

DAFLON AS A NEW DRUG IN TREATING MURINE EXPERIMENTAL CRYPTOSPORIDIOSIS

By

TAHANY A. M. AL-ATTAR^{1*}, AMIRA M. MATAR¹, MONA A. KORA³
AND MONA F. FAHEEM^{1,2**}

¹Department of Medical Parasitology, Faculty of Medicine, Menoufia University,

²Department of Basic Medical Sciences, Faculty of Medicine, King Salman International University, South Sinai, and ³Department of Pathology, Faculty of Medicine, Menoufia University, Menoufia Egypt

(*Correspondence: tahany.alattar@yahoo.com; **monafaheem87@gmail.com).

Abstract

Cryptosporidiosis is a major zoonotic health problem especially in immunocompromised hosts. The current study investigated the effect of Daflon (DFL) as an alternative therapy or combined with Nitazoxanide (NTZ) against cryptosporidiosis in immuno-competent and immuno-suppressed mice. Fifty mice were divided into two major groups: GI included immuno-competent mice and GII included drug-induced immuno-suppressed mice. Each group of five mice was subdivided equally into five subgroups. Mice subgroups were either in GI or GII as follows: A: Control negative neither infected nor treated, B: Control positive *C. parvum* infected but not treated, C: *C. parvum* infected NTZ treated, D: *C. parvum* infected and DFL treated & E: *C. parvum* infected combined treated (NTZ+ DFL). Drug efficacy was done by parasitological, biochemical, histopathological, and immunohistochemical analysis. All drugs significantly reduced oocysts' number compared to their counterparts infected mice. There was an increase in SOD and a decrease in MDA serum levels in mice received combined treatment. Histopathologically, all tissues in mice received combined treatment more or less retained their normal architecture. Also, iNOS expression in treated groups turned into weak cytoplasmic expression in all tissues with the best effect in mice received combined treatment.

Keywords: Cryptosporidiosis, Mice, Daflon, Nitazoxanide, Oxidative stress, iNOS.

Introduction

Cryptosporidium species is a zoonotic coccidian protozoan that affects body's mucosal surfaces, mainly in gastrointestinal tract (Abenoja *et al*, 2021). *Cryptosporidium* is the second major cause of moderate to severe diarrhea in children younger than two and an important cause of mortality worldwide, commonly during waterborne epidemics and in immunocompromised hosts (Bones *et al*, 2019). Besides, a treatment is sub-optimum, and does not currently have robust preventative measures, but generated self-limiting watery diarrhea in immuno-competent hosts (Abouel-Nour, *et al*, 2016). However, cryptosporidiosis may be associated with chronic symptoms, malnutrition, and other complications in high-risk factor for gastrointestinal tract (GIT) malignancies, particularly *C. parvum* (El-Kersh *et al*, 2019). Egyptian infants and toddlers were the most vulnerable as cryptosporidiosis causes diarrhea

in up to 16.7% of children (El-Sayed and Fathy, 2019). The zoonotic species were *C. hominis* and *C. parvum* (Helmy *et al*, 2013). The disease severity was primarily determined by patient's immunological condition (Atia *et al*, 2021).

The only FDA-approved drug for cryptosporidiosis is Nitazoxanide[®] (CDC, 2023). But, in malnourished children showed limited efficacy (El-Ashkar *et al*, 2022), the response rate in Zambian children was 56% (Amadi *et al*, 2002). Thus, declaration the urgent need of more effective medications was a must (Sparks *et al*, 2015).

Daflon (DFL) is an oral micronized purified phlebotonic flavonoid fraction containing 90% diosmin and 10% hesperidin manufactured by Laboratoires Servier used to treat or manage disorders of the blood vessels (Ramelet, 2005). Fahmy *et al*. (2021) in Egypt reported promising actions of Daflon 500mg as an anti-giardiasis treatment.

