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RESEARCH

The therapeutic effects of vonoprazan against giardiasis in rats: parasitological, pathological, and immunological aspects

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Abstract

Giardiasis is a serious public health issue caused by *Giardia lamblia*. The medications used in its treatment induce unfavorable adverse effects. Furthermore, treatment failure and drug resistance are other challenges in its management. Vonoprazan is a safe, recently approved potassium-competitive acid blocker. The current study aimed to assess the potential therapeutic effects of vonoprazan, alone or in combination with metronidazole, on giardiasis. The efficiency of the treatment was evaluated by parasitological, histopathological, immunohistochemical, scanning electron microscopic, and biochemical studies. According to the results, the combination treatment significantly reduced the number of cysts in the stool and trophozoites in the small intestinal wash and ameliorated the intestinal pathological alterations. The expression of TNF-α and caspase-3 in the intestinal tissues was reduced. Also, there were reductions in the levels of IL-6, iNO, and MAD and an elevation in TAC levels in the serum. The combination therapy exhibited better efficacy than metronidazole alone. To sum up, this study underlines the suitability of using vonoprazan as an adjuvant to metronidazole in treating giardiasis.

Keywords *Giardia lamblia* · Vonoprazan · Scanning electron microscopy · TNF-α · Caspase-3 · IL-6 · INO · MAD · TAC

Introduction

Giardia lamblia is a zoonotic extracellular parasite. It lives mainly in the upper part of the small intestine of its vertebrate hosts (Cernikova et al. 2018). Although giardiasis is a cosmopolitan disease, its prevalence in underdeveloped countries (8–30%) is higher than in developed countries (0.4–7.5%) (Mahdavi et al. 2022). Giardiasis can present as either acute or chronic infections, while some cases may be asymptomatic. The disease manifestations include diarrhea, abdominal pain, and loss of weight. Furthermore, severe infections may develop into malabsorption syndrome, dehydration, and electrolyte disturbances (Panaro et al. 2007; Halliez and Buret 2013).

There are numerous contributing factors to the pathophysiology of giardiasis. Inflammation, oxidative stress, apoptosis, and direct damage produced by the parasite are all important factors in the battle between *G. lamblia* and the host (Liu et al. 2020; Kapczuk et al. 2020). IL-6 and TNF-α are pro-inflammatory cytokines that have a role in the immune response against *G. lamblia*. IL-6 is essential for the clearance of *Giardia* as it induces the development of Th17 $CD4^+$ T cells, which mediate early adaptive immune mechanisms (Blaschitz and Raffatellu 2010; Dreesen et al. 2014; Grit et al. 2014). Moreover, early in giardiasis, TNF-α plays a protective role. Its anti-parasitic effects are primarily through apoptosis and stimulation of cell recruitment to the site of infection (Kapczuk et al. 2020; Lopez-Romero et al. 2015). In contrast, excessive TNF-α production impairs epithelial integrity, inducing intestinal ulceration (Tourani et al.

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2018). Additionally, NO is expressed by intestinal epithelial cells in response to microbial products (Eckmann 2003; Müller and Allmen 2005). Its level increases in patients infected with *G. lamblia* (Matowicka-Karna et al. 2011). Furthermore, in vitro studies demonstrated that NO hinders the excystation process and induces a cytostatic effect on *G. lamblia* trophozoites (Eckmann et al. 2000).

Oxidative stress plays a crucial role in controlling and clearing invading pathogens, including parasites (Mohan and Gupta 2018). Alternatively, numerous studies have demonstrated that parasitic infections can induce oxidative stress-mediated host cell apoptosis (Giri and Roy 2016; Xu et al. 2015). It has been reported that excessive production of reactive oxygen species can induce cell apoptosis via a mitochondria-mediated apoptotic pathway (Sinha et al. 2013). The role of oxidative stress and lipid peroxidation was established in patients infected with *G. lamblia* (Ismail et al. 2022).

Metronidazole is the mainstay treatment for giardiasis (Watkins and Eckmann 2014). Nevertheless, its administration is accompanied by adverse effects, such as headache, parageusia, nausea, dizziness, high liver enzymes, and yellowish skin discoloration (Leitsch 2015; Lalle and Hanevik 2018). Additionally, treatment failure and the emergence of drug-resistant strains of *Giardia* are other challenges (Leitsch 2015; Nabarro et al. 2015). These factors raised the researcher's attention to the importance of searching for new, safe, and effective drugs.

It is remarkable how pH and proton pumps influence the viability and development of numerous parasites and microbes (Huynh and Grinstein 2007). Proton pump inhibitors (PPIs) are usually prescribed for treating gastroesophageal hyperacidity (Ghebre and Raghu 2016). Their antibacterial effects were reported against tubercle bacilli (Rybniker et al. 2015), *Salmonella* typhi (Puiac et al. 2009), and *Clostridium difficile* (Kaur et al. 2007). Moreover, they exhibited anthelminthic activity against *Schistosoma mansoni* (Ellakany et al. 2019) and antiprotozoal effects against *Trichomonas vaginalis* and *Entamoeba histolytica* (Sheele 2017). Furthermore, PPIs have potent anti-inflammatory and anti-oxidative stress effects (Kedika et al. 2009; Alfahad et al. 2021).

G. lamblia uses glycolysis as the chief metabolic pathway of ATP synthesis because it cannot perform oxidative phosphorylation. PPIs were found to deactivate glycolytic enzymes, for example, triosephosphate isomerase enzyme; thus, they could hinder the growth and survival of *G. lamblia* (Han and Collins 2012; Reyes-Vivas et al. 2014).

Vonoprazan is a potassium-competitive acid blocker that has a rapid and persistent acid-suppressive effect (Mousavi et al. 2022). It is a recently approved new generation of PPIs that is safer and overcomes the undesirable side effects of older ones (Dong et al. 2017; Sun et al. 2023). Furthermore, it showed high effectiveness against *Helicobacter pylori* (Kiyotoki et al. 2020). The in vitro effectiveness of vonoprazan against *G. lamblia* was formerly assessed with encouraging results (Sallam et al. 2024). Hence, this study was designed to investigate the efficacy of vonoprazan compared with metronidazole against *G. lamblia* infection in experimentally infected rats.

Material and methods

Experimental animals and ethical considerations

This study was conducted on 25 laboratory-bred parasite-free male Swiss albino rats (3–4 weeks old and weighing 150–200 g at the time of infection). They were maintained following the institutional and national guidelines. The study was approved by the Ethics Committee of Tanta University, Faculty of Medicine (approval code: 36,166/12/22).

Parasite and infection

Collection, purification, and preparation of G. lamblia cyst inoculum

Giardia lamblia cysts were collected from fresh diarrheic stool samples from patients attending the outpatient clinic of Tanta University Hospitals, Tanta, Egypt. Repeated saline washing and centrifugation at 2000 rpm for 5 min three times were used to concentrate and sediment the samples. After the last washing, the deposit was thoroughly mixed with normal saline, and the number of *G. lamblia* cysts in 1 mL of stool sediment was counted using a hemocytometer. The average of three counts was estimated to adjust the required infecting dose (10^5 cysts/ml). According to Vinayak et al. (1979), 1 mL of *G. lamblia* cyst suspension containing 10^5 cysts was given orally to each rat in the infected groups. One milliliter of normal saline was given to each rat in the negative control group. The infection was verified by three distinct stool examinations for each rat, beginning on the 3rd day post-infection (P.I.) using unstained direct smears and Lugol's-iodine stained smears, as described by Venkatesh et al. (2016).

