ORIGINAL ARTICLE

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Protective Effects of ACE/NEP Dual Inhibitor Omapatrilat for Indomethacin-induced Gastric Ulcer

ACE/NEP Dual İnhibitörü Omapatrilatın İndometazinle İndüklenen Mide Ülserinde Koruyucu Etkileri

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ABSTRACT

Aim: The renin-angiotensin-aldosterone system (RAAS) plays important roles in oxidative stress and various gastroenterological mechanisms. Omapatrilat, an RAAS-acting agent, inhibits both neprilysin neutral endopeptidase (NEP) and angiotensin converting enzyme (ACE) and may therefore affect protective mechanisms against gastric ulcer. Therefore, this study examined the gastroprotective role of omapatrilat in a mouse model of gastric ulcer induced by indomethacin to reveal pharmacological and biochemical changes resulting from omapatrilat treatment.

Materials and Methods: Forty-two BALB/c mice were divided into seven groups: control, 40 mg/kg omapatrilat only, 25 mg/kg indomethacin only, indomethacin and 40 mg/kg famotidine, and three groups with indomethacin and 10-40 mg/kg omapatrilat. All chemicals were administered by oral gavage in 0.5 mL of 0.9% NaCl solution at the determined doses. Stomach ulcers were induced by indomethacin in mice treated with famotidine (40 mg/kg) and omapatrilat (10-40 mg/kg). Stomach tissue samples were examined macroscopically. Oxidative stress biomarkers of malondialdehyde (MDA), glutathione (GSH), NEP and ACE levels as well as superoxide dismutase (SOD) activity were measured.

Results: The best antiulcer activity was measured with 40 mg/kg omapatrilat, where the gastric damage observed in the ulcer groups was significantly reversed, and gave the most similar results to the specific famotidine treatment. In relation with the increasing omapatrilat dose, SOD activity was corrected as well as GSH and MDA levels. Also the levels of ACE and NEP decreased back towards those measured in the control group. Therefore, these macroscopic and biochemical findings indicating reversal of gastrotoxicity and gastric ulcer indications demonstrate the role of omapatrilat's NEP and ACE inhibition in indomethacin toxicity, and its strong gastroprotective potential.

Conclusion: Dual inhibition of NEP and ACE by omapatrilat may suppress oxidative stress associated with indomethacin-induced gastric ulcer. Therefore, the protective effect of omapatrilat in the treatment of ulcers may lead to the search for new treatment strategies.

Keywords: Gastric ulcer, indomethacin, neprilysin, oxidative stress, omapatrilat

ÖZ

Amaç: Renin-anjiyotensin-aldosteron sistemi (RAAS), oksidatif stres ve çeşitli gastroenterolojik mekanizmalarda önemli roller oynar. RAAS etkili bir ajan olan omapatrilat, hem neprilisin nötr endopeptidazı (NEP) hem de anjiyotensin dönüştürücü enzimi (ACE) inhibe eder ve bu nedenle mide ülserine karşı koruyucu mekanizmaları etkileyebilir. Dolayısıyla bu çalışmada, omapatrilat tedavisinden kaynaklanan farmakolojik ve biyokimyasal değişiklikleri ortaya çıkarmak için farelerde indometazin tarafından indüklenen bir mide ülseri modelinde omapatrilatın gastroprotektif rolü incelendi.

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Gereç ve Yöntem: Kırk iki BALB/c faresi yedi gruba ayrıldı: Kontrol, sadece 40 mg/kg omapatrilat, sadece 25 mg/kg indometazin, indometazin ve 40 mg/kg famotidin, diğer üç grup ise indometazin ve 10-40 mg/kg omapatrilat. Tüm kimyasallar belirlenen dozlarda 0,5 mL %0,9 NaCl solüsyonu içinde oral gavaj ile verildi. Mide ülserleri, famotidin (40 mg/kg) ve omapatrilat (10-40 mg/kg) ile tedavi edilen farelerde indometazin tarafından indüklendi. Mide dokusu örnekleri makroskobik olarak incelendi; ve oksidatif stres biyobelirteçleri malondialdehit (MDA), glutatyon (GSH), NEP, ACE seviyeleri ve ayrıca süperoksit dismutaz (SOD) aktivitesi ölçüldü.