Diosmin is an anti-oxidant, anti-inflammatory, and anti-microbial phyto-compound prevalent in citrus fruits and acts as a free radical scavenger (Islam *et al*, 2020), and hesperidin is the most abundant flavonoid in citrus fruit peels, especially lemons and oranges (Aghel *et al*, 2008). Both diosmin and hesperidin proved to be beneficial in treating hemorrhoids and chronic venous insufficiency, demonstrating pharmacological synergy and great therapeutic potential (Mustafa *et al*, 2022). So, Daflon has a wide range of medical applications, such as anti-bacterial, antioxidant, and anticancer (Park *et al*, 2008; Natarajan *et al*, 2011; Lee *et al*, 2012; Roohbakhsh *et al*, 2015). Hoseiny *et al*. (2020) in Iran found that in toxoplasmosis infected male rats; oxidative stress caused inducer of in reproductive system. But, to avoid oxidative stress damage, a balance between reactive oxygen species (ROS) and antioxidant primary defenses is a must primarily comprise antioxidant scavenging enzymes such as superoxide dismutase (SOD), glutathione peroxidase and catalase (Birben *et al*, 2012). The oxidative stress from *Cryptosporidium* was reported to cause tissue damage in pigs (Ujjwal *et al*, 2014), and in mice (Bhagat *et al*, 2017). Decreasing oxidative stress has a functional immune pool capable to remove pathogens and limit host tissue damage (Tsikas, 2017).

The present study aimed to evaluate efficacy of Daflon[®] as a safe alternative or co-drug for cryptosporidiosis in a mouse model.

Materials and Methods

Animals: This study used fifty laboratory-bred pathogenic-free, Swiss-albino male mice, 6-8 weeks old, weighed 20-25gm. Mice were purchased from Theodor Bilharz Research Institute, Biology Supply Center (Giza). Mice were examined for three consecutive days before experimental study to accommodate the conditions (Abdou *et al*, 2013). They were kept in the experimental animal house of TBRI, maintained at room temperature of (22±2°C) with free access to the usual food and water.

Ethical committee, all animal care and procedures were conducted in accordance with international ethical guidelines (Helsinki declaration, 2008). Ethics Committee for Scientific Research, Faculty of Medicine, Menoufia University approved the study protocol IRB No. 5\2023 PARA13.

Study design: Mice were divided into two main groups: GI included immunocompetent mice and GII involved drug-induced immunosuppressed mice. Each group was subdivided equally into five subgroups (A, B, C, D, & E) with five mice each. Mice groups were: GIA: immunocompetent non-infected control negative, GIB: immunocompetent *C. parvum* infected control positive, GIC: immunocompetent *C. parvum* infected NTZ treated, GID: immunocompetent *C. parvum* infected DFL treated, GIE: immunocompetent *C. parvum* infected combined (NTZ+ DFL) treated, GIIA: immunosuppressed non-infected or control negative, GII B: immunosuppressed *C. parvum* infected, but not treated or control positive, GII C: immunosuppressed *C. parvum* infected NTZ treated, GII D: immunosuppressed *C. parvum* infected DFL treated, & GII E: immune-suppressed *C. parvum* infected combined treated (NTZ+DFL).

Immune suppression animals: Mice of GII (immunosuppressed) were subjected to induce immune suppression by oral synthetic dexamethasone (Dexazone tablets 0.5mg, Al Kahira Pharmaceuticals & Chemical Industries Co., Egypt) at a dose of 25µg/g/day dissolved in 200µl of distilled water/mouse by an esophageal tube. The immune suppression started 14 days before *C. parvum* infection and continued throughout the study.

Cryptosporidium parvum oocysts were obtained from naturally infected calves (at Slaughterhouses). Intestinal contents and ileal mucous membrane scraps (Anderson, 1985) were stained by modified Ziehl-Neelsen (MZN) staining and microscopically examined for oocysts. Concentration of oocysts by floatation in Sheather's sugar solution, sediment was collected and stored at 4°C in a 2.5% potassium dichromate solution (Cur-

rent *et al.*, 1983). For mice infection, *C. parvum* oocysts were concentrated and counted in phosphate-buffered saline (PBS) solution by a hemocytometer and were adjusted to have a concentration of 1×10^5 oocysts in 200 μ l PBS using a gastric gavage (Benamrouz *et al.*, 2012). Before mice inoculation, 12 hours fasting period was required to facilitate infection procedure. Stool samples were regularly examined for oocysts to confirm the infection, using formol-ether concentration method, stained by MZN, and examined by light microscope (Youssef *et al.*, 2008).

Treatment: 1- Nanazoxide[®] (Nitazoxanide 500mg tablets) are available from Medizen Pharmaceutical industry, Utopia Pharmaceuticals, Egypt. It was administered orally via a gastric tube in a dose of 100mg/kg/day for five consecutive days after the establishment of infection. 2- Daflon[®] 500mg tablets purchased from Servier, Servier Benelux, Egypt Industries limited Company, given orally via a gastric tube in a dose of 100mg/kg/mouse diluted in DMSO for 7 days. Treated groups were either treated with NTZ alone at a dose of 100mg/kg/day or Daflon alone at a dose of 100mg/kg/mouse diluted in DMSO, or combination of Daflon & NTZ (~1/3 of dose).

