In Vivo Inhibition of Inducible Nitric Oxide and Evaluation of the Brain Tissue Damage Induced by *Toxocara canis* Larvae in Experimentally Infected Mice

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Abstract

Nitric oxide (NO) is known to be produced by macrophages, endothelial cells and neurons and synthesized by an enzyme called nitric oxide synthase (NOS). Various effector mechanisms and infections can affect the NO production. Excessive amount of NO will lead to biochemical reactions, which cause toxic effects. In this study the role of NO has been evaluated in larval toxocarosis, which is a systemic parasite infection caused by *T. canis* larvae. Infection was established in the Balb/c mice with or without inducible NOS (iNOS) inhibition and the effects of infection and NOS inhibition were observed according to the results of SOD and LPx measurements in brain tissue and NADPH-diaphorase (NADP-d) histochemistry. Results of NADPH-d histochemistry indicate that iNOS inhibition has protective effect on the brains of infected mice and that larval *T. canis* infection could be related to oxidative stress, and NO production and iNOS inhibition can protect the tissue from damage in this infection.

Key Words: *T. canis*, iNOS, oxidative stress, NADPH-d

Introduction

Toxocara canis is a common roundworm of dogs. Humans may become infected and result in condition known as visceral larva migrans (VLM). Transmission occurs by ingestion of infective eggs of parasites (12, 25, 31) or consuming raw or undercooked organs of infected paratenic hosts such as chicken, sheep and cow (21). In humans, *T. canis* may lead to VLM or ocular larva migrans (OLM), both due to the systemic migration of the larval form of the helminth. VLM is characterized by hypereosinophilia, hepatosplenomegaly, pneumonitis, fever and hyperglobulinemia. Symptoms depend on the site and the extent of larval migration which is closely related to the duration of the infection. OLM symptoms include leucocoria, loss of vision in the affected eye, eye pain and strabismus. VLM can rarely be fatal (1, 12, 25). As a systemic infection, VLM affects most of the body systems and causes responsive changes in the infected individuals (1, 12, 14, 17, 25, 29).

Nitric oxide (NO) is an endogenously secreted molecule and produced by macrophages, endothelial cells and neurons. It is synthesized by an enzyme

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Received: April 13, 2004; Revised: June 18, 2004; Accepted: July 31, 2004.

called nitric oxide synthase (NOS) and produced and secreted in small amount in normal conditions (22, 23). In triggered production, excessive amounts of NO would lead to biochemical reactions which cause toxic effects (16). The role of inducible nitric oxide synthase (iNOS) is much complicated in immune system as having prophylactic and also toxic properties. Based on these specifications, iNOS plays a crucial role in pathological conditions such as infectious and autoimmune diseases (3). Various studies have shown the participation of NO in anti-parasite defense (2, 6, 26, 27). The role of NO in several parasitic diseases has also been studied extensively (5, 8, 11, 18, 19, 28). NO may be host-protective (28) or may be responsible for pathological disorders in some diseases caused by parasites (5, 8, 19). NO is claimed to mediates intestinal pathology but not immune expulsion during *Trichinella spiralis* infection in mice (19). Various antigens obtained from the adults and larvae of *T. canis* were proved to stimulate *in vitro* production of NO in rat alveolar macrophages (8). Infection of mice with *Trypanosoma brucei* has been demonstrated to enhance endothelial NOS (eNOS) gene expression in the vascular endothelium (34). iNOS deficiency was proved to increase resistance to chronic infection with *Echinococcus multilocularis* (6). The role of iNOS was analyzed in organ specific and stage dependent parasite control *in vivo* in *Leishmania major* infection. The impact of iNOS is most striking in the skin, remarkable in the lymph node and marginal in the spleen, and iNOS is one of the essentials for parasite control *in vivo* (2). NOS gene knockout mice were infected with *Toxoplasma gondii* and time of death was seen prolonged; distal small intestine and liver were observed normal, as compared with the iNOS+control mice where these tissues appeared damaged (18). In mice with *Schistosoma mansoni infection*, iNOS inhibition caused exacerbated liver pathology. This suggests that NO limits hepatocyte damage (27). Cytoplasmic signaling pathways involved in the generation of NO after stimulation with adult secretory/excretory antigens of *T. canis* and direct activation of NO production of rat alveolar macrophages are demonstrated after stimulation with *T. canis* antigens (9).

