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ORIGINAL ARTICLE

Prolonged *Cryptosporidium Parvum* Infection Can Be a Risk Factor for Intestinal Malignancy Even In Immunocompetent Host

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ABSTRACT

Background: *Cryptosporidium (C.) is a widespread opportunistic protozoan parasite that ranks with Giardia as the most common cause of diarrheal outbreaks globally. In addition to diarrheal manifestations, it was claimed to be a risk factor for GIT malignancy in immunocompromised animals.* ***Objectives:*** *studying the link between experimental C. parvum infection and ileocecal neoplasia. We investigated the effect of immune status, treatment and duration of infection on oncogenesis and its dependency on β-catenin/Wnt signaling pathway abnormalities.* ***Methodology:*** *C. parvum infected mice groups (immunocompetent and immune-suppressed; untreated and treated) were studied regarding oocyst shedding, dysplastic changes, cellular and genetic expression of β-catenin.* ***Results:*** *neoplastic changes were detected at higher rate and earlier time in C. parvum infected-immune-suppressed (ISP) mice with increased β-catenin expression at both cellular and genetic levels. Despite that, low grade dysplasia was detected in immunocompetent (ICP) mice as time progressed (i.e. 75 d.p.i.). No dysplasia was detected in nitazoxanide treated groups.* ***Conclusion:*** *our results demonstrate that, C. parvum infection is a risk factor for ileocecal dysplasia. The resulting pathology depends on the sintensity and duration of infection in addition to immune status of the host. Competent immune system is not an absolute protecting element against incidence of dysplasia but rather postpones it. β-catenin/Wnt signaling pathway is involved in C. parvum-induced intestinal dysplasia.*

INTRODUCTION

Cryptosporidium (*C.*) is a protozoan parasite that ranks with *Giardia* as the most common cause of major diarrheal outbreaks globally [1] with a prevalence ranging from 1-3% - in developed countries- to 10% -in developing countries-. The problem of eradicating this pathogen lies in its resistance to many chemotherapeutic agents that are usually effective in treatment of intestinal parasites [2]. Incidence of foodborne and waterborne outbreaks of cryptosporidiosis is not uncommon. Because of its resistance to chlorination, it usually contaminates water sources in areas of poor sanitary conditions with higher incidence in infants and neonates [3,4,5]. Also, its transmission through contaminated swimming pools is an issue of great concern in many developed countries [6]. The danger of this highly prevalent pathogen is that, the associating pathology of intestinal cells extends beyond diarrhea. Many studies involved *Cryptosporidium* to be a risk factor for gastrointestinal tract (GIT) cancers especially *C. parvum* species. It was reported to cause malignancy in immune-suppressed experimental animals with the use of very low inoculums. Even one oocyst was capable of inducing malignancy [7,8]. Its association with malignancy was also reported in human studies [9].

The mechanism of incidence of GIT malignancies is a complex process that can occur through many different and interacting pathways. Wnt-signaling pathway abnormalities is one of the common mechanisms of oncogenesis that are even targeted during cancer therapy. WNTs are glycoproteins that regulate proliferation, survival, migration, polarity, fate, and self-renewal of cells [10,11,12,13]. Intestinal cells are one of the main sites of Wnt pathway activity. So, genetic mutations in components of this pathway usually underlies tumorigenesis in these tissues. Activation of β-catenin dependent Wnt signaling pathway is a cascade of events that starts by a membranous receptor protein called β-catenin. This protein acts both as a transcriptional co-regulator and an adaptor protein for intracellular adhesion. In normal conditions, the intracellular levels of β-catenin are regulated by multiprotein complex kinases called 'destruction complex' that prevent cytoplasmic and nuclear accumulation of β-catenin. Aberrant activation of this pathway leads to the accumulation of β-catenin in the nucleus and promotes the transcription of many oncogenes with resulting uncontrolled proliferation and differentiation of intestinal epithelial cells. This leads to the disruption of tissue architecture and the formation of tumor growths [14,15,16,17,18]. Many studies reported a link

between *C. parvum* infection and occurrence of intestinal dysplasia in immune-suppressed animals. Part of these studies even linked the resulting dysplasia to Wnt signaling pathway abnormalities and oncogene mutations [8,19].

