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IN VITRO SUSCEPTIBILITY OF *GIARDIA LAMBLIA* TO VONOPRAZAN

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Abstract

Vonoprazan (Vono) is used to manage duodenal and gastric ulcers, reflux esophagitis, and prevention relapse. Its' a mechanism of action similar to proton pump inhibitors (PPIs), with a more rapid and prolonged acid-suppressive action, unaffected by diet and gene polymorphisms.

This study assessed the therapeutic effect of Vono on *Giardia lamblia in-vitro* study as compared with metronidazole (MTZ). The study used five cultures of 10ml of medium each and were divided into: Culture I: medium only (positive control), culture II: inoculated with 10^4 trophozoites, culture III: inoculated with 10^4 trophozoites and treated with MTZ 50mg/ml, culture IV: inoculated with 10^4 trophozoites and treated with Vono 50µm/ml, and culture V: inoculated with 10^4 trophozoites and treated with the same of concentration both drugs. All cultures were subjected to trophozoites count and scanning electron microscope study. The mean number of trophozoites in all treated cultures: MTZ (MTZ), Vono, and combination cultures showed significantly decreased compared to positive control. The mean trophozoite counts in both Vono culture and combination culture were significantly lower than that in MTZ culture. Also, the mean trophozoites count in combination culture was significantly lower than in Vono culture and MTZ culture. SEM showed marked destruction of trophozoites morphology in the combination group as contrasted to the positive control group.

Keywords: Vonoprazan, Metronidazole, *Giardia Lamblia*, *In-vitro,* RPMI media, SEM.

Introduction

Giardia lamblia (G. lamblia) infection is one of the most prevalent causes of gastrointestinal disorders worldwide. It affects a large scale of animal hosts as well as humans (Li *et al*, 2007). The condition is a significant public health issue, particularly in developing nations where its prevalence rate may reach up to 50% (Painter *et al*, 2011). Up to 30.2% of people in Egypt have human giardiasis (El-Badry *et al*, 2017).

Giardiasis might be asymptomatic or may cause chronic or acute diarrhoea accompanied by abdominal discomfort, intestinal lesions, weight loss, and mal-absorption syndrome, which might persist for days to many months (Halliez *et al*, 2013). The immunocompromised people, travelers, international adoptees to the highly endemic areas with inadequate sanitation are the most giardiasis risky populations (Takaoka *et al*, 2016). But, the infants and children are at the higher risk suffering from the severe infection (Skogen *et al*, 2018). The chemotherapeutics, such as metronidazole, tinidazole, nitazoxanide, and paromomycin are used in giardiasis treatment (Watkins *et al*, 2014).

Regarding *G. lamblia*, Glycolysis is seen as a viable biological target for the development of innovative anti-giardiasis drugs (Reyes-Vivas *et al*, 2014). This metabolic route is regarded as a primary source of adenosine triphosphate (ATP) generation for Giardia, since it doesn't perform oxidative phosphorylation (Han and Collins, 2012). The enzyme triosephosphate isomerase is among *Giardia's* glycolytic enzymes and is a potential drug target (García-Torres *et al*, 2016). Proton pump inhibitors (PPIs) like pantoprazole and omeprazole are commonly prescribed for gastroesophageal hyperacidity. Their antiparasitic activity was investigated against many parasites (Sheele *et al*, 2017). They showed effectiveness against helminthes such as *Schistosoma mansoni* (Ellakany *et al*, 2019). Besides they exhibited antiprotozoal activity against *G. lamblia*, *Trichomonas vaginalis,* and *Entamoeba histolytica* (Reyes-Vivas *et al*, 2014). The PPIs can inactivate glycolytic enzymes such as triosephosphate isomerase enzyme of these parasites (Haastrup *et al*, 2018).

Vonoprazan (Vono) is a novel potassium-competitive acid blocker inhibits the gastric

acid secretion by preventing binding of potassium to gastric hydrogen-potassium ATPase (Echizen *et al*, 2016). It is used in the management of duodenal and gastric ulcers, reflux esophagitis and prevention of their relapse. Furthermore, it showed high effectiveness in the eradication of *Helicobacter pylori* (Echizen *et al*, 2016). It has a mechanism of action similar to proton pump inhibitors (PPIs), but more rapid and prolonged acid-suppressive action, unaffected by diet and gene polymorphisms (Echizen *et al*, 2016).

This study aimed to assess the impact of Vono on *Giardia lamblia* by *in vitro* study in comparison with MTZ.

