Journal of the Egyptian Society of Parasitology, Vol. 51, No.1, April 2021
J. Egypt. Soc. Parasitol. (JESP), 51(1), 2021: 89 – 98
(Online: 2090-2549)

ROLE OF APOPTOSIS IN EXPERIMENTAL CRYPTOSPORIDIUM PARVUM INFECTED ALBINO MICE

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Abstract

Cryptosporidium is one of the commonest opportunistic parasites. But, its chemotherapeutic options are limited, with controversy about the role of apoptosis in cryptosporidiosis. Caspases are key molecules of apoptosis. The study assessed the role of apoptosis in cryptosporidiosis by studying the relationship between the effects of phenyl vinyl sulfone (PVS), nitazoxanide (NTZ) and combined therapy on *Cryptosporidium parvum* infection and caspase 3 immunostaining. Ninety female laboratory bred Swiss Albino mice were divided into two major groups; immunocompetent and immunosuppressed. Both groups were divided into the following subgroups, for each one respectively: negative control (1 & 6), infected control (2 & 7), infected treated with PVS (3 & 8), infected treated with NTZ (4 & 9) and infected treated combined (5 & 10). Sacrification were done on 18th day post infection.

Histopathological study of ileum was done and endogenous developmental stages were counted. Also, caspase 3 immunohistochemical staining was carried out on ileal sections. Combined therapy in groups 5 &10 showed the highest reduction in mean number of endogenous developmental stages as in G10 (*P <0.05)*. Caspase expression in PVS treated groups (3 & 8) showed significant decrease when compared to their control groups *(P < 0.05).* While there were significant increase in caspase expression in groups treated with NTZ (4 & 9) when compared to their contorls *(P < 0.05).* Caspase 3 epithelial overexpression was evident in infected immunocompetent G2 than immunosuppressed G7 (*P<0.05)*.

Key words: Apoptosis, Caspase 3, *Cryptosporidium*, Cysteine protease inhibitors, Nitazoxanide, Phenyl vinyl sulfone.

Introduction

Cryptosporidium species are protozoan parasites that infect a broad range of hosts causing diarrhea in humans, second only to rotavirus (Kong *et al*, 2017). Parasite exists in the environment as an resistant oocyst with four sporozoites. When ingested by a host, excystation occurred in the small intestine. Motile sporozoites attach to intestinal epithelium and enveloped by its apical membrane forming an intracellular extracytoplasmic parasitophorous vacuole (Di Genova and Tonelli, 2016). On oocysts ingestion, they infect the villus epithelial cells, rapidly shed and causing profound villus blunting (Ferguson *et al*, 2019). The host immune status plays a critical role to determine the cryptosporidiosis severity. It presents as a self-limited diarrhea in immunocompetent hosts, but can be severe and devastating led to dehydration, starvation, or even death in immunocompromised patients (Zhang *et al*, 2020).

In coeliac disease and parasitic infections, apoptosis increased in the villus epithelial cells, indicating its important roles not only in physiological villous epithelial cells replacement, but also in pathological conditions (Hyoh *et al*, 1999). Apoptosis mechanisms of *C. parvum* is complicated one involved unknown virulence factors (e.g., *C. parvum* enterotoxin) or mediators released from infected cells (Sasahara *et al*, 2003). Liu *et al*. (2008) showed that cryptosporidiosis caused low-level activation of multiple members of the caspase family, and caspase activation kinetics was correlated with apoptosis. Liu *et al*. (2009) added that in early infections (6 & 12hr), anti-apoptotic genes were up-regul-

ated and apoptotic genes were down-regulated, later (24, 48 & 72hr), pro-apoptotic genes were induced and anti-apoptotic genes were down-regulated, suggesting a biphasic regulation of apoptosis, without consensus about the cryptosporidiosis apoptosis. Widmer *et al*. (2000) suggested that apoptosis could be involved in the pathogenesis and propagation of *C. parvum* infection and played a role in immunity against infection

Nitazoxanide, is the cryptosporidiosis accepted drug, but with limited value in the immuno-compromised patients (Amadi *et al*, 2009), as well as in malnourished children <12 months of age (Amadi *et al*, 2002).