Samples collection: All the surviving mice were euthanized by cervical dislocation on 30th day post-infection (dpi). Intestinal lumens of euthanized mice were flushed with ice-cold saline to wash out food particles. One cm of the terminal ileum, whole lung, and liver tissues were taken from each mouse and fixed in 10% formalin (for histopathological & immunohistochemical studies).

Blood samples were collected in 2ml Eppendorf tubes, centrifuged at 10³ rpm for 10 min., and sera were kept at -20°C until needed for biochemical analysis (Muñoz-Carrillo *et al.*, 2021).

Parasitological assessment: Fresh fecal pellets were collected on last experimental day (30th dpi), from each mouse separately, suspended in 10% formalin, homogenized, stained with MZN for shedding oocysts, and counted microscopically as mean in 10 high

power fields. Reduction % was calculated for each drug by Hosking *et al.* (1996) equation: Efficacy (%) = mean value of positive mice - mean value of all infected treated mice / mean value of infected untreated mice \times 100.

Biochemical oxidative stresses: 1- Serum level of malondialdehyde (MDA), as a lipid peroxidation product was estimated by a commercial colorimetric assay kit (CAT. No. ab118970, Kiazist). The thiobarbituric acid (TBA) reactive substances (TBARS) quantity was detected as a MDA production index in sera based on nmol/ml. MDA standard absorption curve was prepared using 1,1,3,3 tetraethoxypropane and values were expressed as mmol/l. 2- Superoxide dismutase activity in sera was determined calorimetrically using commercial kit (CAT. No. RK03200 Wuhan, China), based on SOD ability to inhibit epinephrine autoxidation of to adrenochrome in an alkaline medium (pH 10.2).

Histopathological examination: One cm of the terminal ileum, lung, and liver tissue were taken from each mouse, fixed in 10% neutral buffered formalin, dehydrated in ascending grades of ethanol, followed by immersion in xylene, and embedded in paraffin. Paraffinized blocks were cut into thin sections (4 μ m), mounted on clean glass microscopic slides, and stained by hematoxylin, and eosin (H&E) stain for microscopic examination (Gamble, 2008).

Immunohistochemical staining of iNOS: Several sections were cut from the paraffin-embedded blocks with subsequent steps of deparaffinization and rehydration in xylene and a graded series of alcohol. Antigen retrieval was done by boiling in 10mL citrate buffer (pH 6.0) for 20min, and then cooled to room temperature. Slides were incubated overnight at room temperature with rabbit recombinant monoclonal anti-iNOS antibody (Catalog No. ab283655 (RM1017); Abcam, Cambridge, UK). Optimal dilution was 1:2.000 using PBS. Then slides were deparaffinized using xylene and rehydrated in decreasing concentrations of ethanol. Antigen

retrieval by microwave heating (20min.; 10mmol/citrate buffer, pH 6.0) after inhibition of endogenous peroxidase activity (hydrogen peroxidase for 15 min) was used. The primary antibody was applied to slides and incubated overnight at room temperature in a humidified chamber. Sections were washed with PBS, incubated with secondary antibody for 15 min, and then by PBS washing. Finally, bound antibody was detected using a modified avidin-biotin labeled reagent followed by 20min. wash with PBS. A 0.1% diaminobenzidine solution was used for 5 min. as a chromogen. Slides were stained with Mayer's hematoxylin for 5-10min. Mice lung and liver tissue samples were used as positive control, and omission of primary antibody as a negative control (Gamra and el-Hosseiny, 2003).

Interpretation of iNOS IHC results: Brown cytoplasmic staining involving any number of cells was considered positive in all cases and controls. Ileal, lung, and liver tissues of all groups were assessed for 1- Expression% or positive cells were counted and given as a % of 200 cells of whole section at 200× magnification (Bahnassy *et al*, 2004), 2- Intensity of staining as graded as mild (+), moderate (++), or strong (+++), & 3- Histo-score (H score) was calculated in all positive specimens as follow: H score = 1×% of mildly stained cells + 2×% moderately stained cells + 3×% of strongly stained cells (Aeffner *et al*, 2017).

