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RESEARCH ARTICLE

BETAINE SUPPLEMENTATION PROTECTS RATS AGAINST ALCOHOL-INDUCED HEPATIC AND DUODENAL INJURY: AN HISTOPATHOLOGICAL STUDY

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ABSTRACT

Betaine is a natural compound synthesized endogenously in animals, plants, and microorganisms and can be intaken by diet. There is a growing body of evidence that suggests betaine has properties that can protect against damage caused by oxidation, inflammation, and cell death in various human diseases. Additionally, recent studies suggest that betaine may help prevent and/or ameliorate tissue damage from alcohol consumption. In the present study, we evaluated histopathological changes in the liver and duodenum tissues stained with hematoxylin and eosin (H&E) in the four groups of twenty-eight *Wistar albino* rats: control group, betaine pre-treated group (250 mg/kg/bw, 21 days, i.g.), acute ethanol ingested group (75% ethanol, 4 ml/kg/bw, i.g.) and betaine+ethanol group (75% ethanol, 4 ml/kg/bw, following betaine 250 mg/kg/bw, i.g.). We found that betaine pre-treatment reduced significantly ethanol-induced hepatocyte degeneration, sinusoidal dilatation, hemorrhage, and inflammatory cell infiltration in the liver ($p < 0.05$). We also showed that betaine protects duodenal mucosa significantly against epithelial damage at the tips of villi and hemorrhage ($p < 0.001$). Collectively, this study indicated that betaine supplementation could protect against histopathological changes induced by ethanol in hepatic and duodenal tissues.

Keywords: *Betaine, Ethanol, Liver, Duodenum, Histopathological changes.*

1. INTRODUCTION

Alcohol is a water soluble toxic organic compound that causes damage to almost all tissues in the body, especially the liver. The impact alcohol has on a person's health can change based on the amount and duration of alcohol consumed. Both short-term and long-term alcohol consumption can lead to a condition called alcoholic liver disease (ALD), which is responsible for 5.3% of global fatalities [1]. ALD is a serious disease with a spectrum that can range from fatty liver to hepatitis and fibrosis, and ultimately to cirrhosis. Although the pathogenesis is not clearly known, it is thought that

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ALD is mainly associated with oxidative stress, lipid peroxidation, and inflammation [2]. The process of ethanol metabolism, as catalyzed by the enzyme alcohol dehydrogenase (ADH) in the liver, results in the production of the cytotoxic compound acetaldehyde. This metabolism also leads to the release of reactive oxygen species (ROS) which subsequently causes lipid peroxidation, endoplasmic reticulum stress, and elicitation of an inflammatory response via the production of cytokines [3]. All these factors play a role in the initiation and progression of liver tissue damage and subsequent dysfunction.

Although the liver is the main organ where ethanol oxidation takes place, it is known that it is also oxidized in the gastrointestinal tract. These catabolism reactions, called first-pass metabolism of ethanol, are mediated by alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) in the gastric and intestinal mucosa and are observed even only in small doses of alcohol consumption. Gastrointestinal metabolism is lower than in the liver, but it could still cause tissue damage by local acetaldehyde production [4]. Although the first-pass metabolism of alcohol is thought to occur mainly in the stomach, it has been shown that the ethanol concentration similar to that of the beverages is only found in the upper small intestine. Due to this high concentration in the lumen of the gastrointestinal tract, ethanol is expected to cause mucosal damage in the duodenum and jejunum rather than the stomach. Indeed, acute alcohol exposure results in mucosal injury, and hemorrhagic damage in the mucosa in the small intestine, more specifically in the duodenum [5]. Though the underlying mechanisms are not fully elucidated, studies suggest that ethanol has a direct toxic effect on the mucosa by leading to ROS production.