Materials and Methods

Ethical approval: The study protocol was approved by the Ethics Committee Faculty of Medicine, Tanta University (approval code: 36166/12/22).

Parasite: Fecal samples were obtained fresh from patients attended to a private laboratory in Tanta. A convenient sampling approach was employed. Analysis of six positive samples showed the existence of four cysts and two trophozoites used in the study. The continuous gradient was employed to purify *Giardia* (Roberts-Thomson *et al,* 1976). The purified cyst suspension was diluted 1:10 in isotonic saline, and 10mM diluted aliquots were sent to a hemocytometer for cyst count.

Cyst viability assessment: The viability of cysts was evaluated for potential excystation utilizing the trypan blue exclusion technique (Bingham *et al,* 1979). These cysts restricted dye penetration, while non-viable ones absorb the dye. The cleaned cyst solution was combined with an equivalent amount of trypan blue dye in a 5ml test tube. Ten microliters of the suspension were deposited onto a haemocytometer under a cover slip. Cysts were examined by light microscope at 40X for dye absorption and counting. The viable cysts were calculated by putting the stained and unstained ones in four huge squares of counting chamber and individually assessed as: Viability% = unstained cysts/total cysts ×100.

Excystation of *Giardia lamblia* cysts was done by using the acid induction technique. Purified cysts were liquefied in duplicate of 0.1ml cyst solution, comprised between 100 and 10,000 viable cysts/ml, was combined with 0.9 ml of 1 M HCL at pH 2 in test tubes, which were incubated at 37°C for an hour, and centrifugation at 30,000rpm for 10 minutes at ambient temperature (Al-Tukhi *et al,* 1991). The specimens were subsequently aseptically transferred to a plate and analyzed for excysted motile trophozoites identification. The supernatant acid was carefully decanted, and the preparation was purified of acid via resuspending in RPMI medium and centrifuging as previously reported.

Drug regimens: 1-Metronidazole suspension 125mg/5ml (Amriya Pharmaceutical Company, Cairo). 2-Vonoprazan tablet: 20mg (Inspire Pharmaceutical Company, Cairo).

Culture media preparation: 1-45ml of complete medium CRPMI: Roswell Park Memorial Institute (RPMI) (Maadi Medical Supplies Company, Cairo) is a basal medium consisting of Vitamins: 35mg inositol; 1 mg each of paraaminobenzoic acid, 3mg choline chloride; folic acid, thiamine hydrochloride andpyridoxine hydrochloride; enicotin-amide, 0.25mg calcium pantothenate; 0.2 mg each one of biotin & riboflavin 0.005mg cyanocobalamin. Amino acids: 300mg glutamine; 200mg arginine; 50mg each of asparagine, leucine, cystine, and isoleucine; 40mg lysine hydrochloride; 30mg serine; 20mg each one of aspartic acid, glutamic acid, proline, hydroxyproline, threonine, tyrosine, and valine; 15mg each of histidine, methionine, and phenylalanine; 10mg glycine; 1mg reduced glutathione; 5mg tryptophan. Salts: 6g NaCl, 2g sodium bicarbonate, 1.521g disodium phosphate, 400mg KCl, 100mg magnesium sulfate, and 100mg calcium nitrate. Glucose: 2g. Glutathione and a pH indicator: 5mg phenol red, lacked neither proteins nor growth-promoting substances; 2-0.5cm (gentamicin 20mg) and 5ml of bovine serum All from Maadi Medical Supplies Company, Cairo.

Establishment of excysted trophozoites *in-vitro* cultures (Elnazeer *et al*, 2016): Excyst-

ed trophozoites were put in duplicate into three sets of flask tubes, each containing 5ml of RPMI medium supplemented with 0.5cm of gentamicin (20mg) and 10% bovine serum. Flasks underwent incubation anaerobically at 37°C, and parasite's count was evalauted by using a haemocytometer over a 24, 48, &72hrs, the old medium was passaged to new one. On growth, MTZ and Vono drugs were added to all cultures and trophozoites were counted after 24hrs to evaluate treatment efficacy

The cultures were divided into Culture I: with 10 ml of medium only, culture II: with 10ml of medium inoculated with 10^4 trophozoites, culture III: with 10ml of medium inoculated with 10^4 trophozoites and treated with MTZ 50mg/ml, culture IV: with 10 ml of medium inoculated with 10^4 trophozoites and treated with Vono 50µm/ml, culture V: with 10ml of medium inoculated with 10^4 trophozoites and treated with MTZ 50mg/ml and Vono 50µm/ml.

