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Arginine reduces *Cryptosporidium parvum* infection in undernourished suckling mice involving both nitric oxide synthase and arginase

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Abstract

Objective—This study investigated the role of L-arginine supplementation to undernourished and *Cryptosporidium parvum*-infected suckling mice.

Methods—The following regimens were initiated on the 4th day of life and given subcutaneously daily: either 200mM of L-arginine or PBS for the *C. parvum*-infected controls. L-arginine-treated mice were grouped to receive either 20mM of NG-nitroarginine-methyl-ester (L-NAME) or PBS. Infected mice received orally 10^6 excysted-*C. parvum* oocysts on day 6 and were euthanized on day 14th at the infection peak.

Results—L-arginine improved weight gain compared to the untreated infected controls. L-NAME profoundly impaired body weight gain as compared to all other groups. Cryptosporidiosis was associated with ileal crypt hyperplasia, villus blunting, and inflammation. L-arginine improved mucosal histology following infection. L-NAME abrogated these arginine-induced improvements. Infected control mice showed an intense arginase expression, which was even greater with L-NAME. L-arginine reduced parasite burden, an effect that was reversed by L-NAME. *C. parvum* infection increased urine NO_3-/NO_2- concentration when compared to

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Authors' contribution:
IBC, BBO, JWSS and BPC conducted most of the crypto infection handling of the experimental mice. JES and RLG contributed with the oocyst shedding PCR analyses. AAML, RLG and RBO contributed with the study design, study analysis and manuscript preparation.

uninfected controls, which was increased by L-arginine supplementation, an effect that was also reversed by L-NAME.

Conclusion—These findings show a protective role of L-arginine during *C. parvum* infection in undernourished mice with involvement of arginase I and nitric oxide synthase enzymatic actions.

Keywords

Cryptosporidium parvum; diarrhea; malnutrition; arginine; growing mice

INTRODUCTION

Cryptosporidium parvum is an enteric protozoan spread by fecal-to-oral transmission that causes debilitating diarrhea in its host. *C. parvum* begins infection after intracellular oocytes release sporozoites that bind to intestinal epithelial cells and produce superficial parasitophorous vacuoles [1,2]. *C. parvum* is found worldwide, but is particularly common in developing areas where inadequate sanitation and water contamination are prevalent. In immune-compromised hosts such as those with HIV, this infection is potentially life-threatening. Mortality and risk of protracted *C. parvum* infection is worse in patients with uncontrolled AIDS [3,4]. In addition, undernourished children are at greater risk for acquiring and spreading this chlorine-resistant protozoan, which has been shown to endure and spread in the environment [5].

L-arginine is considered an essential amino acid in growing children whose anabolic protein requirement is continuously high [6]. In addition, arginine deficiency has been associated with severe undernutrition and with other catabolic states, like trauma and sepsis, and during heavy infection burdens [7]. L-arginine has a myriad of biological roles, since it is a substrate to two enzymatic systems, nitric oxide synthase (NOS) and arginase isoforms, which lead to either nitric oxide or to ornithine and polyamine synthesis, respectively. L-arginine is therefore a key constituent of complex immunological, inflammatory, and wound healing responses [8,9].

Recently, we have demonstrated that early post-natal malnutrition worsens *C. parvum* infection in C57BL6J mice in a model of maternal-offspring separation [10], somewhat analogous to premature weaning seen in Brazilian shantytown areas [11].

During suckling time, breast-milk-derived arginine is not sufficient to overcome the higher demand of this nutrient during bursts of growth [12,13]. In this current study, we investigate whether L-arginine supplementation improves intestinal adaptation following *C. parvum* challenge in undernourished suckling mice. We also explore the involvement of arginase and inducible nitric oxide synthase (iNOS; NOS2) in controlling the infection. Our findings support an arginine-based therapy to prevent the devastating and lasting effects of the vicious cycle of diarrhea and malnutrition in the developing world.

Materials and methods

Malnutrition protocol

The protocol described herein is in accordance with the Institutional Animal Care and Use Committee (IACUC) policies of the University of Virginia. Pregnant inbred mice (C57BL6J) were purchased from Charles River Lab. (Wilmington, MA) and monitored daily before delivery with free access to standard chow diet and water (12 h cycle/light and dark). After birth, litter size was adjusted to 6–8 pups. Their first day of life was recorded as post-natal day (D)1. Half of the offspring was separated from their lactating dam for increasing

intervals starting on D4 of life, as described previously [14,15]; four hours at D4, eight hours at D5 and then twelve hours from D6 until D13. Separated pups were kept in an incubator box under controlled temperature (28 ± 2C). Severely underweight pups (< 1.8 g) at D4 were not included. Nourished controls stayed with their mothers. Litters were kept undisturbed during D1 to D4 to reduce stress and to assure full colostrum intake. Body weight was recorded daily until mice were euthanized. Care was taken to keep the same level of animal handling for all groups. The maternal-offspring separation model described here is analogous to the early weaning often seen in Brazilian shantytown children [11].