Drug regimens

Metronidazole (Amrizole; suspension of 125 mg/5 mL, Amriya Pharmaceutical Company, Cairo, Egypt) was given orally at a dose of 7 mg/rat/day for 7 consecutive days (Mahmoud and Shalaby 2006). Vonoprazan (Vonaspire; 20 mg tablets, Inspire Pharmaceutical Company, Cairo, Egypt) was given orally at a dose of 20 mg/kg/day after

dissolving in distilled water for 7 consecutive days (Chen et al. 2020). The treatment started on the 8th day P.I.

Experimental groups and design (Fig. 1A)

The rats were divided into five groups (five rats each) as follows: G I, non-infected, non-treated (Negative control); G II, infected, non-treated (Positive control); G III, infected and treated with metronidazole only (MTZ group); G IV, infected and treated with vonoprazan only (VONO group); and G V, infected and treated with both drugs, metronidazole and vonoprazan (combination group).

After the end of treatment administration (on the 15th 16th, and 17th days P.I.) and before sacrificing the rats, stool samples were collected from each rat in the infected groups.

On the 18th day P.I., all rats were anesthetized and sacrificed. Blood samples were collected. The serum was separated and kept at − 80 °C for biochemical analysis. Each rat's duodenum and proximal jejunum were removed, utilized to quantify trophozoites in the infected groups, and then preserved in 10% formalin solution for histopathological and immunohistochemical analysis of each group under study.

Parasitological study

Fecal G. lamblia cyst count

Stool samples were prepared for parasite counting according to Venkatesh et al. (2016) as follows: Three stool samples of each rat were collected after the end of the course of treatment in clean, wide-mouthed tubes with tight-fitting covers. One gram of the pooled stool samples of each rat was emulsified with saline solution and mixed well in a centrifuge tube**.** Centrifugation was done at 2000 rpm for 2 min.

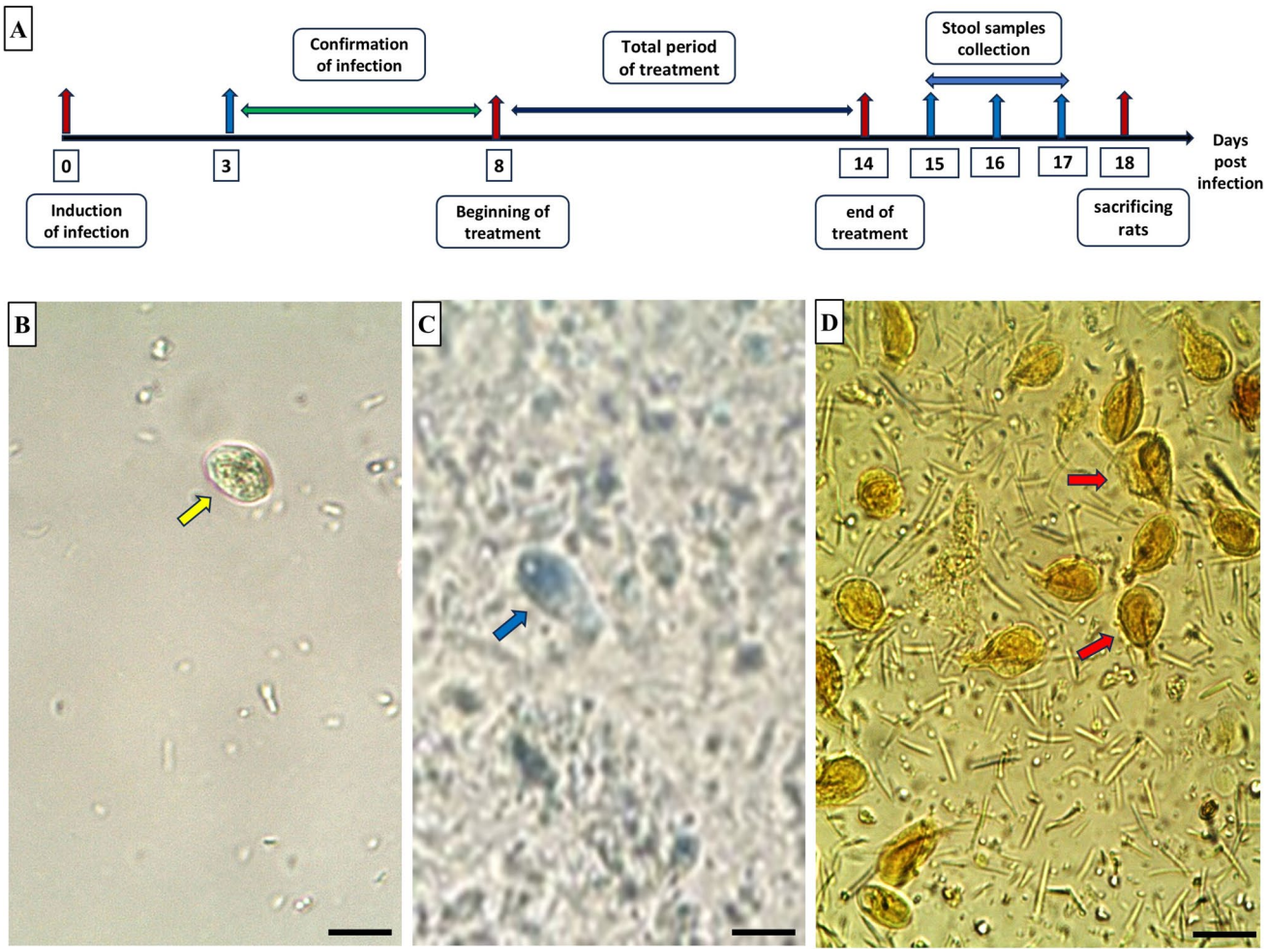

Fig. 1 **A** A scheme shows the duration of treatment and the timing of sample collections. **B** Trypan blue-stained fecal smear shows a viable *G. lamblia* cyst (yellow arrow) (×1000). **C** Trypan blue-stained fecal smear shows a dead *G. lamblia* cyst (blue arrow) (×1000). **D** *Giardia lamblia* trophozoites in an iodine-stained smear of rat stools (red arrows) (×1000). Scale bar = 10 µm

After centrifugation, the supernatant was poured off, and the sediment was examined to estimate the parasite burden. For each rat, 10 high-power fields (40×) were inspected using a hemocytometer, and cysts were counted in each field, and then the mean number of cysts/HPF was calculated (Fathy 2011).

Cyst viability testing

A 0.1-g sample of freshly voided stool was taken from each rat in the infected groups. After carefully homogenizing each sample in 1 mL of saline, 0.3% Trypan Blue stain was applied. The dark blue-stained (dead cysts) (Fig. 1C) and unstained (viable cysts) (Fig. 1B) were counted on a hemocytometer following Hamdy et al. (2019). The percentage of viability was calculated as follows: % Viability = unstained cysts/total cysts × 100. Viability was calculated for each of the three collected stool samples of each rat.

G. lamblia trophozoite count

Trophozoites (Fig. 1D) were counted in the intestinal wash of the infected rats. The duodenum and upper jejunum of each sacrificed rat were submerged in a tube containing 1 mL sterile chill-iced phosphate-buffered saline (PBS) for 15 min. Tubes were vortexed for 30 s to ensure the release of the trophozoite from the intestinal wall. A hemocytometer was then used to count the trophozoites that were separated from the intestinal tissues using gauze filtering. Each rat sample was counted on four different grids.

One parasite on one grid corresponded to 10^4 trophozoites/mL. The total count was expressed as the number × 10^4 trophozoites/mL of intestinal wash (Mahmoud et al. 2014; Al-Ghandour et al. 2020).

