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ORIGINAL ARTICLE

Priming enhances the protective effects of intestinal CD4 and iNOS in the jejunum of *Cryptosporidium* -malnourished mice

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Key words:
Cryptosporidiosis; Malnourishment; iNOs; CD4; priming. PCR

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ABSTRACT

Background: *Cryptosporidiosis constitutes one of the most common causes of severe diarrhea in immunocompromised and malnourished individuals. It is considered as a life threatening for both of them.* ***Objective:*** *the present work aimed to assess the effect of priming with 10^2 Cryptosporidium parvum (C.parvum) oocysts on the immune response in the jejunum of experimental malnourished mice trying to restore the host immune defense against the parasite.* ***Methodology:*** *The experiment lasted for 23 days. Fifty (50) female Swiss mice aged 4weeks were randomly and equally divided into five groups: sham control; infected; malnourished; malnourished –infected and primed protected groups. All groups were followed for weight, oocyst shedding and intestinal parasite tissue load. At the end of the experiment, jejunums were collected and prepared for histological, morphometrical, immunohistochemical and cluster of differentiation CD4 & inducible Nitric Oxide Synthase (iNOS) gene expressions.* ***Results:*** *In compare to malnourished –infected group, primed protected group showed evident clinical, histopathological & immunological improvement. Body weight loss, oocyst shedding & parasite tissue burden were reduced in primed protected group. Also, an increase in iNOS and CD4 gene expressions and immune reactions in primed group reflected as an increased villous length and absence of dysplastic changes in the jejunum compared to malnourished –infected group.* ***Conclusion:*** *These outcomes deepen the insight of potential role of C. parvum priming on both innate and adaptive immunity in malnourished infected mice jejunum for further studies.*

INTRODUCTION

In vertebrates, the priming has been considered as efficient, specific and less costly immune initiator. It increases the survival and strength of the immune response against the pathogens to which the host has been previously exposed [1]. In developing countries, malnutrition and cryptosporidiosis form a vicious cycle and lead to acute and long-term growth impairment in children [2] as well as intellectual capacity [3]. The immune status of the host plays an important role in susceptibility, severity and resolution of the disease [4]. The small intestine is considered as a common primary site of *Cryptosporidium* infection in humans [5]. Malnutrition can damage intestinal architecture and the host's immune system, resulting in increased vulnerability to infection [6]. The impairment of cell mediated immunity has been developed in millions of malnourished children and immuncompromised individuals [5].

Both innate and adaptive immunity have a significant role in cryptosporidiosis [4]. In the early stages of infection, the innate immune mechanisms play an important role in host resistance to infection by preventing attachments or by neutralizing parasite molecule[7]. *Cryptosporidium* causes both increased permeability of the epithelial cell barrier and trigging of the innate inflammatory responses. Epithelial cells play a vital role in immunity by stimulating the expression of chemokines, cytokines, production of Nitric oxide(NO) and antimicrobial peptides[8]. Nitric oxide produced through the induction of nitric oxide synthase (iNOS) plays an important role in epithelial innate immunity against cryptosporidiosis[9]. On the other side, the adaptive immunity is important to clear the infection.CD4 T cells are a major component of the human intestinal lamina propria [10]. Cell mediated immunity particularly CD4 lymphocytes and interferon-gamma (IFN-Y) play an important role in resolution of infection [11].

Many drugs are not effective in treatement of cryptosporidiosis in immunocompromised patients [4, 12]. It is not a surprise to say that; immunity repair might provide protection or reduce the severity of infection in patients with low immunity as immunocompromised person and malunouished children in developing countries. This work aimed to test the ability of pre challenge by 10^2 *C.parvum* oocysts on the immune system of cryptosporidiosis- malnourished mice through parasitological, histological, molecular and immunohistochemical studies.

METHODOLOGY

Experimental design

Fifty female Swiss mice aged 4 weeks & weighted about 12-15 gram were obtained from the *Schistosoma* Biological Supply Center (SBSC), Theodor Belharz Research Institute (TBRI), Giza, Egypt. They were laboratory bred.

Mice were acclimated to fed a regular diet for 4 days and then randomly and equally (10/group) divided into five experimental groups (G) as follows:

G1 (sham control): continuous on a regular diet.

G2 (infected): challenged with 10^7 *C.parvum* oocysts on 20th day of the experiment.

G3 (malnourished): received protein deficient diet from the 5th day till the end of the experiment.