Thus, searches for specific and effective cryptosporidiosis therapy was a must (Graczyk *et al*, 2011). The cysteine protease inhibitors were evaluated for cryptosporidiosis treatment as EDTA, iodoacetic acid, E-64 & phosphoramidon (Nesterenko *et al*, 1995) and combination of PMSF & E-64 (Forney *et al*, 1996a), as well as protease inhibitors including antipain, aprotinin, leupeptin, SBTI and PMSF in a cell culture system, which caused parasitic significant reduction (40-50%) with leupeptin, SBTI and PMSF (Forney *et al*, 1996b).

This study aimed to evaluate the apoptosis role in cryptosporidiosis *parvum* treated by phenyl vinyl sulfone (PVS), nitazoxanide (NTZ) and combined therapy on infection and caspase 3 immunostaining in both immunocompetent and immunosuppressed Swiss Albino mice.

Materials and Methods

All the experiments were carried out in accordance with the guidelines recommendations for the care and use of laboratory animals of the Ethics Committee of Menoufia University, and Theodor Bilharz Research Institute.

Ninety Swiss male albino mice (about 20-25g) were divided into ten groups; G1: uninfected and untreated; G2: infected with *C. parvum* oocysts and untreated, G3: infected and treated with PVS (CPIs), G4: Infected and treated with NTZ, G5: infected and treated combined, G6: immunosuppressed with dexamethasone (DEX) and uninfected, G7: immunosuppressed, infected, and untreated, G8: immunosuppressed, infected, and treated with CPIs, G9: immunosuppressed, infected, and treated with NTZ, and G10: immunosuppressed, infected, and treated combined (all groups were 10 mice each except groups 1 & 6 were five mice each). Mice were scarificed by cervical dislocation on the 18th day PI.

Immunosuppression was induced by DEX (Dexazone 0.5mg), purchased from Kahira Pharmaceuticals and Chemical Industries Company (Cairo), was given orally at a dose of 0.25µg/g/day for 14 successive days before infection, and maintained on DEX throughout the experiment (Sadek and El-Aswad, 2014).

Cryptosporidium oocysts were obtained by scrapings ileal mucous membrane and caecal contents from naturally infected calves as proved by by modified Ziehl-Neelsen stained stool smears. Positive calf stool specimens were preserved with an equal volume of 2.5% potassium dichromate solution at 4°C as infecting inoculum (Reese *et al*, 1982). The oocysts were counted in 1ml of specimen and their numbers were adjusted to approximately 1×10^5 oocysts per each mouse orally (El Shafei *et al*, 2018).

Nitazoxanide (NTZ) as Nitazode powder 100mg/kg/bodywt as oral suspension (Al Andalous Western Company), started 10th day post infection (PI) and continued for 10 days (Theodos *et al*, 1998). A stock solution from Phenyl vinyl sulfone powder (Sigma, USA.) was prepared (Ndao *et al*, 2013), and given orally at a dose of 35mg/kg/bodywt twice per day started on 10th days PI and continued for 10 days as well.

Endogenous developmental stages of *C. parvum* in H & E stained sections: Terminal 2cm of mice ileum was fixed in 10% buffered formalin, processed to paraffin blocks, 5µm thickness serial sections were H&E stained and examined under a light microscope. Endogenous stages in ten villous crypt units

were counted and the mean numbers per villous crypt unit per mouse were calculated (Fayer and Xiao, 2008).

Immunohistochemistry technique: **P**araffin block sections were mounted on positively charged glass slides for immunohistochemical staining using streptavidin-biotin amplified system. The primary antibody (0.5ml) was an Ani-active caspase-3 (CPP32), the primary rabbit polyclonal antibody (Thermo Fisher Scientific, Tudor Road, Marior Park, Runcorn, UK). Ultra V blocked the non-specific background staining. Antigen retrieval was done using Tris-EDTA (pH 9). The kit was ultravision detection system antipolyvalent HRP/DAB (cat. #TP-015-HD; Lab Vision Corporation, Fremont, California). Finally, reaction was visualized by appropriate substrate/chromogen (Diaminobenzidine, DAB) reagent with Mayer's hematoxylin as a counterstain. Staining procedure included positive tissue control (normal human tonsil tissues) and negative tissue control without primary antibodies.