Bulgular: En iyi antiülser aktivite 40 mg/kg omapatrilat ile ölçüldü, burada ülser gruplarında gözlenen gastrik hasar önemli ölçüde tersine çevrildi ve spesifik famotidin tedavisine en benzer sonuçları verdi. Artan omapatrilat dozuna bağlı olarak, SOD aktivitesinin yanı sıra GSH ve MDA seviyeleri düzeldi. ACE ve NEP seviyeleri kontrol grubundaki seviyelerle benzerdi. Dolayısıyla; gastrotoksisite ve gastrik ülser endikasyonlarının tersine çevrildiğini gösteren bu makroskobik ve biyokimyasal bulgular, omapatrilatın NEP ve ACE inhibisyonunun indometazin toksisitesindeki rolünü ve güçlü gastroprotektif potansiyelini göstermektedir.

Sonuç: NEP ve ACE'nin omapatrilat tarafından ikili inhibisyonu, indometazinle indüklenen mide ülseri ile ilişkili oksidatif stresi baskılayabilir. Dolayısıyla; omapatrilatın ülser tedavisinde koruyucu etkisi yeni tedavi stratejileri arayışlarına yol açabilir.

Anahtar Kelimeler: Gastrik ülser, indometazin, neprilisin, oksidatif stres, omapatrilat

INTRODUCTION

Indomethacin, which is a non-steroidal anti-inflammatory drug (NSAID), is frequently used in the treatment of severe inflammatory diseases such as osteoarthritis, rheumatoid arthritis, tendinitis, ankylosing spondylitis and traumatic synovitis[1]. Indomethacin is widely prescribed for the treatment of inflammatory pain, but its anti-inflammatory effects are overshadowed by the fact that it causes significant gastrointestinal ulceration[2]. Indomethacin has also been found to increase oxidative stress and impair blood flow in the stomach[3]. Patients with gastric ulcer prefer to discontinue indomethacin treatment because of its side effects[4].

Indomethacin is a known inhibitor of cyclooxygenase enzymes (COX-1 and COX-2) and its effects on stomach ulceration have been shown to be specifically tied to its COX-1 inhibition[3]. The renin-angiotensin-aldosterone system (RAAS), a multi-hormonal system that coordinates blood pressure regulation and electrolyte balance, has also recently been shown to play a role in the etiology of gastric ulcers[5,6]. RAAS also takes part in pathophysiological phenomena such as inflammation and oxidative stress[7-9]. Angiotensin II (Ang II), the end product of RAAS, is also produced during vascular inflammation and endothelial dysfunction due to oxidative stress. Ang II production increases vascular nicotinamide adenine dinucleotide phosphate oxidase activity and further supports superoxide formation. The resulting superoxide raises oxidative stress and initiates endothelial damage[7].

Omapatrilat, a RAAS-active agent of which Ang II is a key mediator, inhibits both neprilysin/neutral endopeptidase (NEP) and angiotensin converting enzyme (ACE)[10]. NEP inhibition results in some benefits such as stronger vasodilation[11], reduced oxidative stress, and reduced expression of inflammatory cytokines[12,13]. Previous research has observed that omapatrilat can prevent endothelial dysfunction[14], and also provide kidney and cardiovascular protection[15,16]. As such, omapatrilat could potentially be an effective treatment against gastric ulcer. However, relationship between gastric ulcer and ACE/NEP inhibition has not been elucidated. In this study, we examine omapatrilat's gastroprotective roles against indomethacin-induced gastric ulcers, the effects of NEP and ACE inhibition by omapatrilat on various oxidant/antioxidant parameters, and the relationship between this dual NEP and ACE inhibition and the antiulcer role of omapatrilat.

MATERIALS AND METHODS

Chemicals

Famotidine, indomethacin, and omapatrilat were purchased from Nobel A.S. (Turkey), Merck Sharp & Dohme Corporation (Turkey), and Sigma-Aldrich (Germany), as powder materials, respectively.

