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RESEARCH ARTICLE

EFFECT OF *CRYPTOSPORIDIUM PARVUM* INFECTION ON DNA DAMAGE AND CYTOKINES GENE EXPRESSION IN BLOOD OF IMMUNOCOMPROMISED MICE

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ABSTRACT

There are few data regarding the genotoxic and immuno-modulatory effects of *Cryptosporidium parvum* (Family: Cryptosporidiidae) infection *in vivo*. Therefore, the present study evaluated the effect of *Cryptosporidium parvum* infection on DNA and gene expressions of interleukin-6 (*Il-6*) and tumor necrosis factor-α (*Tnf-α*) in blood of the immunocompromised mice. Fifty adult male Swiss albino mice were used in the present study; these were randomly divided into five groups (10 mice/each group) as follows: group I served as a healthy, uninfected control group; groups II-IV were immunosuppressed by dexamethasone, and uninfected, or infected (orally) with 3.0×10^3 (low dose) or 1.0×10^4 (high dose) *Cryptosporidium parvum* oocysts, respectively; group V did not receive dexamethasone and infected with 1.0×10^4 *Cryptosporidium parvum* oocysts. DNA damage of leucocytes was assessed by flow cytometry and comet assay; the gene expression of the cytokines was performed using real-time polymerase chain reaction. The results revealed that infection with the low or the high dose of *Cryptosporidium parvum* induced DNA damage in the leucocytes of the immunosuppressed mice, as revealed by the elevations in tail length and tail moment of the comet assay compared with the healthy and immunosuppressed, uninfected, mice. In addition, significant decreases and increases in the expression of *Il-6* and *Tnf-α* genes, respectively, were recorded in leucocytes of the immunosuppressed, *Cryptosporidium parvum*-infected mice in comparison with the healthy and immuno-suppressed, uninfected, mice. In conclusion, *Cryptosporidium* infection induced DNA damage and augmented the changes induced in the *Il-6* and *Tnf-α* gene expressions in the leucocytes of the immunosuppressed mice.

INTRODUCTION

Cryptosporidium is a microscopic intra-cellular protozoan belongs to a diverse group of apicomplexan parasites that infect the epithelial cells lining the luminal surfaces of gastrointestinal and respiratory tracts in humans and animals[1]. *Cryptosporidium* was first recognized as

a pathogen in human, nearly 70 years after the first *Cryptosporidium* was detected in mice[2]. The risk of developing a severe disease differs according to the status of the immune system of infected host[3]. In immunocompetent hosts, infection usually results in self-limiting diarrhea that resolves spontaneously and considered relatively non-inflammatory. However, in children cryptosporidiosis is often associated with mild inflammation especially in endemic area[4]. In the other hand, in immuno-compromised hosts (such as those suffer from immune deficiencies, undergo cancer chemotherapy, or after bone marrow transplantation) the parasite induces severe clinical significance, since it may cause a potent persistent disease that may be life-threatening[3,5]. Since cryptosporidiosis has a significant effect on health of immunocompromised patients in developing countries, further study to the pathogenesis of the parasite is warranted. Immuno-compromised mice model has been used to study pathogenic potential of *Cryptosporidium* by many scientists[6]. Due to unavailability of appropriate *in vivo* evidence on the genotoxic and immuno-modulatory effects of *Cryptosporidium* infection, the current study aimed to evaluate the effect of *Cryptosporidium* infection on DNA damage and cytokines gene expression in blood of chemically-immunocompromised mice.

MATERIAL AND METHODS

Ethics approval

All experiments were performed in accordance with the ethical guidelines for animal experimentation as established by the ethical research committee of Theodor Bilharz Research (TBRI) and approved by Schistosome Biology Supply Center (SBSC) at TBRI, Giza, Egypt.

Animals

Fifty adult male Swiss albino mice (*Mus muscullus*, CD1 strain, 3-4 weeks old, weighing 20-25 g) were obtained from the SBSC at TBRI, Giza, Egypt. The animals were humanely handled and fed on a standard commercial pelleted diet and water *ad libitum*. They were kept in cages in air conditioned animal house at 20-25°C, and acclimatized on the laboratory conditions for one week prior to experimentation.

