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EVALUATION OF IMMUNE RESPONSES IN CHILDREN INFECTED WITH *ENTAMOEBA HISTOLYTICA* AFTER TREATMENT WITH METRONIDAZOLE

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Abstract

This study was designed to evaluate the immune responses in children infected with *E. histolytica* after treatment with metronidazole. Immunological evaluation was performed by measurement of secreted cytokines (interleukin, IL-10-tumor necrosis factor-α in *blood* of children, on the other hand *E. histolytica* cyst output in children stool was measured. The present investigation is directed to study the therapeutic effect of different doses of metronidazole a treatment known for controlling of E. *histolytica* infection in children. The children infected with *E. histolytica* were divided into three group according *to* the dose of metronidazole (Flagyl syrup). The 1st, second and third group were treated with 30, 25 and 15 mg /kg per day for 7 days, in 3 doses respectively. The results of the present study revealed a significant reduction in cyst count in stool of children treated by metronidazole at dose of 30 mg /kg in comparison with control un treated. The data also revealed that serum IL-10 and TNF-alpha were significantly increased as an immunological response for children treated with metronidazole at dose of 30 mg/kg body weight in comparison with children of dose 15 mg and untreated control group. Also the NO level revealed significant decrease in children at dose 30 mg / kg in comparison with 25 and 15 mg/kg group.

Key words *: IL10 – TNF-alpha – NO-* metronidazole

Introduction

Amoebiasis, the disease caused by pathogenic parasite *Entamoeba histolytica*, is most common in developing countries. Infection, acquired by oral ingestion of cyst-containing food or water, resulting in development of amebic colitis and liver abscess, is second after malaria as a protozoan cause of death **(**Gil **et al ., 2008).** Although the number of registered amoebiasis cases has gradually increased It is estimated that annually 40–50 million individuals are infected worldwide, resulting in 100,000 deaths. *Entamoeba histolytica* is a common cause of endemic and epidemic diarrhoea throughout the world. Infection with *E. histolytica*, includes acute self-limited diarrhea and a chronic syndrome of diarrhea with malabsorption and weight loss **(Ferreira et al ., 2006)**. Children are more liable to clinical amoebiasis than adults. *Entamoeba histolytica* infection leads to amebic colitis and amebic liver abscesses Ameba colonization is an essential process in establishing infection. Invasive disease only occurs in 10% of cases and accumulating evidence

indicates that some humans are predisposed to invasive disease whereas others are resistant **(**Henriques **et al ., 2007).**

Metronidazole is usually the preferred drug in amoebiasis treatment throughout the world. However, the side-effects of this drug and its 7 d treatment protocol are important factors affecting compliance, especially in children (5). Its oral absorption is almost complete, with bioavailability of\90% and *t*max ranging between 2 and 4 h **(**Hall **et al., 2006).**

Metronidazole is used primarily as a drug for the treatment of infections due to *Entamoeba histolytica*, *Trichomonas vaginalis*, and *Giardia lamblia*. Metronidazole has also been used to treat Vincent's infection and acne rosacea. It has been prescribed for invasive intestinal amoebiasis or amoebic hepatic abscess. Metronidazole can also be used as a trichomonacidal agent in veterinary medicine (IARC1977). Metronidazole may be administered orally (capsules or tablets), topically (gels or creams), or by injection (Kihara **et al . , 2006**).

In mouse models of amebiasis, resistance is associated with the inability of the parasite to establish infection within the host and the beneficial physiological effects of the immunoregulatory cytokine IL-10. These results are likely to impact the understanding of amebiasis in humans. However, this will depend on the applicability of these findings to humans, the only relevant hosts of *E. histolytica* **(Schoop et al . , 2006).**

T cells are an important source of IL-10, which can modulate gut function. To elucidate the protective mechanism of this cytokine in amebic infection **(Denis , 2005).**

IL-10 is known to be involved in the maintenance of gut homeostasis and a deficiency in this cytokine can alter the epithelial response to luminal antigens. In fact, it was first reported that IL-10-deficient mice develop chronic intestinal inflammation, which is characterized by mucosal hyperplasia and aberrant MHC class II expression on epithelial cells **(Asgharpour, 2008).** Although growth of these mice is retarded, they have normal T and B cell development and intestinal inflammation is thought to be because of an increase in T-cell induced intestinal damage and Fas expression in epithelial cells **(Samuelson, 2007) .**

Hamano, (2006) reported that IL-10 here are marked differences between allergies and parasitic infections, there are certain parallels. For instance, with regard to environmental and helminth allergens, the immune response is similarly affected. Both are associated with elevated levels of IgE, tissue eosinophillia, mastocytosis, mucus hypersecretion and CD4+ T cells responses that preferentially secrete Th2 cytokines, IL-4, IL-5 and IL-1014 Consequently, some experts have suggested a cause-effect relationship between helminth infections and allergies. Intestinal worms may provide a protective mechanism against the development of allergic diseases (Bull et al., 2007).