Betaine (trimethylglycine) is a natural compound found in almost all organisms, from microorganisms to plants and animals. While betaine could be synthesized endogenously from choline in the liver and kidneys, dietary betaine from sources such as whole grains, beets, spinach, and various seafood has a crucial role in the total betaine content of the body. Essential functions of betaine include acting as a methyl donor in transmethylation reactions and protecting cells against environmental stress by serving as an osmolyte [6]. Studies have suggested that betaine is a potent protective and therapeutic agent against pathological conditions such as neurodegenerative, hepatic, renal, and cardiovascular diseases owing to its ability to scavenge free radicals, increase antioxidant capacity and reduce apoptosis [7]. In addition, *in vivo* studies under ethanol-induced stress conditions have shown that betaine protects the cerebellum and testes against oxidative stress, prevents gastric ulcers, and improves hepatic steatosis by reducing homocysteine levels [8-10]. Moreover, a reduction in hepatic tissue damage has been reported in mice treated with betaine simultaneously with acute alcohol [11]. Similarly, it has been revealed that betaine pre-treatment reduces oxidative stress by increasing the antioxidant level and reducing the oxidant level, thus protecting the gastric tissue from the detrimental influences of ethanol [12]. However, the prophylactic influences of betaine against ethanol-induced damage to the liver and duodenum still need to be clarified. In this study, we aimed to determine whether betaine supplementation could protect against histopathological alterations induced by ethanol ingestion in the hepatic and duodenal tissues of the rats.

2. MATERIALS AND METHODS

2.1. Animal treatment

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This study was approved by the Animal Experiments Local Ethics Committee of Kütahya Health Sciences University (2021.02.07). The experiments were performed on 28 healthy adult female *Wistar albino* rats (average weight 250-300 g). Animals were housed in controlled laboratory conditions with a 12 h dark/light cycle. They were fed a standard diet throughout the experiment and water was accessed ad libitum. The rats were allocated into 4 experimental groups (n=7) namely control, betaine, ethanol, and betaine+ethanol groups. The rats in the control group received 4 ml/kg of distilled water, which is the solvent of betaine. 250 mg/kg/body weight (bw) betaine was ingested into the rats in the betaine group for consecutive 21 days. 75% ethanol (4 ml/kg/bw) was given as a single dose to the rats in the ethanol group. Finally, a single dose of 75% ethanol was administered to the betaine+ethanol group after 21 days of betaine treatment. All applications were done intragastrically. All rats were decapitated under ketamine/xylazine anesthesia (90/10 mg/kg, intraperitoneally) and liver and duodenum tissues were taken. There were no excluded animals during the experiments and data points during the analysis.

2.2. Histopathological Analysis

The liver and duodenal tissues of rats were fixed for 48 hours in 10% neutral buffered formaldehyde solution. At the end of the fixation process, the tissues were dehydrated in the increasing alcohol series of 80%, 95% and 100%, then cleared in xylene. Sections at 4µm thickness were cut with a microtome (Thermo Electron Corporation, Shandon Finesse E, Germany). Following deparaffinization in xylene, the sections were rehydrated in descending series of alcohols and in water, respectively. Afterward, the tissues were taken into hematoxylin solution for 5 minutes to stain the cell nuclei. To stain the cell nuclei, the tissues were then taken into hematoxylin solution for 3 minutes. Tissues immersed in acid-alcohol were kept in eosin for 3 minutes for cytoplasmic staining after washing with tap water. Stained sections were mounted with entellan after soaking in increased alcohol series and keeping in xylene [13]. The prepared slides were examined in a blind manner by a histopathologist under a Nikon Eclipse 80i light microscope (Nikon, Germany) at x200 magnification and photographed.

To quantify the severity of the hepatic injury, each H&E-stained cross-section was scored for evidence of hepatocyte damage (swollen hepatocytes, vacuolated cytoplasm, pyknotic nuclei), sinusoidal dilatation, hemorrhage, and inflammatory cell infiltration based on the report by Akbulut. et al. [14]. Scoring for these histopathological features was performed as follows: score 0-no injury (no lesion observed), sore 1-mild injury (less than 10% of tissue was affected), score 2-moderate injury (less than 50% of tissue was affected), and score 3-severe injury (more than 50% of tissue was affected). Accordingly, the mean histopathological score was scored cumulatively for each section, with a maximum score of 12 for the most severe hepatic injury. Mucosal injury of the duodenum was graded according to Ewer et al., 2004 [15], as follows: score 1- no damage, score 2- mild damage (superficial mucosal erosion, patchy mucosal hemorrhage and acute inflammatory infiltrate within the lamina propria), score 3-moderate damage (mucosal ulceration, mucosal hemorrhage and intact muscularis propria), score 4- localized severe damage (ulceration of the mucosa and focal hemorrhage), score 5-generalized severe damage (ulceration of the mucosa and diffuse hemorrhage).