Animals

42 BALB/c male mice weighing 40-50 grams were held in steel cages under standard conditions (7 am-8 pm light period, 55% relative humidity, and 21±2 °C) throughout the experiments and they were given standard pellet feed and tap water ad libitum. All animal care and protocols were confirmed by Experimental Animal Ethics Committee of Atatürk University (decision no: 157, date: 02.08.2018).

Preparation and Treatment

Mice were separated into seven groups (n=6), fasted for 24 hours, and administered the following chemicals:

Group 1: Control,

Group 2 (OMA): Omapatrilat (40 mg/kg),

Group 3 (ULCER): Indomethacin (25 mg/kg),

Group 4 (ULCER+FAMO): Indomethacin (25 mg/kg) + Famotidine (40 mg/kg),

Group 5 (ULCER+OMA10): Indomethacin (25 mg/kg) + Omapatrilat (10 mg/kg),

Group 6 (ULCER+OMA20): Indomethacin (25 mg/kg) + Omapatrilat (20 mg/kg),

Group 7 (ULCER+OMA40): Indomethacin (25 mg/kg) + Omapatrilat (40 mg/kg).

All chemicals were given through oral gavage of 0.5 mL of 0.9% NaCl solution. Omapatrilat was administered at doses of 10, 20 and 40 mg/kg, as it was determined in previous experimental studies that omapatrilat could affect oxidative stress parameters at similar doses[17,18]. The applied dosage of indomethacin was selected according to the standard literature information[3]. This indomethacin (25 mg/kg)[19] dosage was applied 10 minutes after famotidine (40 mg/kg)[19] or omapatrilat (10, 20 and 40 mg/kg)[17,18] were administered[20]. The mice were then sacrificed after a 6-hour period with a 50 mg/kg lethal dose of thiopental anesthesia. The stomachs of the mice were removed, opened along the larger curvature surface, and then washed with saline solution (0.9% NaCl). Macroscopical images of the ulcerous areas on the collected stomach tissue samples were measured on millimeter paper[21]. Antiulcer activities were calculated as the percentage decrease of ulcerated area in each group compared to the control.

Biochemical Measurements

Following the surgical steps, about 75 mg of ground gastric tissue was homogenized in 1 mL of phosphate buffered saline in eppendorf tubes using a homogenizer (TissueLyser II by QIAGEN) and then centrifuged. Total protein concentrations were evaluated using the Lowry method. (Sigma Aldrich Total protein kit TP0300-1KT).

MDA levels[22] and SOD activity[23] were evaluated in standards and each sample's supernatant at room temperature in duplicate according to modified methods of the methods of the enzyme-linked immunosorbent assay (ELISA) reader as previously described[24,25].

ACE and neprilysin activities and GSH level were determined using YLA0163MO, YLA1760MO, and YLA0167MO ELISA kits, respectively, all at room temperature and in duplicate. All ELISA kits were used according to manufacturer's instructions and measured using a BioTek Epoch Microplate Spectrophotometer.

Statistical Analysis

Biochemical quantities were compared using one-way analysis of variance (ANOVA) and Duncan's multiple comparison test with the help of IBM Statistical Package for the Social Sciences software version 20.0. Differences among the groups were considered to be significant at $p<0.05$. All results were expressed as mean±standard deviations.

RESULTS

Gastric Ulcer in Stomach Samples

Macroscopic images of stomach tissues from each experimental group are shown in Figure 1. Tissues obtained from Groups 1 (healthy control), 2 (healthy+omapatrilat), and 4 (ulcer+famotidine) showed no signs of ulcer formation and no visually significant differences. As expected from indomethacin administration, numerous ulcerated areas of different shapes and sizes comprised of mucosal defects were observed throughout the whole stomach tissue samples obtained from Group 3 mice (Indomethacin control group). These foci presented with distinct boundaries and were mostly surrounded by swollen spots. The number and sizes of these spots were reduced with increasing omapatrilat dosage in Groups 5 (OMA10), 6 (OMA20), and 7 (OMA40), pretreated with 10, 20, and 40 mg/kg of omapatrilat, respectively. These findings are given in Table 1 with measured areas of ulcerated spots and corresponding anti-ulcer activity in each experimental group.

