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ORIGINAL PAPER

Comparative study of nitric oxide (NO) production during human hydatidosis: relationship with cystic fluid fertility

Razika Zeghir-Bouteldja · Manel Amri · Samia Bouaziz · Dalila Mezioug · Chafia Touil-Boukoffa

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Abstract Human hydatidosis is characterized by a prolonged coexistence of *Echinococcus granulosus* and its host without effective rejection of the parasite. This parasitic infection constitutes a major health problem in Algeria. In this study, we investigated in vivo production of nitrite (NO_2^- + NO_3^-) in sera of Algerian patients carrying different cyst locations. Nitrite (NO_2^- + NO_3^-) levels were evaluated by the Griess method. Our results indicated that the levels of nitrite were significantly higher in the sera of hydatic patients than those of healthy controls supporting the involvement of nitric oxide (NO) in antihydatic action. The levels of nitrite in sera of the patients with hepatic hydatidosis were significantly higher than those with pulmonary infection. The lower serum (NO_2^- + NO_3^-) levels were observed in the relapsing cases. In addition, (NO_2^- + NO_3^-) levels of fertile hydatic fluids were significantly higher compared to infertile fluids. Our results suggest that the presence of NO products in hydatic fluids seems to be related to the location and the fertility of hydatic cysts. The assessment of protein concentration in hydatic fluids showed that the concentration of proteins was not exclusively dependent on the fertility but on the cyst locations. The assessment of (NO_2^- + NO_3^-) production in hydatic patients may be a useful tool to evaluate effector mechanisms of NO and clinical manifestations.

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Abbreviations

NO	Nitric oxide
NOs2	Nitric oxide synthase 2
IFN-γ	Interferon gamma
PBMCs	Peripheral blood mononuclear cells
NO_3−	Nitrate
NO_2−	Nitrite
E. granulosus	*Echinococcus granulosus*

Introduction

Human hydatidosis is an endemic parasitic disease caused by the larval stage of the tapeworm of *Echinococcus granulosus*. It occurs practically worldwide, including countries of Central and South America, Western and Southern/Southeastern Europe, the Middle East and North Africa, some sub-Saharan countries, Russia and adjacent countries (Eckert and Deplazes 2004), and China (Wen and Yang 1997). It constitutes a major health problem in North Africa, particularly in Algeria. It is considered as a disease for obligatory declaration by the National Institute of Public Health. The annual incidence is 1.47–2.3/100,000 Algerian population. The transmission of *E. granulosus* in the dog–sheep cycle is known to occur most frequently in rural and grazing areas. Larval infection is characterized by long-term growth of hydatic cysts in the intermediate host (Zhang et al. 2003).

Variability and severity of clinical expression of hydatidosis are not only associated with the duration and intensity of infection but also with the variety of human immunological responses to the antigens of larval hydatic cyst with detectable

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humoral and cellular responses against *E. granulosus*. Touil-Boukoffa et al. (1998) have reported a correlation between in vivo and ex vivo interferon-γ and nitric oxide (NO) production during human hydatidosis. NO is an important regulator and mediator in many physiological and pathophysiological events. It has been implicated in neurotransmission, vasodilatation, and immune regulation (Moncada and Higgs 1991). Although small amounts of NO are constitutively released by endothelial and neuronal nitric oxide enzymes (eNOs and nNOs, respectively), inducible NO synthase, located mainly in immune cells, produces NO in considerable amounts after exposure to cytokines (Nussler et al. 1992). NO and its stable metabolites (NO_2^- and NO_3^-) have been identified as major effector molecules during the majority of parasitic infections (Liew 1992; Ascenzi et al. 2003). Production of NO has been shown to be induced by interferon gamma (IFN-γ) during human hydatidosis, suggesting the relevant role of NO in the host defense (Touil-Boukoffa et al. 1998; Ait Aissa et al. 2006; Amri et al. 2007). Our proposal was supported by in vitro observations that hydatic cysts are susceptible to the products of NO such as NO_2^- and $ONOO^-$. These metabolites showed in vitro scolicidal activity and degenerative effects on germinal and laminated layers of human hydatid of *E. granulosus* (Zeghir-Bouteldja et al. 2009).