Histopathological study

Each rat's duodenum and the proximal portion of its jejunum were sampled (1–2 cm pieces), fixed in 10% formalin, and then processed to create paraffin block sections. Hematoxylin and eosin (H&E) staining was applied to the slides following Ammar et al. (2014). A semiquantitative scoring system of histopathological changes was done in a blinded fashion as follows: inflammatory cell infiltration within the core of the intestinal villi (0 = normal; 1 = mild infiltration; 2 = moderate infiltration; 3 = severe infiltration), epithelial hyperplasia (0 = normal; 1 = mild; 2 = moderate; 3 = extensive), epithelial erosions and ulcerations (0 = absent; 1 = present), goblet cells hyperplasia (0 = absent; 1 = present), and muscle thickening (0 = normal; 1 = mild; 2 = moderate; 3 = severe) (Dann et al. 2018).

Immunohistochemical study

Assessment of duodenal expression of TNF-α and caspase-3

Duodenum tissue sections were deparaffinized and hydrated. The immunohistochemical study was done using Immune Cruz™ rat labeled streptavidin–biotin (LSAB) Staining System (Code: sc-2050). Rodent anti-TNF-α monoclonal antibodies in a dilution of 1:50 (clone No. MP6-XT22, eBioscience, USA) and rabbit recombinant monoclonal caspase-3 p12 antibody (clone No. EPR16888, Abcam, USA) in a dilution of 1:2000 were used as primary antibodies. Untreated sections with primary antibodies were used as negative controls. The immunostaining of both antibodies was evaluated blindly and scored. This evaluation was performed on 10 distinct high-power fields (×400 magnification) per section. For each rat, two duodenal sections were examined separately.

Immunopositivity of TNF-α was scored ranging from grade 1 to grade 4 by assessment of the extent of immunostaining (1 = 25%; 2 = 25–50%, 3 = 50–75%, 4 = 75–100%), and the staining intensity was scored either grade: 1 (mild), 2 (moderate), or 3 (strong) immunoreactivities. The extent and intensity of immunostaining were multiplied to find the final score, which ranged from 0 to 12. The final grades were as follows: 0 (negative), 1 (mild or weak) (score 1 to 4), 2 (moderate) (scoring 5 to 8), and 3 (strong) (score 9 to 12) (Kaemmerer et al. 2012).

The following scores were assigned to the assessed average staining intensity of caspase-3 in intestinal tissues: 0 = negative, 1 = weak, 2 = moderate, and 3 = strong. The percentage of stained cells at each intensity level was graded as 0 (less than 5%), 1 (5–25%), 2 (26–50%), 3 (51–75%), or 4 (more than 75%). The final scores (0–7) were then calculated by adding the intensity score and the percentage of positive cells. A score of 0 denoted negative or absent caspase-3 expression, a score of ≤ 4 meant low expression, and a score > 4 meant high expression (Liu et al. 2017).

Scanning electron microscopic study of the duodenal villi (SEM)

Small duodenal pieces from each rat were immediately fixed in cold glutaraldehyde for 2 h, followed by fixation in 2% osmium tetroxide for 2 h, dehydration in serial rising ethanol concentrations, and drying using liquid CO_2. After that, the samples were placed on stainless steel holders, sputter-coated with a thin layer of gold (Fathy 2011), and finally inspected at 5 kV using a JEOL (JSM-5400LV) SEM at Alexandria University, Faculty of Science.

Biochemical study

Enzyme-linked immunosorbent assays

ELISA kits (Boster Biological Technology, Pleasanton, CA, USA, Catalog # EK0412 and MyBioSource, San Diego, USA, Catalog # MBS263618) were used to test the serum levels of interleukin-6 (IL-6) and inducible nitric oxide (iNO), respectively. Every ELISA procedure was carried out following the manufacturer's instructions and read at 450 nm using a correction wavelength set at 570 nm on a microplate reader (Stat Fax®2100, Fisher Bioblock Scientific, France).

Assessment of the oxidant\antioxidant status in the serum

Biochemical assay of malondialdehyde (MDA) was done using commercial kits supplied by Biodiagnostic, Giza, Egypt. The total antioxidant capacity (TAC) was analyzed as formerly explained by Koracevic et al. (2001).

Statistical analysis

Data were presented as mean ± standard deviation (SD). The normality of data distributions was assessed with the Shapiro–Wilk test. Analysis of variance (ANOVA) was used to compare more than two groups, and the post hoc test (Tukey) was used for pairwise comparisons in the case of normally distributed quantitative variables. The Kruskal–Wallis test was used to compare more than two studied groups, and post hoc (Dunn's multiple comparisons test) was used for pairwise comparisons in the case of abnormally distributed quantitative variables. The Monte Carlo exact test for chi-square was used for the analysis of histopathological and immunohistochemical scores. The reduction percentage was calculated by the equation: Reduction percentage = the mean value of the infected control group − the mean value of the infected treated group/the mean value of the infected control group × 100. *P*-value < 0.05 was considered to be significant. Statistical analysis was performed using IBM SPSS Statistics for Windows, Version 20.0 (Armonk, NY: IBM Corp).

Results

Parasitological study

G. lamblia cyst count/HPF in the stool and cyst viability

All treatment groups exhibited a significant decrease in mean cyst counts compared to the positive control group. The percentages of reduction in cyst counts were 54.29%, 71.43%, and 92.86% in the MTZ, VONO, and combination groups, respectively. Compared to the MTZ group, the cyst count in the VONO group was significantly lower. Moreover, the cyst count in the combination group was significantly lower than in other treated groups (Table 1).

Regarding the viability of cysts in stool samples of the infected groups, the positive control group showed the highest percentage of viable cysts (96%) compared to the treated groups. However, the combination group showed the lowest percentage of viable cysts (0%) compared to the other treated groups. MTZ and VONO groups showed 23.8% and 32.0% percentages of viable cysts, respectively (Table 1).

Table 1 *Giardia lamblia* cyst count in stool and trophozoite count in intestinal wash

	n = 5	G II (positive control)	G III (MTZ)	G IV (Vono)	G V (combination)
Cyst count/HPF	**Mean ± SD**	$14.0^{bcd} \pm 1.87$	$6.40^{acd} \pm 0.89$	$4.0^{abd} \pm 1.0$	$1.0^{abc} \pm 0.71$
	% Reduction		54.29	71.43	92.86
	Percentage of viability	13.40 ± 1.52 (96.0%)	1.60 ± 0.55 (23.8%)	1.20 ± 0.45 (32.0%)	0.0 ± 0.0 (0.0%)
	F	105.378*			
Trophozoite counts ($\times 10^4$/mL)	**Mean ± SD**	$4.0^{bcd} \pm 1.0$	$0.80^{acd} \pm 0.45$	$0.60^{abd} \pm 0.55$	$0.0^{abc} \pm 0.0$
	% Reduction		80.0	85.0	100.0
	H	15.114 *			

F *F* for one-way ANOVA test, *H H* for Kruskal Wallis test, *n* number of studied rats in each group

HPF high power field

[a]Significant compared to G II

[b]Significant compared to G III

[c]Significant compared to G IV

[d]Significant compared to G V

*Statistically significant at $p \leq 0.05$

G. lamblia trophozoite count in the intestinal wash (× 10^4 / ml)

The mean trophozoite counts showed significant reductions in the groups that received treatment compared to the positive control group. The percentages of reduction in trophozoite counts were 80.0%, 85.0%, and 100.0% in MTZ, VONO, and combination groups, respectively. The mean trophozoite counts in both VONO and combination groups were significantly lower than that of the MTZ group. Similarly, the mean trophozoite count in the combination group reduced significantly compared to the VONO group (Table 1).