In the current work, we aimed to study the link between *C. parvum* infection and ileocecal neoplasia in experimental animals and if the duration of infection can increase risk of their occurrence. We also investigated whether this risk is confined to immune-suppression or not. The third aim was to assess if early treatment of infection can abort incidence of malignancy. The fourth aim was to investigate dependency of the resulting neoplasia on Wnt signaling pathway abnormalities.

METHODOLOGY

Experimental animals and ethics statement

Male laboratory bred, pathogen free Swiss-albino mice (6–8-week-old, 18-22 gm weight) were purchased from Theodor Bilharz Research Institute, TBRI (Giza, Egypt). All animal experiments were performed at TBRI. Mice were kept under standard housing conditions in the animal house of TBRI and were maintained on a commercial diet. Room temperature was kept at 20-22°C. Experimental procedures were performed in accordance with the international ethical guidelines after the approval of the institutional ethical committee of TBRI.

Study design

Mice were divided into 6 groups. The 1st 3 groups were immunocompetent (IC) while the remaining 3 groups underwent drug induced immune-suppression. Mice groups were as follows; group I (IC) IC-non-infected control; group II (ICP) IC-*C. parvum* infected; group III (ICPT) IC-*C. parvum* infected and NTZ treated; group IV (IS), non-infected immune-suppressed (IS) control; group V (ISP) IS-*C. parvum* infected; and group VI (ISPT) IS-*C. parvum* infected and NTZ treated mice. Mice of each group were euthanized at three-time intervals, 25-, 50- and 75-days post infection (d.p.i.) Each group consisted of 20 mice except ISP and ISPT which consisted of 50 and 30 mice, respectively. Number of euthanized mice in the 1st 4 groups was 6, 6, 8 mice at the 3-time intervals, respectively. Mice of ISP group were euthanized in numbers of 8,8, 24 respectively. Mice of ISPT group were euthanized in numbers of 10, 10, 10, respectively.

Immune-suppression of the experimental animals

Mice of groups IV, V and VI (i.e. IS, ISP & ISPT) were subjected to drug induced immune-suppression using oral dexamethasone (Kahira Pharmaceuticals & Chemical Industries Company, Egypt) at a dose of 0.25 µg/g/day dissolved in 200 µl of distilled water/ mouse using esophageal tube. Treatment started 14 days prior to *C. parvum* inoculation and continued daily throughout the experiment [20].

Parasite and mice infection

Oocysts of *C. parvum* were obtained from naturally infected calves (at slaughter houses). Intestinal contents and ileal mucous membrane scrapings [21] were stained by modified Ziehl–Neelsen (MZN) and microscopically examined for the presence of oocysts. Oocyst's DNA was genotyped as *C. parvum* by PCR of *Cryptosporidium* oocyst wall protein (COWP) gene [22]. For experimental inoculation, *C. parvum* oocysts were suspended in PBS and were adjusted to obtain a concentration of 1×10^5 oocysts in 200 µL of phosphate buffered saline (PBS) which is the infecting dose for each mouse. Before animal inoculation, 12 hours fasting period -including water restraint- was required to facilitate the infection procedure. The trophozoites were inoculated directly into the duodenum of mice using a gastric gavage [7]. Stool samples were regularly examined for oocysts to confirm the establishment of infection. Samples were concentrated using formol-ether concentration technique [23], stained by MZN and were examined by light microscope every three days.

Nitazoxanide treatment

Mice of ICPT and ISPT groups were treated with nitazoxanide, NTZ (Medizen Pharmaceutical industry, Utopia Pharmaceuticals, Egypt) in a dose of 500 mg/kg/day orally for 6 successive days after the establishment of infection [20,24].