In a mice model, infection by helminthes induced the release of the cytokine, IL-10, which exerted an anti-inflammatory action, thereby diminishing anaphylactic symptoms as measured by histamine levels **(Kuhn , 2007).** Moreover, helminth infection also suppressed the development of allergen-induced airway inflammation, which was induced by the release of eosinophils. This effect may be mediated by the transmission of IL-10 Thus, helminthes may have a role in anaphylactic treatment or prevention.

While the anti parasitic effects of NO have now been documented for a vast array of target organisms **(Kagnoff and Gillin , 2005).** One of the earliest demonstrations of its anti parasitic activity was a study of the mechanism of macrophage cytotoxicity toward *E. histolytica*

Although immunity to *E. histolytica* is complex and improperly understood, a number of different effector mechanisms have been implicated, including nitric oxide (NO) **(Farber, and Raychaudhuri, 2007).** It is suggested that a cascade of reactions leading to NO production are involved in *E. histolytica*. There are some contradictory reports about the role of NO and related molecules in *E. histolytica* Some researchers propose NO is involved in the development of severe malaria, whereas others argue a protective role for NO **(Keller and Chadee, 2002).**

Cytokines play an important role in the defense against *E. histolytica* and some have long been recognized to have anti-parasitic effects on different stages of *E. histolytica*. This protective effect was further demonstrated by administration *in vivo* of some key cytokines **(Tannich, (2003).** A large number of cytokines appear to be involved in *E. histolytica* , *ie* TNF- alpha, IFN-gamma , GM-CSF, IL-1, IL-4, IL-6, IL-8, and IL-10 [8]. NO production during *E. histolytica* infection is regulated *in*

vivo by the Th1- cytokines (TNF- alpha and IFN-gamma), but not by IL-4, which is a Th2 cytokine. IFN γ, TNF-α induce high amounts of NO involved in controlling the peak level of parasitemia [36]. NO is known to affect the production of more than 20 cytokines, including IL-1, IL-6, IL-10, IL-12, IFN γ, TNF-α, and TGF-β by various immune cells, *eg* macrophages, T-lymphocytes, natural killer cells (NKC) and endothelial cells **(Keller and Chadee, 2002).** Conversely, more than 30 cytokines or cytokine-like factors have been described that increase or inhibit the expression of iNOS activity in cells participating in the immune response: macrophages, microglia, Kupffer cells, neutrophils, eosinophils, mast cells.

Materials and Methods

Study area. Preschool children 3–5 years of age enrolled in this study were from Elsaf – Giza. The majority of the inhabitants are of Giza origin, who settled in villages of Elsaf. The area is densely populated with approximately 200,000 residents. The use of human subjects was approved by Elsaf Hospital Central. Informed consent was obtained from the parents of the children. Forty children was divided into 4 groups , the 1st group was treated with a dose of metronidazole (Flagyl,) at 30 mg:kg per day for 7 days in 3 doses , the second group was treated with a dose at 25 mg/kg per day for 7 days in 3 doses , the third group was treated with a dose at 15 mg/ kg per day for 7 days in 3 doses , the fourth group was control non infected .

Sample collection. A list of all children 3–5 years of age was made and stool samples were collected using wide mouth stool containers. Serum samples were collected by trained personnel using sterile butterfly needles and 5-ml syringes and were placed into 5-ml serum separator tubes. Blood samples were centrifuged for 5 min at 1000 g and the resulting sera were frozen until assayed for IL-10 and TNF-alpha

Enumeration of cyst output. Stool samples were collected from children in the study. The cyst output children was estimated using the technique described by **Danciger and Lopez** (2005). Briefly, 10 g of stool was placed in a container, weighed, and emulsified in 5 ml of phosphate buffered saline (PBS). Three 50-ml aliquots of the fecal suspension were placed on slides and mounted with a 22 3 22-mm cover slips. The preparation was examined using a bright field microscope at 40x in the following manner: starting at the upper left corner of the cover slip and

moving in a straight line to the right edge of the cover slip, then moving down the width of one field and going back to the left edge in a straight line. Total number of cysts for each sample (cover slip) was determined and an average number of cysts for the three aliquots calculated and expressed per gram of feces.