The present study was designed to assess the production of (NO_2^- + NO_3^-) in sera of Algerian hydatic patients in relation with clinical status (before and after surgery), cystic location, and cystic status. We also examined (NO_2^- + NO_3^-) and protein levels in fertile and infertile hydatic fluids collected after surgery from cysts of different anatomical sites.

Materials and methods

Patients

The group of Algerian patients (33±2, 5 years old, and 55 % men) is composed of hydatic patients with different cyst locations. Regions of origin of patients are the provinces of high plateau, the northeastern, north-central provinces, and center of Algeria. They were tested for circulating (NO_2^- + NO_3^-) levels. Patients were tested before and after surgery (1 week before and 24–72 h after surgery). All patients were admitted to the Department of Surgery (Mustapha Bacha Hospital, Algiers, Algeria). None of the patients had received pharmacological treatment. Subjects with other acute or chronic diseases, smoking, and alcoholism were not included in this study. Serological reaction against parasitic antigen (antigen 5) was tested in each case by immunoelectrophoresis. The diagnosis was radiologically and surgically confirmed for all patients. Indirect hemagglutination test was used for detecting hydatidosis antibodies. Stool examination was made on both patients and controls groups to exclude intestinal parasitic infections.

Healthy controls (n=20, mean age 37±6 years, 50 % males) from the same Algerian region (Algiers) are composed of adult volunteer blood donors who presented no inflammatory disease nor sign of infection at the time of blood sample collection. In addition, none of the subjects had ever received blood transfusion or any medication. All subjects were informed on the study and have signed an official consent, which was conducted according to the guidelines of the local Ethics Working Group.

Serum collection

Blood samples collected from healthy donors and hydatic patients were centrifuged at 2,000×g for 10 min to separate the serum. Hemolysis serum was excluded from this study.

The serum samples were classified into several groups (Table 1). This classification was based on clinical stage (before and after surgery, n=60), patients who relapsed after 18 months of surgery (n=15), cyst location (lung n=24; liver n=32), cyst states (intact cysts n=56; calcified cysts n=7; broken cysts n=4), and double location (liver+lung n=6).

Hydatic fluids collection

Forty-six hydatic fluid samples were obtained after surgery. Hydatic fluids (HFs) were collected after aseptic dissection of the intact hydatic cysts. They were centrifuged at 3,000×g for 30 min at +4 °C, and the sediment was checked for the presence of protoscolices. Cyst fertility was determined by the presence of viable free protoscolices in hydatic fluid.

Table 1 Serum levels of (NO_2^- + NO_3^-) in Algerian hydatic patients

Groups	Population (n)	(NO_2^- + NO_3^-) concentration (μM)
Healthy controls	20	30.01±1.96
Patients (before surgery)	60	77.37±7.24
Patients (after surgery)	60	49.70±6.89
Patients with intact cysts	56	74.31±3.87
Double location (lung and liver)	6	83.53±14.48
Patients with lung cysts	24	59.90±4.14
Patients with liver cysts	32	85.12±5.31
Patients with relapsed infection (liver and lung)	15	20.17±1.62
Patients with calcified (liver and lung cysts)	7	28.64±2.55
Patients with broken lung cysts	4	105.81±14.74