Euthanizing animals and sample collection

On days 25, 50 and 75 p.i., mice were euthanized by decapitation. The intestinal lumens of euthanized mice were flushed with ice-cold saline to wash out food particles. Ileocecal region was cut out and divided into 2 parts, one part was fixed in formalin 10% (for histopathological and immunohistochemical studies) and the other part was homogenized in Tri-zol reagent (for further gene expression studies).

Assessment of *C. parvum* oocyst shedding

Fresh fecal pellets were collected separately starting at the 1st d.p.i and repeated every 3 days until the end of the experiment. Samples were suspended in 10% formalin, homogenized and stained with MZN. Oocysts were counted using a hemocytometer as described by Sayed et al. [25].

Histopathological examination of ileocecal region

Paraffinized blocks of ileocecal region was cut into thin sections, mounted on clean glass microscopic slides and stained with haematoxylin and eosin (H&E) stain [26]. The slides were examined using a multi-head microscope, Olympus SC100, and analySIS getIT software. Pathologic changes were classified according to the Nomenclature for Histologic Assessment of Intestinal Tumors [27] as follows, 0= No lesion; 1= Inflammation and/or regenerative changes; 2= Low grade intraepithelial neoplasia (LGIEN) or low-grade dysplasia; 3= High grade intraepithelial neoplasia

(HGIEN) or high-grade dysplasia. In this category, adenoma with HGIEN, carcinoma in situ (limited to the epithelium) or intra-mucosal adenocarcinoma (invasion of the basal membrane of glands) were also included.

Assessment of cellular B-catenin expression

Abnormal expression of β-catenin in intestinal cells was detected by immunohistochemical staining using anti-β-catenin antibodies (Abcam, USA). Positive cells for β-catenin immune stain appeared as brown punctuations. Because β-catenin is normally localized at the membranes of the cell–cell borders with no cytoplasmic or nuclear expression in normal epithelial cells, any expression out of this description was considered abnormal [28]. Immunohistochemical grading of β-catenin staining was calculated using histo score (H-score) method. The intensity of membrane staining was given a number from (0, 1+, 2+ or 3+). Percent of stained cells in each tissue was multiplied by the intensity of staining. 1 × (% cells1+) + 2 × (% cells 2+) + 3 × (% cells 3+). Then, a score of 0-300 was given for each field followed by a mean score for all fields [29].

Assessment of genetic β-catenin expression

To ensure that the increase and abnormal location of β-catenin is original (i.e. primary) and not a result of malfunctioning destruction system, genetic β-catenin expression was assessed by Quantitative Real-time PCR (qRT-PCR). The ileocecal tissues were homogenized, and RNA was extracted using the Direct–zol RNA Miniprep Kit (Zymo research, USA.). Extracted RNA was reverse transcribed into cDNA using QuantiTect Reverse Transcription Kit (Applied Biosystems, USA). Quantitative Real-Time PCR was performed using QuantiTect SYBR Green with low ROX PCR Kit (Qiagen, USA). All steps were done according to the manufacturer's protocol. The reaction conditions were: a 15-minutes initial denaturing at 95°C, followed by 45 cycles of denaturing at 94°C for 15 seconds (s), annealing at 60°C for 30 s. and extension at 72°C for 34 s., and the final extension step at 72°C for 10 minutes. Melting curve analysis was used to confirm the specific PCR products. Data analysis using Applied Biosystems 7500, software version 2.0.1. Housekeeping gene, Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an endogenous reference. The $2^{-\text{delta delta Ct}}$ method was used to calculate the differences of the expression level of β-catenin. The primer sequences were as follows: **β-catenin gene**: Forward (F): 5' – GCTGACCTGATGGAGTTGGA- 3'. Reverse (R): 5' - GCTACTTGCTCTTGCGTGAA- 3'. **GAPDH gene:** Forward (F): 5'-ACCACAGTCCATGCCATCAC- 3'. Reverse (R): 5'-TCCACCACCCTGTTGCTGTA- 3' [30].