Statistical analysis: Data were represented in mean ±SD and median, by using Statistical Package for Social Science, version 26 (SPSS Inc. Released 2019, IBM SPSS statistics for Windows, version 23, Armonk, NY, Corp.). After Homogeneity, one-way ANOVA (F), with LSD as post-Hoc and Kruskal Wallis (K) with Tamhane as Post Hoc were used to compare continuous variables among different groups. Two-sided P value <0.05 was considered statically significant.

Results

There was a decrease in oocyst count for all groups of treated mice compared to in-

fectured untreated mice. In immune-competent group, lowest oocysts mean number passed was (22.0±5.61) in GIE (DFL+NTZ treated) when compared to GIB (106.0±9.97) with significant difference (P<0.001). Reduction was 79.2%. But, in GID, mean oocysts passed was 81.0±6.81, without significant (P= 0.100) with a reduction of 32.6%. In immune-suppressed group, oocytes reduction of (85.5%) was detected in GIIE with a mean oocyst count of 27.0±10.17 compared to GIIB (204.0±17.81). In GIID, mean oocysts passed was 148.0 ± 8.03 with significant difference compared to GIIB (P= 0.040) and reduction of 27.4%

Cryptosporidium infection increased the serum level of MDA in both GIB & GIIB (5.23±0.46 & 6.1±0.97, respectively), with a decrease in mean serum level of MDA in all treated mice. In immune-competent mice, mean serum MDA level in GIE showed least 1.99 ± 0.38 level compared to GIB followed by GIC & GID (2.43±0.42 & 2.6±0.82, respectively). Immune-suppressed mice given combined treatment mean serum level was 3.09±0.62 compared to GIIB 6.1± 0.96 then GIIC & GIID (3.12±0.81 & 3.99±0.84, respectively).

Infection decreased SOD serum level in GIB & GIIB (42.44±7.71 & 49.35±4.55, respectively) with increase in mean serum SOD level in treated mice. In immune-competent mice, mean serum MDA level in GIE gave highest level (91.77±2.89) compared to GIB, then GIC & GID (82.97±4.79 & 76.71±7.49, respectively). Immunosuppressed mice given combined treatment mean serum level detected (80.43±5.02) compared to GIIB then GIIC & GIID (72.98±5.003 & 68.18±5.69, respectively).

Histopathological examination of GI ileum, GIA showed preserved crypt-villous ratio with long villi, intact epithelial lining, regular brush border, and normal goblet cells. GIB showed disturbed villous architecture with blunted villous tips and broadening of villi, ulceration and focal sloughing of villous tips in the lumen, focal or total loss of

brush border and many oocysts were embedded in villous epithelium together with inflammatory cells infiltration in lamina propria and congestion. GIC showed a sort of improvement in surface epithelium with partial restoration of crypt-villous ratio and regular brush border. Focal blunting, thickening of some villi, inflammation, and congestion were still present. DFL treated mice were more or less similar to GIC improvement. NTZ+DFL treated mice showed marked improvement with restoration of villous architecture, brush border, goblet cells, minimal inflammation, but without oocysts. In GII, GIIA showed similar morphology as GIA. GIIB showed more pathological picture than GIIA. GIIC showed some improvement as GIC. GIID showed nearly same improvement as GIIC. GIIE showed marked improvement compared to GIIC or GIID.

Regarding lung GI histopathological examination, sections from GIA showed normal alveoli and bronchi without pulmonary hemorrhage or interstitial inflammation or oocysts at epithelial lining. GIB showed destruction of intra-alveolar septa, pulmonary hemorrhage and heavy lymphoplasmacytic inflammatory infiltrate of bronchial lamina propria. GIC showed improved picture with restored intra-alveolar septa, minimal pulmonary hemorrhage and interstitial inflammation. GID showed slight better improvement than GIB. GIE showed good improvement with nearly normal morphology. In GII, sections from GIIA showed normal morphology as GIA. GIIB showed a more marked deterioration in morphology than GIB. GIIC showed improvement compared to GIIB. GIID showed nearly same morphology as GIIC. GIIE showed marked improvement nearly similar to normal.

Regarding liver, histopathological examination of GIA showed preserved hepatic architecture with normal hepatocytes and portal tract. GIB showed liver congestion and marked lymphocytic infiltrate in portal tract. GIC showed some improvement with mild congestion and mild inflammatory infiltrate

in portal tract. GID showed more or less same improvement as GIC with less congestion and portal tract inflammation. GIE showed marked improvement with neglected congestion and portal tract inflammation. GIIA showed same morphology as GIA. GIIB showed more pathological picture than GIIA. GIIC showed some improvement compared to GIIB. GIID showed more or less same improvement as GIIC. GIIE showed best improvement as to GIIC or GIID with very minimal congestion and inflammation.