Data are means of the (NO_2^- + NO_3^-) levels ± standard errors

n number of patients

The viability of the protoscolices was assessed by staining with 0.1 % aqueous eosin solution. Other parameters of cyst fertility were considered such as a whitish color of the laminated layer and limpidity of hydatic fluid. The fertility was variable for the cysts located in the same organ. The fertile fluid (+) was characterized by the presence of a high number of protoscolices and daughter vesicles. The hydatic fluid had a milky aspect and whitish color. The number of protoscolices was more than 1,000 parasites/ml. Less fertile fluid (+/−) was characterized by the presence of reduced number of protoscolices. The hydatic fluid was clear and had whitish color, and the number of protoscolices was less than 500 parasites/ml. Infertile fluid (−) was characterized by total absence of protoscolices and daughter vesicles and was limpid and clear. The hydatic fluids were classified into several groups. This classification is based on fertility and cyst location [liver HFs (Table 2) (+) HF n=13, (+/−) HFs n=5, and (−) HFs n=7; lung HFs (+) HFs n=10, (+/−) HFs n=4, (−) HFs n=4), and (−) muscular HF n=1; (+) splenic HF n=1; and (−) pancreatic HF n=1].

Nitric oxide assay

NO production was assessed by determination of the end products of NO oxidation. Total concentration of (NO_2^- + NO_3^-) was quantified by the Griess reaction as described in 1879 (Sun et al. 2003). Briefly, the Griess Reagent System was based on a reaction which uses sulfanilamide and *N*-1-napthylethylenediamine dihydrochloride under acidic conditions. NO_2^- was assayed after reduction of NO_3^- by nitrate reductase containing *Pseudomonas oleovorans* bacteria (ATCC 8062). One hundred microliters of each serum or hydatic fluid to be tested was mixed with 50 μl of 1:10 dilution of pelleted bacteria cells. In parallel, the NO_2^- standard for calibration curves was treated similarly as the samples. More than 90 % of NO_3^- was converted to NO_2^- under these conditions. After incubation at 37 °C for 90 min, the sample was centrifuged and mixed with 100 μl of the Griess reagent (0.5 %*N*-1-naphtyl-ethylenediamine in 20 % HCl, 5 % sulphanilamide in 20 % HCl (1 *v*/*v*). After 20 min of incubation at 22 °C, the formation of chromogenic derivative was detected by spectrophotometry at 540 nm as described by Touil-Boukoffa et al. (1998).

Table 2 (NO_2^- + NO_3^-) levels in liver hydatic fluids according to the degree of fertility

Cyst location	Degree of fertility	n	(NO_2^- + NO_3^-) concentration (μM)
Liver	+	13	29.76±7.52
	+/−	5	7.88±0.66
	−	7	4.92±0.43

Data are means ± standard errors of the concentration of (NO_2^- + NO_3^-) detected in hydatic fluid. Symbols (+), (+/−), and (−) indicate fertile, less fertile, and infertile fluids, respectively

n number of hydatic cysts

Protein assay

The protein concentration in hydatic fluids was determined by the Bradford method (Bradford 1976). A convenient standard curve was made using a series of dilutions of bovine serum albumin (BSA) with the concentrations of 10–100 μg/μl in phosphate-buffered saline. The concentration of protein was assessed by the addition of 100 μl of each sample to 3 ml of Bradford reagent. When mixed with a protein solution, the acidic Coomassie dye reagent changes color from brown to blue in proportion to the amount of protein present in the sample (Coomassie stains; Sigma-Aldrich). After incubation for 5 min at 22 °C, the absorbance was read at 595 nm. The average absorbance value of each concentration of the BSA was plotted. For this study, the hydatic fluid samples were treated similarly, and 100 μl of hydatic fluid was mixed with 3 ml of Bradford reagent. The concentration of proteins was determined by extrapolation of the value of optic density on the BSA standard curve.

Statistical analysis

All values are expressed as means ± standard error. Data analysis was performed using the Origin 7.5 software (OriginLab 2003, USA). Differences between means were analyzed by student's t test. Values for P<0.05 were considered statistically significant.