We have examined the role of NO in the larval toxocarosis to clarify whether the release of NO during infection has a host-protective effect, and whether NO secretion plays a role in the pathology of the infection.

Materials and Methods

Preparation of Parasite Eggs

T. canis eggs were obtained from the uteri of female nematodes collected from the naturally infected dogs. Eggs were incubated in 0.5% formalin solution at 28˚C for 4 weeks. Embrionated eggs were kept at +4˚C until used (10, 30). Mice in the VLM groups were each infected with 2500 embrionated eggs.

Inducible Nitric Oxide Synthase (iNOS) Inhibition

Aminoguanidine (Sigma) was used as the specific iNOS inhibitor. It was applied intraperitoneally 3 times/day for 1, 2 and 7 days according to the groups at the dose of 100 mg/kg (24).

Experimental Animals

Three months old, seventy-two albino Balb/c mice (25-30 g) were used in the experiments. They were kept in 12 hours light-12 hours dark routine and led to consume standard mouse food and tap water *ad libitum*.

Experimental Design

Group A (n=6): Aminoguanidine was applied intraperitoneally 3 times/day for 24 hours at the dose of 100 mg/kg to each mouse in the group.

Group L (n=6): Each mouse was infected with 2500 embryonated *T. canis* eggs by oral inoculation.

Group LA (n=6): Each mouse was infected with 2500 embrionated *T. canis* eggs by oral inoculation, and aminoguanidine was applied intraperitoneally 3 times/day for 24 hours at the dose of 100 mg/kg.

Group C (n=6): Physiological saline was applied in the same schedule as aminoguanidine for establishing the same handling stress to control animals.

First groups were sacrificed 24 hours post infection (pi) and this group design was organized for the 48 hours pi and 7 days pi groups in the same schedule.

NADPH-Diaphorase Histochemistry

On the necropsy of control and experimental mice, brains were removed, and 1/3 of each right cerebrum which is close to cerebellum was fixed in 4% paraformaldehyde. For histochemical staining, sections in 8 µm were taken on the glass slides and NADPH-diaphorase histochemistry was applied to these cryostat sections. Slides were incubated in 0.1 M phosphate buffer containing 0.3% Triton X-100, 0.01% Nitroblue tetrazolium (NBT), and 0.1% β-NADPH for 25-30 min. The catalytic activity of NOS was demonstrated by enzymatic reduction of NBT in the presence of NADPH (NADPH-diaphorase reaction) (15).

Biochemical Methods

Frontal lobes (anterior 1/3 of the cerebrum) of each animal were removed and used for the determination of lipid peroxidase (LPx) levels and superoxide dismutase (SOD) activity. LPx values and SOD activity in brain tissues were determined with the thiobarbituric acide (4) and nitroblue tetrazolium inhibition (32) methods, respectively.

Brain tissue homogenization: Tissue samples were weighed (wet weight) and homogenized in ice-cold NaCl 0.9% to produce a 1/10 (w/v) homogenate. Tissue homogenization was performed with a tissue grander fitted with a Teflon pestle. The homogenate was sonicated with MSE sonicator twice at 30 seconds intervals in ice, with a power output of 38 Watts. The sonicated homogenates were centrifuged at 2000 rpm for 10 min for the measurement of lipid peroxidation and at 15,000 rpm for 15 min for the measurements of the SOD activity at 4˚C. Supernatants were used for the biochemical assay performing.

Measurement of the lipid peroxidation: Tissue TBARS (thiobarbituric acid reacting substances) levels as the marker of lipid peroxidation were determined with the spectrophotometric method described by Buege and Aust (4). Stock solution: 15% trichloroacetic acid (TCA), 0.375% thiobarbituric acid (TBA), 0.25 N hydrochloric acid (HCl). The samples were heated in a water bath for 20 min and after cooling, centrifuged at 2,000 rpm for 15 min. The formation of pink color, as a result of the reaction in-between one molecule of TBARS and two molecules of TBA, was measured at 560 nm spectrophotometrically.