Experimental Groups

Data from nourished and undernourished mice are presented; the latter had shown approximately 10-fold heavier infections than their nourished sibilings given the same ammount [10]. We therefore expected that the effect of intervention with L-arginine in the undernourished groups would be greater and more quantifiable with more severe infections with malnutrition.

In this study, we have used the following six groups: uninfected nourished controls (N=11); uninfected undernourished controls (N=16); infected nourished controls (N=7); infected undernourished controls (N=11), infected undernourished treated with 200 mM of L-arginine (N=14), and infected undernourished treated with 200 mM of L-arginine and 20mM of NG-nitroarginine-methyl-ester (L-NAME) (N=7).

Treatment regimens were initiated on the 4th day of life. Based on a previous dose-response curve (data not shown), a dose of 200 mM of L-arginine was chosen for this study. L-NAME treatment was given immediately prior to L-arginine injection. Treatments were given subcutaneously in different dorsal areas of the animals daily starting D4 of life until D13, at volumes of 40 μl during D4–D10 and 80μl during D11 until D13. L-NAME treatment was given twice daily, each dose given at half of the volume administered for the single L-arginine treatment dose.

Cryptosporidium parvum infection

At D6, 1 hour prior to parasite inoculation, pups were separated from their lactating dams to empty their stomachs. Each pup received an oral inoculum of 10^6 excysted *C. parvum* oocysts in 10μl of PBS (pH 7.2). Oocysts were obtained from experimentally infected calves (Iowa isolate; Waterborne Inc., New Orleans, LA). Littermate controls were kept and handled separately and received PBS orally at the same time that the infected mice received their inoculation. Body weight was measured daily.

Tissue collection and histology

Animals were euthanized at D14, 8 days post-inoculum, at the peak of the infection, after being anesthetized with sodium pentobarbital (8 mg/100g i.p.), followed by cervical dislocation. After opening the abdominal cavity, approximately 1 cm of ileum (proximal to the ileocecal valve) was removed and prepared for histology (24 hr in 10% zinc formalin). Another 2 cm-segment, proximal to the first removed segment, was removed and milked free of stool to perform quantitative real-time PCR for cryptosporidial DNA (frozen and stored in −20°C). A final 1-cm ileal segment was harvested and prepared for immunoblotting, as described below (frozen immediately in liquid nitrogen and stored in −80°C).

Digital micrographs of intestinal histology were taken using a high resolution microscope and Photoshop CS software (BH-2, Olympus, Tokyo, Japan). In order to address mucosal villus surface and crypt hyperplasic response, villus height and crypt depth and area were

measured using Image J software, at low magnification (100X), from ileum samples at day 14 (8 days post-inoculation), following calibration, as described elsewhere [16]. Recognizable longitudinal sections of crypts and villi were measured per ileum. In order to perform direct visualization of oocysts, and to confirm infectivity, higher magnifications were used (400X).

Immunoblotting

In brief, ileal segments (0.5 cm to the ileocecal valve) were harvested and immediately frozen in liquid nitrogen. Thawed specimens were pulverized in glass homogenizers, containing lysis buffer and then transferred to test tubes with protease inhibitor and centrifuged at 14 000 rpm. Supernatants were assayed using the bicinchoninic acid method, BCA Protein Assay Kit (Pierce, Rockford, IL) to standardize 50 μg of protein product. Samples were loaded into 15% denaturating polyacryamide gels (Biorad, Hercules, CA), and gels were transferred overnight and then blotted onto nitrocellulose membranes. Membranes were incubated with rabbit arginase I IgG antibody (at dilution of 1:500) for 2 hours and then rinsed 3 times in rinsing buffer then incubated in a horseradish peroxidase-conjugated secondary antibody (1:1000) and rinsed as described above. Each membrane was washed and exposed to Kodak X-Omat AR film (Kodak, Rochester, NY). Following stripping, blots were further incubated with GAPDH for densitometry normalization.

Immunohistochemistry

Four-micrometer sections were cut from each paraffin tissue block (n = 4 for each group). Immunohistochemical staining was performed using the avidin-biotin complex technique (VectaStain Kit, Vector Laboratories, Burlingame, CA), using the automated DakoCytomation System (Carpinteria, CA). The rabbit polyclonal antibody Arginase I (Santa Cruz,Biotechnology, Santa Cruz, CA) was used at 1:100 dilution after antigen retrieval. Briefly, sections of specimens and controls were incubated in a 0.01 mMol/L sodium citrate buffer (pH 6.0) in a pressure cooker at full steam pressure for 10 minutes. Positive control included normal human liver. Negative controls for each experiment omitted the primary antibody. Negative controls, which consisted of omission of the primary antiserum, were uniformly negative. Positive controls were used to confirm immunostaining (brown). Slides were counterstained with Harris' hematoxylin (blue).