Results

Production of nitrite in sera of patients

As shown in the Table 1, sera (NO_2^- + NO_3^-) levels were higher in the presurgical hydatic patients group (77.37± 7.24 μM) than those of healthy subjects (30.01±1.96 μM). The (NO_2^- + NO_3^-) levels were significantly higher in the patients group before surgery (77.37±7.24 μM) than in the patients group after surgery (49.70±6.89 μM); P=0.01023), whereas the relapsing patients group showed lower nitrite levels (20.17±1.62 μM) in comparison to healthy individuals. There are no significant differences between relapsing patients and controls (P=0.0008). The nitrite levels detected in sera of patients with hepatic hydatidosis (85.12± 5.31 μM) were higher than those with pulmonary infection (59.90±4.14 μM; P=0.029). Indeed, the comparative study of production of (NO_2^- + NO_3^-) in sera of patients carrying intact cysts showed significant higher levels compared to

patients with calcified cysts (28.64±2.55 μM). The (NO_2^- + NO_3^-) level of patients with calcified cysts did not differ from the control group (28.64±2.55 μM versus 30.01± 1.96 μM; P=0.7111). While the (NO_2^- + NO_3^-) sera level of patients with broken cysts was significantly higher (105.81±14.74 μM) compared to those with calcified cysts, the difference between the two groups was statistically significant (P=0.00007).

The assessment of (NO_2^- + NO_3^-) production in hydatic fluids showed higher levels of (NO_2^- + NO_3^-) in fertile liver fluids with (29.76±7.52 μM) compared to the infertile fluids (4.92±0.43 μM; P=0.00004). The same observations were also noted in pulmonary cysts with (26.55±4.25 μM) in fertile fluids versus infertile fluids with (4.53±0.62 μM; P=0.00322) (Fig. 1). (NO_2^- + NO_3^-) levels were low in infertile fluids of muscular and pancreatic cysts. (NO_2^- + NO_3^-) production in splenic hydatic fluid was higher than that in the liver or lung.

Protein levels detected in fertile liver fluids were significantly higher (0.93231 ± 0.10435 μg/μl) than those in infertile fluids (0.21 ± 0.026 μg/μl; P = 0.0005) (Fig. 2). This result was also observed in pulmonary hydatic fluids (0.695± 0.07166 versus 0.18167±0.01078 μg/μl, P=0.00001) (Fig. 2).

In addition to the data shown in Table 2, our results indicated that the concentration of proteins was not exclusively dependent only on the cyst fertility but also on the cyst location, the environment of exchange between the host and parasite, and host tissue vascularity (Table 3).

Discussion

Our comparative study showed nitrite concentration variations in all hydatic patients tested. These differences could

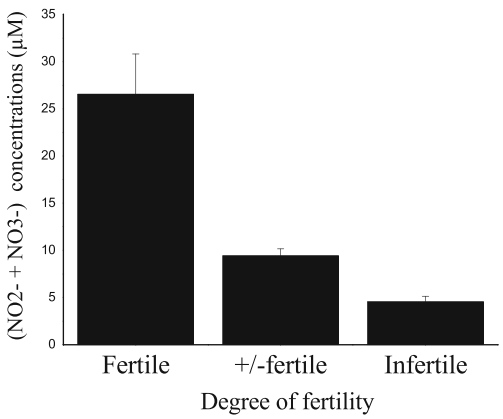

Fig. 1 Comparison of (NO_2^- + NO_3^-) levels in pulmonary hydatic fluids according to the degree of fertility. Data are means ± standard errors of the means for each point. P=0.00322 for (NO_2^- + NO_3^-) levels detected in fertile hydatic fluids (26.55±4.25 μM; n=10) versus infertile fluids (4.53±0.62 μM; n=4). Values for P<0.05 were considered statistically significant

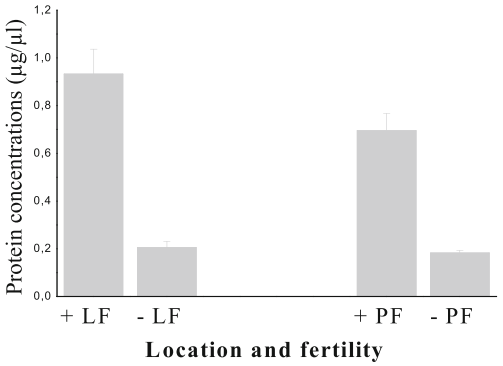

Fig. 2 Protein levels in hydatic liver and pulmonary fluids according to the fertility. Data are means ± standard errors of the means for each point. P=0.0005 for protein levels detected in fertile liver hydatic fluids (0.93231±0.10435 μg/μl; n=13) versus infertile fluids (0.21± 0.026 μg/μl; n=6). P=0.00001 for pulmonary fertile fluids (0.695± 0.07166 μg/μl; n=8) versus infertile fluids (0.18167±0.01078 μg/μl; n=6). + Fertile, − infertile, *LF* liver fluid, *PF* pulmonary fluid. Values for P<0.05 were considered statistically significant