Immunostaining interpretation: Assessment of ileal epithelial and stromal cells was done. Positive cells for caspase-3 immune stain showed brownish cytoplasmic with some nuclear staining (Vyas *et al,* 2007). Expression intensity was ranked as: 0=no staining, +1=mild, +2 =moderate, and +3=strong intensity (Samaka *et al,* 2016). H score system was applied (Han *et al,* 2014), where the intensity and positivity percent were considered using the following formula: H score = (3×% of strong intensity) + (2 ×% of moderate intensity) + (1 ×% of mild intensity).

Statistical analysis: Data were collected, tabulated and analyzed by using Statistical Package for Social Science (SPSS) program, version 20. Descriptive statistics; means and standard deviation (SD). Student t-test and ANOVA test were used. Significant was at P-value < 0.05 (Bryman, 2011).

Results

The results were shown in tables (1, 2, 3, 4 & 5) as well as in figures (1, 2, 3 &4)

Table 1: Endogenous developmental stages of *C. parvum* / villous crypt unit in groups.

Groups	Developmental stages/villous unit	t- test	P-value
G1	8.55± 1.65		
G3	7.8 ± 0.99	0.77	P_1=0.44
G4	3.50±2.79	4.90	P_2<0.05
G5	3.31±1.75	6.49	P_3<0.05
G7	13.81 ± 2.06		
G8	12.37±1.92	1.44	P_4=0.17
G9	11.11± 1.96	2.76	P_5<0.05
G10	10.11±1.90	3.84	P_6<0.05

P_1: compared between G2 & G3, P_2: compared between G2 & G4, P_3: compared between G2 & G5, , P_4: compared between G7 & G8, , P_5: compared between G7 & G9 , P_6: compared between G7 & G10.

Table 2: Comparison between immunocomptent and their immunosuppressed groups as to endogenous developmental stages of *C. parvum* / villous crypt unit

Groups	Developmental stages/villous unit	t- test	P-value
G2 G7	8.55± 1.65 13.81 ± 2.06	6.00	P<0.05
G3 G8	7.8 ± 0.99 12.37±1.92	5.98	P<0.05
G4 G9	3.50±2.79 11.11± 1.96	6.783	P<0.05
G5 G10	3.31±1.75 10.11±1.90	7.63	P<0.05

There were significant differences between infected immunocompetent Gs 2, 3, 4 & 5 as regards as epithelial and stromal H scores of immunohistochemical staining of Caspase 3 (P <0.05). PVS treatment caused a significant reduction of both Caspase 3 epithelial and stromal H scores when compared with infected control mice. NTZ treatment caused a significant increase in both Caspase 3 epithelial and stromal H scores. A combined ones

showed no significant changes (Tab. 3).

Significant differences between infected immunosuppressed Gs (2, 3, 9 & 10) were identified regarding Caspase 3 both epithelial and stromal H scores =P < 0.05 (Tab. 4**).**

Caspase 3 epithelial and stromal immunohistochemical expression were down regulated in immunosuppressed mice than immunocomptent ones. Significant differences between G1 & G6 as to epithelial and stromal H scores (P <0.05). A significant difference between G2 & G7 regarding Caspase 3 epithelial H score was evident (P <0.05). Significant differences were betw-een G4 & G9 or G5 & G10 (P <0.05) as to Caspase 3 stromal H score, without signific ant difference between other groups (Tab. 5).

Table 3: Immunohistochemical staining of caspase 3 in immunocompetent mice infected with *C. parvum*.