Quantitative real-time PCR

DNA from intestinal samples was extracted using a Qiagen Tissue kit (Valencia, CA). Quantification of the infection burden was done by comparing the curves with a known amount of DNA derived from a standard curve. Master mix was prepared by mixing 12.5μl of SYBR green mastermix (Biorad, Hercules, CA), 5.5 μl of nuclease free water (Fisher Scientific, Pittsburgh, PA) and 1.0 μl of both forward and reverse primers. The primers are specific for the 18s rRNA gene of the parasite (forward: 5'- TAG AGA TTG GAG GTT GTT CCT – 3'; reverse: 5'- CTC CAC CAA CTA AGA ACG GCC – 3') (GenBank no. X64341). The total reaction mixture for each sample was 20μl, to which 5μl of the DNA sample was added. DNA amplification was previously described in detail elsewhere [17].

Nitrate and Nitrite Colorimetric Assay

A reliable measurement of total NO is the sum of both its oxidative metabolites, the nitrate and nitrite concentrations in urine since NO is scavenged rapidly (t1/2 = 4 seconds). The first step is the conversion of nitrate to nitrite utilizing nitrate reductase followed by the addition of the Griess reagent that converts nitrite into a deep purple azo compound. Photometric measurement of the absorbance due to this azo chromophore accurately determines NO2- concentration. Urine samples from anesthetized animals, prior to

euthanasia, were collected by urinary bladder puncture using a 1-ml syringe and stored at −80°C until analysis. Samples were thawed and diluted ten times with the Assay Buffer solution for standard curve reading. Following dilution, 80μL of each sample were plated in triplicate, and 20μL total of an enzyme cofactor and nitrate reductase were added into each well. The plate was covered and incubated at room temperature for 1 hour. After incubation, Griess Reagent R1 was added and immediately followed by Griess Reagent R2. The plate was incubated for 10 minutes at room temperature and the absorbance was read at 550nm using an ELISA plate reader. All materials were purchased from Cayman Chemical (Ann Arbor, Michigan).

Statistical analyses

The data analysis and graphs were performed with GraphPad 4.01 for Windows. All statistical analyses were done from raw data using either 1-way analyses of variance with post-hoc Bonferroni's comparison test or Student's unpaired *t*-tests, as appropriate. A *P*-value < 0.05 was considered significant. Data are represented as either mean ± standard error for graphs or mean ± standard deviation in table 1.

RESULTS

Physical Growth

We analyzed the daily weight gain of the experimental mice over a 9 day-period. As expected, undernourished and *C. parvum* infected mice had significant reductions in the body weight gain during most of the experimental time course as compared with the uninfected undernourished control (Fig. 1A). In undernourished mice, treatment with 200mM of L-arginine significantly improved weight gain as soon as the first days post-inoculum, as compared to the untreated infected control, with growth advantages (Fig. 1B). L-NAME treatment (20mM) profoundly impaired the relative body weight gain over the same period, reducing weight gains by 30% at the end point, as compared to the infected PBS control, thus reversing the L-arginine protective effect against *C. parvum*. Most notably, L-NAME treatment (20mM) reduced weight gains from the first day of treatment and worsened during the post-infection period - decrements that were maintained until the end of the experimental course (Fig. 1).

Histology—As previously described [15], the undernourished non-infected mice showed profound crypt derangement and shortening, with almost complete mitotic blunting. Rarely Paneth and goblet cells were found within the crypt. In addition, villi were thin and scattered (Fig. 2B) as compared to the nourished control (Fig 2A). Cryptosporidial infections were associated with crypt hyperplasia, villus blunting and inflammation in the lamina propria and submucosa (Fig. 2C–F). Oral arginine treatment (200mM) resulted in improved mucosal histology to the level of the uninfected undernourished control group (Fig 2E). We found significant improvements in villus height in L-arginine treated groups in the ileal mucosa (Table 1). L-NAME abrogated the histological improvements seen with L-arginine treatment. In addition, the L-NAME-treated group showed marked inflammatory infiltrates in the submucosa. The ileal histology from 200 mM L-arginine + L-NAME showed profound villus flattening, accompanied by surrounding crypt enlargement (Fig. 2F).

Immunoblotting and immunohistochemistry—To assess the activity of arginine enzymatic pathway, we performed immuohistochemistry and western blots to determine ileal arginase I (ARG I) expression. Interestingly ARG1 immunostaining was found in the ileum of undernourished uninfected mice and among the ileum of infected mice, in the vicinity where *C. parvum* oocysts clustered, namely in the apical surface of infected enterocytes (Fig 3A). Additionally, the *C. parvum* parasites were seen in large numbers in

the ileum of L-NAME-treated animals. ARG1 immunolabeling was markedly seen in lining epithelia, in the apical region of the enterocytes nearby *C. parvum oocysts* but the immunostaing was found weaker within crypts.

Unexpectedly, expression of ARG I protein was seen in the ileum of undernourished uninfected mice, as detected by western blotting. A marked increase of ileal ARG I expression was seen when *C. parvum* infection was present (Fig 3B). Undernourished mice treated with L-arginine showed reduced expression of ARG1, as compared to infected undernourished controls ($p<0.05$). This effect was reversed by L-NAME treatment (Fig 3B).