Statistical analysis:

Data entry, coding and analysis were conducted using an IBM personal computer with Statistical Package of Social Science (SPSS) version 22 (SPSS, Inc, Chicago, Illinois, USA). Data of this study were of both quantitative and qualitative types. Quantitative data were expressed in mean ($\bar{X}$) and standard deviation (SD). Kruskal Wallis and Mann Whitney's tests were used to estimate the difference between means of quantitative variables. Qualitative data were expressed as number and percent (%). Spearman's correlation (r) was used to measure the association between quantitative and qualitative (categorical) variables. The level of significance of the present data was 95%, so, p-value >0.05 was considered a non-statistically significant difference, while p-value < 0.05 was considered a statistically significant difference.

RESULTS

Shedding of C. parvum oocysts is controlled by a properly functioning immune response.

Regarding control infected groups, shed oocysts were lower in ICP than ISP group with a statistically significant difference between both groups at all times of euthanasia ($p<0.05$). Moreover, NTZ treated groups showed a statistically significant reduction of oocyst shedding ($p<0.05$). Although NTZ treatment reduced shed oocyst compared to infected controls, comparing both treated groups revealed a statistically significant higher reduction in ICPT group than the ISPT one at all times of euthanasia ($p<0.05$) (figure 1a).

Dysplastic changes appear earlier and are more frequent with immune-suppression

LGIEN changes of the ileocecal region were detected earlier in ISP than ICP group. They appeared 50 d.p.i in 87.5% of ISP mice, while ICP group was totally free of LGIEN. With time progression (i.e. 75 d.p.i.), HGIEN was detected in 25% of ISP mice and 25% of ICP mice developed LGIEN changes. Compared to positive controls, NTZ treated groups revealed total absence of dysplastic changes in both groups. Changes detected in ISPT group were restricted to mild inflammatory changes while ICPT group was totally free of inflammation (fig. 1b & fig. 2). A statistically significant correlation was detected between number of shed oocysts and degree of pathological changes (fig. 1c&d).

Abnormal ileocecal β-catenin expression is more with infection and immune-suppression

Although the abnormal β-catenin expression in the ileocecal region was increased in both sole *C. parvum* infected groups (i.e. ICP and ISP), it was significantly higher in ISP group than the ICP one ($p<0.05$). Treatment with NTZ was associated with a statistically significant reduction in abnormal β-catenin expression compared to the untreated groups. ISPT group showed lower reduction of abnormal β-catenin expression compared to ICPT group with a statistically significant difference between both groups ($p<0.05$) (fig. 1e and fig. 3).

Genetic expression of β-catenin is increased with infection and immune-suppression

Like its cellular expression, genetic expression of β-catenin in the ileocecal region was higher in the untreated *C. parvum* infected groups (i.e. ICP and ISP) compared to either control or treated groups. Moreover, it was higher in ISP than ICP group with a statistically significant difference between both groups ($p<0.05$). A statistically significant reduction of abnormal genetic expression of β-catenin was detected in both treated groups although it was higher in ICPT group than the ISPT one where genetic expression approached control levels (fig. 1f). Level of β-catenin gene expression correlated positively with degree of dysplasia in ileocecal tissues (fig. 1g).