Induction of immunosuppression

Dexamethasone phosphate (DEXP; Merck, Germany) used as an immunosuppressive agent for mice. Animals were administered DEXP dissolved in distilled water by gavage at a concentration equivalent to 0.25 mg/kg for 14 successive days prior to inoculation with *Cryptosporidium parvum* (Family: Cryptosporidiidae) oocysts[7].

Collection of *Cryptosporidium parvum* oocysts

Oocysts of *Cryptosporidium parvum* were obtained from neonatal calves, aged from 3 to 14 days, at governmental farm at Abu-Rawash, Giza, Egypt. Freshly collected clean oocycts were used to infect 2-day old female goat kids (10^6/animal) for maintenance and ampli-fication of *Cryptosporidium* oocysts[8]. After the shedding of oocysts, feces were collected daily, mixed with an equal volume of 2.5% potassium dichromate (for preservation of oocysts), and stored at 4°C. Feces were then sieved sequentially through stainless steel screens. Oocysts were purified by discontinuous sucrose gradients[8] and counted using hemocytometer under bright field microscopy, and then stored in 2.5% potassium dichromate at 4°C.

Experimental design

The animals were randomly allocated into five similar groups each of 10 mice. Group I was left without treatment as a healthy immunocompetent control group. Three additional groups (groups II-IV) were received DEXP for 14 days. These immuno-suppressed groups were arranged as follows: group II served as an immunosuppressed, uninfected group; groups III and IV were inoculated per mouth with 3.0×10^3 or 1.0×10^4 *Cryptosporidium parvum* oocysts,

respectively. Group V did not receive DEXP, but was inoculated with 1.0×10^4 *Cryptosporidium parvum* oocysts. Success of infection in each mouse was proved by tracking the rate of shed oocysts in its feces post inoculation. Fourteen days after infection, mice were killed and blood samples were collected. The infected, immunocompetent group (group V) was found to be not significant in all measured parameters (DNA damage and cytokines gene expression in blood), as the healthy, uninfected control group (group I). Therefore, the data of group V was not shown in the results.

Assay of apoptotic cells by flow cytometry

Assay of apoptotic cells was performed with the annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit and propidium iodide (PI) (Immunotech SAS, Marseille, France) according to the manufacturer's instructions. Briefly, cell samples were washed with phosphate-buffered saline and subjected to 5 min centrifugation at 300 ×*g* and 4°C. The supernatant was discarded, and the cell pellet was resuspended in ice-cold diluted binding buffer at 5×10^5-5×10^6 cell/mL. The cell suspension (100 µL) was stained with annexin V-FITC solution (1.0 µL) and PI solution (5 µL) for 10 min in ice-cold tubes in the dark; then, 400 µL of ice-cold diluted binding buffer was added to the cell sample. Measurement of apoptotic cells, which had high FITC and a low PI signal, was performed within 15 min by a 4-color flow cytometer (Coulter Epics XL-MCL, Beckman Coulter, Miami, FL, USA) with EXPO 32 ADC software (Beckman Coulter).

Alkaline comet assay

Alkaline comet assay was performed to measure the level of leucocytes DNA damage according to the protocol described by Tice *et al*.[9] with minor modification. Whole blood (10 µL) is mixed with low melting agarose (0.5%) and spread on a slide coated with normal melting agarose (1%). Slides were left until agarose is hardened and incubated in cold lysis buffer (2.5 mol NaCl, 100 mmol EDTA, and 10 mmol Tris, pH = 10, with freshly added 10% DMSO and 1% Triton X-100) at 4ºC for 24 hours in darkness. After lysis, slides were incubated in a fresh alkaline buffer (300 mmol NaOH and 1 mmol EDTA, pH>13) for 20 minute and electrophoresed at 25 V and 300 mA for 30 minute. Slides were dipped in 0.4 mol Tris base (pH = 7.5) for neutralization, fixed in 100% cold ethanol, left to dry, and stored at room temperature until scoring. Slides were stained with ethidium bromide prior imaging and 50 comet cells per animal were analyzed using v1.5 Comet Score™ software (Tritek Corp, Sumerduck, VA, USA). The tail length and tail moment were used as endpoints for evaluating DNA damage.