Intensity of cryptosporidial infection—In order to better evaluate the anti-parasitic effect of L-arginine supplementation in *C. parvum*-infected undernourished mice, we determined the intensity of cryptosporidial infection in the ileum using Real Time-PCR. L-arginine treatment at dose of 200mM significantly reduced intensity of cryptosporidial infection per mg of ileal tissue ($p<0.001$), as opposed to the infected PBS control mice. The non-selective nitric oxide synthase inhibitor L-NAME (20mM) significantly reversed the effects of L-arginine ($p<0.05$), as depicted in Fig. 4A. Nourished infected mice showed much lower ileal oocyst shedding as opposed to the undernourished controls.

Nitrate and Nitrite Quantification—In order to assess the production of nitric oxide synthase-derived nitric oxide, we measured its oxidative metabolites in the urine, nitrate ($NO3^-$) and nitrite ($NO2^-$). *C. parvum* infection significantly increased urine NO3- and NO2- concentration when compared to non-infected controls. 200mM L-arginine treatment significantly increased nitric oxide metabolites when compared to infected and non-infected controls. The combined treatment of 200mM of L-arginine and L-NAME (20 mM) reversed this observed effect (Fig. 4B).

DISCUSSION

Cryptosporidiosis is one of the most important etiologies of persistent and debilitating watery-diarrhea in children in the developing world and profoundly jeopardizes physical growth and cognitive performance [18,19,20]. Undernourished children living in crowded households without adequate sanitation are at particular risk of acquiring and spreading this enteric protozoan [5]. The vicious cycle between malnutrition and diarrhea creates a self-amplifying loop which may have a lasting impact on physical and cognitive development that may extend over an entire life-span and even through generations [2,21].

In an earlier study, we demonstrated that undernutrition caused by breastfeeding restriction during the suckling time in rodents could lead to severe stunting, poor weight gain [15] and much heavier cryptosporidial infections [10]. In the current study, we have evaluated the potential benefit of the conditional amino acid L-arginine during intestinal adaptations against *C. parvum* infection in suckling mice afflicted with early post-natal malnutrition. This malnutrition model is particularly valuable to dissect the components of the vicious cycle of infection during early post-natal development since L-arginine is not present in sufficient amounts in rodent (or human) breast-milk to provide full support to the rapid growth during the suckling time [22], a demand in which might be increased even more during a catabolic state like heavy cryptosporidiosis. Suckling mice have been shown to be susceptible to *C. parvum* infection in the ileum tissue [23,24] and malnutrition induced by maternal-offspring separation was found to determine profound and lasting effects on the intestinal barrier function [25]. Supplementation of L-arginine has been shown to enhance growth in suckling piglets [13] and growing arginase I overexpressing transgenic mice, which develop severe and selective arginine deficiency, have impaired immunity and lymphoid tissue formation [26].

Additionally, experimental cryptosporidiosis causes a significant reduction in amino acid absorption even after clearance of the parasite with concomitant up-regulation of intestinal peptide transporters [27].

Since intestinal Th1-directed pro-inflammatory cytokines are critical to eradicate *C. parvum* in the small intestine [28,29], the rationale for L-arginine supplementation (NOS substrate to generate nitric oxide, a potential inducer of IFN-γ and TNF-α) to control *C. parvum* infection includes its greater need at a time of its increased demand, as seen during catabolic states.

Our findings clearly demonstrate the beneficial role of L-arginine supplementation against *C. parvum* infection, as depicted by reduced ileal- *C. parvum* oocyst shedding as compared to controls and as shown by elsewhere studies in athymic nude mice [30] and neonatal piglets [31]. Gookin et al, showed the role of NO-mediated prostaglandin synthesis in maintaining intestinal barrier function (14). We found similar levels of NO metabolites in *C. parvum*-challenged mice at day 14, as shown by Leitch and He [32] emphasizing the importance of NO production in controlling infection. Further evidence comes from iNOS knock-out murine models and prolonged treatment with NO donor compounds, in which the rate of infection and intestinal epithelial integrity is significantly impacted and protected, respectively [32].

L-arginine is a substrate to two enzymatic pathways, arginase and NOS isoforms. Interestingly, arginase (ARG) is poorly expressed in the murine neonatal small intestine until day 18, when thereafter there is a peak of enzymatic activity in growing mice, corresponding to the intestinal adaptation to the weaning [33,34]. In fact, unlike adults, the neonatal small intestine is a main source of arginine synthesis for which demand is heavy during the anabolic state of the growth burst [35]. Thus, poor arginase expression in the neonatal small intestine further emphasizes the need of the amino acid arginine for protein synthesis during intestinal development. In our study, we demonstrated the expression of arginase I (ARG I) in the ileum of uninfected undernourished mice, suggesting that higher ARG I activity is required for the compensatory mechanisms accompanying the intestinal adaptation to malnutrition. Additionally, increased ileal ARG I expression was directly correlated with the inflammatory and infection states associated with ileal cryptosporidiosis in mice, especially following treatment with L-NAME. Although, clearly documented with a Th-2 anti-inflammatory response, ARG I was found to be up-regulated together with iNOS in the colon of *Citrobacter rodentium* [36].