Immunostaining for iNOS showed moderate to strong cytoplasmic staining of inflammatory cells in villous cores and lamina propria. As to ileal tissue of GI, showed lowest mean value of iNOS-positive cells% & H score (10.0 ± 6.12 & 17.0 ± 13.96 , respectively) in GIE compared to GIB (90.0 ± 0.0 & 270.0 ± 0.0 , respectively) with $P < 0.001$ for both. In descending order, a significant reduction in percentage of iNOS-positive cells and H score compared to GIB was in GID (24.0 ± 13.41 & 46.0 ± 28.80 , $P = 0.017$ & 0.003 respectively), followed by GIC with a mean of 25.0 ± 6.12 & 58.0 ± 12.54 , respectively with $P < 0.001$. In GII, GIIE showed lowest mean value for iNOS-positive cells & H score (18.0 ± 5.70 & 28.0 ± 19.55 , respectively) with significance difference compared to GIIB (89.0 ± 4.18 & 266.0 ± 10.83 , respectively) with $P < 0.001$ for both. In descending order, a significant reduction in iNOS positive cells & H score compared to GIB was in GIIC with a mean of 29.0 ± 4.18 & 65.0 ± 9.35 , respectively & $P < 0.001$ for both), then GIID with a mean of 34.0 ± 5.47 & 76.0 ± 21.90 , respectively & $P < 0.001$ for both. No significant difference was found between GIC & GID, GIIC & GIID, GIC & GIIC, GID & GIID, or GIE & GIIE ($P \geq 0.05$).

Lung tissue immunostaining for iNOS in GI & GII showed moderate to strong cytoplasmic staining in inflammatory cells in bronchi lamina propria and interstitium between alveoli. Regarding GI, showed lowest mean value for both iNOS-positive cells &

H score (00.0±00.0) was in GIC, GID, & GIE with significant difference compared to GIB (46.0±5.47 & 138.0 ±16.43, respectively with P =0.002 for all). In GII, gave lowest mean value for iNOS-positive cells & H score (1.0 ± 2.23 for both) was observed in GIIE with statistical significance compared to GIIB (58.0±4.47 & 266.0 ± 10.83, respectively) with P<0.001 for both). In descending order, the reduction was significantly recorded (compared to GIIB) in GIIC with a mean of (0.0±0.0 for both) and P <0.001 for both), then GIID with a mean of (8.0±4.47 and 12.0±8.36, respectively) and P <0.001 for both).

Liver tissue iNOS immunostaining showed moderate to strong cytoplasmic staining within hepatocytes and interstitial tissue in between. GI showed lowest mean value for both percentage of iNOS-positive cells and

H score (0.0±0.0) in GIE (44.0±5.47 & 88.0±10.95, respectively) with significance difference compared to GIB (P=0.003 for both). In descending order, a significant reduction in percentage of iNOS-positive cells and H score compared to GIB was in GIC with a mean of 2.0±2.78 and P <0.001 for both, then GID with a mean of 7.0±2.73 & 14.0±5.47, respectively with P=0.001 for both. GII gave lowest mean value for both percentage of iNOS-positive cells & H score (00.0±00.0) was in GIIC, GIID, & GIIE with significant difference compared to GIIB (28.0±2.73 & 56.0±5.47, respectively) with P<0.001 for all. But, no significant difference was between GIC & GID, GID & GIE, GIIC & GIID, GIID & GIIE, GIC & GIIC, GID & GIID or GIE & GIIE (P ≥ 0.05).

Details were given in tables (1, 2 & 3) and figures (1, 2, 3, 4, 5, 6, 7, 8 & 9).