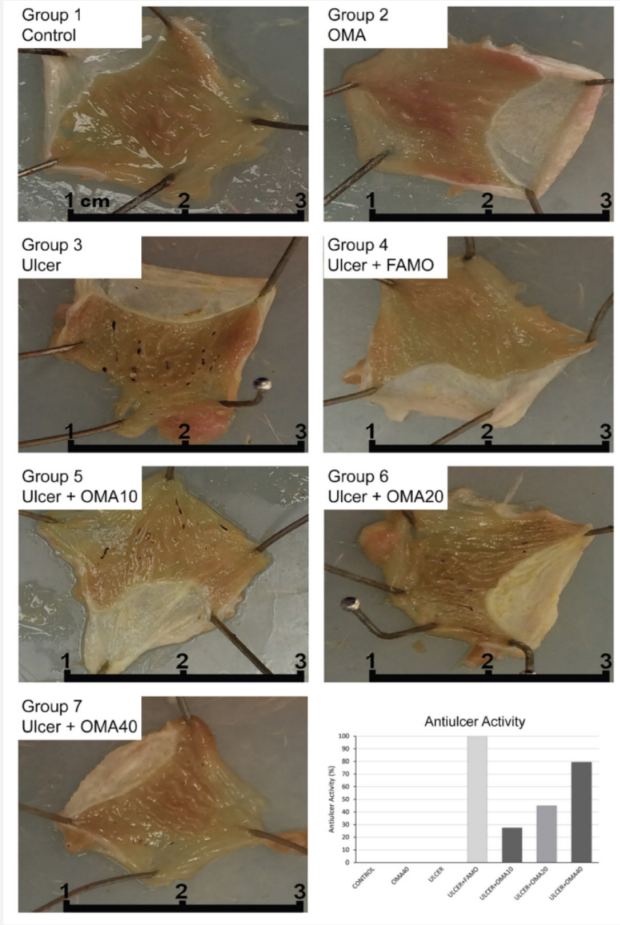

Figure 1. Macroscopic images of stomach tissue samples from mice groups where ulcerations are observed (scale bars indicate a length of 2 cm)

Table 1. Measurements of ulcerated areas and antiulcer activities

Groups	Ulcerated area (mm2)	Antiulcer activity (%)
Control	0.0[a]	-
OMA 40	0.0[a]	-
ULCER	29.0±2.0[e]	-
ULCER+FAMO	0.0[a]	100.0
ULCER+OMA 10	21.0±2.0[d]	27.5
ULCER+OMA 20	16.0±1.2[c]	44.8
ULCER+OMA 40	6.0±0.3[b]	79.3

Results quoted as mean ± standard deviation. Antiulcer activities were calculated as the percentage decrease of ulcerated area in the corresponding group in comparison with the control. Comparisons performed using one-way ANOVA and Duncan's multiple comparison test. Means with the same letter are not significantly different p<0.05

Biochemical Results

SOD, MDA, and GSH measurements are graphically shown in Figures 2, 3, 4. SOD activity measured in the treatment groups with 20 and 40 mg/kg omapatrilat were the closest to those measured in healthy groups (1 and 2). The least SOD activity was measured with indomethacin application in Group 3. In addition, Group 5, which was pre-treated with 10 mg/kg of omapatrilat, exhibited similar SOD activity as with in Group 3. Similar results were obtained for the GSH measurements. The highest GSH level was measured in the Groups 1 and 2. GSH

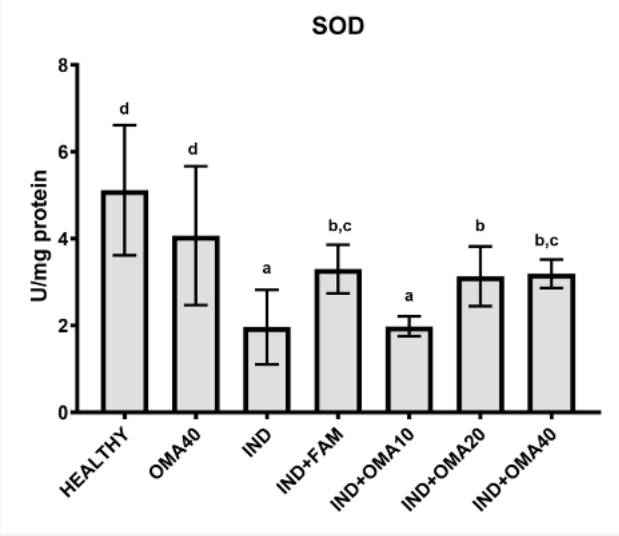

Figure 2. Effects of omapatrilat treatment on SOD activity in indomethacin-induced gastric ulcer (results quoted as mean±standard deviation. Comparisons performed using one-way ANOVA and Duncan's multiple comparison test. Means with the same letter are not significantly different p<0.05)