Arginine is predominantly transported across the intestinal membrane via a Na+-independent CAT-1 (cationic transporter) system induced by mitogen-activated protein kinase [8,37]. Following the *C. parvum*-induced inflammatory response, the increased ARG I expression we observed may associate with ongoing mucosal healing since ARG I is co-localized with ornithine decarboxylase (ODC) in the cytosol. This is significant because ARG I may regulate the conversion of ornithine to polyamines [38,39], to enhance cell proliferation and mucosal repair during *C. parvum* infection. In addition, arginine-derived polyamines have been shown to support migration by a focal adhesion kinase mechanism in intestinal cell lines during monolayer wound healing, which may aid in repair of the intestinal epithelial barrier following its disruption by the infection [40].

Arginine supplementation in our model may provide sufficient amino acid substrate for both enzymes (iNOS and ARG 1) and thus efficiently enhancing the innate immune system to fight against the infection or alternatively improving ARG-I dependent RhoA activity, which has been associated in human inflammatory bowel disease (IBD) with submucosal tissues near microvessels [41].

Recently, it has been shown that apoptotic cells through TGF-β might induce anti-inflammatory responses and reduce NO synthesis via increased arginase (ARG) breakdown of arginine in these cells [42]. ARG has also been found to protect against mucosal damage in a murine model of colitis [36]. Therefore, L-arginine treatment might help to control the severe pro-inflammatory responses due to *C. parvum* infection and initiate earlier mucosal healing. This effect appears to be mediated in part by increased intestinal ARG activity.

Our findings further support the importance of NO-mediated responses against cryptosporidial infections and document for the first time the benefit of L-arginine treatment when malnutrition is present with *C. parvum* infection, and an arginase-dependent role in mucosal healing. Furthermore, we show that *Cryptosporidium* infection induces increased expression of arginase I in the ileum during suckling, and that this increase is localized around the apical membrane of the enterocytes near the site of parasite attachment, suggesting that *C. parvum* may induce higher enterocyte's expression of ARG1 to limit the availability of arginine to the iNOS pathway as a survival mechanism. This feature was found in heavily infected ilea, as the enterocyte's apical membrane infected with *C. parvum* oocysts is highly immunostained by arginase I within the ileal epithelia, suggesting that in addition to the scarcity of iNOS driven pro-inflammatory cytokines to kill the parasite due to inhibitory action of L-NAME, the *C. parvum* parasite may indirectly induce L-arginine breakdown, as noted above. Such survival advantage of increased *C.parvum* driven-arginine uptake by the ileal epithelium and by shifting arginine from iNOS toward the arginase pathway was recently described by Gookin et al. for arginase II [43], findings which warrant further studies to dissect their relative importance. Interestingly, we also found some immunolabeling within the parasite itself, which may represent antigenic cross-reactivity, since *Cryptosporidium* species do not express arginase I but rather arginine decarboxylase [44].

In addition, a marked elevation in arginase activity found with *C. parvum* infection in untreated undernourished mice may reduce NO synthesis in the ileal tissue by diverging arginine away from iNOS, thereby contributing to parasite survival. Similar findings were described by Gaur and colleagues in a model of murine cutaneous Leishmaniasis [45]. Furthermore, plasma arginase levels may increase after being released from challenged tissues driving systemic arginine deficiency [46], which may even be further compromised by undernutrition.

In summary, our findings support the concept of testing arginine-based therapy against cryptosporidiosis in growing children afflicted by infection and malnutrition, and suggest that arginine may provide a double benefit of both antiparasitic and mucosal restitution effects.