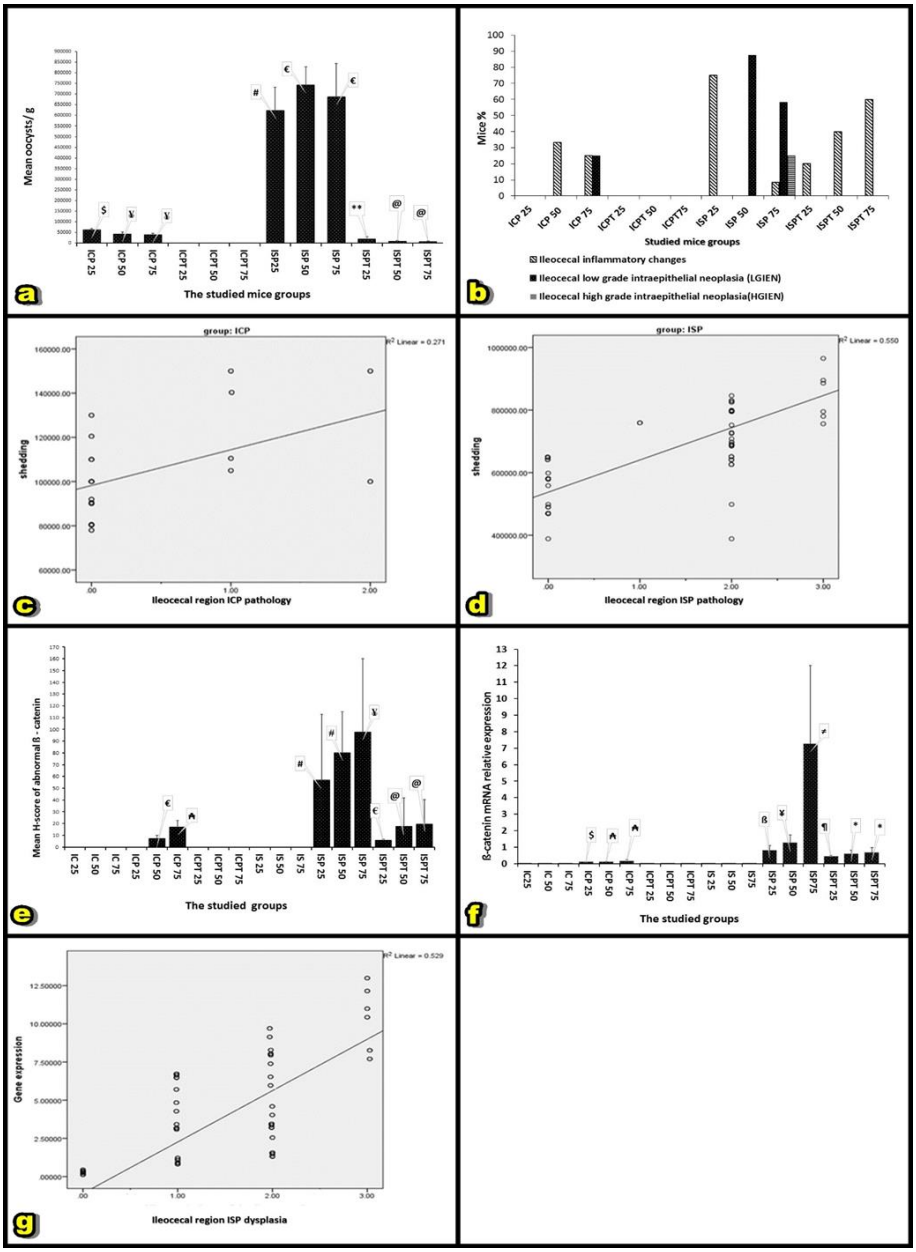

Fig. 1: Comparison of the studied groups regarding: a) Counts of shed *C. parvum* oocysts. b) Pathological changes of ileocecal region. c) Correlation of counts of shed *C. parvum* oocysts and pathological changes of ileocecal region of ICP group. d) Correlation of counts of shed *C. parvum* oocysts and pathological changes of ileocecal region of ISP group. e) Mean H-scores of abnormal β-catenin expression. f) Levels of β-catenin RNA expression. g) Correlation of levels of β-catenin RNA expression and degree of dysplasia.

N.B. Symbols present on columns of fig. a,b,e & f refers to the statistical differences between groups. Columns with similar symbols has no statistically significant difference. Columns with different symbols has statistically significant difference.