G4 (malnourished- infected): received protein deficient diet from the 5th day & challenged with10^7 *C.parvum* oocysts on 20th day.

G5 (primed): received protein deficient diet from 5th day, prechllenged with 10^2 *C.parvum* oocysts on the 8th day and rechallenged with 10^7 oocysts on day 20thday.

At the beginning of the experiments, the body weight for all mice was recorded. The stools were collected daily to confirm clearance of parasite shedding before rechallenge with 10^7oocysts in G5. On the 23rd day, the animals were weighted again and the fecal pellets were collected and then scarification was done by cervical dislocation[7]. The mice jejunums were removed immediately and prepared for histopathological and immunhistochemical studies. Samples for RT-PCR were frozen at -70ºC. All experimental procedures were conducted with the approval of the Research Ethics Committee, Faculty of Medicine, and Menoufia University.

Preparation of the diet

For nourished groups a normal chow was taken while isocaloric chow with 3% protein was used for malnourished groups[12]. The chow was prepared in Biological Supply center (SBSC), theodor Belharz Reasearsh Institute (TBRI), Labs), Giza, Egypt.

Preparation and administration of *Cryptosporidium* infection:

The *Cryptosporidium* oocysts were gotten from infected calves (from slaughter houses) by scraping the contents of the intestinal mucousa [13]. The samples were examined to confirm the presence of the parasite by modified Ziehl–Neelsen staining method [14]. The oocysts of *Cryptosporidium* were purified by centrifugal flotation method [15]. The purified oocysts were stored in phosphate-buffered saline (PBS) at 4 C. for DNA extraction and subsequent PCR.

PCR technique was performed at the Central lab, Biochemistry Department, Faculty of medicine, Menoufia University. Amplification of a specific DNA fragment at 550 bp with C. parvum outer wall protein (COWP) was done by the forward primer CRY-15 (5'-GTA GAT AAT GGA AGA GAT TGT G -3') and the reverse primer Cry-9 (5'- GGA CTG AAA TAC AGG CAT TAT CTT G -3'), according to (16). The PCR products and a size marker of 550 bp ladder (Pharmacia Biotech Sweden) were electrophoresed in 5% ethidium bromide stained agarose gels and trisborate buffer using Biorad gel elecrophorcsis and viscuahzed on a UV transilluminator[17]. The oocysts were prepared for infection according to Reese et al.[18]

They were measured using a hemocytometer [19] and re-suspended in PBS to get a final concentration (10^7 oocysts / 100 µl or as 10^2oocysts/100 µl). Each mouse in G2,G4,G5 received the oocysts via oral gavage directly into the stomach. The control received 100 µl PBS alone [20].

Methods:

Stool examination:

Each collected fecal sample was prepared by formol ether concentration method[21], stained with a modified Ziehl–Neelsen stain[14] and examined under the microscope in 10 high power fields. For each animal, oocysts were counted per mg of the stool, and calculated for each group [22] (Fig.1).

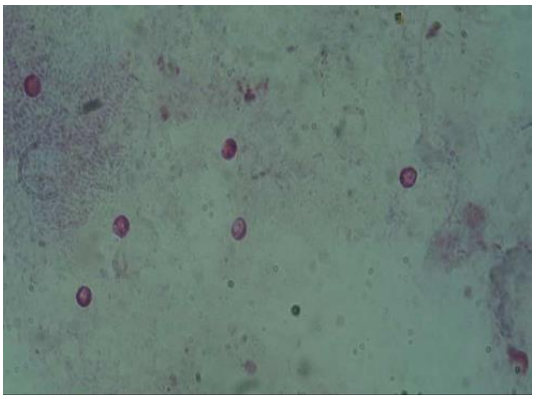


Fig. 1: *Cryptosporidium* oocysts with Ziehl –Neelsen stain x1000.

Histopathological study:

Animals jejuneal specimens were prepared and stained with H&E stain [23].The number of parasites in the intestinal epithelium was counted in 10 villus crypt unit to asses the severity (tissue burden)of infection in each group[22].

Immunohistochemical study (IHC):

Jejunum, sections (n = 10) were prepared and subjected to (IHC) staining .The following antibodies were used, rabbit monoclonal anti CD4 (abcam, USA, Cat#, ab183685, 1:1000), Rabbit polyclonal anti-inducible nitric oxide synthase (iNOS) (abcam, Cambridge, USA, Cat#:ab3523, 1:500). All the primary

antibodies were validated to be used with mice. Counterstaining was performed using Mayer's hematoxylin (Bio-Genex, Menarini Diagnostics, Antony, France Cat #, 94585) [24].

iNOS and CD4 mRNA expression in the jejunum:

For both CD4 and iNOS genes expressions, the extracted RNA was done according to[25].