Evaluation of drugs *in vitro*: *G. lamblia* trophozoites were counted after 24hrs using a haemocytometer. Total trophozoites numbers in ml were calculated as mean of five counts (Pintong *et al*, 2020).

Scanning electron microscope (SEM): Assessed trophozoites morphological changes before and after treatment. Trophozoites were washed with PBS, fixed for 1hr in2.5% glutaraldehyde in PBS, & adhered to poly-lysine-coated coverslips. The fixed cells were washed three times with PBS and then post-fixed for 1hr in 1% osmium tetroxide in deionized water. The cells were rinsed with PBS, dehydrated in ethanol, to critical point dryness with Co2, and coated with gold (Shareef *et al*, 2014). SEM assessment was done Electron Microscopy Unite, Faculty of Science, at Alexandria University.

Statistical analysis: Data were computerized and analyzed by SPSS v26 (IBM Inc., Chicago, IL, USA). Quantitative was displayed as mean and standard deviation (SD) and contrasted with different groups using ANOVA (F) test with post hoc test (Tukey). $P < 0.05$ was considered significant.

Results

The trophozoites in treated cultures: MTZ (3617.0±403.7), Vono (5088.0±130.4), and combined culture (100.0±173.2) showed significantly decreased ($P < 0.001$) compared to positive control (9340.0±1502.7). Reduction in trophozoite counts in MTZ, Vono, and combined cultures were 61.27%, 45.52%, & 98.93%, respectively. Mean trophozoites count in Vono culture ($P= 0.041$) and in combined one ($P <0.001$) were significantly lower than in MTZ culture. Mean trophozoites count in combined culture was significantly lower than in Vono one ($P <0.001$).

SEM showed trophozoites in positive control culture were pear shape, four pairs of flagella, and adhesive disc on ventral surface. Trophozoites from MTZ culture showed loss of pear shape, depressions, dimples, irregularities, and perforation on the outer surface, bur that of Vono culture showed loss of pear shape, loss of some flagella, erosions, and deformities on trophozoite outer surface. Combined culture showed cell depressions with loss of pear shape, complete flagella, cellular contents, and erosions on touter surface indicating their death.

Details were given in table (1) and figures (1 & 2)

Table 1: Comparison between tvarious cultures groups as to total trophozoite counts

Variations	Culture II	Culture III	Culture IV	Culture V	F
Trophozoite total No. /m	1502.7±9340.0	3617.0±403.7	5088.0±130.4	100.0±173.2	118.600*
Reduction%		61.27	45.52	98.93	
p_1		<0.001*	<0.001*	<0.001*	
p_2			0.041*	<0.001*	
p_3				<0.001*	

. *Significant P <0.05, P1 value compared with Culture II, P2value for comparison with Culture III, P3alue for comparison with Culture IV, Culture II: Positive control, Culture III: MTZ group, Culture IV: Vono only Culture V: Combined drugs.

Discussion

Generally speaking, *Giardia lamblia* (=*G. duodenalis* and *G. intestinalis*) is among the most prevalent causes of gastrointestinal disorders worldwide (El Shazly *et al*, 2007). Giardiasis was reported in the Eastern Mediterranean Countries and North Africa (Hijjawi *et al,* 2022), Egyptian Children (Morsy

et al, 2023), Jordan (Nimri, 1994), Lebanon (Arslanian, 1960), Libya (Saaed and Ongerth, 2019), Saudi Arabia (Awadallah and Morsy, 1974), Syria (Almerie *et al,* 2008) and other regional countries.

Person-to-person transmission can occur in settings with animals fecal incontinence and poor hygiene, such as childcare centers, risk of acquisition or transmission was greatest for young children who didn't yet toilet trained; who can also serve as a source for secondary cases within the households as well as, can be transmitted via heterosexual or homosexual anal-oral sexual contact (CDC, 2021). Apart from man, giardiasis are common in pigs, cattle, sheep, goats, elks and deer, other ruminants, and dogs and cats as well as man (Cacciò *et al,* 2018)

In Egypt microscopy-based prevalence of giardiasis was 24.2% in Benha City school children (Curtale *et al,* 1998), which was consistent with previous Egyptian estimations that ranged from 24.7% to 27.9% or even lower prevalence rate (7.9%) in Mansoura City rural inhabitants (el-Beshbishi *et al,* 2005). Ahmad *et al.* (2020) in Upper Egypt reported that among children Giardia assemblage A (45.7%), assemblage B and mixed A & B infections were detected in 31.4% & 22.8% respectively, but assemblage E was not detected. They added that assemblage A was dominant among children with diarrhea and abdominal cramps. Abd El-Latif *et al.* (2020) in Alexandria reported that *Giardia* assemblages A & B were in diarrheic children and in raw water samples.