Measurement of the tissue SOD activity: Tissue super oxide dismutase activity was measured by the modified method of Sun (32). This assay for super oxide dismutase (SOD, EC 1.15.1.1) activity involved inhibition of nitroblue tetrazolium (NBT) reduction, with xanthine oxidase used as a super oxide generator. One unit SOD was defined as the amount of protein that inhibits the rate of NBT reduction by 50%. Reaction mixture: 40 ml of 0.3 mmol/l xanthine solution, 20 ml of 0.6 mmol/l EDTA solution, 20 ml of 150 µmol/l nitroblue tetrazolium solution, 12 ml of 400 mmol/l Na_2CO_3 solution, and 6 ml of bovine serum albumin. The final concentration of xanthine oxidase was 167 U/l. The production of formazon was determined at 560 nm, spectrophotometrically.

Evaluation of the Organs for the Presence of Larvae

The rest of the brain tissue samples of the infected mice was inspected by the squash preparations. Liver, lung and other tissue samples were digested in the pepsin-HCl solution and screened for the presence of the larvae for the confirmation of the visceral larva migrans (30).

Statistical means among the groups and with the controls were evaluated with the Student-*t* and Tukey tests.

Results

Macroscopic pathological differences were found in the inspection of the brain of parasite inoculated mice, but these differences were not clear at 24th hour of necropsy (Figure 1A and 1B). On the cortex of the mice brains which have been inoculated with *T. canis* eggs, hemorrhagic areas were seen on motor cortex (indicated as upper arrow in 1D), sensory areas (lower arrow in 1C) and cerebellum (lower arrow in 1D) on the 48th h and 7th day, respectively (Figure 1C and 1D). Hemorrhagic areas were denser in the *T. canis* infected mice than those in the aminoguanidine and *T. canis* eggs administered groups (Figure 1D and 2D).

Aminoguanidine, which is the specific iNOS inhibitor, was found to have protective effect on the brains of animals at 24 h and 48 h after the parasite inoculations (Figure 2B and 2C). Brains of only aminoguanidine-administered mice were normal (Figure 2A) and similar to those of the control mice (Figure 1A). Hemorrhagic spots were not found at the 24th and 48th hours in the brains of aminoguanidine-treated infected mice (Figure 2A and 2B). However, on the 7th day, areas of hemorrhagic spots were still present in the brains of this group (Figure 2 D), but numbers of spots were less dense as compared to the the mice which received only parasite eggs (Figure 1D). In contrast, mice which were only aminoguanidine administered showed no pathological changes in their brain.

In the inspection of NADPH-d histochemistry applied to brain sections, an increased reaction was seen in parasite inoculated mice. Number of the cells with NADPH-d reaction increased with statistically significant difference ($P < 0.001$), as compared with the control animals (Table 1). In the brain slides of the mice which were parasite inoculated, at the 24th, 48th hours and on the 7th day, significantly increased cell counts with NADPH-d reaction were observed. In the mice which were given aminoguanidine and parasite inoculated in the same time cell counts decreased ($P < 0.001$). However, compared with the control mice, the amount of stained cells was still higher ($P < 0.001$). There was no difference in the brain sections of the control mice and the mice given only aminoguanidine. These results are summarized in Table 1 and the appearance of the stained sections is provided in Figure 3. When NADPH-d histochemistry of the experimental mice was compared with the control mice (Figure 3A and 4A), reaction density of the cells increased, while simultancously the increment of the number of cells results in NADPH-