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2.3. Statistical Analysis

Histopathological scores showing the severity of injuries in liver and duodenum tissues were evaluated using statistical techniques in SPSS 21. The normality of the data was checked using the Shapiro-Wilk test and then ANOVA and Tukey test were applied to those data which met the normality assumptions. The final results were presented as mean and standard deviation and any differences were determined to be statistically significant if the *p*-value was less than 0.05.

3. RESULTS

For histopathological analysis, liver and duodenum sections from all experimental groups were assesed by H&E staining. Morphological changes in liver tissues are shown in Figure 1. Microphotographs of the control group showed that the rats in this group had normal liver architecture with hepatocytes arrayed in cords around the normal central vein and had normal hepatic parenchyma. The cytoplasm of hepatocytes was well preserved, they had a clear nucleus and distinct cell borders (Fig. 1A). Liver tissues of betaine-treated rats contained normally located hepatocytes and regular parenchyma, similar to the control group. Some animals in the betaine group showed mild signs of degeneration, but these findings were not statistically significant when compared to the rats in the control group ($p = 0.1263$) (Fig. 1B). The livers of the ethanol-treated rats showed more severe histological changes than both control and betaine groups. The most prominent findings in this group were hepatocyte degeneration, sinusoidal dilatation, hemorrhage, and inflammatory cell infiltration around the central vein (Fig. 1C-E). The pathological alterations triggered by ethanol in the livers of rats given betaine+ethanol were remarkably improved. Sinusoidal dilatation and inflammatory cell infiltration were less in this group than the ethanol group, and hemorrhage was not observed (Fig. 1F). Accordingly, the histopathological score of the liver increased in the ethanol group compared to the control group ($p < 0.001$) and reduced significantly in the betaine+ethanol group versus the ethanol group ($p < 0.05$) (Fig. 2).

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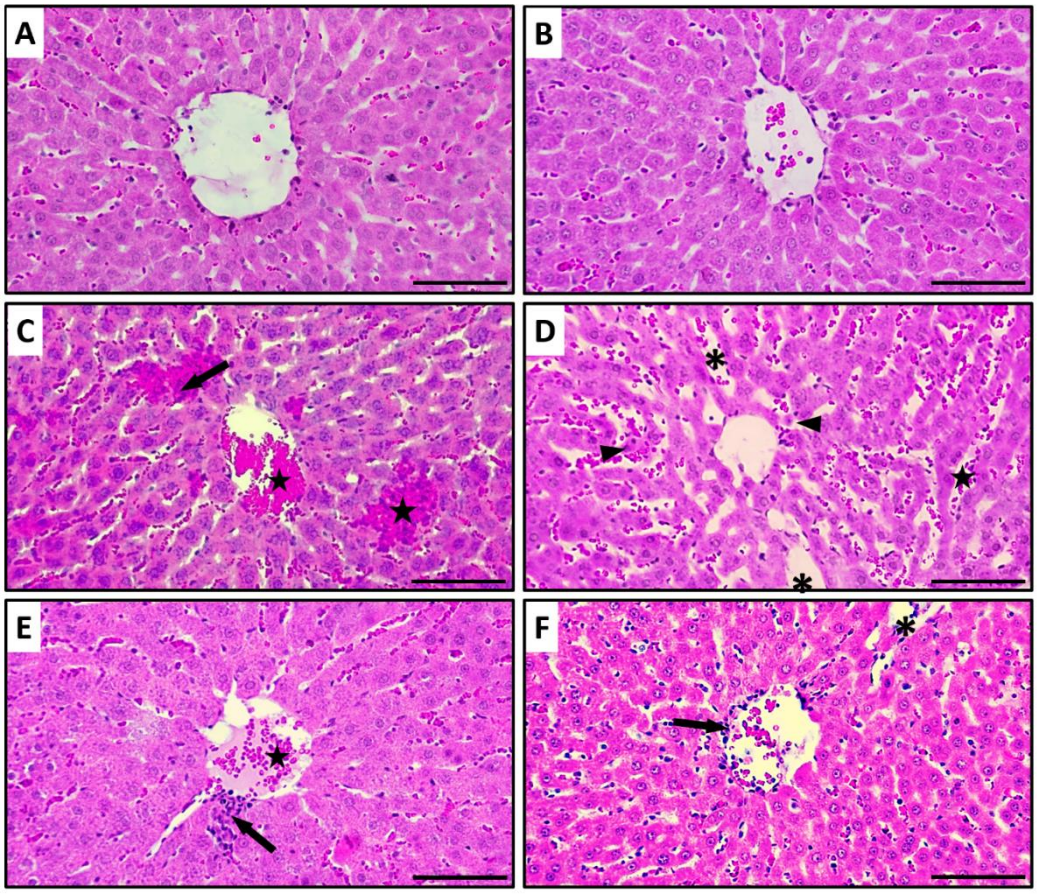