(CD4 F: GAGAGTCAGCGGAGTTCTC;
CD4 R: CTCACAGGTCAAAGTATTGTTG),
(iNOS F; TCCTGGACATTACGACCCCT;
iNOs R: AGGCCTCCAATCTCTGCCTA)] primers (Invitrogen, Carlsbad, California). The run did in the presence of β-actin gene
(F; AATTTCTGAATGGCCCAGGT-
R; TTTGTGTAAGGTAAGGTGTGC) were used. Samples were spun before loading in the rotor's wells. Cycling conditions were was performed according to [25]. Initial denaturation at95 ˚C for 10min; 40 cycles of 64˚C for 15s and extension at72 ˚C for 30sData acquisition performed during the extension step. Each RT-PCR was repeated three times with similar results. using Rotor-Gene6000system (QIAGEN, USA). Quantitation data analysis was performed:

Ratio target gene expression = Folded change in target gene expression (sample/control)

Folded change in reference gene expression (housekeeping gene/control)

Morphometric study:

Image analysis for villus height and crypt depth, well-oriented sections of jejunum for 10 mice. For each animal, a total of 10 villi and 10 crypts of jejunum tissue, were measured with a micrometer. In addition, (IHC) findings were quantitatively estimated by two blind investigators. Briefly, 5 rep-resentative fields scattered in the preparation (×400, Olympus light microscope BX51TF; Olympus, Tokyo, Japan) from 3 non-adjacent sections were analyzed for each animal (n = 10) [26].

Statistical analysis

Stool shedding (parasites / mg of stool) and tissue burden (parasites/mg of tissue) and image analysis data were performed using SPSS 17.0 software (Inc., Chicago, IL, USA). Statistical analyses were performed using ANOVA with Bonferroni post-hoc correction. Data were presented as mean ± SEM and $P \leq 0.05$ was considered significant.

RESULTS

Biological activities

At the end of the experiments (23 day), G1 showed insignificant increase ($p \geq 0.05$) in the body weight, the percentage of increase was 0.87%. Conversely, G2,G3, G4, G5 showed significant ($p \leq 0.001$) weight loss, the percentage of weight loss was 7.6;12.13; 19.9 and 5.4 % respectively (Table.1).

Comparing to G2, the animals of the G4 showed significantly ($P \leq 0.0001$) increase in *C.parvum* oocysts shedding and tissue burden. However, G5 resulted in significance ($P \leq 0.0001$) decrease in both oocyst shedding and tissue burden (Table.1).

Table 1: Data represented morphometric and clinical criteria in the experimental groups.

Groups / *Mean ±SD*	*Control G1*	*Infected G2*	*Malnourished G3*	*Infected-malnourished G4*		*Primed G5*
Villous height (µm)	420±80	502±35***	256±46***	a	200±73***	414±03 ooo
				h	564±17***	
Crypt depth (µm)	120±51	133±05***	80±4***	a	55±06***	114±42 ooo
				h	125±09***	
Weight in gm day 0	14.89±0.328	14.86±0.427	14.51±0.515		14.79±0.357	14.90±0.454
Weight in gm day23rd	15.02±0.209***	13.73±0.476***	12.75±0.380***		11.84±0.350***	14.10±0.319***
Oocysts shedding	00±00	453.8±27.2	00±00		858.3±139.1# # #	110.8±25.4ooo
Tissue burden	00±00	19.3±6.3	00±00		34.0±5.9# # #	11.5±3.8 ooo

Footnote. The results are expressed as mean ± SEM of 10 mice per group. ** P ≤0, 001;
*** P ≤0.0001 significance in relation to control group.
P ≤0.0001 significance in relation to infected group. ooP ≤0. 001; ooo P ≤0.0001 significance in relation to Infected - malnourished group.