Histopathological study

Histopathological examination of the small intestinal sections from the negative control group exhibited a normal villous pattern and healthy mucosa. No histopathological changes were observed (Fig. 2A). However, those from the positive control group showed evident structural changes in the intestinal mucosa, including mucosal ulceration, villous atrophy, infiltration of the lamina propria with inflammatory cells, mainly lymphocytes, and raised number of goblet cells (Fig. 2B). The MTZ group showed mild inflammatory changes, mild improvement of villous pattern, and mild goblet cell depletion

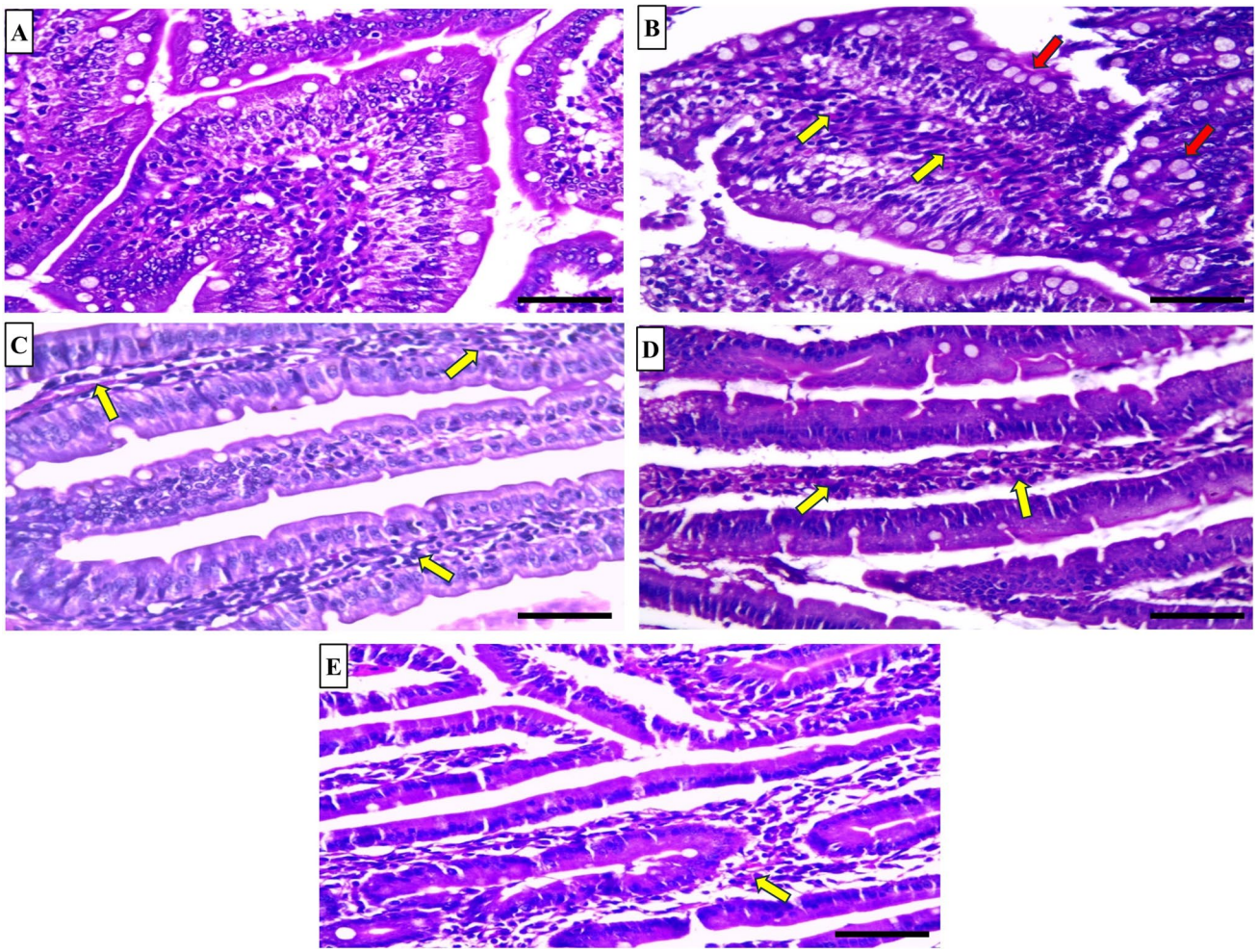

Fig. 2 Small intestinal sections (H&E × 400). **A** The negative control group shows a normal villous pattern, healthy mucosa, and no inflammatory reaction in the lamina propria. **B** The positive control group shows intense infiltration of the lamina propria with inflammatory cells mainly lymphocytes (yellow arrows) and an increased number of goblet cells (red arrows). **C** The MTZ group showed mild inflammatory changes (yellow arrows) and mild improvement of the villus pattern. **D** The Vono group showed minimal inflammatory cells and infiltration of the lamina propria (yellow arrows), and **E** the combination group showed a normal villous pattern and mild inflammation (yellow arrow). Scale bar = 50 µm

(Fig. 2C). Similarly, the VONO group showed improvement of the histopathological lesions in the form of less inflammation of the lamina propria, marked improvement of villous pattern, and less mucosal ulceration (Fig. 2D). Moreover, small intestinal sections from the combination group revealed a normal villous pattern and architecture (Fig. 2E) (Table 2).

Immunohistochemical results

Expression of TNF-α in small intestinal tissues (Table 3)

Immunohistochemical assessment of TNF-α expression revealed strong expression (grade 3) in the positive control

Table 2 Semiquantitative scoring of histopathological changes in the small intestinal sections of the studied groups ($n=10$)

Score	Examined group					χ^2	^{MC}p
	G I (negative control)	G II (positive control)	G III (MTZ)	G IV (Vono)	G V (Combination)		
Inflammatory cell infiltration of the mucosa							
0	10	0	0	0	10	87.099*	< 0.001*
1	0	0	0	8	0		
2	0	0	9	2	0		
3	0	10	1	0	0		
Epithelial hyperplasia							
0	10	0	1	2	5	52.779*	< 0.001*
1	0	1	9	8	5		
2	0	1	0	0	0		
3	0	8	0	0	0		
Epithelial erosions and ulcerations							
0	10	0	7	8	10	30.987*	< 0.001*
1	0	10	3	2	0		
Goblet cell hyperplasia							
0	10	0	7	8	10	30.987*	< 0.001*
1	0	10	3	2	0		
Muscle thickening							
0	10	0	0	0	9	83.825*	< 0.001*
1	0	0	0	8	1		
2	0	0	9	2	0		
3	0	10	1	0	0		

χ^2 Chi-square test, MC Monte Carlo, *p* *p*-value for comparing the studied groups, *n* number of the studied samples in each group. One duodenal and one proximal jejunal sample were assessed for each rat

*Statistically significant at $p \leq 0.05$

Table 3 Immunohistochemical score of TNF-α expression in duodenal tissues in all studied groups ($n=10$)

Groups	TNF-α score							
	Negative		Grade 1		Grade 2		Grade 3	
	No	%	No	%	No	%	No	%
Group I (negative control)	10	100.0	0	0.0	0	0.0	0	0.0
Group II (positive control)	0	0.0	0	0.0	1	10.0	9	90.0
Group III (MTZ)	0	0.0	3	30.0	6	60.0	1	10.0
Group IV (Vono)	1	10.0	4	40.0	5	50.0	0	0.0
Group V (Combination)	9	90.0	1	10.0	0	0.0	0	0.0
χ^2	62.221*							
^{MC}p	< 0.001*							

$\chi 2$ Chi square test, MC Monte Carlo, *p* *p*-value for comparing the studied groups, *n* number of studied duodenum tissue sections/group. For each rat, two duodenal sections were examined separately

*Statistically significant at $p \leq 0.05$

group (Fig. 3B) compared to the negative control group (Fig. 3A, F). The MTZ group displayed mild to moderate expression with grade 1 (30%) and grade 2 (60%) (Fig. 3C, F). Similarly, the VONO group showed mild to moderate expression with grade 1 (40%) and grade 2 (50%) (Fig. 3D, F). The lowest expression was noticed in the combination group, with 90% negative expression and 10% weak expression (grade 1) (Fig. 3E, F).