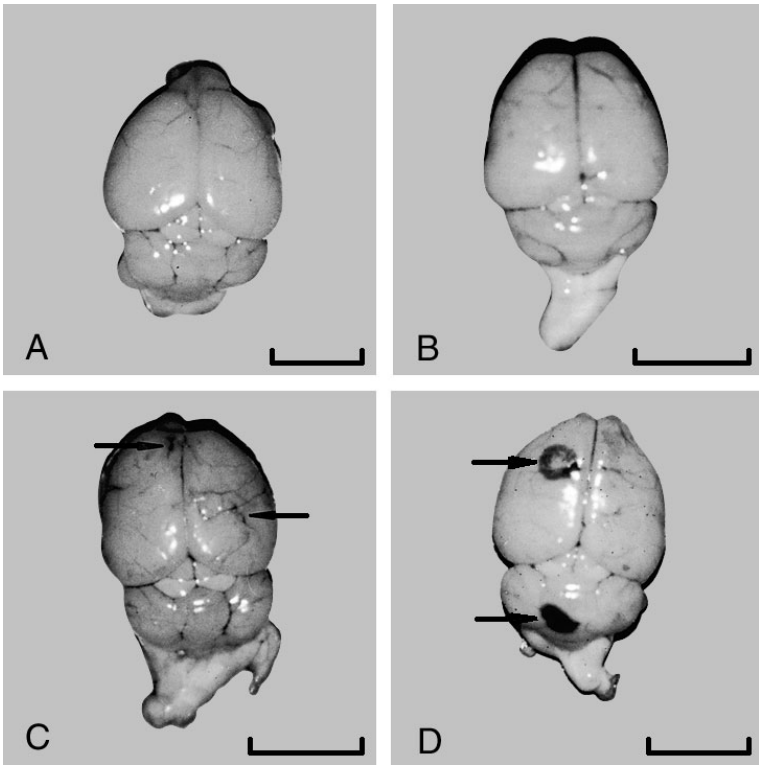

Fig. 1. Total brain images showing differences in the control and *T. canis* infected mice. (A) brain of the control mice. (B) 24 hours, (C) 48 hours and (D) 7 days after egg inoculation. Arrows show bleeding areas. Scale bar: 1 cm.

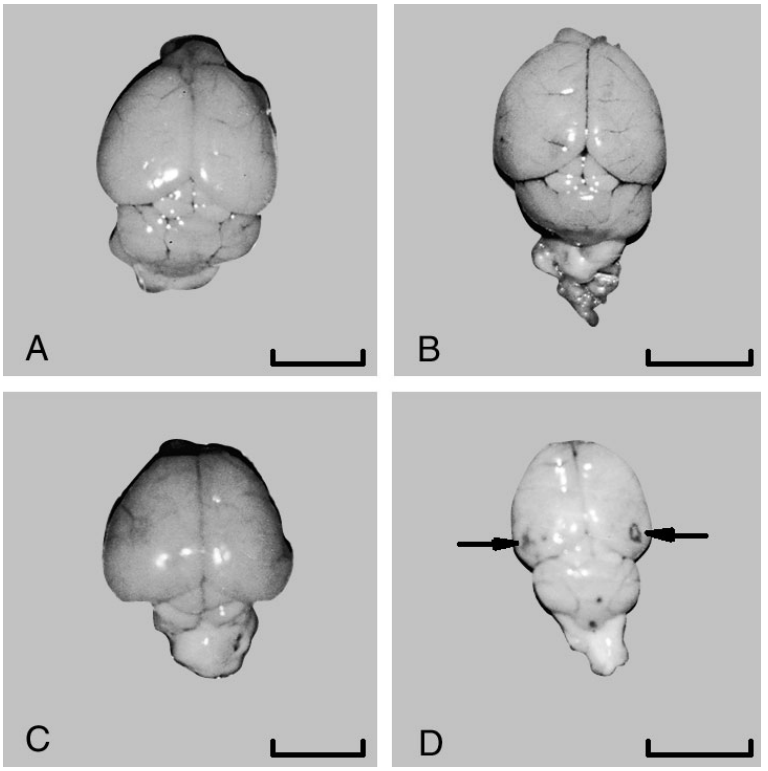

Fig. 2. Brain photographs taken from control and aminoguanidine-treated mice (A) control, (B) 24 hours (C) 48 hours and (D) 7 days after *T. canis* inoculation in the aminoguanidine treated mice Arrows show bleeding points. Scale bar: 1 cm.

Table 1. The number of cells showing NADPH-d reaction in the brain sections of the experimental mice

Groups	Cell numbers with NADPH-d reaction		
	24th hour	48th hour	7th day
A (n=6)	23.7±3.36	36.3±2.45	32.2±1.93
L (n=6)	47.4±2.71*	48.8±2.89*	61.1±3.38*
LA (n=6)	33.7±2.16*	37±2.16*	44.2±2.53*
C (n=6)	22.8±0.78	33.7±1.63	29.9±3.03

A: only aminoguanidine-administered, L: inoculated with embryonated *T. canis* eggs, LA: aminoguanidine-administered and-inoculated with embryonated *T. canis* eggs, C: control. Student-*t* and Tukey tests were used for comparison of the groups.