Nitric Oxide measurement

Nitric oxide in serum was measured by technique according to **Nussler et al . (1993)**. A standardized colorimetric assay for nitrite (NO2-) as an indirect index of NO production using the Greiss reagent. Briefly, 100 ml of sample was mixed with an equal volume of Greiss reagent(1% sulfanilamide, 0.1% naphthylene diamine dihydrochloride, 2.5 % phosphoric acid) and incubated at room temperature for 10 min. Sodium nitrite (NO2-)(Sigma) was used as the standard from 0.156 mM – 20 mM concentration. The absorbance at 550nm was measured in a microplate reader with a correction wavelength of 650 nm. The sensitivity of this assay is <0.5 mM.

Cytokine measurement

1- Measurements of Cytokine IL-10 was described by Mosmann ,1994 and Defu ,1998).

2- Measurements of Cytokine TNF – alapha according to Thomas ,1994 and Friedman, 1997).

Statistical Analysis

Data were analysed with SPSS for Windows (Version 13.0). All data are expressed as means ± SEM, and significant differences between the patient and control groups were analysed using Student's *t*-test.

Results

Evaluation of cyst output (Table 1) demonstrates the effect of metronidazole on cyst output in children infected by *Entamoeba histolytica*. The results in table (1) indicate that the number of total cyst in the children treated at dose 30 mg/kg group was extremely lower than those in the untreated control group. It also revealed moderate percentage of resistance in(25 and 15 mg/kg) groups in comparison with the untreated control group. The recorded percentage of resistance ranged from 88.5 to 78.6%. ($P < 0.01$) consequently.

Evaluation of NO level: (Table 2) demonstrates the effect of metronidazole on NO level in children infected by *Entamoeba histolytica* .The data obtained in table 2 indicated that NO levels were significantly increased in all investigated groups. The more pronounced increase was observed in the (15mg/kg) group reaching 162.4% as compared to non treated control group. It is worthy to mention that NO levels were significantly increased in (30 mg/kg) group as compared to the control group at 151.1% (P <0 .001)

Evaluation of Cytokine (IL-10)

(Table 3) demonstrates the effect of metronidazole on cytokine (IL-10) level in children infected by *Entamoeba histolytica* . The data obtained revealed a higher significant increase in serum IL-10 level in the (30 mg/kg) group amounted 136.1% (P <0 .001) as compared to control non infected group. It is worthy to mention that IL-10 level was revealed a significant increase in (15 mg/kg) group amounted 171.1% (P <0 .001) in contrast non treated control group were recorded 99.1% (P < 0.05).

Evaluation of Cytokine (TNF- alpha)

(Table 4) demonstrates the effect of metronidazole on cytokine (TNF- alpha) level in children infected by *Entamoeba histolytica* . A significant increase was recorded in serum TNF- alpha level (179.8%, p<0 .001) in the (30 mg/kg) as compared to control group. In addition,(25,15 mg/kg) groups were showed a significant increase (p<0 .001) as the percent changes recorded 150.2 and 141.6% respectively in comparison with the control group .

Table (1): Evaluation of cyst out put in children infected with *Entamoeba histolytica* and treated with metronidazole

Group Item	Children non treated control	Children treated at dose 30 mg/kg metronidazole	Children treated at dose25 mg/kg metronidazole	Children treated at dose 15 mg/kg metronidazole
	±SE Mean	±SE Mean	±SE Mean	Mean±SE
Cyst	±3.7 97.65 100 %	±2.9 50.58 * 51.7%	±3.8 86.43 ** 88.5%	± 2.7 77.79 ** 78.6%

Table (2): Evaluation of Nitric Oxide in children infected with *Entamoeba histolytica* and treated with metronidazole

Group	Control non	Children non treated	Children treated at dose	Children treated at metronidazole	Children treated at dose 15 mg/kg

Item	infected	control	30 mg/kg metronidazole	dose25 mg/kg	metronidazole
	±SE Mean	±SE Mean	±SE Mean	±SE Mean	Mean±SE
Nitric Oxide (µmol/L)	120 .48±5.2 100%	±4.7 110.63 * 91.8 %	±6.5 167.21 *** 151.1%	±5.5 135.43 ** 122.1%	± 4.9 179.72 *** 162.4%

Table (3): Evaluation of cytokine (IL-10) in children infected with *Entamoeba histolytica* and treated with metronidazole