Figure 1. Histopathological evaluation of liver tissues. **(A)** Normal liver morphology in the control group. **(B)** Undamaged normal liver tissue in the betaine-treated group. **(C-E)** Hepatic injury characterized by hepatocyte damage (arrowheads), sinusoidal dilatation (asterisks), hemorrhage (stars), and inflammatory cell infiltration (arrows) in ethanol-administered rats. **(F)** Reduced hepatic injury with less inflammatory cell infiltration (arrow) and dilated sinusoids (asterisk) in the betaine+ethanol group. H&E staining, Scale bar=100 µm.

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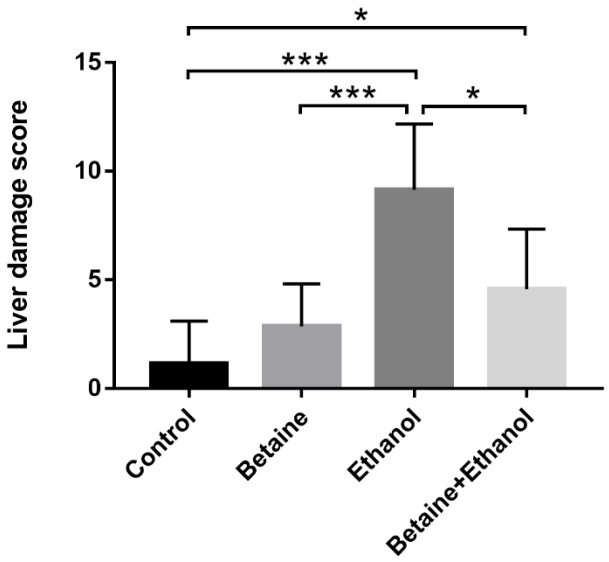

Figure 2. Comparison of histopathological scores of hepatic injury between groups. n=7, *$p < 0.05$, ***$p < 0.001$.

In the control group, the duodenum had a normal morphological appearance, the overlying epithelium of the villi was intact, crypts were spared, and lamina propria and lamina muscularis layers were regular (Fig. 3A, B). The histological structure of the duodenum in the betaine group was similar to that of the control group, only mildly sloughed epithelial cells were found in the tips of some villi, however, it did not cause a statistically significant difference when compared to the control group ($p = 0.1089$) (Fig. 3C, D). Ethanol ingestion caused severe mucosal damage characterized by the exfoliation of epithelial cells to the lumen and hemorrhage in the lamina propria (Fig. 3E, F). Betaine pre-treatment prevented ethanol-induced mucosal injury substantially. Both villous tip damage and hemorrhage were significantly less in the betaine+ethanol group than the ethanol group ($p < 0.001$) (Fig. 3G-H). Comparison of duodenal mucosal damage scores between groups is given in Figure 4.