Expression of caspase-3 in small intestinal tissues (Table 4)

Immunohistochemical assessment of caspase-3 immunoreactivity showed negative expression in the negative control group (Fig. 4A). However, the expression was high (90%) in the positive control group (Fig. 4B, F). Forty percent of rats in the MTZ group showed low expression, and another 40% showed high expression (Fig. 4C, F). The VONO group showed negative expression in 60% and low expression in 30% of rats (Fig. 4D, F). Furthermore, the combination group showed negative expression in 80% of rats and low expression in 20% of the studied rats (Fig. 4E, F).

Scanning electron microscopic study of the duodenal villi The scanning electron microscopy of the small intestinal mucosa of the negative control group showed a normal appearance of intestinal villi with no vesicle formation (Fig. 5A). However, the positive control group revealed marked destruction of the intestinal villi with disorganization and small and large vesicle formation (Fig. 5B–D). The small intestinal mucosa of both the MTZ group (Fig. 5E–G) and the VONO group (Fig. 5H, I) showed mild to moderate improvement of the intestinal villi and mild improvement of the degenerative changes with few vesicle formation, especially at the villi tips. The combination group (Fig. 5J, K)

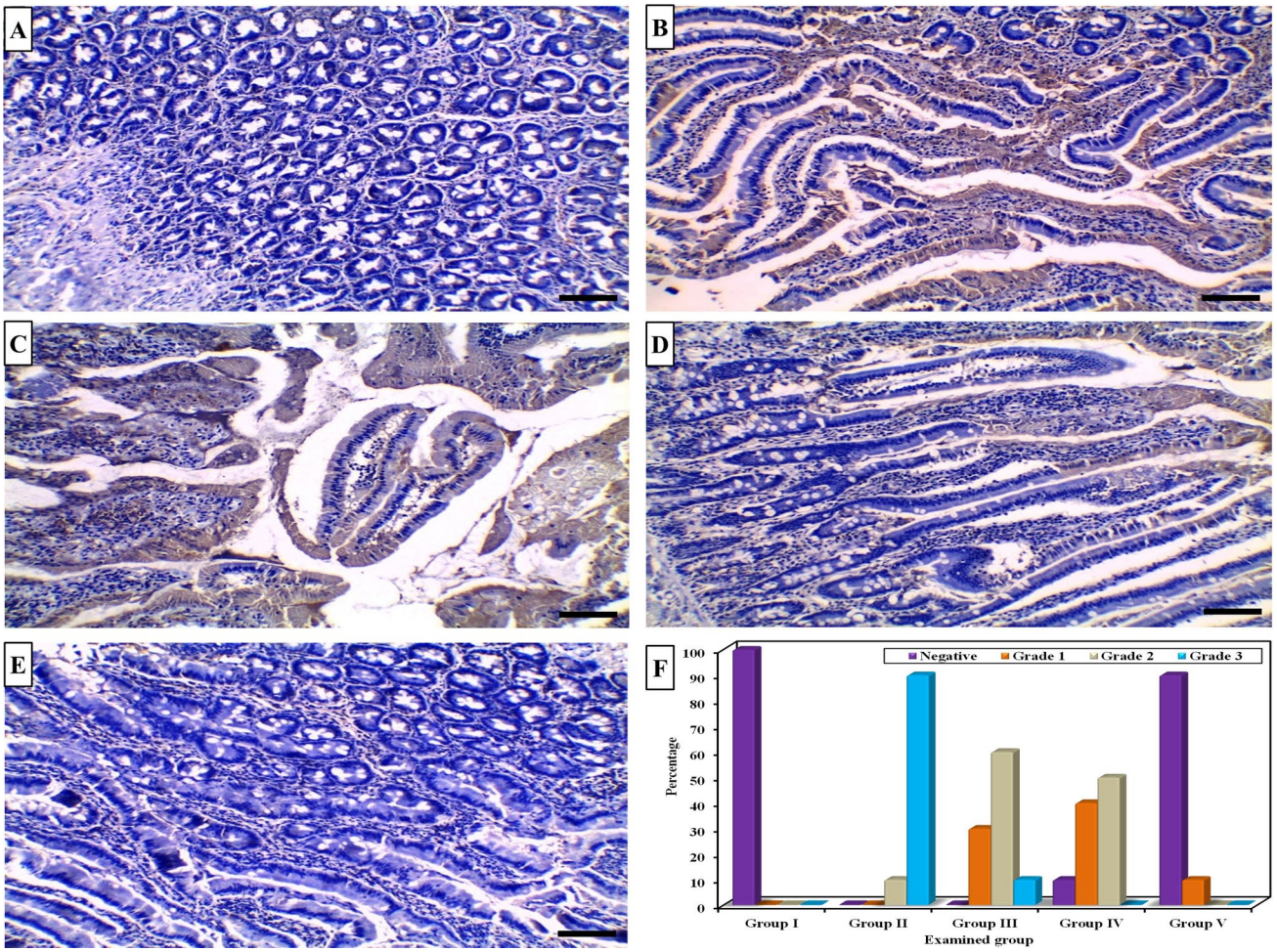

Fig. 3 Immunohistochemical expression of TNF-α in duodenal tissues (×200). **A** The negative control group shows negative expression of TNF-α. **B** The positive control group shows strong cytoplasmic expression grade 3. **C** The MTZ group shows moderate cytoplasmic expression grade 2. **D** The Vono group shows mild cytoplasmic expression grade 1. **E** The combination group shows negative cytoplasmic expression of TNF-α and **F** immunohistochemical expression grades of TNF-α in all studied groups. Scale bar = 50 µm

Table 4 Immunohistochemical score of caspase-3 expression in duodenal tissues of all studied groups ($n = 10$)

Groups	Caspase-3 score					
	Negative		Low expression ≤ 4		High expression > 4	
	No	%	No	%	No	%
Group I (negative control)	10	100.0	0	0.0	0	0.0
Group II (positive control)	0	0.0	1	10.0	9	90.0
Group III (MTZ)	2	20.0	4	40.0	4	40.0
Group IV (Vono)	6	60.0	3	30.0	1	10.0
Group V (Combination)	8	80.0	2	20.0	0	0.0
χ^2	36.286*					
^{MC}p	< 0.001*					

$\chi 2$ Chi square test, MC Monte Carlo, *p* *p*-value for comparing the studied groups, *n* number of studied duodenum tissue sections/group. For each rat, two duodenal sections were examined separately