SOD: Superoxide dismutase

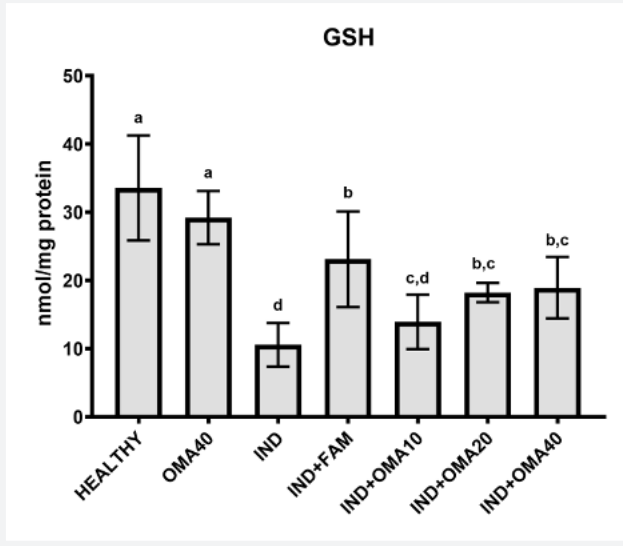

Figure 3. Effects of omapatrilat treatment on GSH level in indomethacin-induced gastric ulcer (results quoted as mean±standard deviation. Comparisons performed using one-way ANOVA and Duncan's multiple comparison test. Means with the same letter are not significantly different p<0.05)

GSH: Glutathione

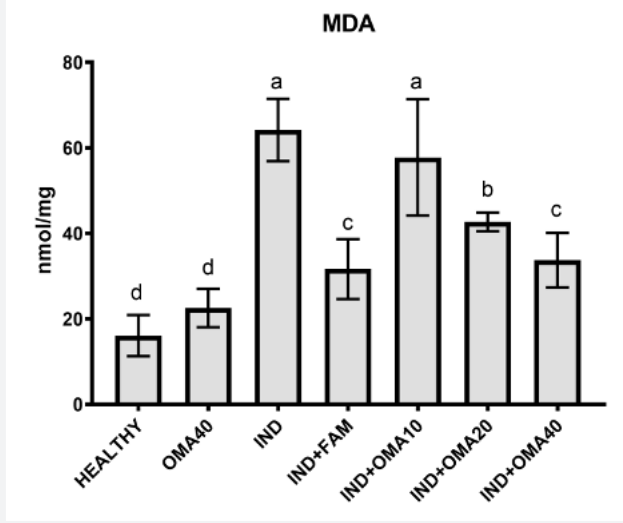

Figure 4. Effects of omapatrilat treatment on MDA level in indomethacin-induced gastric ulcer (results quoted as mean±standard deviation. Comparisons performed using one-way ANOVA and Duncan's multiple comparison test. Means with the same letter are not significantly different p<0.05)

MDA: Malondialdehyde

level in Group 3 was significantly reduced due to indomethacin administration, and was mostly recovered in Group 4 with the standard famotidine treatment. In omapatrilat-treated groups, while Group 5 only showed minor improvement in the GSH level, in Groups 6 and 7 GSH levels were statistically similar to that measured with the standard treatment in Group 4. Omapatrilat's effectiveness against oxidative stress was also observed in the MDA measurements. While Groups 1 and 2 exhibited the lowest MDA levels, the highest MDA levels were measured in Groups 3 and 5. Standard treatment in Group 4 and the 40 mg/kg omapatrilat treatment in Group 7 resulted in the closest MDA levels to those recorded in Groups 1 and 2. These results in SOD activity as well as in GSH and MDA levels indicate omapatrilat's gastroprotective potential against ulcer.