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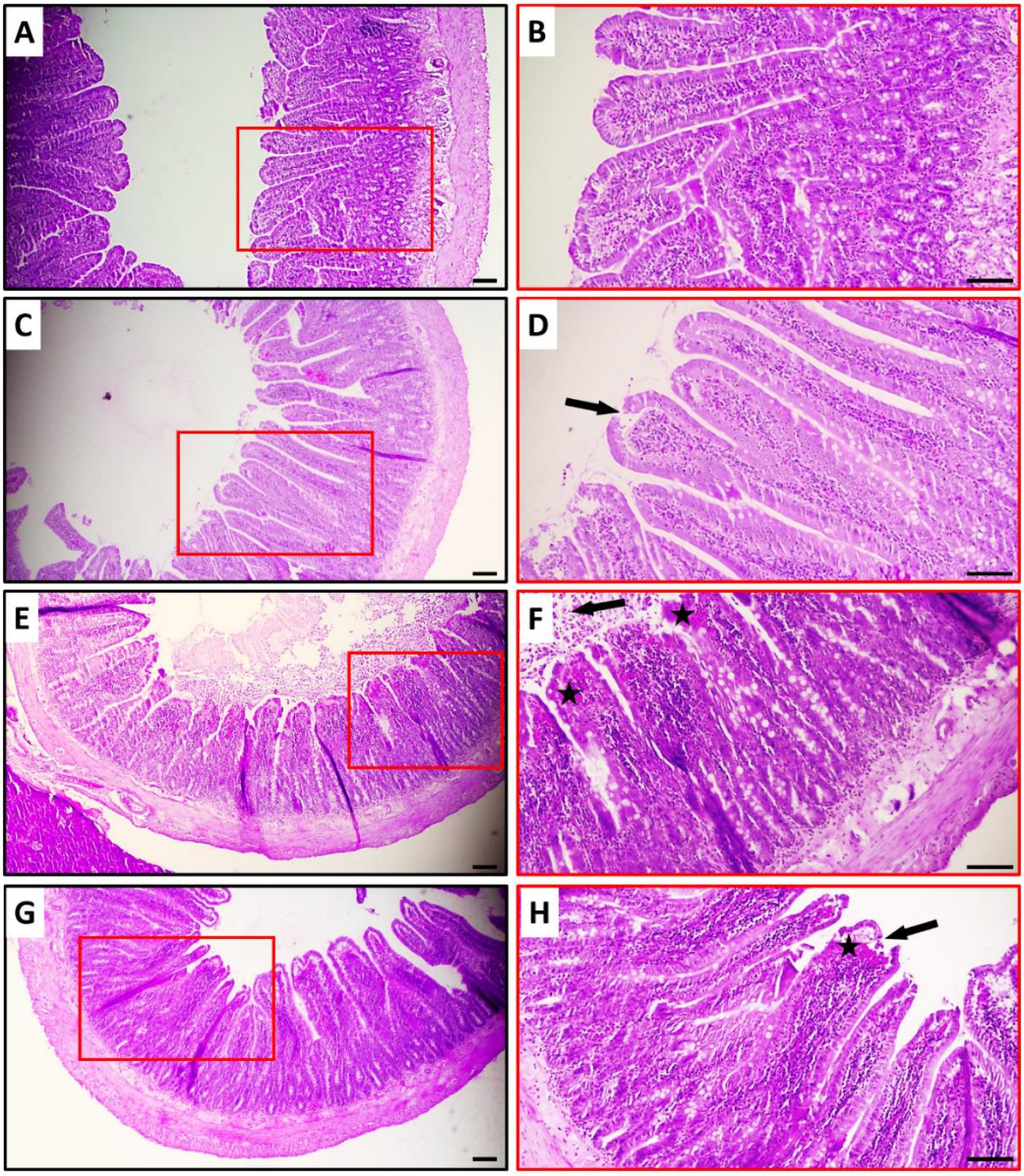

Figure 3. Histopathological evaluation of duodenum tissues. **(A-B)** Normal mucosa of the duodenum in the control group. **(C-D)** Nearly normal duodenal mucosa with only mildly sloughed epithelial cells (arrow) at the tips of some villi in the betaine-treated group. **(E-F)** Severe mucosal damage

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characterized by exfoliation of epithelial cells to the lumen (arrow) and hemorrhage (stars) in the lamina propria in the ethanol-ingested group. **(G-H)** Reduced duodenal mucosal injury with villous tip damage (arrow) and hemorrhage (star) in the betaine+ethanol group. H&E staining, Scale bar=100 µm.