*Statistically significant at $p \leq 0.05$

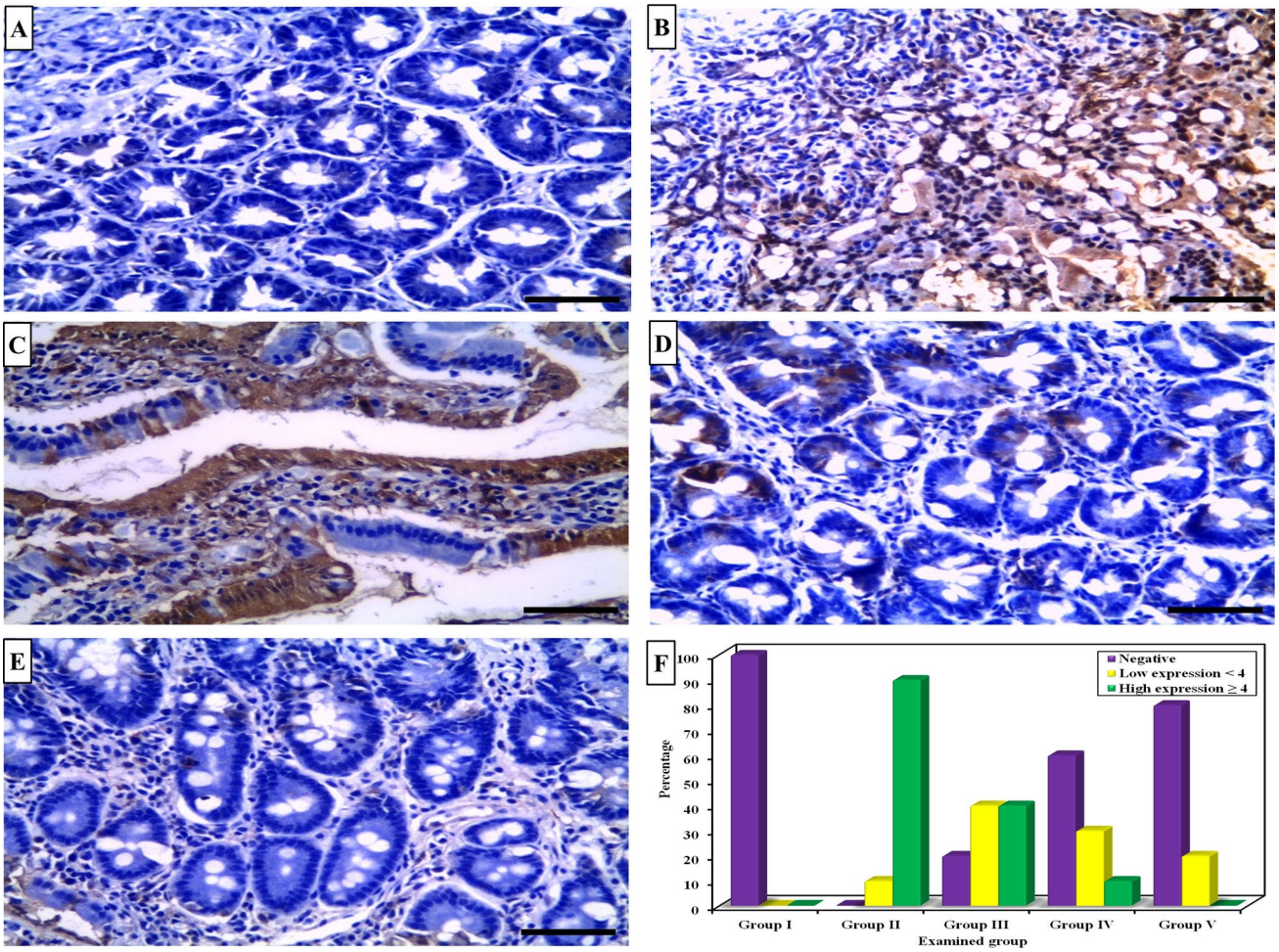

Fig. 4 Immunohistochemical expression of caspase-3 in duodenal tissues (×400). **A** The negative control group shows negative nuclear expression (score 0). **B** The positive control group shows high nuclear expression (score > 4). **C** The MTZ group shows low nuclear expression (score < 4). **D** The Vono group shows negative nuclear expression of less than 10% (score 0). **E** The combination group shows negative nuclear expression (score 0) and F immunoexpression grades of caspase-3 in all studied groups. Scale bar = 50 µm

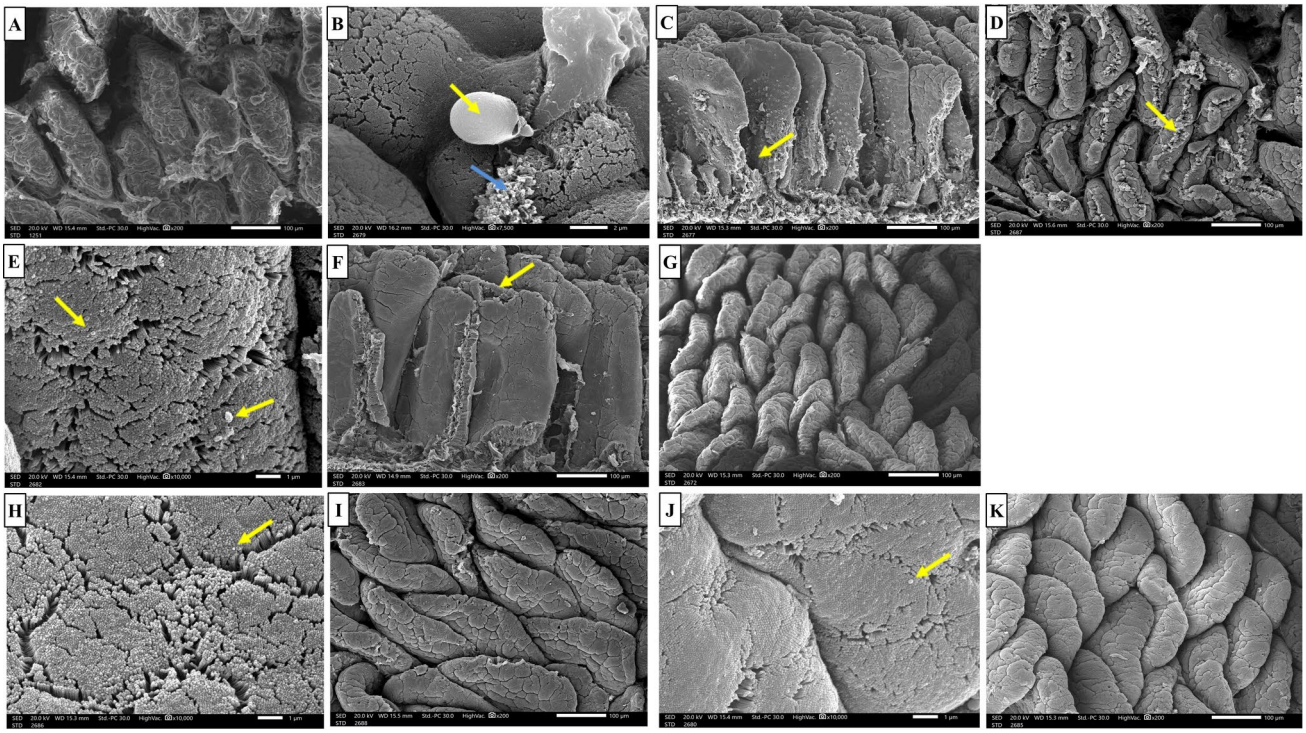

Fig. 5 Scanning electron microscopic study of the duodenal villi. **A** The negative control group shows normal appearance of the intestinal villi and no vesicle formation. **B** The positive control group shows degeneration of the tips of the villi with small (blue arrow) and large vesicles (yellow arrow) formation. **C** The positive control group shows marked destruction of the intestinal villi especially at the base and middle of the villi. **D** The positive control group shows degeneration of the lateral borders of villi. **E** The MTZ group shows the presence of a few vesicles at the tips of the villi. **F** MTZ group shows mild improvement of degenerative changes with necrosis of the tips of the villi. **G** The MTZ group shows mild improvement of degenerative changes with necrosis of the tips of the villi. **H** The Vono group shows minimal vesicle formation on the tips of the villi. **I** The Vono group shows moderate improvement of the intestinal villi. **J** The combination group shows good organization of the intestinal villi with minimal vesicle formation, and **K** the combination group shows a nearly normal appearance of the intestinal villi

showed nearly normal intestinal villi with good organization and minimal vesicle formation.