* Significant *vs.* control ($P < 0.001$)

Table 2. LPx values in brain tissue homogenates of the groups

Groups	LPx values		
	24th hour	48th hour	7th day
A (n=6)	78.51±1.15	57.15±1.67	59.55±1.62
L (n=6)	85.82±1.88*	79.56±3.40*	64.11±2.18
LA (n=6)	86.5±1.15*	81.51±1.95*	75.16±1.54
C (n=6)	61.98±1.83	56.43±1.56	60.30±0.40

A: only aminoguanidine-administered, L: inoculated with embryonated *T. canis* eggs, LA: aminoguanidine-administered and-inoculated with embryonated *T. canis* eggs, C: control. Student-*t* and Tukey tests were used for comparison of the groups

* Significant *vs.* control ($P < 0.001$)

d reaction in the parasite inoculated mice (Figure 3B and 4B). Aminoguanidine application to the infected mice did not change the NOS amount histochemically but decreased the number of active cells including the NOS (Figure 3C and 4C). NADPH-d histochemistry and the numbers of these cells were similar in the controls and in the mice which were given only aminoguanidine (Figure 3D and 4D).

Lipid peroxidase levels increased in the brain homogenates of mice given *T. canis* eggs. This increment was significantly high in the all infected groups and all experimental periods in comparison with the control groups ($P < 0.001$). However, LPx levels were much higher 24 hours after inoculation in comparison with the other groups ($P < 0.001$). Aminoguanidine application to the infected mice did not change the LPx level; it was still higher than that of the controls. If only the effects of aminoguanidine were considered, there was no significant difference between the aminoguanidine-administered and control mice (Table 2).

SOD activity was found to decrease in the aminoguanidine group, but this value was not significantly different from that of the control mice. Aminoguanidine cause a significant decrease in SOD activity in the *T. canis* infected mice, as compared to the control mice and the mice inoculated with only parasite ($P < 0.001$). It was also lesser at the 24 and 48 h post infection in the aminoguanidine-administered mice than the controls, but aminoguanidine application increased the SOD level in the brain tissue when compared with the controls on the 7th day. (Table 3).

Discussion

NO and some other reactive oxidants such as superoxide, hydrogen peroxide are involved in complex interactions when humans or animals are infected with microorganism or parasites (20). The role of NO is quite scarce in toxocarosis infections. It's known that microorganisms increase the iNO in humans and animals (3). Parasite infections cause the increment of iNO and maybe other isoforms of NO. *In vivo* production of NO induced by infection with *T. canis* resulted in direct host damages (7). *T. canis* larvae migrate from intestine to several organs such as liver, lung, kidney and brain (1, 12). We have seen the effects of this migration on the brain as bleeding areas, and they were most obvious 7 days after parasite inoculation. On the 7th day, pathological spots were severe in the *T. canis* infected mice, but specific iNOS inhibitor aminoguanidine seemed to have protected the mice partly from iNO effect in aminoguanidine administered infected animals. Results showed that brain tissue can be less damaged if iNOS was inhibited by specific inhibitors in visceral larva migrans. Some studies also report that parasites such as *T. gondii* infections prolonged the time of death in the iNOS knockout mice, compared with controls. Histological analysis of tissue from infected mice shows scattered small foci of inflammation with parasites in various tissues of iNOS-/-mice, whereas tissue from wild type mice show more extensive tissue inflammation (18). In mice with metazoan parasite, *B. malayi* infections, aminoguanidine can get less worm burdens (26). It is not clear that *T. canis* infections can be related to NO production in the brain tissue. nNO (neuronal NO) is produced normally in the nerve cells, but in the induction of immune system by microorganism, iNO can be produced in the brain cells besides other cells, such as macrophages, epithelial cells, endothelial cells, etc. (13). In this study, NADPH-d histochemistry was