NEP and ACE levels are shown in Figures 5, 6. The lowest NEP and ACE levels were measured in Groups 1 and 2; and the highest levels were measured in Group 3 due to indomethacin, as expected. By increasing the dosage of omapatrilat from 10 to 40 mg/kg, these enzyme levels were significantly reduced, indicating that omapatrilat dually inhibits both NEP and ACE. Both ACE and NEP enzyme levels were increased in line with ulcer formation.

DISCUSSION

In this study, we have observed that omapatrilat, an inhibitor of both NEP and ACE that acts through the RAAS pathway, reduces indomethacin-induced gastric ulcer damage. Omapatrilat's connection with oxidant/antioxidant parameters was demonstrated. The relationship between gastrotoxic effect mechanism of indomethacin and the antiulcer effect of omapatrilat was also studied. Omapatrilat was shown to reduce both NEP and ACE levels in the stomach tissue. It was observed that omapatrilat treatment increased levels GSH and SOD activity while decreasing MDA levels. Therefore, omapatrilat was shown to prevent the damage induced by oxidative stress in gastric ulcer.

Indomethacin, which is used in the treatment of many inflammatory diseases, is a NSAID. A well-known side effect of indomethacin, gastric ulcer formation, is commonly used in experiments to induce inflammation. As such, in our experiments, indomethacin is used not only as an anti-inflammatory drug, but also to create an ulcer model in animals[26]. Indomethacin has been shown to cause significant damage to gastric tissue in animals at doses of 10, 20, and 25 mg/kg[27]. Similarly, our results also showed clearly visible ulcer areas in the group receiving only the 25 mg/kg indomethacin. No ulcer formation was observed in the famotidine group. It was seen that omapatrilat showed an antiulcer effect in a dose-dependent manner. The best antiulcer effect was recorded at an omapatrilat dose of 40 mg/kg.

Ang II, which induces inflammation and oxidative stress in RAAS, is an important end product[28]. In addition, Ang II narrows

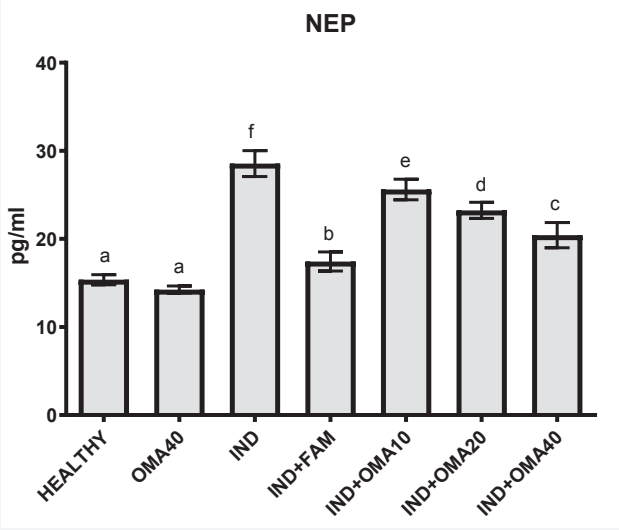

Figure 5. Effects of omapatrilat treatment on NEP levels in indomethacin-induced gastric ulcer (results quoted as mean±standard deviation. Comparisons performed using one-way ANOVA and Duncan's multiple comparison test. Means with the same letter are not significantly different $p<0.05$)