Biochemical results

Levels of IL-6 and iNO in the serum

The IL-6 levels in the positive control group and all treated groups were significantly elevated compared to the negative control group. In comparison to the positive control group, its levels significantly decreased in all treated groups. Between the MTZ and VONO groups, there was no significant difference. Comparing the combination group to the other treated groups, the mean level of IL-6 was significantly lower (Table 5).

The mean levels of iNO in the positive control group and all treated groups were significantly higher than those of the negative control group. In contrast, its levels in the MTZ, the VONO, and the combination groups were significantly reduced compared to the positive control group. The difference in its levels was not significant between the VONO and MTZ groups. The combination group showed a significant reduction compared to the MTZ and VONO groups. So, the mean level of iNO in the combination group was the lowest among all treated groups (Table 5).

Levels of MAD and TAC in the serum

The mean levels of MAD in the positive control group and all treated groups were significantly higher than in the negative control group. Its levels in all treated groups were significantly lower than those in the positive control group. Its levels did not significantly vary between the MTZ and VONO groups. Comparing the combination group to the other treated groups, a significant reduction was reported. On the other hand, the mean levels of TAC in the positive control group and all treated groups were significantly lower than those of the negative control group. However, in the MTZ, VONO, and combination groups, its levels were significantly higher than those of the positive control group. There was no significant difference between the VONO and

Table 5 Levels (mean ± SD) of IL-6 and iNO in the serum ($n = 5$)

		G I (negative control)	G II (positive control)	G III (MTZ)	G IV (Vono)	G V (Combination)
Interleukin-6 (IL-6) (pg/ml)	Mean ± SD	$205.1^{bcde} \pm 16.51$	$775.7^{acde} \pm 66.13$	$448.5^{abe} \pm 29.22$	$436.3^{abe} \pm 30.57$	$301.4^{abcd} \pm 21.15$
	F	**169.796***				
Inducible nitric oxide (iNO) (Mmol/L)	Mean ± SD	$18.56^{bcde} \pm 2.57$	$82.97^{acde} \pm 6.51$	$50.25^{abe} \pm 4.77$	$48.58^{abe} \pm 3.19$	$30.59^{abcd} \pm 4.11$
	F	**150.695***				

F, *F* for one-way ANOVA test; *n*, the number of studied rats in each group

[a]Significant compared to G I

[b]Significant compared to G II

[c]Significant compared to G III

[d]Significant compared to G IV

[e]Significant compared to G V

*Statistically significant at $p \leq 0.05$

MTZ groups. The combination group exhibited a significant rise compared to MTZ and VONO groups (Table 6).

Discussion

Giardiasis is a serious public health issue caused by *G. lamblia*. The medications used to treat it cause unfavorable side effects. Besides, drug resistance is another challenge in its management (Auriostigue-Bautista et al. 2022). In the present study, we assessed the effectiveness of vonoprazan as a potential anti-giardiasis treatment compared with metronidazole.

The MTZ group in the present work demonstrated a 54.29% decline in the count of *G. lamblia* cysts in the stool. These findings align with several earlier studies that revealed reduced effectiveness of MTZ against *G. lamblia* (Bezagio et al. 2017; El-Kady et al. 2021; Baz et al. 2022; Shady et al. 2024). This could be explained by the emergence of MTZ resistance, as documented by Ansell et al. (2017), who proposed the downregulation of MTZ activation enzymes that convert MTZ into reactive toxic intermediates as one of the resistance mechanisms in giardiasis. In contrast, the VONO group displayed a greater reduction percentage in cyst count (71.43%) and trophozoite count (85%). According to the in vitro study by Sallam et al. (2024), vonoprazan caused *G. lamblia* trophozoites to entirely lose their pear shape, elongate, lose some of their flagella, and develop erosions on their dorsal surface. These results support the findings of the present study.

The highest reduction percentages in the cyst (92.86%) and trophozoite counts (100.0%) were reported in the group that received the combination treatment. This indicates that the combination therapy (MTZ-VONO) was more effective than either MTZ or vonoprazan alone at killing *G. lamblia*, pointing to a synergetic action of MTZ and vonoprazan. In the same way, Sallam et al. (2024) described a better efficacy of the combination

Table 6 Levels (mean ± SD) of MDA and TAC in the serum ($n = 5$)

		G I (negative control)	G II (positive control)	G III (MTZ)	G IV (Vono)	G V (Combination)
Malondialdehyde (MDA) (nmol/L)	Mean ± SD	$58.61^{bcde} \pm 4.29$	$185.2^{acde} \pm 12.01$	$117.1^{abe} \pm 7.58$	$114.2^{abe} \pm 8.94$	$81.42^{abcd} \pm 7.23$
	F	**162.759***				
Total antioxidant capacity (TAC) (mm/L)	Mean ± SD	$0.20^{bcde} \pm 0.02$	$0.04^{acde} \pm 0.01$	$0.10^{abe} \pm 0.02$	$0.11^{abe} \pm 0.01$	$0.16^{abcd} \pm 0.01$
	F	**89.833***				

F F for one-way ANOVA test, *n* the number of studied rats in each group

[a]Significant compared to G I

[b]Significant compared to G II

[c]Significant compared to G III

[d]Significant compared to G IV

[e]Significant compared to G V

*Statistically significant at $p \leq 0.05$

(MTZ-VONO) against *G. lamblia* trophozoites than either drug alone.

The inhibition of oxygen consumption and the damage to the DNA of the parasite are the established main mechanisms of action of metronidazole against *G. lamblia* (Argüello-García et al. 2020; El-Gendy et al. 2021). So far as we know, this is the first investigation of vonoprazan's efficacy against giardiasis in vivo. The mechanisms of action of vonoprazan against this parasite have not been recognized yet. In addition to its direct lethal effect on the trophozoites, we propose that it may interfere with the adhesion and fixation of *G. lamblia* trophozoites, as evidenced by Zhang et al. (2023), as a mechanism of action of vonoprazan against *Helicobacter pylori*. Furthermore, it may manipulate the pH level. It raises the intragastric pH by causing a quick, significant, and long-lasting reduction of gastric acid secretion (Kiyotoki et al. 2020). *G. lamblia* excystation is a pH-dependent process initiated by acidic circumstances encountered in the stomach, so vonoprazan can prevent the excystation process (Travers et al. 2016). Moreover, vonoprazan may act similarly to PPIs such as omeprazole by inhibiting giardial triosephosphate isomerase. This impairs metabolic processes critical for trophozoite survival (Enríquez-Flores et al. 2011; Reyes-Vivas et al. 2014; López-Velázquez et al. 2019).

Histopathological study of the intestinal mucosa of rats from the positive control group revealed marked inflammatory changes, increased goblet cells, marked epithelial hyperplasia, mucosal erosions and ulcerations, and thickening of the mucosal muscle layer. These findings are consistent with Amaral et al. (2021).

Both MTZ and VONO groups showed partial improvement of mucosal architecture, fewer inflammatory and goblet cells, less epithelial hyperplasia, fewer erosions and ulcerations, decreased thickness of the mucosal muscle layer, and less damage and inflammation. Likewise, other researchers reported incomplete restoration of the mucosa following the administration of metronidazole in giardiasis (Aly et al. 2013; Ammar et al. 2014). Nevertheless, in the current work, the improvement in intestinal pathology and inflammation was higher in the VONO group than in the MTZ group. Furthermore, the combination group (MTZ-VONO) showed the best results in the histopathological study. There was nearly normal mucosal architecture, reduced inflammatory and goblet cell counts, absence of mucosal ulceration, and normal muscle thickness. Vonoprazan potentiated the efficacy of MTZ in alleviating intestinal pathology. Interestingly, vonoprazan is used in the treatment of gastric and duodenal ulcers (Garnock-Jones 2015). It has antiulcer properties because it inhibits stomach hydrogen-potassium adenosine triphosphatase (ATPase), reverses K + -competitive ionic binding, and prevents the crucial last stage of gastric acid secretion that may enhance the repair of mucosal erosions and ulcerations (Kagawa et al. 2016).