Variable	G2 (n=10)	G3 (n=8)	G 4 (n=10)	G5(n=8)	ANOVA	P –value	Post Hoc
Epithelial H score	222 ± 22.87	95 ±23.29	275 ± 32.65	203.75 ± 35.43	58.96	< 0.05	P_1< 0.05 P_2<0.05 P_3=0.19 P_4<0.05 P_5<0.05 P_6<0.05
Stromal H score	57 ± 6.32	30 ±7.55	130.00±26.66	67.50±4.62	73.06	< 0.05	P_1<0.05 P_2<0.05 P_3=0.153 P_4<0.05 P_5<0.05 P_6<0.05

P_1: compared between G2 & G3, P_2: compared between G2 & G4, P_3: compared between G2 & G5, P_4: compared between G3 & G4, P_5: compared between G3 & G5, P_6: compared between G4 & G5.

Table 4: Immunohistochemical expression of caspase 3 in immunosuppressed mice infected with *C. parvum*

Variable	G7 (n=8)	G8 (n=8)	G9 (n=9)	G 10 (n=9)	ANOVA	P –value	Post Hoc
Epithelial H score	140±27.77	93.75±13.02	259.44±35.21	188.88±30.18	53.981	< 0.05	P_1< 0.05 P_2< 0.05 P_3< 0.05 P_4< 0.05 P_5< 0.05 P_6< 0.05
Stromal H score	55.62±6.23	33.75±4.43	70 ±7.07	60±7.07	48.447	< 0.05	P_1< 0.05 P_2< 0.05 P_3= 0.16 P_4< 0.05 P_5< 0.05 P_6< 0.05

P_1: compared between G7 & G8, P_2: compared between G7 & G9, P_3: compared between G7 & G10, P_4: compared between G8 & G9, P_5: compared between G8 & G10, $P_{6.}$: compared between G9 & G10.

Table 5: Comparison between immunocompetent groups and immunosuppressed groups regarding epithelial H score and stromal H score:

Variables	Epithelial H score	t- test	P-value	Stromal H score	t- test	P-value
G1 G6	59 ± 11.4 45 ± 6.1	2.419	P<0.05	48 ± 8.3 28 ± 8.3	3.780	P<0.05
G2 G7	222 ± 22.8 140 ± 27.7	6.877	P<0.05	57 ± 6.3 55.62 ± 6.2	0.461	P=0.651
G3 G8	95 ± 23.2 93.7 ± 13	0.13	P=0.89	30 ± 7.5 33.75 ± 4.4	1.21	P=0.24
G4 G9	275 ± 32.6 259.4 ± 35.2	0.99	P=0.33	130 ± 26.6 70 ± 7	6.529	P<0.05
G5 G10	203.7 ± 35.4 188.8 ± 30.1	0.93	P=0.36	67.5 ± 4.6 60 ± 7	2.54	P<0.05

Discussion

In vitro studies showed the essential role of apoptosis for the pathogenesis of *C. parvum*. Paradoxically, either increasing apoptosis by silencing Bcl-2 or decreasing apoptosis by the pan-caspase inhibitor impaired *C. par-*

vum infection (Liu *et al*, 2009). Caspase-dependent apoptosis was undoubtedly increased by *C. parvum* in both *in vitro* and *in vivo* models (Ojcius *et al*, 1999; Sasahara *et al*, 2003; Foster *et al*, 2012; Di Genova and Tonelli, 2016). It still unclear whether the occurrence of apoptosis helped the parasite or the cryptosporidiosis host. Removal of infected epithelial cells by apoptosis assisted the host in maintaining the epithelial barrier integrity (Sasahara *et al*, 2003). Moreover, in order to limit the spread of infection, infected hosts may eliminate infected cells through activating caspases and inducing apoptosis (Uchiyama and Tsutsui, 2015). On the other hand, the cells undergoing apoptosis were limited to those directly infected by the parasite and dependent on the activation of caspases (McCole *et al*, 2000). Apoptosis of intestinal epithelial cells, are common events in the *C. parvum* infected ileum. When the ileum *C. parvum* oocysts number was maximal, few apoptotic epithelial cells were found in the basal crypts where *C. parvum* proliferated. Villous structure changes and apoptotic epithelial cells were scarcely detected in duodenum, cecum, and colon of infected mice suggested that epithelial apoptosis have a significant role in the cryptosporidiosis pathogenesis (Sasahara *et al*, 2003).