Group Item	Control non infected	Children non treated control	Children treated at dose 30 mg/kg metronidazole	Children treated at metronidazole dose 25 mg/kg	Children treated at dose 15 mg/kg metronidazole
	±SE Mean	±SE Mean	±SE Mean	±SE Mean	Mean±SE
IL-10 (pg/ml)	400 .74±8.6 100%	±3.7 96.395 NS .% 98.8	±9.3 695.87 *** 174.3%	±6.9 552.67 *** 137.9%	± 8.6 685.47 *** 171.1%

Table (4): Evaluation of cytokine (TNF – alapha) in children infected with *Entamoeba histolytica* and treated with metronidazole

Group Item	Control non infected	Children non treated control	Children treated at dose 30 mg/kg metronidazole	Children treated at metronidazole dose25 mg/kg	Children treated at metronidazole dose 15 mg/kg
	±SE Mean	±SE Mean	±SE Mean	±SE Mean	Mean±SE
TNF – alapha (pg/ml)	220.37±6.4 100%	±4.2 218.17 *** 99.1 %	±5.7 395.87 *** 179.8%	±5.1 330.57 ** 150.2%	± 6.7 310.75 *** 141.6%

* Significant difference from infected group P < 0.05
** Significant difference from infected group P < 0.01
*** Significant difference from infected group P <0 .001

Discussion

In the present study the experimental work has indicated a crucial role for IL- 10 and TNF- alpha to enhance the immune responses for elimination of *Entamoeba histolytica* this is in agree with the result obtained by **Finkelman (2008**) who stated that cytokines IL-10, IL-4, and IL-5, which regulate the characteristic mastocytosis, IgE, and eosinophilia, respectively in parasite infections . On the other hand TNF-alpha which regulated Th2-controlled effector mechanisms are responsible for parasite loss remains a matter of. There is a relative paucity of field-based

observations on the immunology of human infections with these parasites, **Haller (2005).**

The particular advantages of the present study were that the evaluation of cytokine with metronidazole in children infected by *Entamoeba histolytica*. In the present study intensity of the infection in children's groups were closed to some extent. In the present study, moderate reduction in cyst out put from children group treated by metronidazole at dose 30 mg/ kg , while in medium and low dose of metronidazole recorded the highly reduction in cyst out put .This result is coincide with **Chairungsi (2008)** who described that the high dose of anti parasitic lead to the reflecting mechanism in immune system of patient ,Hence the of circulating Th1 and Th2 may be suppressed . The Th1 and Th1 leading to cytokine IL-10 immune modulator against eggs of intestinal parasite.

In the present study the children treating at 25 mg/kg metronidazole have shown the great reduction in cyst count reached 88.5 % , as well as this reduction in cyst out paralleled with significant increased in NO and cytokine IL-10, such results is in agreement with Sabin ,(2006) who found that extremely strong correlations were observed between reduction in cyst output in children treated by metronidazole , and IL-10, this indicates that the early potency by metronidazole for enhancement the immune response. Th1 and Th2 is a good indicator for stimulant other immune regulation not only of reduction intensity of infection but also of the reduction number of *Entamoeba histolytica* eggs accumulating in the intestine of children **Chwaya et al ., (2006).**

The results of the current study help to clarify the controversies of studies on the cytokine parasite relationship. A different dose of metronidazole facilitated cellular immunity in patients chronically infected with *Entamoeba histolytica* Observations presented in this study suggest that repeated metronidazole treatment further supported cellular reactivity to improved the production of IL-10 and TNF –alpha Thl-type cytokines. This agree with the report of **Hamano et al.**, (2007) he mentioned that the contribution of IL-10 to innate resistance and propose that the cytokine has physiological effects on intestinal epithelial cells and induces a protective barrier to infection. Some epithelial cells could be predisposed to intestinal invasion and would then rely on the inflammatory cells below to clear the infection.

Asgharpour et al., **(2008)** observed that IL-10 resulted in reduced neutrophil infiltration and epithelial inflammation; however, this was accompanied by neutrophil depletion decreased protection against intestinal amebiasis in children At the IL-10 level, a recent study revealed that significant increased in IL-10 in children's were treated by metronidazole . The elevation of serum IL-10 attributed to amoebic invasion where produced high levels of Th2 cytokines, on the other hand metronidazole enhanced the effect of intestinal Th2 response. These findings were also agree with **Lockhart, (2009)** who stated that the early Th1-type response is an essential component of the acute response against amoebiasis, and that immune modulation occurs as a result of the down-regulation of the Th1 response **,** and therefore a potentially beneficial effect of a Th1 response We have shown that a Th1 and Th2 response, characterized by high interleukin (IL)-2 and tumour necrosis factor (TNF), and high IL-10 protein production by Fab cells in immure receptors.