Histopathological Results:

Histopathological findings in the jejunal tissue of the control mice (G1) showed mucosa with normal villi and crypts. In *(G2)*, there was hyperplasia epithelial cells and significant hypertrophy (P ≤0.0001) in both villi and an increase in crypts' depth (P ≤0. 0001). The villi showed apical sloughing, while inflammatory cellular infiltration could be seen in its lamina propria. The crypts were widened with necrotic debris in the lumens. Different forms of *C.parvum* were detected at the brush border of the villi with increased vascularity in the submucosa while in (G3) showed distorted villi and crypts with significant shorting (P ≤0.0001). The tip of the villi showed severe sloughing and ulceration with hemorrhagic areas. Moreover, the jejunal tissue showed many inflammatory cells, edematous areas, dilated and congested blood capillaries (Fig.2, Table.1).

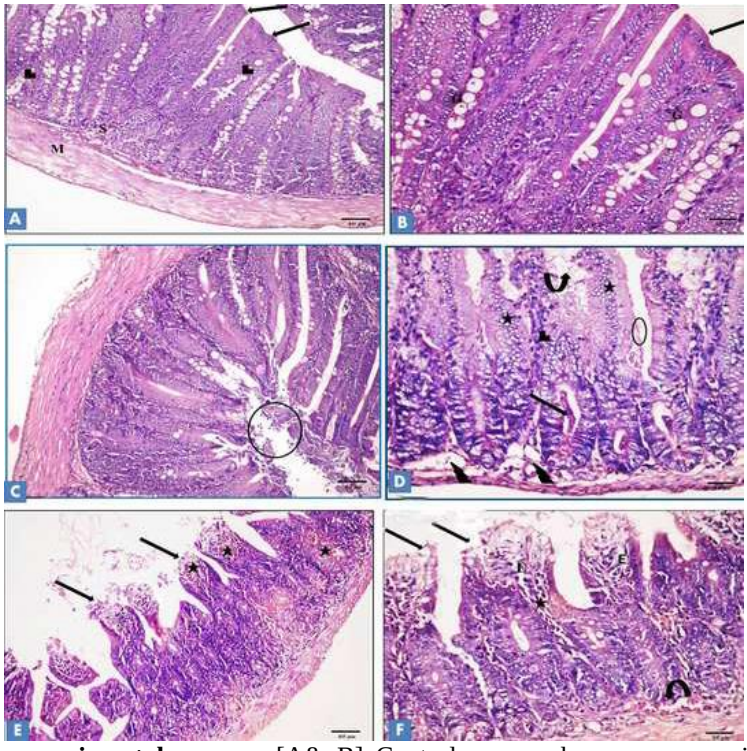


Fig. 2: Jejunum of the experimental groups: [A& B] Control mouse shows mucosa with apparent normal villi (arrows) and crypts (arrowsheads). The submucosa (S) is resting on muscularis externa (M). Both villi and crypts are lined by columnar epithelium, with brush border, (arrow) and goblet cell (G). [C&D] Infected mouse shows *Cryptosporidium* oocysts in the lumen (circle).Some of the epithelial cells shows hyperplasia (stars), while others shows partial lysis (curved arrow).The crypts are widened with presence of necrotic debris in their lumens (arrows). The lamina propria (LP) shows infiltrating inflammatory cells (arrow head). Notice. A number of *Cryptosporidium* oocysts attached with epithelial surface (circle) and numerous b.v in the submucosa (zigzag arrows). [E&F] Malnourished mouse shows distorted atrophic short villi with apical sloughing (arrows) and focal hemorrhagic areas (stars). Ulceration of the villous epithelium (arrows)with presence of the underlying inflammatory cellular infiltration (star) and edematous spaces (E). Notice. Dilated and congested blood capillary (curved arrow) (H&E x250 &400).

The jejunum of (G4), showed epithelial hyperplasia. Some villi appeared significantly short (P ≤0.0001) and distorted while others appeared significantly hypertrophied (P ≤0.0001), thickened with flat surface. Some of *C.parvum* oocysts attached to the distorted epithelium and others admixed with the mucus and food debris between villi. Some crypts showed irregular nuclei, increased N/C ratio, nuclear hyperchromasia. The submucosa showed increased vascularity while musculosa became thinner. The primed group (G5), the showed apparently normal luminal & mucosal architecture and significant increase in the villus length and muscle thickness. Only few luminal *C.parvum* forms & few hyperplastic cells were noticed (Fig. 3, Table.1).