Measuring the expression level of *Il-6* and *Tnf-α* genes

Real-time polymerase chain reaction (qPCR) was performed to measure the expression level of interleukin-6 (*Il-6*) and tumor necrosis factor-α (*Tnf-α*) genes in mice blood. The whole RNA was extracted using Gene JET RNA purification kit (Thermo Fisher Scientific, Waltham, MA, USA); DNase I was added to ensure the removal of remaining genomic DNA. The purity and concentration of extracted RNA were detected using Nano Drop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific) at 260 nm and 280 nm. Pure RNA has an absorbance ratio equals 1.8-2.0 at 260 nm: 280 nm. The extracted RNA was converted into complementary DNA (cDNA) using Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). The expression level was finally measured by performing SYBR green-based qPCR (Thermo Fisher Scientific) using the 7500 Fast qPCR System (Applied Biosystem 7500, Clinilab, Egypt). Each PCR reaction (12 µL) contains 1.0 µL of cDNA sample, 0.5 µL of forward primer (50 pmol/µL), 0.5 µL of reverse primer (50 pmol/µL), 6 µL of SYBR green master mix (2x), and 4.0 µL of deionized DNase and RNase free water.

The qPCR reaction was started with an initial denaturation for 15 minute at 95ºC, then thirty five cycles of denaturation at 95ºC for 15 second, annealing for 30 second, and extension for 1.0 minute at 72ºC using the primers sequences listed in Oh *et al*.[10] (Table 1). The expression level of *Tnf-α* and *Il-6* genes was normalized against the expression of β actin gene[11], as house-keeping gene, and quantified using the comparative cycle threshold (ΔΔCt) method[11].

Statistical methods:

Data were tabulated, statistically analyzed by one way analysis of variance ANOVA (followed by Duncan's multiple range test to compare between different groups), and expressed as mean ± standard deviation. A *P*-value ≤ 0.05 was considered significant.

Table 1: List of primers' sequences and annealing temperature used in the real-time polymerase chain reaction (qPCR)

Gene	Primers	Sequence	Annealing
Il-6[10]	Forward	5′-CATGTTCTCTGGGAAATCGTGG-3′	58°C
	Reverse	5′-AACGCACTAGGTTTGCCGAGTA-3′	
Tnf-α[10]	Forward	5′-AGCACAGAAAGCATGATCCG-3′	55°C
	Reverse	5′-GTTTGCTACGACGTGGGCTA-3′	
Beta actin[11]	Forward	5′-GCA CCA CAC CTT CTA CAA TG -3′	58°C
	Reverse	5′-TGC TTG CTG ATC CAC ATC TG-3′	

RESULTS

Cryptosporidium parvum infection induced apoptosis and DNA damage in leucocytes of the immunocompromised mice

Cryptosporidium parvum infection increased markedly the apoptotic cells (Figure 1) and induced DNA damage (Table 2) in leucocytes of the immunosuppressed mice, as revealed by significant elevations (*P*<0.001) in the tail length and tail moment compared with the healthy and immunosuppressed, uninfected mice. The level of DNA damage induced by heavy infection in group IV is statistically higher (*P*<0.001) than that induced by light infection in group III (Table 2).

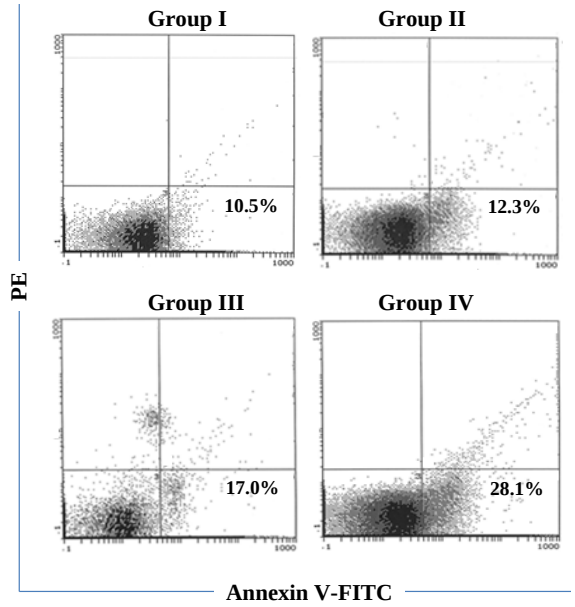

Figure 1: Flow cytometric analysis of apoptotic cells in the immunosuppressed mice infected with 3.0×10^3 and 1.0×10^4 *Cryptosporidium parvum* oocysts (groups III and IV, respectively) compared with the healthy (group I) and immunosuppressed, non-infected (group II) mice. Apoptotic cells showed high annexin V-fluorescein isothiocyanate (FITC) and low propidium iodide (PI) signal. The flow cytometric data represented three animals from each group, and indicated that the apoptotic cells increased markedly in infected groups, especially group IV.