be related to several parameters. The presence of significantly high levels of (NO_2^- + NO_3^-) in the serum of hydatic patients before surgery supports the possible involvement of NO in antihydatic action. NO level increase seems to be associated with the stimulation of the cell-mediated immune system during a long-term parasitic infection. In previous studies, our team has reported that during human hydatidosis, IFN-γ led to the elevated production of nitrite through the NOs2 pathway (Touil-Boukoffa et al. 1998; Ait Aissa et al. 2006; Amri et al. 2007). A correlation between (NO_2^- + NO_3^-) and IFN-γ levels indicates that NO production by the host is induced in large part by IFN-γ, knowing that this Th1 cytokine is highly required in the upregulation of nitric oxide production by the monocyte/macrophage system (Paul-Eugène et al. 1994; Touil-Boukoffa et al. 1998).

The formation of NO and its products has been reported in a majority of parasitic infections. Significant increase of (NO_2^- + NO_3^-) was detected in the serum of patients infected with *Entamoeba coli* (Karaman et al. 2009). In addition, human schistosomiasis results in an increase in the production of serum (NO_2^- + NO_3^-) (Abost-Shousha

Table 3 (NO_2^- + NO_3^-) and protein levels according to the fertility of hydatic fluid in uncommon cyst locations

Infrequent cyst location	Degree of fertility	n	(NO_2^- + NO_3^-) concentration (μM)	Protein levels (μg/μl)
Muscle of left leg	−	1	4.10	0.31
Spleen	+	1	31.11	1.29
Pancreas	−	1	4.76	1.80

Symbols (−) and (+) indicate infertile and fertile fluids, respectively
n number of hydatic cysts

et al. 1999). An increase nitrite concentration in the serum was also reported in experimental toxocariasis. Mice infected with *Echinococcus multilocularis* demonstrated an increase in NOs2 expression in peritoneal macrophages (Dai and Gottstein 1999).

However, in our study, concentrations of (NO_2^- + NO_3^-) declined after surgery in the absence of postoperative complication such as an infection. This decline may have a role in hydatidosis pathophysiology returning to normal levels (Ait Aissa et al. 2006; Parsak et al. 2007).

Interestingly, we observed that patients with intact and viable cysts showed elevated (NO_2^- + NO_3^-) levels compared to those with calcified cysts. This result was likely related to the inhibition of the immunogenicity. The calcification of the cyst indicated the loss of viability, while the detection of high levels of nitrite in sera of broken cysts is due to a probable increase of cyst antigen stimulation and, in turn, more activation of immune cells. This hypothesis is supported by marked hypereosinophilia after breakdown of cysts associated to allergic manifestations and production of IgE interacting with CD23 inducing human NOs2 (Paul-Eugène et al. 1994; Bell 1996).

In the present study, a significant increase of (NO_2^- + NO_3^-) concentrations was observed in the serum of patients with liver cysts in comparison with those bearing cysts in the lung. This difference is probably related to several factors. Hepatic cysts were expected to provide the highest amount of antigen, probably because the high vascularity of this major filtering organ functionally promotes hydatid development. In addition, Ait Aissa et al. (2006) reported the presence of NOs2 in hepatocytes and Kupffer cells from liver biopsies of hydatic patients. It has been reported that human PBMCs and leukocytes constitute the cellular source of NO during parasitic infection (Touil-Boukoffa et al. 1998; Ait Aissa et al. 2006). The production of high levels of nitrite in hepatic infections could be correlated with the stimulation of the cell-mediated immune system by *E. granulosus* antigens.