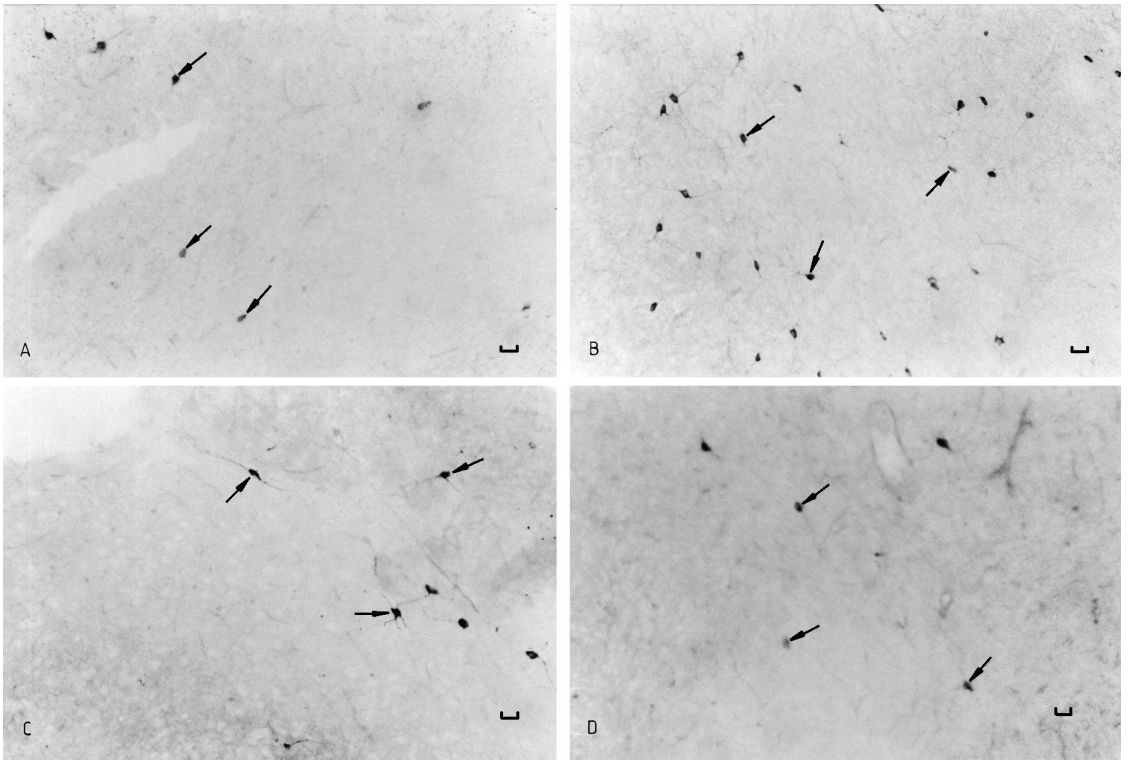

Fig. 3. End of the 7th day , NADPH-d histochemistry in the brain sections (right cerebrum) of experimental groups. (A) control, (B) *T. canis* inoculated, (C) *T. canis*-inoculated and aminoguanidine-treated and (D) only aminoguanidine-treated animals. Arrows show NADPH-d histochemically stained cells. Scale bar: 10 μm.