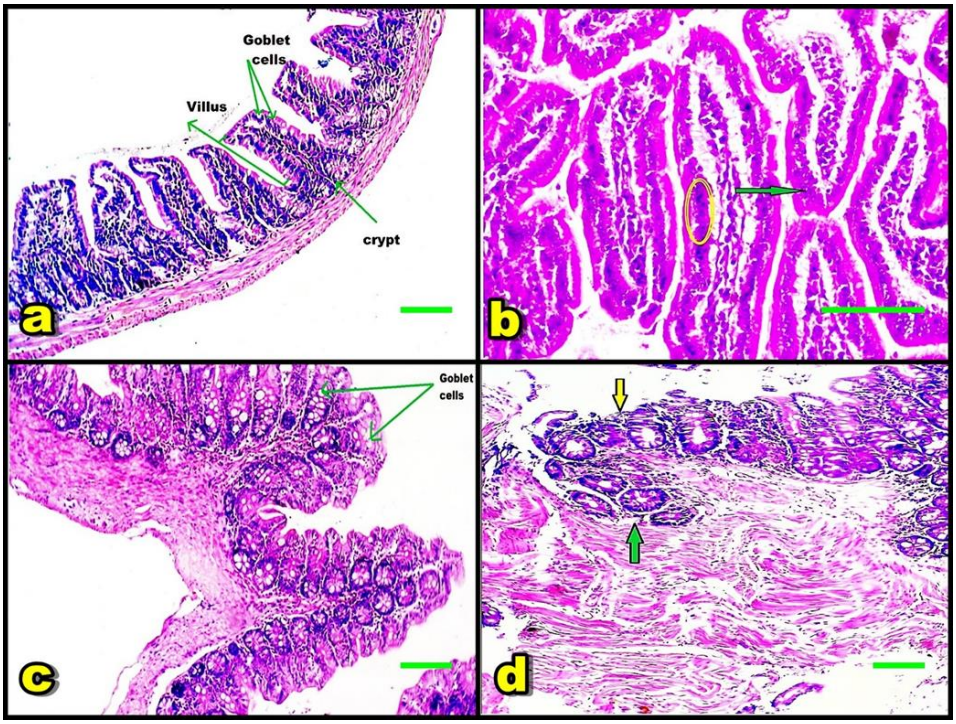

Fig. 2: H & E stained sections of ileocecal region (scale bar 100 µm). a) Ileum of IC group showing normal villous architecture with preserved goblet cells. b) Ileum of ICP group (75 d.p.i.) showing LGIEN in the form of nuclear stratification (green arrow), hyperchromatic nuclei (illustrated with yellow circle) and goblet cell depletion. c) Cecum of IC group showing normal mucosa with preserved goblet cells. d) Cecum of ISP group (75 d.p.i.) showing HGIEN in the form of mucosal ulceration (referred by yellow arrow) with glandular invasion of muscle layer (referred by green arrow).

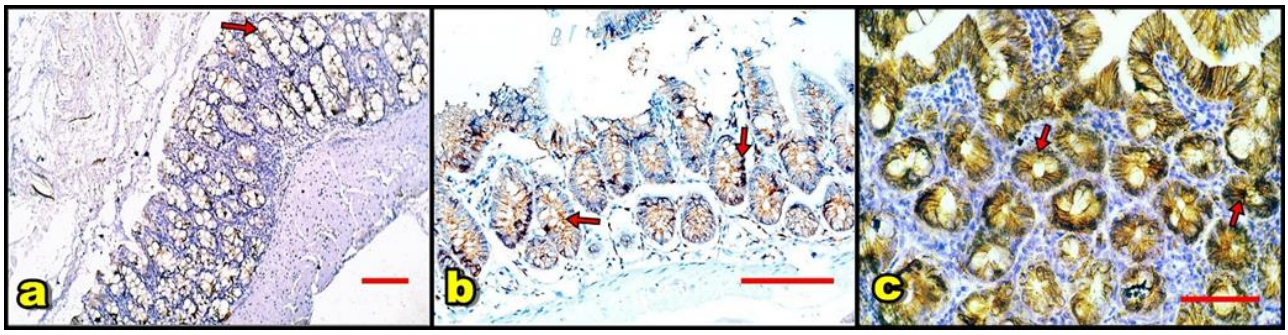

Fig. 3: Immune histochemical staining of β-catenin expression (referred by red arrows) in ileocecal region (scale bar 50 µm). a) Normal pattern of β-catenin expression in IC group. b) Mild increase in abnormal β-catenin expression in ICP group (75 d.p.i). c) Strong abnormal β-catenin expression in ISP group (75 d.p.i).