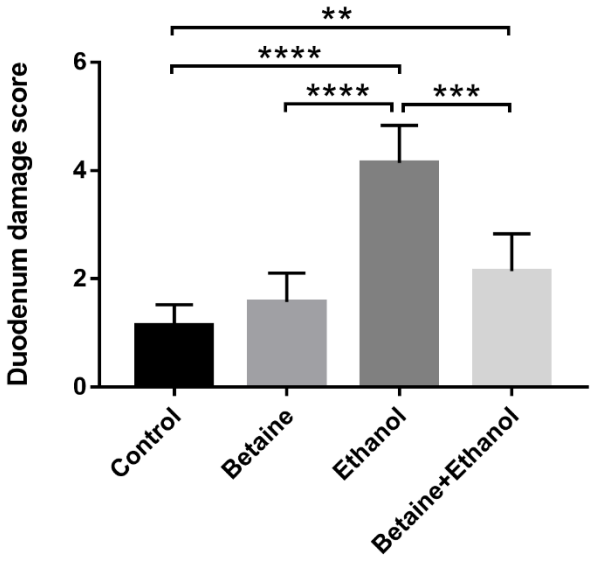

Figure 4. Comparison of histopathological scores of duodenal injury between groups. n=7, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

4. DISCUSSION

Alcohol consumption is an important factor that threatens health and quality of life since it affects pathophysiological conditions in various organ systems including gastrointestinal, urinary, cardiovascular, and nervous systems [16-18]. ALD, which is the most crucial pathology related to alcohol, causes a series of liver disorders that might result in steatosis, fibrosis, cirrhosis, and even carcinoma and liver failure. The catabolism of ethanol to acetaldehyde, mediated by alcohol dehydrogenase (ADH) and cytochrome P450 2E1, takes place mainly in the liver. The gastrointestinal tract, where the first-pass metabolism of ethanol takes place, contains the organs that are most affected by the damage of alcohol after the liver. In this catabolic reaction of ethanol and acetaldehyde, ADH and aldehyde dehydrogenase (ALDH) of the gastric and intestinal mucosa are the main mediators [4]. Acetaldehyde produced in these reactions acts as an oxidative stress inducer by disrupting the balance of pro-oxidants and antioxidants and causes tissue damage by interacting with the proteins in the gastric and intestinal mucosa [19].

In our current study, consistent with previous studies, ingestion of rats with 75% ethanol caused hyperemia, dilatation of sinusoids, infiltration of mononuclear cells, and degeneration of hepatocytes.

When duodenal damage was examined, we showed that acute ethanol administration caused loss of epithelial cells in the villus tips and bleeding in the mucosa. Studies in rodents and humans have shown that the morphological changes induced by ethanol in the liver are characterized by steatosis, necrosis, fibrosis, mononuclear cell infiltration, hyperemia, dilatation of sinusoids, various degrees of degeneration of hepatocytes (swelling, vacuolization and hydropic degeneration), and Kupffer cell hyperplasia [20-23]. Moreover, morphological examinations in experimental animal models have reported that oral or intragastric ethanol intake poses a risk of bleeding in the gastric and duodenal mucosa. In addition, ethanol causes loss of epithelium at the tips of the villi in the duodenal mucosa, thus reducing the mucosal surface. It also leads to mononuclear cell infiltration to the epithelial layer and goblet cell hyperplasia [24-26]. In our study, the absence of changes such as steatosis, necrosis, and fibrosis in the liver is due to the fact that ethanol was not administered chronically, and only its acute effect was evaluated. Similarly, acute alcohol did not cause mononuclear cell infiltration and an increase in goblet cell count in duodenal mucosa as in chronic alcohol intake.

In the present study, in which we assessed the effects of pre-treatment of betaine for 21 days, we first showed that betaine exhibits hepatoprotection by reducing acute ethanol-induced hyperemia, sinusoidal dilatation, inflammatory cell infiltration, and hepatocyte degeneration. Next, we demonstrated that betaine also prevents the exfoliation of epithelial cells at the tips of the villi and hemorrhage in the lamina propria that is, it protects the duodenal mucosa from the toxic effects of ethanol. We speculate that this protective effect of betaine is related to the antioxidant, anti-apoptotic, and anti-inflammatory effects of this natural compound [27,28]. Previous studies investigating the effects of betaine against alcohol-induced tissue damage have shown that this natural compound provides protection through the methionine-homocysteine cycle [29]. In this cycle, betaine plays a role in converting homocysteine to methionine, thus enhancing the homocysteine level while decreasing the methionine concentration. Increased methionine is involved in antioxidation; it could be used in the synthesis of reduced glutathione (GSH) by hepatocytes as well as alleviate oxidative stress through chelation. In addition, betaine increases the level of GSH precursor S-adenosylmethionine (SAM) via sulfur-containing amino acid metabolism [29]. Betaine has also been shown to increase the activity of enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) in the brain, testis and liver tissues [8,9,30]. Moreover, in our previous report, we showed that betaine improves total antioxidant status (TAS) and reduces total oxidant status (TOS) in betaine ethanol-treated rats [12].