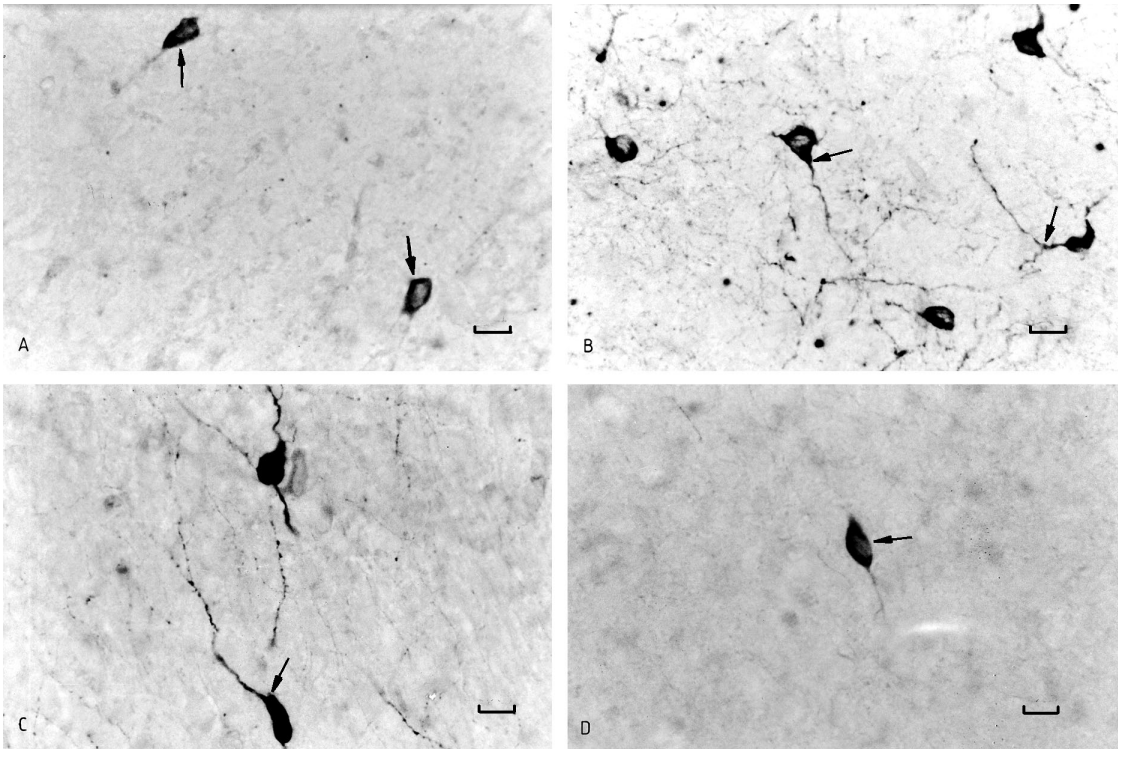

Fig. 4. Same results such as figure 3 are shown in higher magnifications (right cerebrum). (A) control, (B) *T. canis*-inoculated (C) *T. canis*-inoculated and aminoguanidine-treated and (D) only aminoguanidine-treated animals. Arrows show NADPH-d histochemically-stained areas. Scale bar: 5 μm

Table 3. SOD values in brain tissue homogenates of the groups

Groups	SOD values		
	24th hour	48th hour	7th day
A (n=6)	90.46±1.24	134.73±0.82	134.89±2.14
L (n=6)	85.93±1.00	111.2±1.54*	101.7±1.54*
LA (n=6)	74.45±1.60	93.13±1.02	65.76±2.51
C (n=6)	99.97±1.02	143.35±0.45	129.27±2.54

A: only aminoguanidine-administered, L: inoculated with embryonated *T. canis* eggs, LA: aminoguanidine-administered and-inoculated with embryonated *T. canis* eggs, C: control. Student-*t* and Tukey tests were used for comparison of the groups.

* Significant *vs.* control ($P < 0.001$)

applied to nerve cells in the brain of *T. canis* infected mice. The number of NOS, including cells, was much higher and NADPH-d histochemistry was also denser in the infected mice than controls. Thus, infection caused the production of iNOS together with nNOS in the brain. Aminoguanidine applied infected animals showed normal cell numbers and histochemically condensations and this finding supports the above-mentioned result. Now we can ask why NADPH-d increased in the aminoguanidine applied mice. iNOS inhibition in the other cells of the body can cause over production of nNOS and eNOS. Some studies support this idea. For example, septic shock increases the iNOS in some organs but, on the other hand it can decrease eNOS in the some vessels (33). *T. canis* infection causes over-production of iNO in several organs such as liver and lungs. Espinoza *et al*. found that high titrations of nitrites in bronchoalveolar lavage fluid (BALF) from infected rats indicate that during experimental toxocarosis there is an effective increase in the production of NO. Treatment of infected rats with aminoguanidine has resulted in a significant decrease of total nitrites in BALF, in comparison with the infected and non-treated rats (7). They have also found that treatment of infected animals with aminoguanidine resulted in the decrease of all pathological signs in both liver and lungs. These results support our findings which include NOS increments in the brain cells of infected mice and NOS decrement in the aminoguanidine treated mice. Our and Espinoza et al's (7) results demonstrated that beneficial effects of aminoguanidine treatment were specifically due to the diminution of iNOS production.

NO, lipid peroxidation, and SOD production are important under the oxidative stress conditions, and many pathological disorders and infections can cause oxidative stress. NO increment in the brain tissue in mice which were infected with *T. canis* was related to increment of LPx levels and decrement of SOD, indicating the oxidative stress. We have seen that the number of NO producing cells decreased in the brain tissue of *T. canis* infected and aminoguanidine treated animals according to the results of NADPH-d histochemistry, but oxidative stress was still present in these mice according to the LPx and SOD values. However, in the mice which were only aminoganidine-administered, the LPx and SOD levels did not change, when compared to the controls. This demonstrated that aminoguanidine could not change the oxidative stress in the brain tissue alone; instead, it could only inhibit the iNOS production. Therefore, it could protect the tissue damage without changing the antioxidant production such as SOD.

As a result, we conclude that parasite infection could be related to oxidative stress and NO production, and iNOS inhibition can protect the brain tissue from pathological damage in VLM infection caused by *T. canis* larvae.

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