DISCUSSION

The aim of the present study was to investigate the link between experimental *C. parvum* infection and occurrence of ileocecal neoplasia. We investigated the effect of immune status, treatment and duration of infection on the process of oncogenesis and its dependency on β-catenin dependent Wnt signaling pathway abnormalities. In the current work, the statistically significant lower shedding of oocysts that was detected in ICP compared to ISP group can be explained by the effect of a perfectly functioning immune elements in ICP group which were able to resist parasitic multiplication and spread to a large number of intestinal epithelial cells. Deficiency of these mechanisms with immune-suppression is the cause of

increased shedding in ISP group [31,32]. The noticed difference in efficacy of NTZ among ICPT and ISPT groups can be related of the dependency of this drug on a well function immune response to perform a perfect action [33]. Similarly, the protective role of immune system in control of cryptosporidiosis was reported in many studies e.g. Certad et al. [34]; Benamrouz et al.[7]; Abdou et al. [20] and Sayed et al. [25].

The increased intensity of infection that associated immune-suppression was reflected on pathology where LGIEN changes of ileocecal region were detected earlier and involved a higher percentage of mice in ISP than ICP group. It even progressed to HGIEN as time passed. Severity of pathological changes correlated positively with intensity of infection. Our results are supported by Certad et al. [27]. They reported that, appearance and severity of dysplastic changes is dependent on both intensity of infection and degree of immune-suppression. Occurrence of dysplasia with ISP mice was similarly recorded in many other studies e.g. Certad et al. [34]; Benamrouz et al. [7]; Abdou et al. [20] and Sayed et al.[25].

The differences in time of appearance and severity of dysplastic changes between our work and others can be regarded to the use of different mice strains which respond differently to *C. parvum* infections [35]. In addition to different protocols of immune-suppression and number of oocyst inoculums used.

Time was also effective in ICP group where LGIEN changes were detected 75 d.p.i. Similarly, Abdou et al. [20] reported appearance of dysplastic changes in ICP mice with time passage which involved lower percentage and were milder compared to ISP groups. In our work comparison of both NTZ treated groups revealed total absence of dysplastic changes in both groups. Changes detected in ISPT group were restricted to mild inflammatory changes. These findings were also in accordance with those of Abdou et al. [20].

The abnormal β-catenin expression that was detected in the ileocecal regions of both sole *C. parvum* infected groups (i.e. ICP and ISP) and was significantly higher in ISP group than the ICP one can explain the difference in pathology among both groups. Increased abnormal β-catenin promotes the transcription of oncogenes with resulting uncontrolled proliferation and differentiation of intestinal epithelial cells [15,16,17,18]. This was confirmed in NTZ treated groups that showed a statistically significant reduction in abnormal β-catenin expression compared to the untreated groups and was totally free of dysplastic changes. Likewise, Benamrouz et al. [8] reported increased abnormal β-catenin expression in ISP mice exhibiting LGIEN or HGIEN and was the highest in mice that developed colonic adenocarcinoma.

Based on the detected abnormalities of β-catenin expression, the Wnt signaling pathway appears to be highly implicated in the *C. parvum*-induced malignancy, so, we were directed to study gene expression of β-catenin to determine if this cellular increase is primary or secondary to malfunctioning destruction system. Gene expression of β-catenin was higher in the untreated *C. parvum* infected groups (i.e. ICP and ISP) and was reduced in both treated groups to approach normal levels in ICPT group. Our results are supported by Liu et al. [19] who recorded potentiating effect of *C. parvum* on expression of the apoptosis-controlling-genes. Also, incidence of mutation in β-catenin gene was absent in the study of Benamrouz et al. [8]. So, oncogenesis occurs in the epigenetic level rather than the genetic one. This point needs to be studied in more depth.

CONCLUSION

Cryptosporidium parvum is a risk factor for ileocecal dysplasia. The resulting pathology depends on intensity and duration of infection in addition to immune status of the host. Competent immune system is not an absolute protecting agent against incidence of dysplasia but rather postpones it. β-catenin/Wnt signaling pathway is involved in *C. parvum*-induced intestinal dysplasia.

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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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