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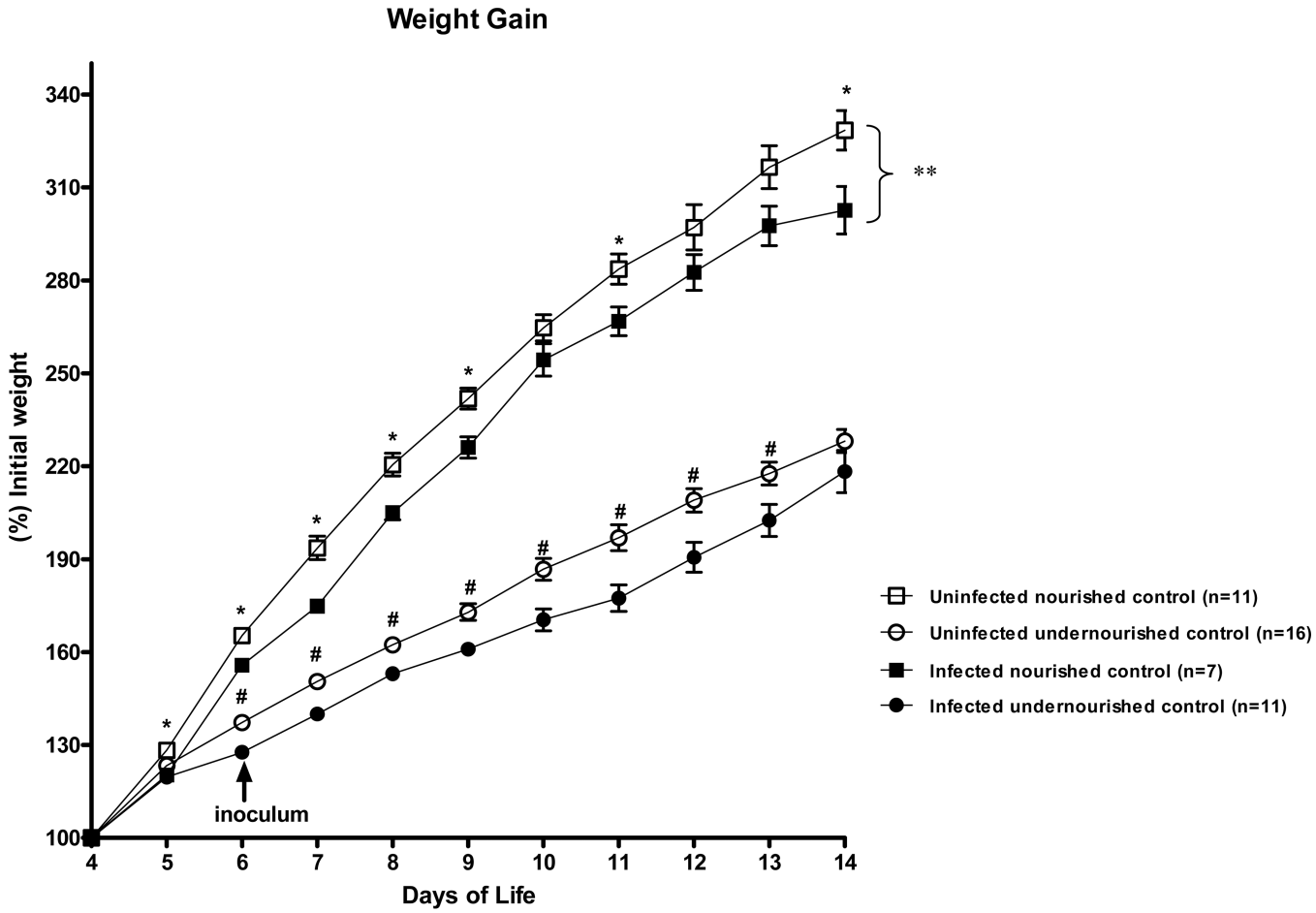
A



# p < 0.05 Infected undernourished control vs Uninfected undernourished control

* p < 0.05 Uninfected Nourished Control vs Infected nourished control (Days 5, 6, 7, 8, 9, 11 and 14)