It has been postulated that alcohol consumption causes an increase in the levels of S-adenosylhomocysteine (SAH), the metabolic precursor of homocysteine, in hepatocytes. SAH is produced in methyl transfer reactions, in which betaine is also involved, and is involved in the regulation of methylation reactions as a methyltransferase inhibitor [31]. Studies have shown that the increase in intracellular SAH is also associated with the induction of apoptosis [32,33]. Moreover, it has been reported that elevated SAH production is responsible for the increase in apoptosis observed following ethanol intake in hepatocytes [31]. It has been reported that betaine administration inhibits apoptosis by suppressing the increase in SAH in rat hepatocytes *in vivo* [34].

Ethanol-induced lesions in the gastrointestinal tract are closely associated with inflammation. Therefore, the use of agents with anti-inflammatory effects to protect tissues from ethanol damage is

considered a suitable approach [35]. Olli et al. showed that betaine reduces pro-inflammatory mediators interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) mRNA levels in human adipocytes under hypoxia [36]. Similarly, Yang et al. reported that betaine supplementation decreases the levels of interleukin-1beta (IL-1β), IL-6, TNF-α, and interferon-gamma (IFN-γ) in serum and increased the anti-inflammatory interleukin-10 (IL-10) in lipopolysaccharide-induced inflammation in rats [37]. Furthermore, Shi et al. proposed that betaine ameliorates ethanol-induced liver injury by reducing TNF-α, IFN-γ, and interleukin-18 (IL-18) levels [38]. These pieces of evidence suggest that the protective effect of betaine against tissue damage, which we observed in our study, is probably due to its ability to regulate inflammatory responses apart from its oxidative stress suppressor and apoptosis inhibitory features.

In conclusion, we demonstrated that betaine supplementation minimizes alcohol-induced injuries in hepatic and duodenal tissue in rats. These findings suggest the potential prophylactic value of betaine in preventing liver and duodenal damage caused by acute alcohol intake and warrant further investigations to reveal precise mechanisms of its protective effects.

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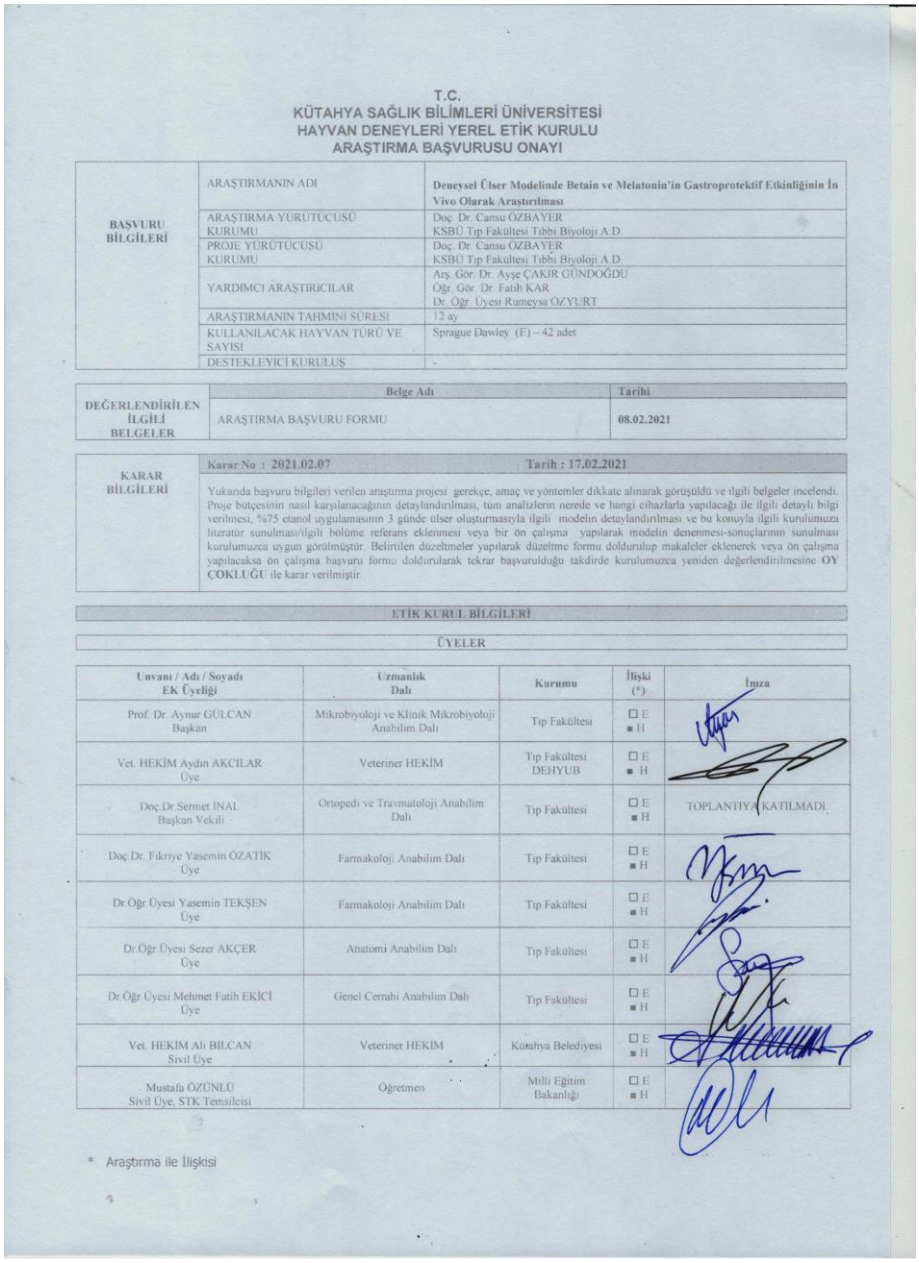
APPENDIX