Table 2: Level of DNA damage in the immunosuppressed mice infected with the low or the high dose of *Cryptosporidium parvum* oocysts (groups III and IV, respectively) compared with the healthy (group I) and immunosuppressed, non-infected (group II) mice.

	Treatment	Tail length (pixel)	Tail DNA (%)	Tail moment
Group I	---	4.69 ± 0.80^{a}	18.69 ± 2.06^{a}	1.13 ± 0.32^{a}
Group II	DEXP	4.55 ± 0.89^{a}	26.83 ± 0.23^{b}	1.68 ± 0.13^{a}
Group III	DEXP + Oocysts (3.0×10^{3})	10.00 ± 2.13^{b}	27.42 ± 1.18^{b}	3.49 ± 0.25^{b}
Group IV	DEXP + Oocysts (1.0×10^{4})	18.82 ± 3.24^{c}	25.57 ± 2.57^{b}	5.80 ± 1.15^{c}
One way ANOVA analysis		F= 32.08, *P*<0.001	F= 15.87, *P*<001	F= 35.20, *P*<0.001

DNA damage was determined by tail length, tail DNA, and tail moment of comet assay. Data are expressed as mean ± standard deviation. Values with different letters in the same column were significantly different ($P \leq 0.05$).

***Cryptosporidium parvum* infection raised the changes in the *Tnf-α* and *Il-6* gene expressions in leucocytes of the immuno-compromised mice**

Immunosuppression of mice with DEXP (group II) resulted in a statistical significant decrease ($P<0.001$) in the expression of *Il-6* gene compared with the healthy control mice (group I, Figure 2). Likewise, the immunosuppressed mice infected with *Cryptosporidium parvum* oocysts (groups III and IV) had a significant decrease in the expression of *Il-6* gene ($P<0.05$), in an infection-dependent manner, more than that of the immunosuppressed, uninfected mice (group II), as shown in Figure (2).

On the contrary, the expression level of *Tnf-α* gene was significantly increased ($P<0.05$) in the immunosuppressed, un-infected mice (group II), as well as the immunosuppressed, infected mice with *Cryptosporidium parvum* oocysts (groups III and IV), compared with the healthy control mice (group I), as shown in Figure (3). However, there were no statistical differences ($P>0.05$) in the level of *Tnf-α* gene expression in the heavy-infected (group IV) and light-infected (group III) immunosuppressed mice (Figure 3).

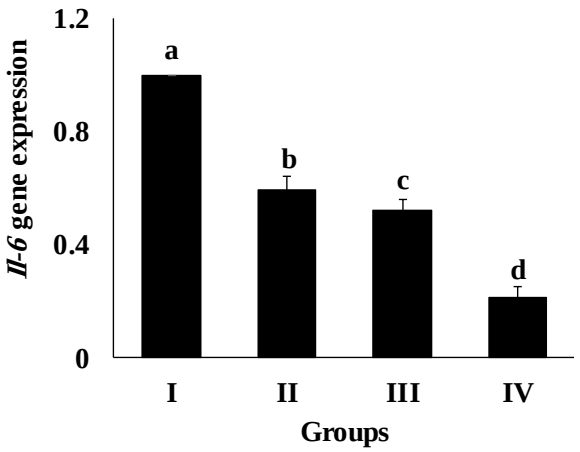

Figure 2: The expression level of interleukin-6 (*Il-6*) in the immunosuppressed mice infected with 3.0×10^{3} and 1.0×10^{4} *Cryptosporidium parvum* oocysts (groups III and IV, respectively) compared with the healthy (group I) and immunosuppressed, non-infected (group II) mice. Data are expressed as mean ± standard deviation. Values with different letters in the same column were significantly different ($P \leq 0.05$).