In the present study, *G. lamblia* trophozoites were effectively grown and sustained in RPMI media. This agreed with (Basco, 2023), who reported that the RPMI medium efficacy *in-vitro* due to its capacity to preserve the viability and some physiological processes.

In the present study, MTZ culture showed a reduction percentage of trophozoite number (61.27%), while Vono culture showed a reduction percentage (45.52%). The combination culture MTZ-Vono showed the highest reduction (98.93 %), indicating that Vonoprazan proved to be anti-*Giardia* effects. These effects may be attributed to the affection of *Giardia lamblia* trophozoite motility via the action of Vono on potassium ion channels. Studies on protozoan parasites reported that Ion channels provide a crucial physiological purpose as controllers of cellular activities and interactions between hosts and parasites (Jimenez *et al*., 2022). Flagellar movement is essential for the movement of *Leishmania* parasites (Beneke *et al*, 2019).

Moreover, the advantage of SEM techniques well assessed the impacts of MTZ and Vono on *Giardia* trophozoites, and irregular shape of the dorsal surface, the existence of membrane bl-ebs, disc and median body fragmentation, and uneven distribution of membrane-bound components. This agreed with Campanati and Monteiro (2002) in Brazil, who reported the good impacts of the anti-protozoal agents MTZ and furazolidone on *Giardia lamblia* trophozoites. Drastic morphological changes in the *Giardia* trophozoites were demonstrated following both MTZ and Vono treatment, which included partial loss of pear shape, elongation, loss of some flagella, and erosions on the outer surface (Benchimol *et al,* 2022)

In the present work, the combination culture (MTZ-Vono) showed complete loss of pear shape, complete loss of flagella, loss of contents and cells death. Therefore, Vono was able to potentiate the effect of MTZ on *Giardia* trophozoites. These effects of Vono on *Giardia* trophozoites may be similar to those of other PPIs such as omeprazole. Omeprazole was reported to target *Giardia* triosephosphate isomerase enzyme and consequently affect glycolysis inducing cytotoxicity toward *Giardia* trophozoites causing the dysfunction of many chemicals and metabolic pathways that together are essential for trophozoites survival (López-Velázquez *et al*., 2019).

In the present work, Vono has giardicidal effects as detected by its ability to reduce the total trophozoite counts and induce morphological changes in the trophozoites. The combination of MTZ and Vono was superior to MTZ alone in killing *Giardia* trophozo-

ites. Vono was able to potentiate the effect of MTZ. This could be attributed to its powerful effects on the motility of *Giardia* trophozoites.

Apart from giardiasis treatment, Chey *et al.* (2022) in USA, reported that the US/FDA, the efficacy of vonoprazan-based dual and triple therapy regimens in the *H. pylori* treatment was evaluated in the randomized, active-controlled, phase III PHALCON-HP trial, conducted in the USA and the European Union. Moreover, Shirley (2024) in New Zealand gave a review study of the effectiveness of Vonoprazan in treating humans infected with Helicobacter pylori He concluded that Vonoprazan is a first-in-class potassium-competitive acid blocker with the potential to provide potent and sustained acid suppression as demonstrated superiority of the vonoprazan-based regimens.

Conclusions

Vonoprazan® was approved by the USA/FDA, and proved its efficacy as a novel anti-*Giardia* drug in *in-vitro* study.

The vonoprazan or vono must be considered as an adjuvant treatment for management of human giardiasis. This can reduce the side effects of metronidazole® and consequently its complications of the long-term use.