** p < 0.05 both nourished groups vs both undernourished groups (from day 5 onwards)

B

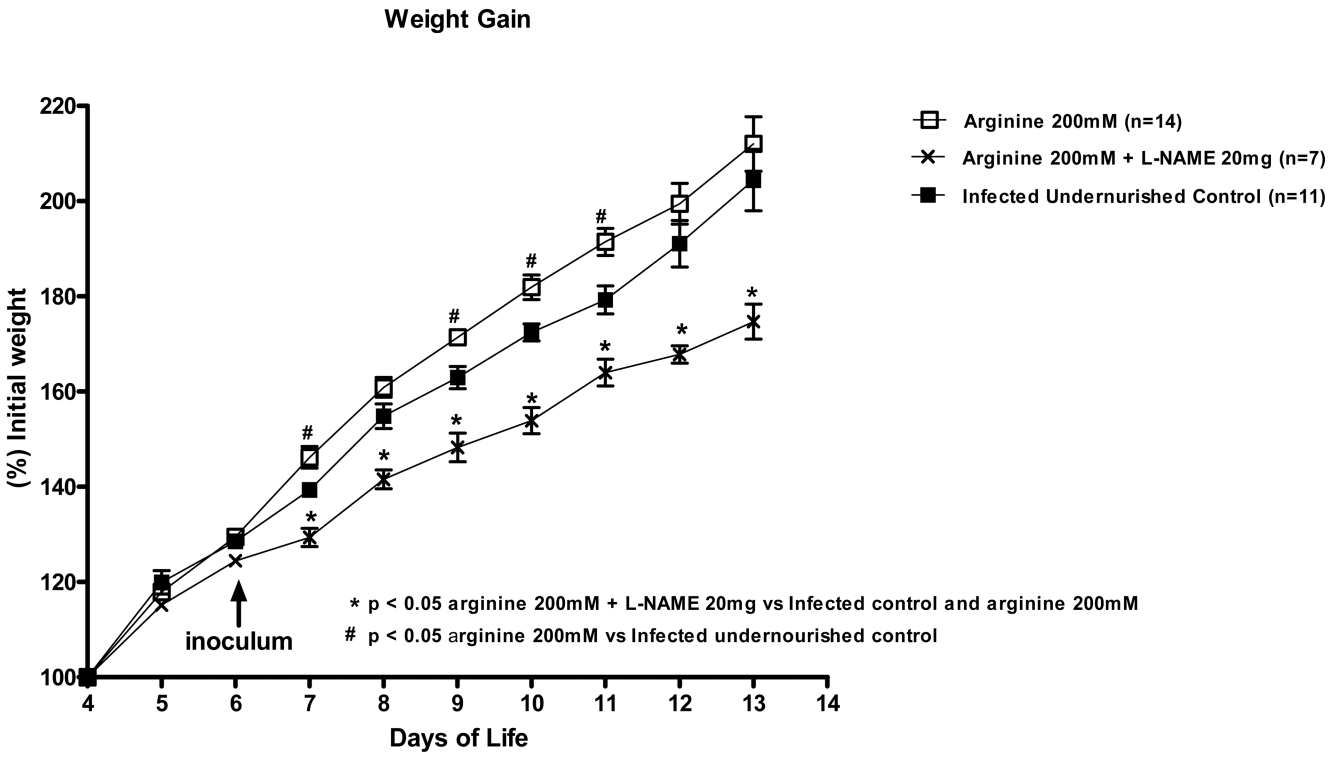


FIGURE 1.

Weight gain curves of the experimental mice challenged by malnutrition from post-natal day 4 until day 13 (% initial weight). (**A**) Infected mice were inoculated with 10^6 *C.parvum* oocysts at post-natal day 6 by gavage with or without L-arginine treatment (200 mM) given subcutaneously. (**B**) A subset of 200 mM L-arginine treated and infected mice received the NOS inhibitor NG-nitroarginine methyl ester (L-NAME), 20 mM. Curves are presented as percentage of initial weight at day 4.