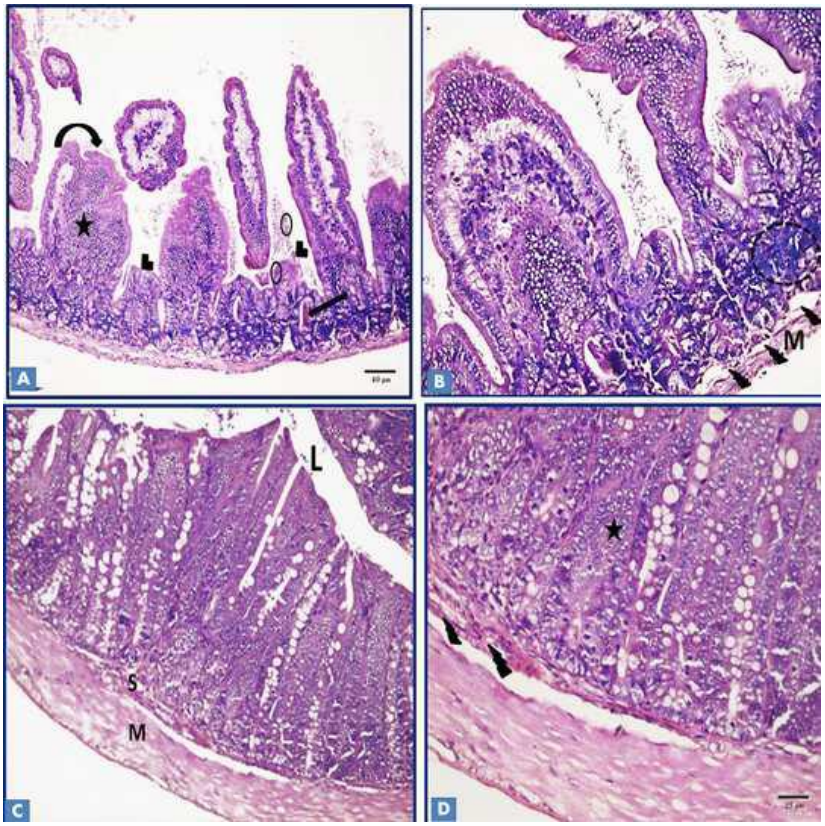


Fig. 3: Jejunum of the experimental groups: [A&B] Malnourished - infected mouse shows marked epithelial hyperplasia (star) with distortion and shortening of the villi (arrows heads). Some villi showing thickening with flat surface (curved arrow). Number of *Cryptosporidium* is attached with distorted epithelial and others admixed with the mucus and food debris between villi (circles). Note. Degenerated cellular debris in the lumen of the crypt (arrow). Some crypts shows Irregular nuclei, increased N/C ratio, nuclear hyperchromasia (dashes circle). Note. Submucosal hypervascularity (zigzag arrows) and musculosal atrophy (M). [C&D] Jejunum of primed mouse shows more or less normal architecture of jejunum, villi and crypts with normal narrow submucosa (S) resting on muscularis externa (M). Note. Few luminal *Cryptosporidium* (L), few hyperplastic cells in villi (star) and vascular submucosa (zigzag arrows) (H&E X250 &400).

Immunohistochemical results:

For iNOs immunstaining marker, the control jejunum mice showed faint diffuse brown immune reaction that increased significantly (*P ≤0. 0001*) in G2 except for positive degenerated area on the top of the villi. malnurished mice showed significant decrease in immune the decrease became markedly in G4 group (*P ≤0. 0001*) and this decrease was significantly (*P ≤0. 0001*) recovered in the G5 (Fig.4).

For CD4 immunstaining marker, the control jejunum mice showed positive light brown reaction in the epithelial cells of the villi and lamina propria. The infected mice, showed significant increase *(P ≤0.0001*) in the immune reaction. However, significant decrease in the immunostaining were detected in malnurished (*P≤0.0001*) and malnurished – infected (*P ≤0. 0001*) groups. In primed malnurished – infected group there was significant increase the in CD4 reaction (Fig.5).