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Authors' contribution: The authors reported that they equally shared in the theoretical and practical studies. They wrote, and revised the manuscript, as well as approved its publication

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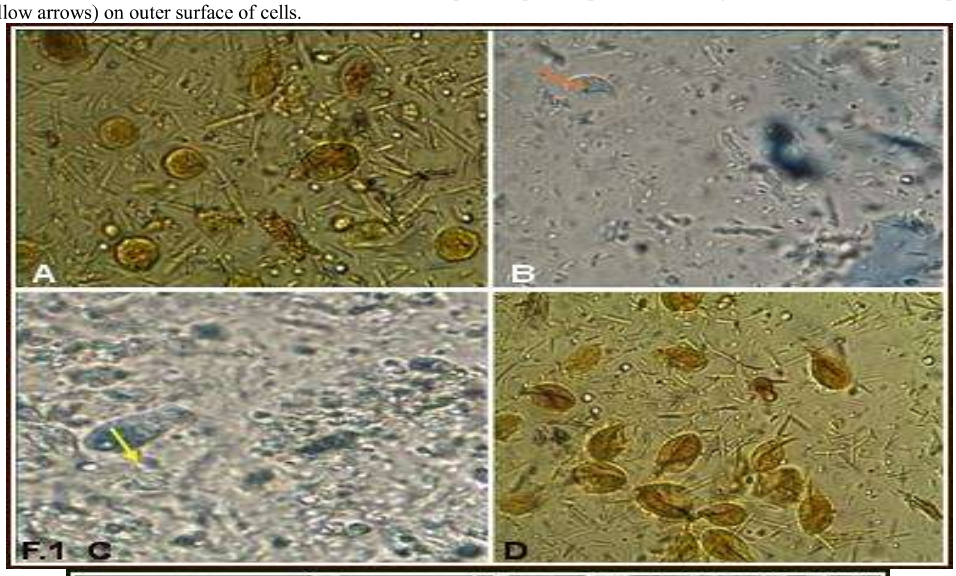
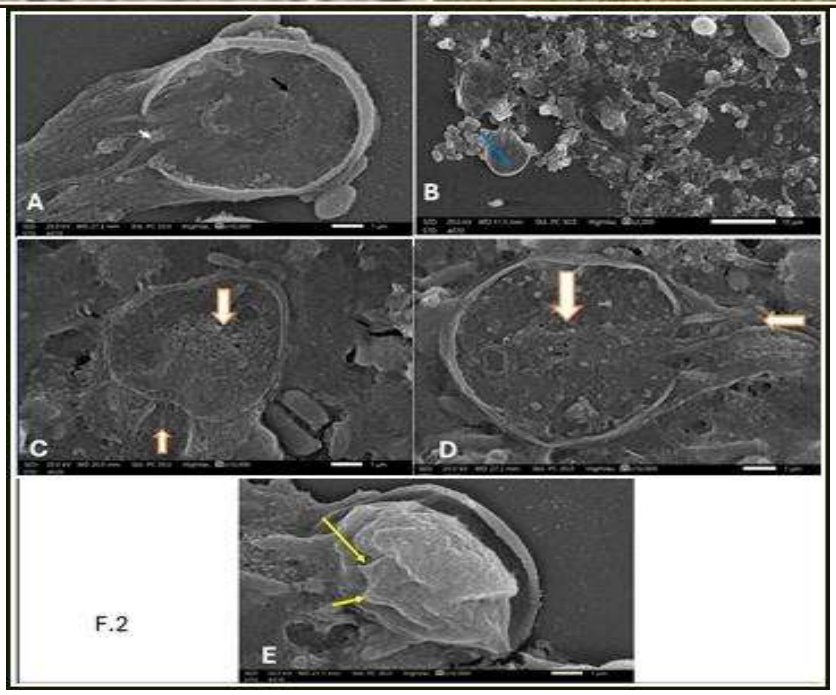
Explanation of figures

Fig.1: A- *Giardia lamblia* cysts in an iodine-stained smear x1000. B- Viability of cysts by trypan blue stain: viable cysts inhibit dye penetration (red arrow) x1000. C- While non-viable ones absorb dye (yellow arrow) x1000. D- *Giardia lamblia* trophozoites after excystation in an iodine-stained smear x1000.

Fig. 2: SEM of trophozoite from different studied cultures: **: A-** Positive control culture showing normal flagella, normal disc, and smooth outer surface of trophozoite. Sucking disc (black arrow) and intact flagella (white arrow). B- Positive control culture showed normal pear shape of trophozoite, C- MTZ culture showed loss of pear shape, irregularities, depressions, dimples on outer surface, erosion surface (thick arrow), and destroyed flagella (thin arrow), D- Vono culture showed loss of pear shape, elongation, erosion surface (thick arrow) and residual flagella (thin arrow),. E- C ombination culture showedl oss of pear shape, complete loss of flagella, loss of contents, compression and dimples yellow arrows) on outer surface of cells.





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