The results of the SEM of the intestinal mucosa supported our histopathological findings. The positive control group showed signs of brush border damage, marked destruction and shortening of the intestinal villi, and multiple vesicle formations. Similarly, many other studies showed the same result in giardiasis (Fathy 2011; Shukla et al. 2019). In the treated groups, all mucosal alterations detected in the positive control group began to return to normal. MTZ and VONO groups showed mild to moderate improvement of intestinal villi and mild improvement of degenerative changes, with few vesicle formations. The decreased efficacy of MTZ against giardiasis was documented in earlier research by Nabarro et al. (2015) and Bezagio et al. (2017). They explained these results by the appearance of resistance to MTZ by *Giardia* parasites. The significant improvement in the pathological alterations in the combination group herein coincides with data shown by García-Torres et al. (2016), Hernández-Ochoa et al. (2017), and Argüello-García et al. (2020), who used other PPIs in the treatment of giardiasis.

The pathogenesis of giardiasis is multifactorial. In addition to the mechanical damage brought on by trophozoite attachment to the host epithelial cells, the most crucial elements include inflammation, apoptosis, and oxidative stress (Hanevik et al. 2007).

Pro-inflammatory cytokines such as TNF-α and IL-6 have been shown to mediate intestinal inflammation in giardiasis (Goyal and Shukla 2013; Zoghroban et al. 2023). Excessive production of TNF-α damages the integrity of the epithelium and consequently induces digestive tract ulceration (Tourani et al. 2018). The positive control group in this study had the highest expression of TNF-α. This result is consistent with that of Khedr et al. (2021). In comparison with the positive control group, the MTZ and VONO groups showed a significant decrease in TNF-α expression. The combination group reported a significant reduction compared to other treated groups. Vonoprazan in the present work potentiated the effect of metronidazole in reducing this inflammatory marker. It is the first time to assess the effects of vonoprazan on TNF-α expression. Its effects may be similar to those of omeprazole, as it helped in the elimination of acute systemic inflammation via suppression of TNF-α production (Balza et al. 2016).

A major factor in boosting the immune response against *G. lamblia* is the proinflammatory cytokine IL-6 (Zhou et al. 2003; Kamda et al. 2012). It alters the IgA response and promotes T-cell proliferation, antibody synthesis, and B-cell differentiation (Vazquez et al. 2015; Groza et al. 2022). Our results revealed a significant upregulation in the serum levels of IL-6 in the positive control group compared with the

negative control. This confirms the preceding reports by Ahmed et al. (2015) and El-Kady et al. (2021).

The treatment with MTZ significantly downregulated IL-6 levels, which matches with Yuan et al. (2019), El-Kady et al. (2021), and Girish et al. (2021). The combination group (MTZ-VONO) displayed the lowest level of IL-6 among all the treated groups. This also may denote a synergistic effect of vonoprazan and MTZ. Vonoprazan might have a suppressive effect on proinflammatory cytokines, including IL-6, similar to lansoprazole and other PPIs (Chanchal et al. 2016; Nishi et al. 2018; Nakatake et al. 2019). Peng et al. (2014) pointed to the role of NF-κB inhibition on proinflammatory cytokines such as IL-6.

Apoptosis is a substantial player in the fight between *Giardia* trophozoites and the mucosal barrier of the host (Kapczuk et al. 2020). *Giardia* induces apoptosis in the intestine of the host by DNA fragmentation and upregulation of caspase-3 (Panaro et al. 2007; Kapczuk et al. 2020). Our results demonstrated strong caspase-3 expression in the small intestinal tissues in the positive control group, which coincides with the prior findings by Perrucci et al. (2019) and El-Kady et al. (2021). Reduced caspase-3 expression was reported in all treated groups with varying scores. Following the administration of combined treatment (MTZ-VONO), its expression became negative. In the same context, preceding studies on lansoprazole attributed its antiapoptotic effects to the reduction in caspase-3 levels (Fornai et al. 2009; Khaleel et al. 2017).

NO is a key factor in the innate immunity against *G. lamblia* infection. It can halt the encystation-excystation processes and the reproduction of *G. lamblia* trophozoites (Pavanelli et al. 2010). However, during *G. lamblia* infection, an increased iNO level contributes to the destruction of intestinal epithelial cells (Ajdary et al. 2009; Prolo et al. 2014). In the current study, iNO serum levels reported a significant rise in the positive control group compared to all other groups. This agrees with Zoghroban et al. (2023). Treatment with MTZ or vonoprazan significantly lowered iNO levels, and the combination group showed the lowest levels among all treated groups. We suggest that vonoprazan might have a mechanism of action similar to PPIs in manipulating NO synthesis. PPIs reduce NO generation by increasing intracellular levels of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NO synthesis (Nolde et al. 2021; Ghebremariam et al. 2013).

During the battle between *G. lamblia* and the host immune response, there is excessive formation of reactive oxygen species and free radicals, which damage the host's intestinal mucosa (Khedr et al. 2021). MDA is the terminal product of lipid peroxidation and an indicator of the change in the redox status (Todorova et al. 2005). In the present study, significant upregulation in MDA and downregulation in TAC were detected in the positive control group. In the same way, numerous prior studies demonstrated supportive findings (Mahdi 2009; Khedr et al. 2021; Ismail et al. 2022). The significant reduction in MDA levels and elevation in TAC in the MTZ group, comparable with the positive control group, went hand in hand with the findings of Ansell et al. (2017). Additionally, the VONO group showed similar results to the MTZ group. This points to the antioxidant effects of vonoprazan, which align with the findings of Shoman et al. (2020) on the effects of PPIs in the protection of cardiac cells. Besides, a significant decrease in oxidative stress in the combination group (MTZ-VONO) was evidenced by the significant lowering in MDA levels and elevation in TAC compared with the other treated groups.

From the above results, we could conclude that the upregulation of TNF-α, IL-6, and iNO levels, the state of oxidative stress, and apoptosis may help explain the pathological alterations in the mucosa in the positive control group in this study. Noteworthy, vonoprazan succeeded in potentiating the efficacy of metronidazole in combating all these factors. This could explain the healing of the intestinal mucosa that appeared in the combination group, compared with the positive control and the other treated groups. Nevertheless, the current work has a certain limitation: the short follow-up duration after treatment. Some parameters (IL-6, iNO, MDA, and TAC) still showed significant differences compared to the negative control, which indicates that recovery should be reassessed in further studies, 1 or 2 weeks later.

To summarize, our findings point to the giardicidal effects of vonoprazan, as it successfully reduced the total cyst and trophozoite counts and combated intestinal pathology. The combination (MTZ and vonoprazan) was superior to MTZ alone in treating giardiasis. This could be attributed to the powerful anti-inflammatory, antiapoptotic, and antioxidant effects of vonoprazan. Therefore, vonoprazan should be regarded as an adjuvant medication for the treatment of *G. lamblia* infection, and more research on its application in humans is worthwhile.

Author contributions K.E. and D. E. designed the study. A.S. and D.E. performed the practical research. H.A. performed histopathology analyses. All authors shared in writing the original draft and revising and editing the manuscript. All authors approved the publication of the version.

Data availability No datasets were generated or analysed during the current study.

Declarations

Consent to participate Done and confirmed.

Consent for publication Done and confirmed.

Competing interests The authors declare no competing interests.

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