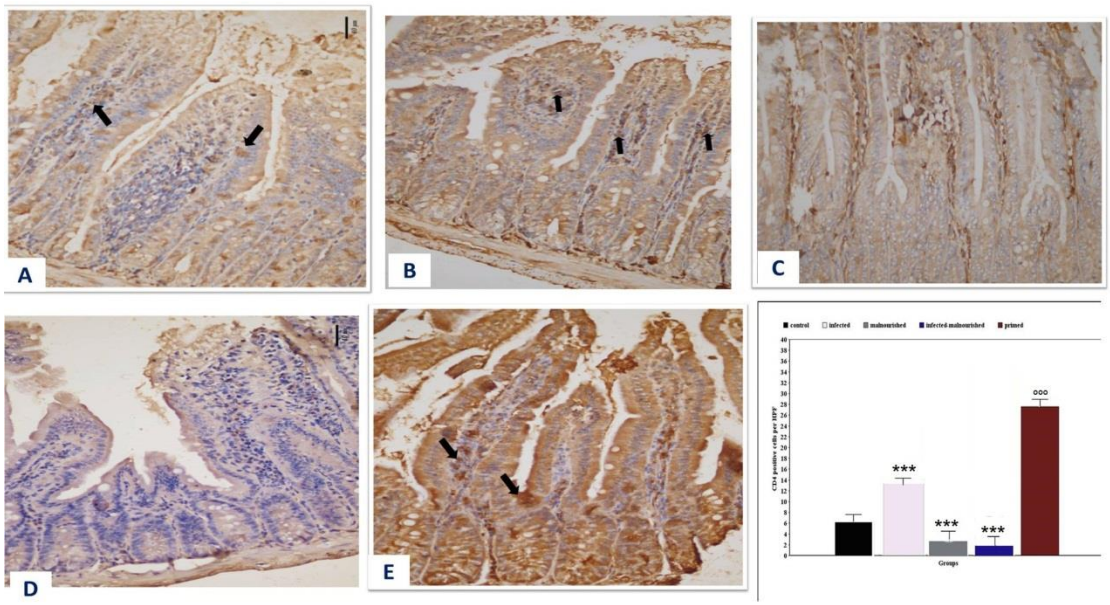


Fig (4): iNOS (indicated arrows head x250) immunohistochemistry of mice jejunum in different groups: [A] Control mice with diffuse negative immune reaction. [B] Infected mice with moderate brown immunostaining in inflammatory cells the lamina propria of the villi as well as base of the crypts. [C] Malnourished mice positive moderate brown reaction in the surface degenerated part of the villi. [D] Malnourished – infected mice with diffuse negative immune reaction. Small positive inflammatory cell reaction is seen in the lamina propria of the villi. [E] Primed mice shows diffuse moderate reaction with strong reaction in the lamina propria and the base of the crypts.
Right; Bar charts showing the number of iNOS per high power field (HPF) of the different animal groups. Difference from control mice, .****p* < *0.0001*; #difference from Malnourished – infected mice, $^{ooo}p < 0.0001$. Scale bars, 25 μm.

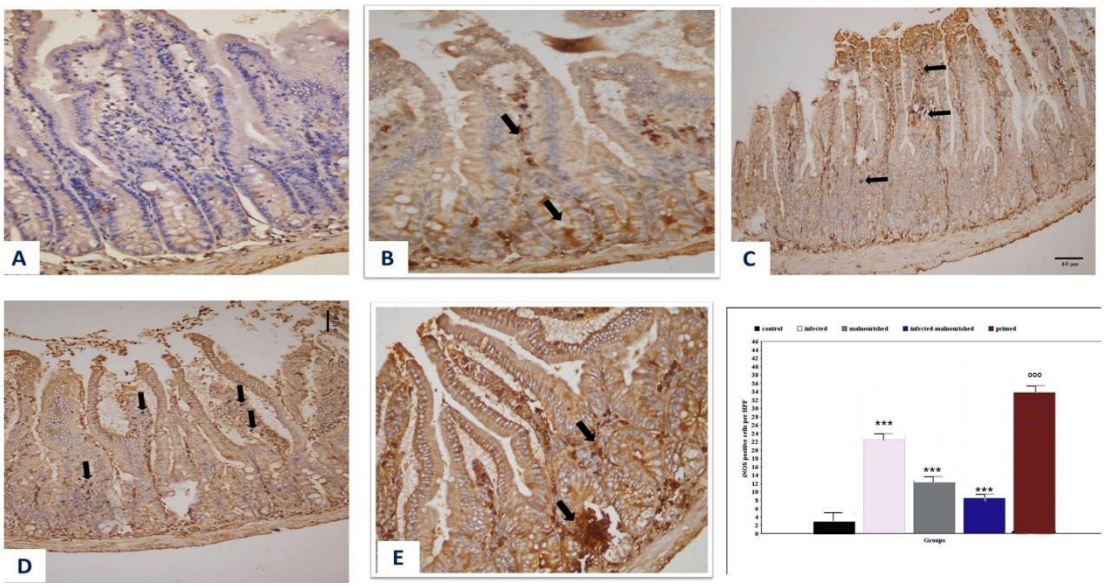
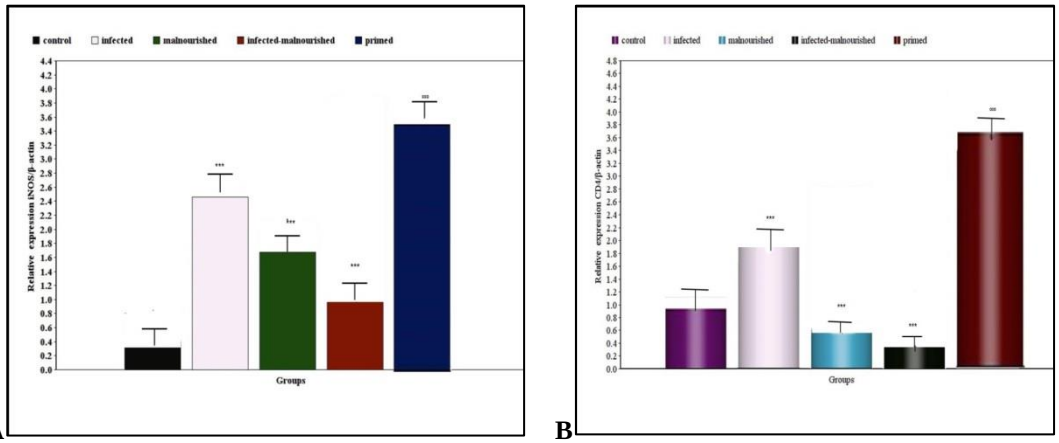