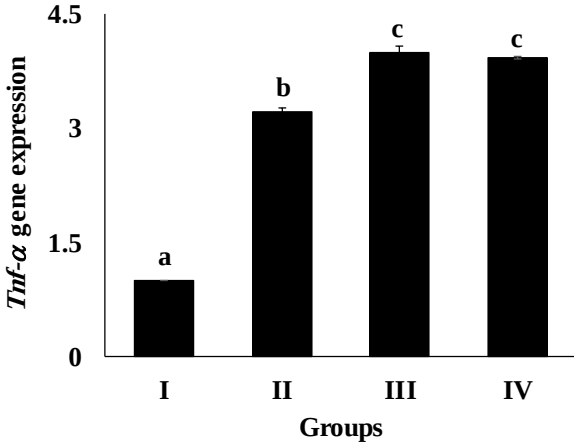

Figure 3: The expression level of tumor necrosis factor-α (*Tnf-α*) in the immunosuppressed mice infected with 3.0×10^3 and 1.0×10^4 *Cryptosporidium parvum* oocysts (groups III and IV, respectively) compared with the healthy (group I) and immunosuppressed, non-infected (group II) mice. Data are expressed as mean ± standard deviation. Values with different letters in the same column were significantly different ($P\leq0.05$).

DISCUSSION

Cryptosporidium sp. is a protozoan parasite that causes significant and major health problems in the immunosuppressed individuals, while self-limiting disease is generally observed in immunocompetent hosts. *Cryptosporidium* sp. is a potent food and drinking water contaminant[12]. The pathogenic effect of *Cryptosporidium* sp. was reported by numerous scientists; it causes several pathological changes in gastrointestinal epithelial cells, especially in the immunocompromised host[13]. This parasite may result in a higher risk of developing colorectal malignancies and can generate invasive cancer in gastrointestinal and biliary epithelia of severe combined immunodeficiency (SCID) mice[14,15]. Moreover, *Cryptosporidium* sp. is considered as one of the infectious agents that may induce intestinal dysplasia, including the high-grade category, which occurs particularly in the presence of immunosuppression states[16]. Several scientists observed that when different groups of protozoa and helminthes interact with their respective host, they caused DNA damage[17,18]. In addition, the current study showed that *Cryptosporidium* sp. infection induced DNA damage in leucocytes of the chemically-immunosuppressed mice; and the DNA damage was proportional to the severity of infection. Bhagat *et al*.[19] reported that experimental infection with *Cryptosporidium parvum* in immunocompromised Swiss albino mice induced oxidative stress and elevated the lipid peroxidation, through enhancing the production of reactive oxygen species (ROS) and decreasing the antioxidant defense system. ROS produced by the infected host, as a defense strategy, not only caused the killing of the parasite but also induced oxidative DNA damage in uninfected cells[20].

The obtained results also showed that there was a significant decrease in the expression of *Il-6* gene in the immunosuppressed mice, which were uninfected or infected with *Cryptosporidium parvum* oocysts (in an infection-dependent manner) in comparison with the healthy control one. On the contrary, the expression level of *Tnf-α* gene was increased in the immunosuppressed mice, which were uninfected or infected with *Cryptosporidium parvum* oocysts compared with the healthy control mice. Innate immune responses are very important in eradicating parasitic infection. In inflammatory response, macrophages are activated and are able to liberate nitric oxide, interferon, TNF-α, IL-6, and reactive oxygen species (ROS). While such mechanisms helped to eliminate infection, they exposed target organs to certain endogenous genotoxic agents[21]. IL-6 is a multifunctional cytokine with diverse actions and can act as anti-inflammatory cytokines under certain circumstances by inhibiting the synthesis of classical pro-inflammatory cytokines of macrophages/monocytes, including TNF-*α*, and