There is evidence that liver cysts grow at a lower rate than lung cysts (Larrieu and Frider 2001). In fact, the presence of hydatic cysts in the lung may cause compression of surrounding structures and lead to the cough reflex, resulting in a release of antigenic material and hemoptysis (Gottstein and Reichen 2002; Blanton 2007). Our results are in-line with these data and support the reasoning that differences in the host immune response are intimately related to the cyst location, suggesting a dependent relationship between cyst location and NO serum levels in hydatic patients.

During the lung infection, molecular interactions between inflammatory cells and larvae were investigated (Muro and Pérez-Arellano 2010). Human epithelial cells and alveolar macrophages may constitute a cellular source of production of NO. However, NO produced by surface epithelial cells may therefore act as an important barrier to invasion of the respiratory tract by inhaled organisms (Liew and Cox 1991; Taylor Robinson et al. 1994).

The reduction of nitrite production in relapsing patients correlates with the lack of IFN-γ production, which is related to a decrease in the CD4+ T cell count and is of predictive value in *E. granulosus* follow-up (Touil-Boukoffa et al. 1998).

The patients with double location showed a significant increase in (NO_2^- + NO_3^-) concentration. This observation could mean an enhanced immunological response when cysts are multiple. This observation was reported also by Refik et al. (2005).

The presence of NO products in the cyst fluid was previously reported by our team (Ait Aissa et al. 2006). This result is probably a consequence of antigenic burden and IFN-γ induction. The free radical can probably diffuse through the cystic walls (Ait aissa et al. 2006). In this context, we suggest the possible involvement of activated macrophages present in the adventitial layer in local innate immune response, knowing that these cells are a major source of inducible NO synthase, and they are highly activated by IFN-γ. This local response could provoke infertility and death of the hydatic cysts (Shepherd et al. 1991; Rigano et al. 1995, 2007; Vuitton 2003; Amri et al. 2007). Our hypothesis correlates with the structure of hydatic cysts. In fact, immune cells that lodged in the adventitial layer are unable to penetrate to the germinal layer due to the physical barrier imposed by the laminar layer. Our data suggest a possible local NO production by the larvae. Currently, more investigations are undertaken by our team to clarify this hypothesis. However, these findings do not exclude the possibility of presence of NOs in the scolex, although larvae NOs expression has not been identified yet (Amanvermez and Celik 2002). In the current study, our results show high levels of (NO_2^- + NO_3^-) in fertile liver and lung fluids compared to infertile fluids. This observation was also reported by Amanvermez and Celik (2002) in cattle hydatic fluids. We have noted with interest that the production of (NO_2^- + NO_3^-) in splenic hydatic fluid was higher than that in the liver or lung. This increase was probably related to the high vascularity of this organ.

Similarities of human hydatic cyst fluid components and the host serum have been reported, but in reduced amounts as compared to serum, suggesting that the host proteins can penetrate the membranes of the hydatic cyst (Goodchild and Kagan 1961; Khorsandi and Tabibi 1978). However, our results indicate that the concentration of proteins is related to the cyst location and organ vascularity. The presence of consistent protein concentrations in hydatic fluids underlines the tight relationship between the host and parasite. Compared to the protein levels, the (NO_2^- + NO_3^-) production appears to be dependent on the fertility of the hydatic cyst.

Collectively, our findings show that the NO production during human hydatidosis depends upon the cyst location, the status and viability of the cyst, the number of cysts, and the clinical stage of hydatic patients. The presence of NO products in hydatic fluids underlines the strong relationship between the immune systems of host and may be associated with the location and fertility of the cyst. Assessment of NO production may be a useful tool in the evaluation of the effector mechanisms and clinical manifestations of hydatidosis and represents a useful marker of the clinical aggressiveness of this parasitic infection. In future studies, we intend to evaluate the clinical usefulness of NO in the treatment of human echinococcosis.

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