Fig. (5): CD4 (black arrows x400) immunohistochemistry of mice jejunum in different groups: [A] Control mice shows scattered week brown immune cell reaction in the surface epithelium and lamina propria. [B] Infected mice shows increase in the positive immune cells in the lamina propria. [C] Malnourished mice shows diffuse negative immune reaction except for scarce positive epithelial cells. [D] Malnourished – infected mice shows diffuse negative immune reaction. [E] Primed mice shows diffuse strong brown immune reaction with scattered tense in the lamina propria.
Right; Bar charts showing the number of CD4+ lymphocytes per high power field (HPF) of the different animal groups.****p* < *0.0001*: Difference from control mice; $^{ooo}p < 0.0001$: difference from malnourished – infected mice,. Scale bars, 25 μm.

CD4 and iNOS mRNA expression challenged Results:

In order to assess key mechanisms involved in cellular and innate immunity in primed mice against *C.parvum* infection with or without malnutrition,we addressed CD4 and iNOs mRNA transcripts in the mice jejunum. Both genes expression were found to increase significantly (*P ≤0. 0001*) in the infected mice and decrease significantly (*P ≤0. 0001*) in malnourished mice while the malnourished -infected mice showed insignificant decrease (P ≤0.0001) in both genes expression comparing to the control. Pre challenge of malnourished -infected mice by 10^2 *C.parvum* oocysts induce significant increase (*P ≤0.0001*) in jejunal CD4 and iNOs mRNA expression in primed group (Fig. 6 a & b).



A **B**

Fig. 6: Real-time PCR assays from experimental mice for the following jejunal mRNA transcripts: (A) cluster of differentiation 4 (CD4) and (B) Inducible nitric oxide synthase (iNOS).. Groups have at least 6 per groups and the results are shown as mean ±EM and expressed after β actin normalization. ****p < 0.0001:* Difference from control mice; $^{ooo}p < 0.0001$:difference from malnourished – infected mice.

DISCUSSION

Malnutrition with immune suppression and infection intensify each other[27]. The protective effect of *Cryptosporidium* priming versus vaccine is a matter of controversy.The current work, was done to evaluate the protective effect of priming with 10^2 *C.parvum* oocysts in *Cryptosporidium*- malnourished mice. This was assessed clinically, histopathologically and immunologically. Both clinical and histopathological improvement were evident with sensitization of both innate and adaptive immune responses.

The effect of priming with 10^2 *C.parvum* oocysts was evident clinically through significant reduction of weight loss, oocyst shedding, and tissue burden of the parasites. On the other hand the infected - malnourished group showed worsening of the clinical signs. The percentage of weight loss was (12.1%), (19.9%), (5.4%) in malnourished & malnourished infected and primed groups respectively. Koethe et al [28] found that there was association between weight loss and clinical outcomes among malnourished patients. Our results were in accordance with[12] as they found that protein malnutrition enhances weight loss (40%), oocyst shedding, and tissue burden of the parasites. Also, some [20] found that pre challenge with 10^6 *C. parvum* reduce the effect on weight loss (5.4%), oocyst shedding, and tissue burden of the parasites. The differences in the percentage of weight loss due to the difference in the dose and the duration of infection.