Table 1: Comparison between means of oocysts count and reduction% in all groups:

Group	Oocyst count		Percent of reduction	P value (K)	Post Hoc
	Mean ±SD	Median			
IA (N= 5)	0.0 ± 0.0	0.0			IB & IC <0.001
IB (N= 5)	106.0 ± 9.97	107.0			IB & ID 0.100
IC (N= 5)	41.0 ± 7.34	40.0			IB& IE <0.001
ID (N= 5)	81.0 ± 6.81	81.0			IB & IIB 0.001
IE (N= 5)	22.0 ± 5.61	21.0	61.3%		IC& IIC 0.763
IIA (N= 5)	0.0 ± 0.0	0.0	32.6%		ID& IC 0.001
IIB (N= 5)	204.0 ±	200.0	79.2%		ID&IE <0.001
IIC (N= 5)	17.81	61.0	70.0%	<0.001	ID & IID <0.001
IID (N= 5)	61.0 ± 14.23	145.0	27.4%		IE & IIE 1.00
IIE (N= 5)	148.0 ± 8.03	28.0	85.5%		IIB & IIC
	27.0 ± 10.17				<0.001
					IIB & IID 0.040
					IIB& IIE <0.001
					IID&IIC 0.001
					IID&IIE <0.001

Table 2: Mean serum level of MDA in all groups:

Group	MDA level (nmol/ml)	P value (K)	Post Hoc
IA (N= 5)	1.74 ± 0.67		IB & IC <0.001
IB (N= 5)	5.23 ± 0.46		IB & ID <0.001
IC (N= 5)	2.43 ± 0.42		IB& IE <0.001
ID (N= 5)	2.6 ± 0.82		IB & IIB 0.067
IE (N= 5)	1.99 ± 0.38		IC& IIC 0.139
IIA (N= 5)	3.004 ± 0.61	<0.001	ID& IC 0.710
IIB (N= 5)	6.1 ± 0.96		ID&IE 0.198
IIC (N= 5)	3.12 ± 0.81		ID & IID 0.004
IID (N= 5)	3.99 ± 0.84		IE & IIE 0.023
IIE (N= 5)	3.09 ± 0.62		IIB & II <0.001
			IIB & IID
			<0.001
			IIB& IIE <0.001
			IID&IIC 0.065
			IID&IIE 0.055

Table 3: Mean serum level of SOD in all groups:

Group	SOD level (nmol/ml)	P value (F)	Post Hoc
IA (N= 5)	52.09 ± 5.12	<0.001	IB&IC <0.001
IB (N= 5)	42.44 ± 7.71		IB & ID <0.001
IC (N= 5)	82.97 ± 4.79		IB& IE <0.001
ID (N= 5)	76.71 ± 7.49		IB & IIB 0.073
IE (N= 5)	91.77 ± 2.89		IC& IIC 0.011
IIA (N= 5)	57.71 ± 5.99		ID& IC 0.103
IIB (N= 5)	49.35 ± 4.55		ID&IE <0.001
IIC (N= 5)	72.98 ± 5.003		ID & IID 0.028
IID (N= 5)	68.18 ± 5.69		IE & IIE 0.004
IIE (N= 5)	80.43 ± 5.02		IIB & IIC <0.001
			IIB & IID <0.001
		IIB& IIE <0.001	
		IID&IIC 0.208	
		IID&IIE 0.002	

Discussion

Generally speaking, there are a worldwide high host burden of cryptosporidiosis, mainly among children & immunocompromised or malnourished people. Microscopy and antigen assays are useful for clinical diagnosis at genus level. Species and subtyping identification are important for outbreaks, epidemiology, burden assessment, and transmission risk. Efforts to develop vaccine are limited by insufficient understanding of the immune responses mediating protection (El-Bahnasawy *et al.*, 2018)

In the current study, the immunocompetent or immunosuppressed mice given Daflon coupled with Metronidazole (NTZ) cured cryptosporidiosis, with oocysts reduction number of 79.2-85.5%. Meanwhile, NTZ alone gave a decrease rate of 61.3-70%. But, mice given Daflon alone gave the lowest oocyst reduction rate among all groups 32.6% in immunocompetent mice and 27.4% in immunosuppressed ones. This agreed with Fahmy *et al.* (2021a), who reported that combined Daflon and MTZ increased the efficacy of individual medications and decreased the quantity of *Giardia lamblia* trophozoites much more than either drug alone. Also, this agreed with Rashed *et al.* (2021), who found that both Piperazine citrate and NTZ reduced *C. parvum* oocysts significantly when compared to positive controls. This also agreed with Fahmy *et al.* (2021b), who found a considerable reduction in the number of *Cryptosporidium* oocysts in the Cyperus-treated group, with the com-

bined NTZ-Cyperus-treated group having the highest percentage reduction (85.4%). Again Mostafa *et al.* (2018) reported that combining artesunate and NTZ had a synergistic effect in reducing *C. parvum* oocysts number shed and improved cryptosporidiosis colonic dysplastic changes in immunosuppressed mice compared to either one alone. Abdel Maksoud *et al.* (2020) found that Asafoetida methanol extract decreased oocysts in infected mice and improved the histological alterations of tiny intestinal villi at ileocaecal area with normal liver architecture. Moreover, antioxidants also scavenge free radicals and protect people and animals from infectious illnesses (Qulez *et al.*, 2013).