In the present study, ileum was chosen as the heaviest site with cryptosporidiosis in both immunocompetent and immunosuppressed mice (Mead *et al*, 1991; You and Mead 1998; Sadek and El Aswad, 2014; Aly *et al*, 2015). Where the biochemical conditions and the presence of specific receptor are favorable for the parasite development (Verdon *et al*, 1998). The expression of caspase 3 in ileal epithelial cells from experimentally infected mice was investigated as a marker of apoptosis to evaluate the role of apoptosis of epithelial and stromal cells during the course of *Cryptosporidium* infections. Activation of caspase 3 (a member of the caspase family) is considered to be a point at which a cell marches toward irreversible apoptotic death (Porter and Janicke, 1999; Abu-Qare and Abou-Donia, 2001).

In this study, immunohistochemical staining of ileal sections of mice was done at 18 days PI during the period of the severest pathological changes (the maximum oocyst shedding, the results not shown). Normal control group (G1) showed positive epithelial and stromal expression of mild intensity. This agreed with Krajewska *et al*. (1997) and Hyoh *et al*. (2002) who found low-level Caspase immunostaining in the cytosol of the absorptive epithelial cells.

Ileal sections from immunosuppressed uninfected and untreated group (G6) showed significant downregulation in caspase expression when compared to normal control group. This agreed with Liu *et al*. (2016) from malnourished uninfected mice, where immunostain showed slight decreased in the expression of caspase 3. Malnutrition is considered as a cause of immunosuppression.

Caspase-3 stained sections from infected control group (G2) showed a strong epithelial and mild stromal immunostaining. These results more or less agreed with Foster *et al*. (2012). and colleagues who reported that *C. parvum* infection in a neonatal piglet model initiated enterocyte apoptosis which was confined to villus tips, and preferentially to infected cells. The epithelial cells undergoing apoptosis were influenced by adjacent cells infected with *C. parvum* via the Fas receptor-Fas ligand death system (Chen *et al*, 2002) and via CD40-CD40 ligand interaction (Cosyns *et al*, 1998).

Ther was a significantly increased expression of Caspase 3 in epithelial cells of infected immunocomptent mice compared with immunosuppressed infected group. This agreed with Liu *et al.* (2016); Coutinho *et al*. (2008) and Guerrant *et al*. (2008) that *C. parvum* infection increased the expression of caspase 3. But, malnutrition was accompanied by significant suppression of *C. parvum*-induced caspase 3 activity, resulting in increased vulnerability to infection.

In the current study, PVS treatment caused significant downregulation of caspase 3 stai-

ning in both immunocomptent and immunosuppressed infected mice when compared to counterpart positive controls. This agreed with Newton *et al*. (2010) who found that vinyl sulfones were potent inhibitors of many clan CA cysteine and inhibitors of caspases that belong to class CD, as well as Glória *et al*. (2011).

In the present study, expression of caspase 3 in response to PVS treatment did not show reduction in parasite load of ilea of either immunocompetent or immunosuppressed infected mice when compared with counterpart infected control mice.

Immunostained ileal sections from NTZ treated mice showed significantly increased expression of caspase in both immunocomptent and immunosuppressed infected mice when compared with counterpart infected control groups. This agreed with Müller *et al*. (2008) who found that NTZ increased apoptosis. Glutathione-S-transferase P1 was identified in human colon carcinoma cells. The enzyme inhibition by NTZ correlated well with its efficacy to induce apoptosis. Suppressing the expression of GST P1 resulted in caspase-dependent apoptosis. This agreed with Müller *et al*. (2011) who concluded that the NTZ efficacy against human cryptosporidiosis was partly due to apoptosis effects on the intestinal cells. Sidler *et al*. (2012) and Arnold *et al*. (2014) explained the action mechanism of NTZ on apoptosis, and antimicrobial and anti-inflammatory actions, NTZ triggered tumor cell apoptosis caused nuclear condensation, DNA fragmentation and phosphatidylserine exposure.