protecting tissues from inflammation[22]. IL-6 is rapidly activate during the initiation of inflammation, when early TNF-α production is also induced, and plays an important regulatory role in various immune responses and inflammatory conditions[23]. It was postulated that TNF-α and IL-6 production in an innate immune response may be negatively regulated by each other; and the TNF-α/IL-6 balance may be a key factor in regulating immune responses[23]. This may explain the decrease in *Il-6* gene expression and the increase in *Tnf-α* gene expression shown in leucocytes of the immunosuppressed, infected mice in the current study. In addition, significant elevations in the expression level of *Tnf-α* gene in the immunosuppressed, uninfected mice can be attributed to the upregulation of the transcription factors nuclear factor (NF)-κB by DEXP, which is responsible for transcriptional induction of pro-inflammatory cytokines including TNF-α, chemokines, and additional inflammatory mediators in different types of innate immune cells[24,25]. Moreover, Liu *et al*.[25] demonstrated a time-dependent change in the TNF-α production upon DEXP administration, as the level of TNF-α decreased significantly in 2-day-old rats treated with DEXP, but it was subsequently increased at the 7th days and 4 weeks of age.

In conclusion, the findings of the present study demonstrated augmentation of the DNA damage and inflammation (due to concurrent significant decreases and increases in the expression level of *Il-6* and *Tnf-α* genes) by infection with *Cryptosporidium parvum* oocysts in the immunosuppressed mice.

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CONFLICT OF INTEREST

The authors have no potential financial conflict of interest.

AUTHORS' CONTRIBUTIONS

MThA conceived and designed the experiments. NISS and HRHM performed the experiments and analyzed the data. All authors discussed and interpreted the results, as well drafted and reviewed the manuscript.

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تأثير العدوى بالطفيلي "*Cryptosporidium parvum*" على السُمية الوراثية والتعبير الجيني للسيتوكينات فى دم الفئران المثبطة مناعيا

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تتوافر قليل من المعلومات المتعلقة بالسُمية الوراثية والتأثيرات المناعية الناتجة عن العدوى بالطفيلي "*Cryptosporidium parvum*" عائلة "Cryptosporidiidae" في الخلايا الحية. لذلك فإن الدراسة الحالية تهدف إلى تقييم مدى تأثير العدوى بهذا الطفيلي على إحداث ضرر في الحمض النووي الديوكسي ريبوزي وتغيرات في التعبير الجينى للإنترلوكين "6" وعامل ألفا لنخر الورم فى خلايا دم الفئران المثبطة مناعيا. واستخدم فى هذه الدراسة خمسين من ذكور الفئران المهقاء "Swiss albino" ووزعت بطريقة عشوائية إلى خمس مجموعات (10 فئران في كل مجموعة) كالآتى: المجموعة الأولى سليمة وغير مَعدية (المجموعة الضابطة)، المجموعة الثانية تم تثبيطها مناعيا بواسطة عقار ديكساميثازون، وكلا من المجموعتين الثالثة والرابعة تم تثبيطها مناعيا ثم إصابتها بعدد "$10^3 \times 0.3$ (جرعة منخفضة)" أو "$10^4 \times 1.0$ (جرعة عالية)" من الطور المُعدي للطفيلي "*Cryptosporidium parvum*" على التوالي، والمجموعة الخامسة كانت غير مُثبطة مناعيا، ولكنها مَعدية بعدد "$10^4 \times 1.0$" من الطور المُعدي للطفيلي. تم تحديد الإضرار بالحمض النووي الديوكسي ريبوزي فى خلايا الدم بواسطة اختبار كوميت، وقياس التعبير الجيني لكل من إنترلوكين "6" وعامل ألفا لنخر الورم بواسطة تفاعل البلمرة المتسلسل الكمي. وأوضحت النتائج أن العدوى بالجرعة المنخفضة والجرعة العالية من الطفيلي "*Cryptosporidium parvum*" أدت إلى حدوث ضرر في الحمض النووي الديوكسي ريبوزي، بالإضافة إلى حدوث إنخفاض ملحوظ في التعبير الجيني للإنترلوكين "6" وكذلك ارتفاع ملحوظ في عامل ألفا لنخر الورم في خلايا دم الفئران المثبطة مناعيا، مقارنة بكل من الفئران السليمة والفئران المثبطة مناعيا غير المَعدية بالطفيلي. وخلصت الدراسة إلى أن العدوى بالطفيلي "*Cryptosporidium parvum*" تحفز تكسير الحمض النووى الديوكسي ريبوزي وتعزز من التغيرات الحادثة في التعبير الجيني للإنترلوكين "6" وعامل ألفا لنخر الورم في الفئران قليلي المناعة.