In the present study, the cryptosporidiosis produced oxidative stress by decreasing activity of SOD, and increasing MDA concentration in control-positive infected mice. This agreed with El-Sayed and Fathy (2019). Also, Elmahallawy *et al.* (2020) described oxidative damage during cryptosporidiosis, noting a reduction in SOD activity. The present early antioxidant capabilities of DFL, IE & IIE mice given combined therapy showed an increase SOD & a decrease MDA. Diosmin has anti-oxidant power to diminish many components involved with oxidative imbalance (Huwait and Mobashir (2022). This agreed with Mustafa *et al.* (2022), who found that Diosmin has a number of medicinal benefits owing to its antioxidant activity. Fahmy *et al.* (2021a) reported that decrease in MDA level and an increase in SOD levels in livers of *Giardia lamblia* infected mice

treated with both MTZ and DFL. El-Kholy *et al.* (2021) reported significant increase in GSH with a decrease in MDA and SGOT in group treated with combined Platelet-Rich Plasma (PRP) & NTZ compared to the positive control infected group of *Cryptosporidium* in immune-suppressed rats. Again, Atia *et al.* (2021) found that mice given prophylactic treatment with Azoximer bromide and NTZ had an increase in SOD, but a decrease in MDA.

In the present study, NTZ+DFL-treated mice showed significant improvement in villous architecture, brush border, goblet cells, limited inflammation, and without oocysts. This agreed with Metawae *et al.* (2020); Atia *et al.* (2021), who found that combined therapy was more effective than NTZ alone. Again, Fahmy *et al.* (2021a) reported that treating *Giardia*-infected mice with Daflon & MTZ gained normal intestinal architecture (crypts and villi) and lamina propria.

In the current work, lung tissue of immunocompetent and immunosuppressed infected mice showed obvious pulmonary hemorrhage, destruction of intra-alveolar septa, and heavy lymphoplasmacytic inflammatory infiltrate of the bronchial lamina propria. But, mice given combined treatment showed marked improvement in the form of normal epithelial lining without oocysts, near-total restoration of intra-alveolar septa, very minimal interstitial inflammation, and pulmonary hemorrhage. Madbouly *et al.* (2017) detected pulmonary hemorrhage and interstitial inflammation in the infected immune-suppressed mice's lung tissue, and these abnormalities improved after a combination of Atorvastatin and NTZ. Heo *et al.* (2018) reported that *Cryptosporidium* can infect epithelial organoids derived from human small intestine and lung.

In the present study, GIB & GIIB showed liver congestion and marked lymphocytic infiltrate in portal tract, but GIE and GIIE given DFL+NTZ showed marked improvement with very minimal liver congestion and portal tract inflammation. This agreed with

Mustafa *et al.* (2022), who found that Diosmin has hepatoprotective effect against ferrous sulfate-induced liver damage in adult male albino rats. Elevated levels of AST, ALT, GGT, ALP, LDH, and bilirubin suggest hepatocyte membrane injury.

In the present study, iNOS IHC showed moderate to strong cytoplasmic staining in all examined tissues of GIB & GIIB (infected control positive). But, iNOS expression in treated mice groups turns into weak cytoplasmic expression in all tissues examined with the best effect in mice that received combined treatment without significant differences. This agreed with Abdelhamed *et al.* (2019), who reported that pigs treated with either a peroxynitrite scavenger or an iNOS inhibitor recovered from cryptosporidiosis. In cryptosporidiosis, NO synthesis was elevated, and the lack or suppression of iNOS dramatically worsens epithelial infection and oocyst shedding (Gookin *et al.*, 2004).

Conclusion

The results showed that Daflon is a promising anti-cryptosporidiosis drug with impact in enhancing intestinal epithelium, lung, and liver tissue and disrupting *Cryptosporidium* when combined with Nitazoxanide. More parameters are in the ongoing study on drug-induced immune response and how Daflon and Nitazoxanide routes vary will be published.

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Explanation of figures

Fig. 1: *Cryptosporidium* oocysts count of all groups

Fig. 2: Mean serum level of MDA in all groups.