NEP: Neutral endopeptidase

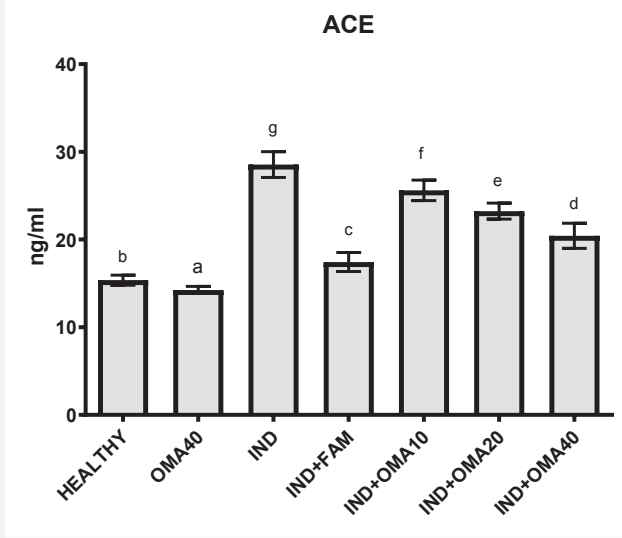

Figure 6. Effects of omapatrilat treatment on ACE levels in indomethacin-induced gastric ulcer (results quoted as mean±standard deviation. Comparisons performed using one-way ANOVA and Duncan's multiple comparison test. Means with the same letter are not significantly different $p<0.05$)

ACE: Angiotensin converting enzyme

the gastric mucosal vasculature by activating the receptor in the gastrointestinal tract[29]. In the gastrointestinal system, RAAS has been reported to have functional effects on the stomach[30], colon[31], and intestine[32]. The effects of RAAS-acting agents including ACE inhibitors and Angiotensin receptor blocker (ARB) drugs on different ulcer models have previously been studied. For instance, captopril, the first ACE inhibitor used in hypertension treatment, showed antioxidant effects on aspirin-induced gastric lesions[33]. It has been determined that ARBs prevent ulcerations by protecting gastric blood flow and reduce sympathoadrenal activation[34]. ARBs have also been shown to suppress gastric damage due to ischemia/reperfusion injury in rats[35]. Angiotensin-(1e7), a physiological antagonist of Ang II, was also effective against stress-induced gastric lesions in rats[9]. According to these results, it is suggested that the inhibition of RAAS at different stages may play an important role during ulcer treatment. Potential anti-inflammatory, gastro-protective or anti-oxidative effects can be cited as underlying causes of the gastroprotective nature of RAAS inhibition in various ulcer models. Similar to these findings, here we show that omapatrilat blocks RAAS by inhibiting ACE and protects the stomach from possible oxidative stress due to Ang II.

It is known that the toxic effect of indomethacin on the stomach is much more severe than that of other NSAIDs[36]. It has been reported that this effect of indomethacin on the gastrointestinal system is due to the inhibition of prostaglandin (PG) synthesis[37]. However, the COX theory, which plays a role in the gastric mucosal damage by NSAIDs, cannot adequately explain this damage[38]. For instance, in some studies, although PGE2 formation was prevented largely with repeated administration of indomethacin, it was observed that the mucosal lesion decreased[39]. This indicates that there may be factors other than PG in the antiulcer activity mechanism. Based on this idea, GSH and MDA levels as well as SOD activity were measured to determine whether oxidant/antioxidant parameters played a role in the gastric mucosal adaptation mechanism.

In some studies, it has been demonstrated that reactive oxygen species (ROS) play a role in the etiopathogenesis of gastric damage caused by indomethacin[40]. Tissue damage due to ROS begins with the formation of lipid radicals in the cell membrane. Then, this radical first transforms into lipid hydroperoxides, and finally into toxic products such as malondialdehyde, alkane, and aldehyde[40,41]. The detrimental effects of ROS have required cells to develop distinct and potent detoxification mechanisms through a regulatory network of antioxidant enzymes and non-enzymatic compounds[42]. Among these antioxidants, GSH prevents tissue damage by maintaining ROS concentrations below certain levels[43]. Therefore, as expected, we observed that the highest GSH level was measured in the control group. In the indomethacin group, where the damage was the highest, the GSH level was measured to be the least. Additionally, it was seen that omapatrilat increased the GSH level in the ulcer groups in a dose-dependent manner. With this increase in the antioxidant levels, it is shown that omapatrilat helps to maintain the oxidant/antioxidant balance.

SOD, another key antioxidant, is shown to act as a protective factor in controlling indomethacin-induced damage[44]. Similar to the GSH results above, we also measured the highest SOD activity in the control group, and the lowest SOD activity in the indomethacin group. As expected, we observed that omapatrilat resulted in an increase in SOD activity in a dose-dependent manner. In our experiments, the increase in SOD activity in ulcerated mice treated with omapatrilat demonstrates omapatrilat's antioxidant and antiulcer effects. Our measurements have shown that 40 mg/kg omapatrilat results in statistically similar findings to those from standard famotidine treatment.