T.C.
KÜTAHYA SAĞLIK BİLİMLERİ ÜNİVERSİTESİ
HAYVAN DENEYLERİ YEREL ETİK KURULU
ARAŞTIRMA BAŞVURUSU ONAYI

BAŞVURU BİLGİLERİ	ARAŞTIRMANIN ADI	**Deneysel Ülser Modelinde Betain ve Melatonin'in Gastroprotektif Etkinliğinin İn Vivo Olarak Araştırılması**
	ARAŞTIRMA YÜRÜTÜCÜSÜ KURUMU	Doç. Dr. Cansu ÖZBAYER KSBÜ Tıp Fakültesi Tıbbi Biyoloji A.D.
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	ARAŞTIRMANIN TAHMİNİ SÜRESİ	12 ay
	KULLANILACAK HAYVAN TÜRÜ VE SAYISI	Sprague Dawley (E) – 42 adet
	DESTEKLEYİCİ KURULUŞ	-

	Belge Adı	Tarihi
DEĞERLENDİRİLEN İLGİLİ BELGELER	ARAŞTIRMA BAŞVURU FORMU	08.02.2021

KARAR BİLGİLERİ	Karar No : 2021.02.07	Tarih : 17.02.2021
	Yukarıda başvuru bilgileri verilen araştırma projesi gerekçe, amaç ve yöntemler dikkate alınarak görüşüldü ve ilgili belgeler incelendi. Proje bütçesinin nasıl karşılanacağının detaylandırılması, tüm analizlerin nerede ve hangi cihazlarla yapılacağı ile ilgili detaylı bilgi verilmesi, %75 etanol uygulamasının 3 günde ülser oluşturmasıyla ilgili modelin detaylandırılması ve bu konuyla ilgili kurulumuza literatür sunulması/ilgili bölüme referans eklenmesi veya bir ön çalışma yapılarak modelin denenmesi-sonuçlarının sunulması kurulumuzca uygun görülmüştür. Belirtilen düzeltmeler yapılarak düzeltme formu doldurulup makaleler eklenerek veya ön çalışma yapılacaksa ön çalışma başvuru formu doldurularak tekrar başvurulduğu takdirde kurulumuzca yeniden değerlendirilmesine **OY ÇOKLUĞU** ile karar verilmiştir.	

ETİK KURUL BİLGİLERİ

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Mustafa ÖZÜNLÜ Sivil Üye, STK Temsilcisi	Öğretmen	Milli Eğitim Bakanlığı	☐ E ■ H	

* Araştırma ile İlişkisi