In the present study, increased expression of caspase after NTZ treatment was associated with significant reductions in parasite load in ilea of both immunocompetent and immunosuppressed infected mice when compared with counterpart infected control mi ce. Combined treatment (PVS & NTZ) in infected immunocompetent group gave increased apoptosis without significant difference as to epithelial or stromal H scores. But, in infected immunosuppressed ones, there were epithelial over expression.

Conclusion

Apoptosis of epithelial cells was a part of the host immune system to eliminate infected cells, where increased expression of epithelial caspase 3 occurred in immunocomptent than immunosuppressed groups. Improvement occurred in mice more than in overexpression of caspase 3 epithelial immunestaining (NTZ treated & combined ones) than those PVS treated, which inhibited apoptosis. Upregulation of caspase in *C. parvun* infected intestine could be an immune mechanism to eliminate infection specially in the immunocompetent groups.

Acknowledgments

The authors would like to thank the National Liver Institute, Menoufia University, for kind financial support and facilitating this work.

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Explanation of figures

Fig 1a: Different developmental stages of *Cryptosporidium* at brush borders of intestinal villi epithelial cells appeared as small round or oval basophilic bodies in G2 (arrows, circle) (H&E ×1000).

Fig 1b: Ileal section from G5 (immunocompetent infected treated combined) showed only one parasite at brush border of intestinal villi (arrow) (H&E ×1000).

Fig 1c: Ileal section from G1 (normal control group) showed mild epithelial expression (red arrows) and mild stromal expression (blue arrows) of caspase 3 (Caspase IHC ×200).

Fig 1d: High power of ileal section from G6 (immunosuppressed control group) showed mild epithelial (red arrow) and stromal expression (blue arrows) of caspase 3 (Caspase IHC ×400).

Fig 2a: Ileal section from G2 (immunocompetent infected not treated) showed strong immunostaining in epithelium (red arrow) and mild degree of staining in stroma (blue arrow) (Caspase ×200).

Fig 2b: Mucosal glands and lining epithelium from G3 (immunocompetent infected treated with PVS) showed moderate immunostaining. Intervening stroma showed mild immunostaining of lymphocytes (Caspase ×200).

Fig 2c: Ileal section from G4 (immunocompetent infected treated with Nitazoxanide) showed strong epithelial expression and moderate stromal expression of caspase 3 (Caspase IHC ×200).

Fig 2d: Mucosal glands and lining epithelium from G5 (immunocompetent infected treated combined) showed moderate immunostaining (red arrows). Intervening stroma showed mild immunostaining of lymphocytes (blue arrows) (Caspase 3 ×400).