Fig. 3: Mean serum level of SOD in all groups.

Fig. 4: Histopathological sections of ileum from GI & GII (H&E). (A) GIA & (G) GIIA showed normal morphology. (B, C) GIB & (H, I) GII B showed villous architecture with blunted villous tips and broadening of villi with inflammation of lamina propria, ulceration of surface epithelium, irregular brush border and many oocysts embedded in villous epithelium. Sloughed villous tips and marked congestion were observed in GIB. (D) GIC & (J) GII C showed improved morphology except blunting and thickening of some villi, moderate lamina propria inflammation, focally irregular brush border and mild congestion. Brush border was regular in GII C. (E) GID & (K) GII D showed slightly better improvement than GIC except broadening of few villi, moderate inflammation and mild congestion, Sloughed villous tip in GII D. (F) GIE & (L) GII E showed marked histopathological improvement (x100 for B, & x200 for A, C, D, E, F, H, I, J& L & x400 for G& K).

Fig. 5: Histopathological sections of lung tissues from GI & GII (H&E). (A) GIA & (F) GII A showed normal morphology. (B) GIB & (G) GII B showed marked pulmonary hemorrhage, interstitial inflammation and marked destruction of intra alveolar septa with more deteriorated picture in GIB than GIB. (C) GIC & (H) GII C showed restored alveolar architecture, minimal interstitial inflammation with moderate pulmonary haemorrhage. (D) GID & (I) GII D showing marked histopathological improvement, less obvious in GII D than GID. (E) GIE & (J) GII E showed marked improvement with nearly normal morphology (x100for C, & x200 for A, B, D, E, F, G, H, I & J).

Fig. 6: Histopathological sections of liver from GI & GII (H&E). (A) GIA & (F) GII A showed normal morphology with preserved hepatic architecture. (B) GIB & (G) GII B showing liver congestion and marked lymphocytic infiltrate within portal tract. Congestion much marked in GIB. (C) GIC & (H) GII C showed improved histopathological picture with mild congestion & inflammation. (D) GID & (I) GII D showed histopathological improvement more or less similar to GIC & GII C with mild congestion and inflammation. (E) GIE & (J) GII E showed best improvement with marked reduction of congestion and inflammation (x 100 for C & D, x200 for B, E, G, H, I & J and x 400f or A & F).

Fig. 7: iNOS IHC staining of ileum from GI & GII. Strong cytoplasmic staining of many inflammatory cells (black arrow) in control infected mice (B&G) compared to NTZ- treated groups (C&H) and DFL-treated groups (D&I). (E&J) Combined subgroup (DFL +NTZ) showed cytoplasmic staining in very few inflammatory cells, nearly as control uninfected group (IHC x400 for A, B, C, D, E & H, x200 for J and x100 for F, G & I). iNOS % & H score of ileum among GI & GII. Data as mean \pm SD (n=5). Post-Hoc test was used for comparison between groups* $P < 0.05$ vs. control infected (K).

Fig. 8: iNOS IHC staining of lung tissue from GI & GII. Strong cytoplasmic staining of many inflammatory cells in interstitial lung tissue (black arrows) in control infected (B & G) compared to control uninfected (A & F), NTZ- treated (C & H), DFL- treated (D&I) and combined subgroups (DFL +NTZ) (E&J) (IHC x400 for B, C, E & F, x200 for D, G, H, I & J and x100 for A). iNOS% & H score of lung tissue among GI & GII. Data as mean \pm SD (n = 5). Post-Hoc test for comparison between groups* $P < 0.05$ vs. control infected (K).

Fig. 9: iNOS IHC staining of liver tissue from GI & GII. GI: Strong cytoplasmic staining of interstitial tissue between hepatocytes in control infected g (B) compared to NTZ- treated (C) and DFL-treated (D). (E) Combined subgroup (DFL +NTZ) negative as control uninfected (A & F) (IHC x400 for B, x200 for C, & D and x100 for A & E). GII: Strong cytoplasmic staining of interstitial tissue between hepatocytes in control infected (G) compared to totally negative staining in control uninfected (F), NTZ- treated (H), DFL-treated (I) and combined subgroups (DFL +NTZ) (J) (IHC x400 for F, G, H, & I x200 for J). iNOS% & H score of liver tissue among immunocompetent and immunocompromised groups. Data as mean \pm SD (n = 5). Post-Hoc test for comparison between groups* $P < 0.05$ vs. control infected (K).