It is reported that excessive toxic oxygen radicals in tissues activate lipid peroxidation that leads to the formation of MDA, an oxidative stress biomarker[45]. MDA directly damages the cell membrane structure and other cell components by producing reactive aldehydes, resulting in tissue damage and various diseases[46]. Previous studies have shown high levels of MDA in ulcerated stomach tissue[47]. Similarly, in our experiments, MDA levels were found to be as much as 4 times higher than the control group in the indomethacin group, and were gradually reduced in groups treated with omapatrilat, again in a dose-dependent manner. As with SOD activities, MDA levels were found to be statistically similar with 40 mg/kg of omapatrilat and the specific famotidine treatment. Similar results were reported in another study where reduction in SOD activity and GSH levels in indomethacin-induced ulcer groups and an accompanying increase in the MDA level were observed. These changes were reversed due to treatment with aliskiren, a renin inhibitor that affects the RAAS pathway[5]. These findings show that omapatrilat, which is also a RAAS-acting agent, contributes to the reduction of oxidative stress by promoting antioxidant activity. Our findings of ACE and neprilysin enzyme levels were also similar to the oxidative stress biomarkers discussed above. In comparison with the control group, it was seen that indomethacin significantly increased ACE and neprilysin levels, which were then subsequently reversed to healthy levels with famotidine. With omapatrilat administration in ulcerated mice, we recorded similar decreases in ACE and neprilysin levels that were proportional to the omapatrilat dosage from 10 to 40 mg/kg. Resulting ACE and neprilysin levels with specific famotidine treatment and with 40 mg/kg of omapatrilat were both significantly less than those measured in untreated ulcerated mice, but still above those measured in the control group. Omapatrilat administration did

not alter ACE or neprilysin levels in healthy mice. As a result, omapatrilat reduced the formation of ulcers by preventing Ang II production, which plays a key role in ulcer etiology. In recent studies, drugs that affect the RAAS, such as aliskiren and enalapril, have been shown to be successful in increasing antioxidant levels. Among these drugs, omapatrilat, which is the first drug known to inhibit both NEP and ACE enzymes simultaneously, is a recently developed RAAS effective agent[10]. As such, we have investigated the possible effects of ACE/NEP pathway on gastric ulcer due to indomethacin, and have seen significant increases in the levels of both enzymes in the ulcer group. This increase in ACE and NEP levels can be interpreted as a defense mechanism accompanying gastric damage.

Study Limitations

The limitations of our study are that the experimental study was conducted only on one ulcer model and the tissues could not be examined histopathologically.

CONCLUSION

In conclusion, the best antiulcer activity of omapatrilat was demonstrated at a dose of 40 mg/kg in our experiments. Our macroscopic results, oxidant/antioxidant levels, and biochemical measurements show that omapatrilat demonstrates significant anti-ulcer effect at 40 mg/kg dosage by inhibiting both neprilysin and ACE levels, which have approached to those measured in the control group. These findings indicate that the dual inhibition of NEP and ACE by omapatrilat suppressed the oxidative stress related to indomethacin-induced gastric ulcer. These results reveal for the first time the combined role of ACE and NEP enzymes in gastric ulcer and the protective effects of omapatrilat against oxidative stress. Therefore, we think that the protective effects of omapatrilat in the treatment of ulcers may lead to the search for new treatment strategies.

Acknowledgements

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Ethics

Ethics Committee Approval: All animal care and protocols were confirmed by Experimental Animal Ethics Committee of Atatürk University (decision no: 157, date: 02.08.2018).

Informed Consent: Animal experiment.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: K.G.E., E.Ç., Concept: R.A.U., Z.B.A.M., A.B., Design: R.A.U., Z.B.A.M., A.B., Data Collection or Processing: K.G.E., E.Y., Analysis or Interpretation: E.Y., A.B., E.Ç., Literature Search: K.G.E., Writing: K.G.E.

Conflict of Interest: No conflict of interest was declared by the authors.

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