. 2). However, in the saline-treated gastric ulcer group, severe damage of surface mucus cells, gastric pits with degenerated glandular cells, hemorrhage, severe inflammatory cell infiltration with granulated and degranulated mast cells and basophils in the mucosa were observed. In the whey-treated ulcer group, moderate damage of surface mucous cells and gastric pits, localized hemorrhage and moderate inflammatory cell infiltration with granulated mast cells were observed.

DISCUSSION
Although there is substantial evidence documenting the health-promoting effects and biological activities of milk protein components [9,10,20,] the potential role of these proteins in the gastrointestinal mucosal defense system is less well elucidated. The present study was designed to examine the effect of whey protein on gastric mucosal and associated hepatic tissue injury in an acute gastric ulcer model. The results demonstrate that whey protein supplementation reduced the generation of free radicals, while the severity of ulcer was attenuated. Moreover, the oxidative gastric and hepatic damage, as assessed by increased MDA content, and the recruitment of neutrophils, evidenced by elevated MPO activity,

were reduced by whey protein pretreatment. Thus, the mechanism of the protective action of whey protein appears to be due to its anti-oxidant effect that involves inhibition of neutrophil accumulation.

The ethanol-induced gastric ulcer model is widely used to evaluate the protective activity of new anti-ulcer agents [21-23]. Ethanol increases production of superoxide anion and hydroxyl radicals as well as lipid peroxidation in the gastric mucosa [24]. Lipid peroxidation, mediated by free oxygen radicals, is believed to be an important cause of destruction and damage to cell membranes. Our results indicate that ulcer induction resulted in increased lipid peroxidation of gastric and hepatic tissues. In addition, gastric luminol (selective for HOCl, H_2O_2, and OH. radicals) and lucigenin (selective for superoxide radical) CL levels that were elevated in the ulcer group were significantly reduced by whey treatment. In accordance with these findings, Korhonen and Reiter [25] have demonstrated that release of H_2O_2 by bovine polymorphonuclear leucocytes was decreased in the presence of milk whey. It was also shown that whey protein decreases oxygen-free radical production in a murine model of chronic iron-overload cardiomyopathy [26]. In the same study, mice receiving iron treatments with whey supplementation had significantly lower concentrations of cytotoxic aldehydes and significantly higher cardiac levels of antioxidant activity than iron-only treated mice. On the other hand, it was demonstrated that the protective effect of whey proteins on the gastric mucosa was related to the sulfhydryl component, particularly cysteine and its link with glutamic acid in the production of glutathione [27]. Thus, whey proteins may be effective in limiting oxidative damage to proteins and lipids due in part to their ability to neutralize a variety of different reactive species, which are generated at different intracellular sites.

Milk is known to be effective in preventing experimental ulcers induced by irritants such as ethanol, hydrochloric acid, aspirin and stress [28,29]. Its gastroprotective effect was assumed to be due to the formation of a protective layer by milk phospholipids or to membrane stabilization and cellular restitution by calcium [30]. An experimental study also reported that whey proteins, particularly α-lactalbumin, exerted a marked protective effect against ethanol-induced gastric damage [31]. In another study, Rosaneli et al. [13] showed 41% reduction in ulcerative lesions caused by ethanol ingestion in rats fed a whey protein concentrate, while 73% reduction rate was observed following repetitive doses of whey. In the present study, whey pretreatment prevented the increase in stomach weight in rats with ulcer, by limiting the extent of inflammatory process and consequent edema of the gastric tissue. Although whey pretreatment did not affect the macroscopic appearance of mucosal ulcer, microscopically observed gastric injury was significantly reduced in the whey-treated animals. Since the alterations in submucosal and glandular structures can be evaluated microscopically, while macroscopic evaluation is limited to the mucosa, a macroscopically apparent improvement was not evident. Clarke et al. [32] prophylactically administered whey extract to protect oral mucosa against chemotherapy-induced damage and showed a combined effect of different growth factors and bioactive proteins with anti-infectious, anti-proliferative and anti-apoptotic activity. Moreover, our results have also demonstrated that whey treatment protects hepatic tissue along with the gastric mucosa against oxidative damage.

It is well-established that oxygen metabolites play a role in the recruitment of neutrophils into injured tissues besides their direct damaging effects on tissues [33]. Activated neutrophils are also a potential source of oxygen metabolites [34] initiating the deactivation of antiproteases and activating cytotoxic enzymes including elastase, proteases, lactoferrin and MPO [35], thereby generating hypochlorous acid. Since activation of neutrophils might lead to the generation of reactive oxygen metabolites, the reduction in tissue neutrophil accumulation may also result in reduced lipid peroxidation and attenuated tissue injury. In the present study, increases in gastric and hepatic MPO activity were significantly decreased by whey proteins concomitant with the improvement in ulcer formation, suggesting that ethanol-induced gastric oxidative damage involves the interaction of neutrophils and the protective effect of whey proteins on the liver and the stomach involves, in part, blockade of neutrophil infiltration.

In conclusion, our results demonstrate that pretreatment with whey protein exhibits a gastroprotective effect on ethanol-induced gastric mucosal injury, implicating that it may be regarded as a powerful dietary protein supplement in the treatment of gastric mucosal lesions. Although the results indicate that the gastroprotective action of whey protein involves

its radical-scavenging and antioxidant effects via the inhibition of neutrophil accumulation, further studies are needed to evaluate the precise mechanisms by which whey proteins exert gastroprotection. Nevertheless, a nutritional approach with a whey-based product might offer an attractive new choice to support the treatment of gastric lesions.

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