In the current study the correlation between NO and production of cytokines in infected children by *E. histolytica* .NO which recorded highly significant increased in groups of children were treated by metronidazole at all doses . This correlation was attributed to NO enhancement the production of Th1 and Th2 immune response against amoebiasis. This result is coincide with **Houpt (2008)** who described that the production NO promotes the Th1 cytokine interferon TNF -α show amoebicidal activity , killing *E. histolytica* trophozoites *via* the production of nitric oxide (NO) , while TNF -α killed trophozoites Furthermore, native possibly act as its own Th1 adjuvant. metronidazole can directly induce TNF -α primed macrophages to produce NO production for the Th1-promoting pro-inflammatory cytokines TNF -α and IL-10 in macrophages.

The principal targets of NO in the amoebiasis appear to be enzymes containing a catalytically active Fe-S group. Since many products of activated effector cells, including NO, can be harmful to the host, mechanisms exist to inhibit their production or restrict their toxic effects several cytokines are known to suppress proliferation or production of IFN-g by Th1 lymphocytes, thus limiting effector cell activation and indirectly inhibiting NO production **Houpt, (2008)**

In view of the data obtained in the present study showed a highly significance increase in serum TNF –alpha of children infected by *E. histolytica* and treated groups by metronidazole reaching 99.1% and 179.8 % , this present change could

be attributed to the significant increased of IL-10 in children , this results are confirmed by **Smith et al., (2006)** they have been reported for Th1 and Th2 response is important in protection against and destroying the intestinal parasites. The presence of anti-parasitic antibodies detected in the serum depended on the secretion of cytokines such as IL-10 and TNF- alpha which is also produced from Th2 cells.

Conclusions

There are previous conflicting reports on whether cytokine are protective or deleterious during *E. histolytica* infection. Cytokine contribute to immune response and immuno induction after *E. histolytica* infection . Other studies have indicated that interleukins have a protective role in amebiasis. . Our data support the latter findings and strongly suggest that IL-10 and TNF- alpha play a protective role in intestinal amebiasis.

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الملخص العربي

تقييم الإستجابة المناعية في الإطفال المصابين بالأنتيميبا هستولتيكا بعد العلاج بالميترونيدازول

أدهم جمال صادق

قسم علم الحيوان - كلية العلوم – جامعة الأزهر

صممت هذه الدراسة لتقييم مدى استجابة الجهاز المناعي في الأطفال المصابين بالأنتميبا هستولتيكا بعد العلاج باستخدام عقار الميترونيدازول. التقييم المناعي تم بقياس السيتوكينات المفرزة داخل الجهاز المناعي للاطفال كنتيجة للتعرض بالاصابة بالأنتميبا هستولتيكا ، وقد تم قياس اثنين من هذه السيتوكينات المؤثرة في الاستجابة المناعية والمتحكمة فيها وهي الانترلوكين -10 و تي – ان – اف – الفا وايضا تم قياس معدل حويصلات الأنتميبا هستولتيكا الموجودة داخل براز الاطفال ، بالاضافة الي قياس مستوي اكسيد النتريك بالدم. وتم تقسيم مجموعات الاطفال الي اربع مجموعات ، حيث قسمت الي ثلاث مجموعات تعامل باستخدام عقار الميترونيدازول عند جرعات 15،25،30 ملي جرام لكل كيلو جرام من وزن الجسم لمدة سبعة ايام ، ثلاث مرات يومياً ، مع وجود مجموعة ضابطة لم تعامل بالميترونيدازول، اظهرت النتائج حدوث اختزال في حويصلات الأنتميبا هستولتيكا الموجودة داخل براز الاطفال المعالجين بالميترونيدازول عند جرعة 30 ملي جرام بالمقارنة مع المجموعة الغير معالجة ، ايضا اظهرت النتائج حدوث زيادة معنوية في معدلات الانترلوكين -10 و تي – ان –اف – الفا في كل المجموعات المعالجة بالميترونيدازول عند جرعة 30، 25 ملي جرام بالمقارنة مع المجوعة المعالجة بالميترونيدازول عند جرعة 15 ملي جرام وهذا يوكد الدور الذي تلعبة السيتوكينات ومنها الانترلوكين في عمل الاستجابة المناعية داخل الجهاز المناعي والسيطرة علي العدوي . ايضا اوضحت الدراسة حدوث انخفاض معنوي في معدل اكسيد النتريك في الاطفال المعالجين بالميترونيدازول عند جرعة 30 ملي وحدوث ارتفاع معنوي عند جرعات 15 ،25 ملي جرام .