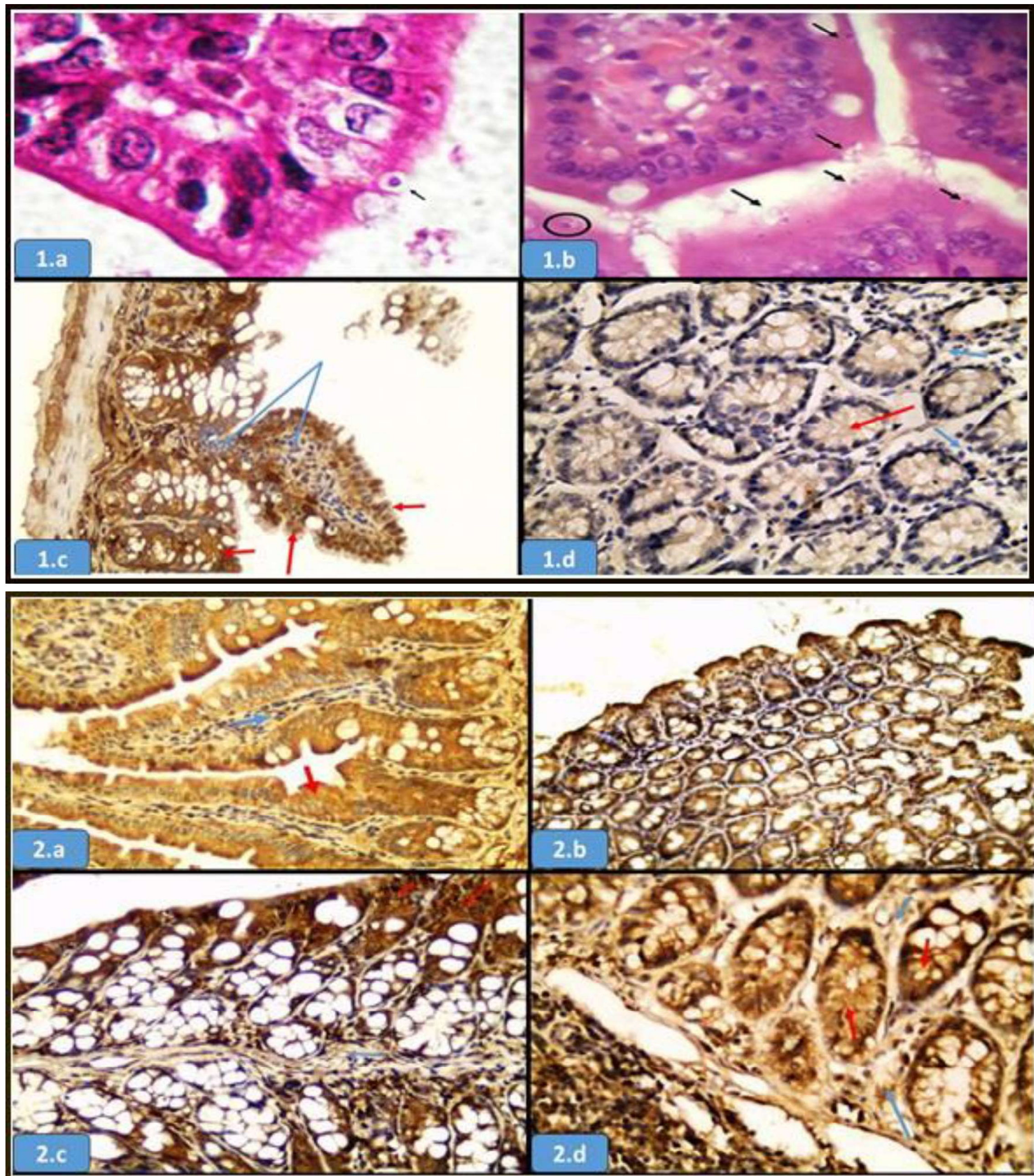
Fig 3a: Mucosal glands and lining epithelium from G7 (immunosuppressed infected not treated) showed moderate immunostaining with caspase 3. Intervening stroma showed mild immunostaining of lymphocytes (Caspase IHC ×200).