In this work, the jejunum of mice in G5 showed restoration in the number and the size of the villi. On the other hand they showed disrupted morphology in G4 which may affect the gut flora. Intestinal flora serves to maintain the integrity of intestinal mucosal epithelia and mucosal immune response. Our results are coincides with others [29]. The villous changes were obvious in malnourished –infected animals as there were atrophy of the villi. This may be due to *C.parvum* toxins that induce cells damage and displace the brush borders [30] and hence decrease the innate immune response[31]. Inversely, the marked protective effect of priming on jejunum tissue against the effect of cryptosporidiosis and malnourishment is very clear through increase in villous height and crypt depth, restoration of intestinal cells and goblet cells. As, intestinal epithelial cells (IECs) are considered as the main target and the first physical barrier against infection[32]. We can take this at the start point of priming effect. As the inflammatory cells noticed in infected animals and increased in malnourished- infected animals might came through chemotaxis produced by *Cryptosporidium* toxins.

These inflammatory cells enhance the production of certain cytokines that increase permeability, causing weakening of the intestinal epithelial barrier[33]. Thus Improvement of intestinal inflammation added a protective effect to intestinal mucosa. The malnutrition effect was improved in primed group and manifested histologically in our work through the increase in villus height .Thus pre challenged of malnourished–infected animals with 10^2 *C.parvum* oocysts can modulate the histopathological cascade in jejunum of infected-malnourished mice. The important role of intestinal epithelium in the activation of innate and adaptive immunity was discussed by others [12,20] who concluded that *Cryptosporidium* infection triggers early innate immune chemokines and cytokines in IECS that activate the immune effector cells .

This work proved that priming with 10^2 *Cryptosporidium* oocysts stimulated the innate immunity in primed group in compare to the malnourished –infected one. This is indicated by a significant increase in iNOS gene and expression. These effects were manifested through the significant decrease in oocyst shedding, and tissue burden of parasites. Our results were in accordance with [34] who proved that inhibition of iNOS led to increase parasitism and oocyst shedding in *C.parvum* -infected piglets. Our study also demonstrated a significant reduction in iNOS concentration in malnourished infected group as compared to the other groups this may be due to the effect of malnutrition on arginine synthesis as the protective effect of iNOS during *cryptosporidium* infection depends on arginine availability in mice. [35] proved the direct toxic effect of iNOS on the parasite and its indirect signaling stimulatory effect of the macrophages [36]. The macrophages produce large amounts of NO that induce cytostatic effects on the parasit [37] or prevent its attachment to the intestinal cells [36,34]. The present findings showed that the effect of pre challenge by 10^2 *C.parvum* oocysts stimulates the immune system of mice through increase both CD4 and iNOS gene and expression. The intimate relation between both types of immunity in the primed group was cleared by [35] who proved that CD4 T cells can induce iNOS in infected cells at distance from their site of antigen recognition through cytokine production.

CD4 T cells have a vital role in the protective immunity against bacterial infections viral infections and protozoan infections [38]. Our results come in line with [39] who detected increasing numbers of CD4 T cells in the intestinal mucosa of AIDS patient recovered from cryptosporidiosis infection in response to highly active antiretroviral treatment. Conversely, some [20] suggested the crucial role of CD8 T cell in clearance of most intracellular infections.

Aguirre et al [40] supported our results as they proved the role of CD4 in clearance of *Cryptosporidium* infection in immunocompetent mice.

In this study, the epithelial lining of the crypts of the malnurtioned –infected group showed irregular nuclei, increased N/C ratio, nuclear hyperchromasia. These signs were proved by some authors as a high-grade intestinal dysplasia accompanied with *C. parvum* and they referred these to suppression of the immune system[22]. Inversely, Absence of dysplastic changes in primed group in this work proved the marked protective effect of priming on the immune system through sensitization of innate and cell mediated immunity. In conclusion, we confirmed that cryptosporidiosis is worsened by malnutrition with biological end points while priming by a low dose change the natural history of the disease and helps CD4& iNOS upregulation giving a new sight to control the disease in vulnerable human populations for further studies.

Conflicts of interest: The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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