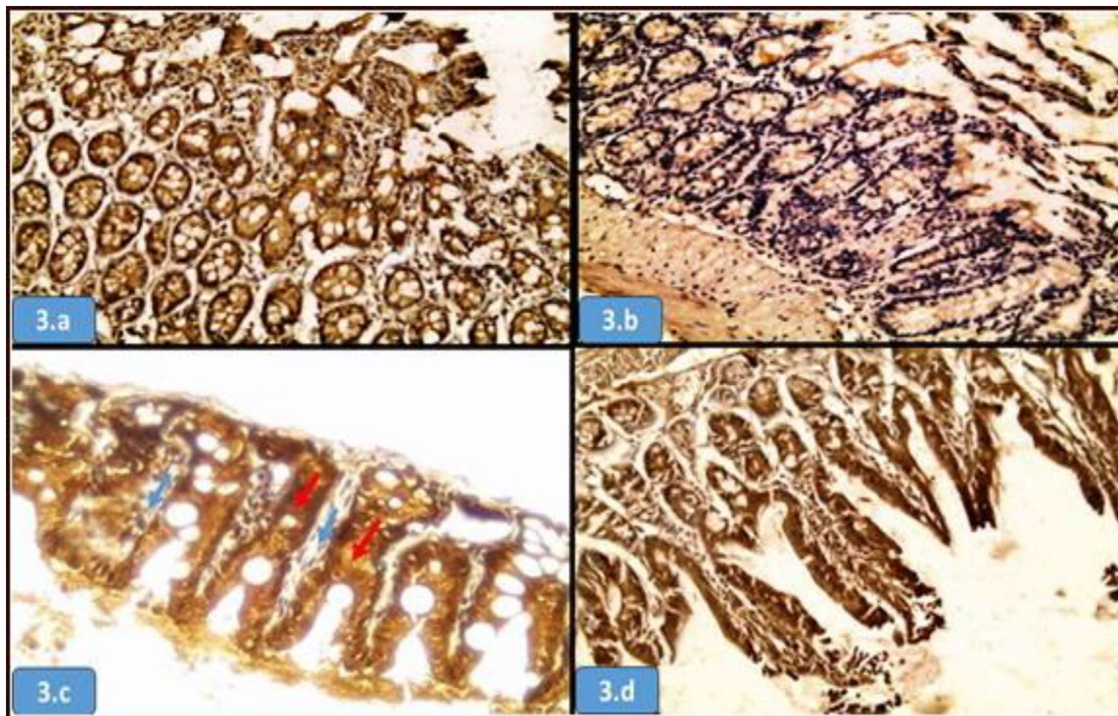
Fig 3b: Ileal section from G10 (immunosuppressed infected treated with PVS) showed mild epithelial expression of caspase 3 and intervening stroma also showed mild immunostaining (Caspase IHC ×200).

Fig 3c: Ileal section from G9 (immunosuppressed infected treated with Nitazoxanide) showed strong epithelial immunostaining (red arrows) and mild stromal immunostaining (blue arrows) (Caspase IHC ×200).

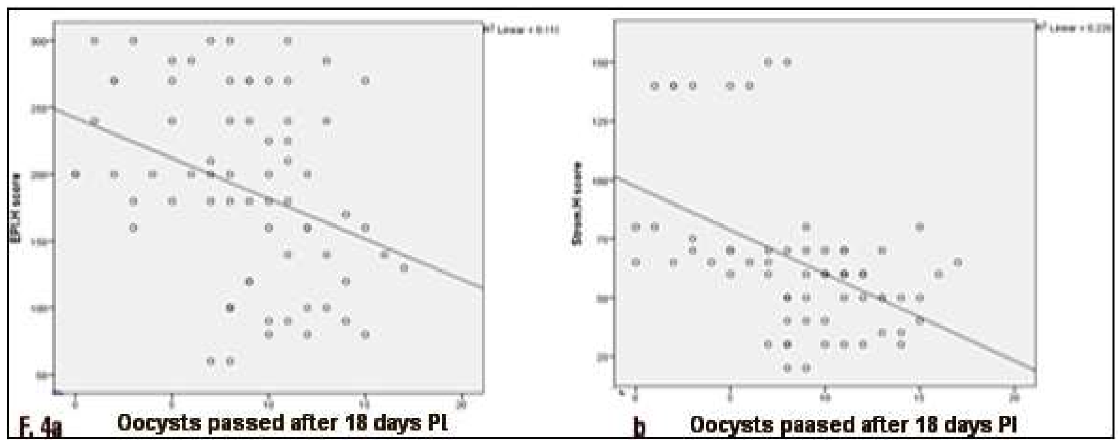
Fig 3d: Mucosal glands and lining epithelium from G10 (immunosuppressed infected treated combined) showed moderate immunostaining. Intervening stroma showed mild immunostaining of lymphocytes (Caspase IHC ×200).

Fig 4 Significant negative correlation (increased caspase3 was associated with decreased parasite load) between oocyst shedding at day 18 PI and both Caspase 3 epithelial H score (4.a) and stromal H scores (4.b).





3.a
3.b
3.c
3.d



F. 4a
Oocysts passed after 18 days PI
b
Oocysts paased after 18 days PI