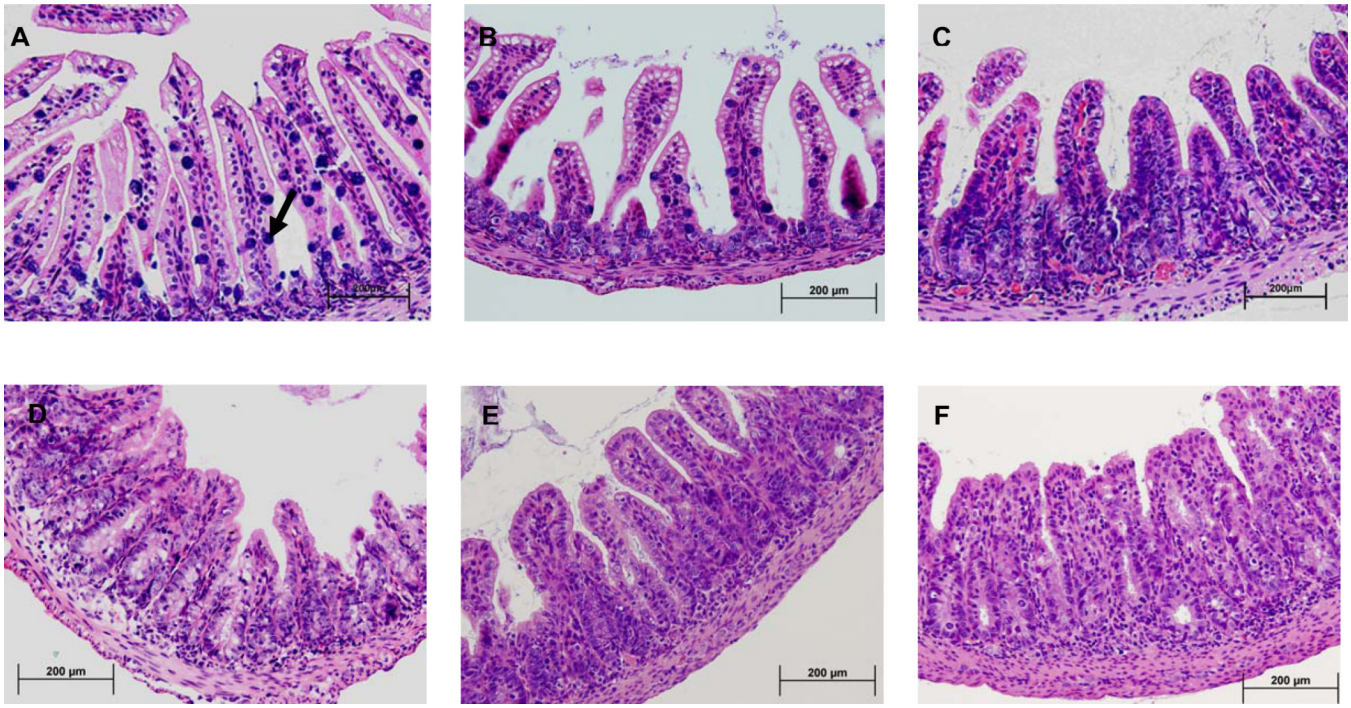
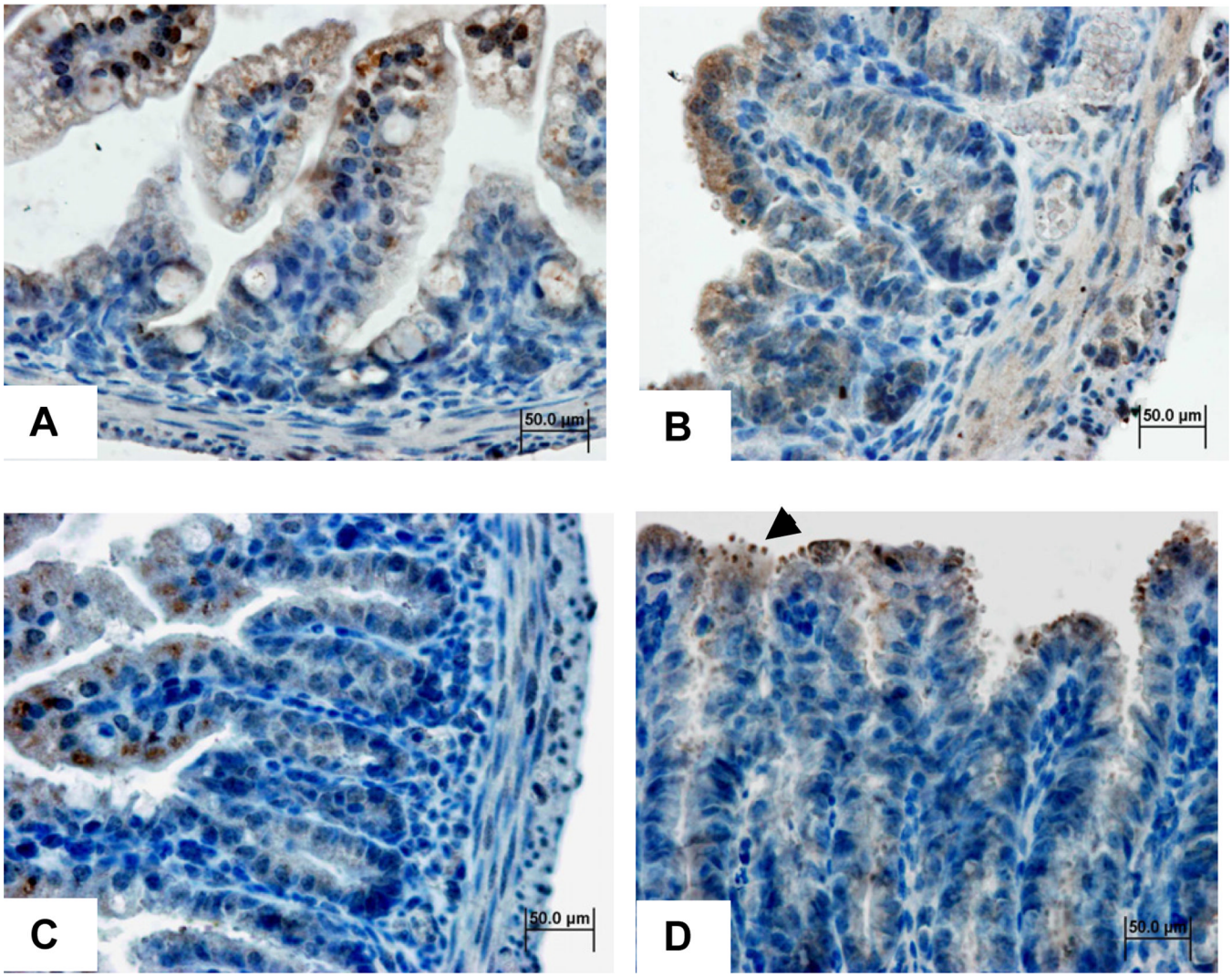


FIGURE 2.

Representative ileal histology of the experimental groups, H&E, 100X. (a) Nourished uninfected control; (b) undernourished uninfected control; (c) nourished infected control; (d) undernourished infected control; (e) undernourished infected mice treated with L-arginine 200 mM; (f) undernourished infected mice treated with L-arginine 200 mM and the NOS inhibitor NG-nitroarginine methyl ester (L-NAME), at the dose of 20 mM. Blue spots represent goblet cells scattered within villi. Note abundance of goblet cells in uninfected nourished controls (arrow) and cell number reductions in the ileum from *C. parvum*-infected mice. Marked villus atrophy, crypt hyperplasia, and inflammatory infiltrates are seen in *C. parvum* infected and undernourished mice. Note improvements with arginine treatment.

A



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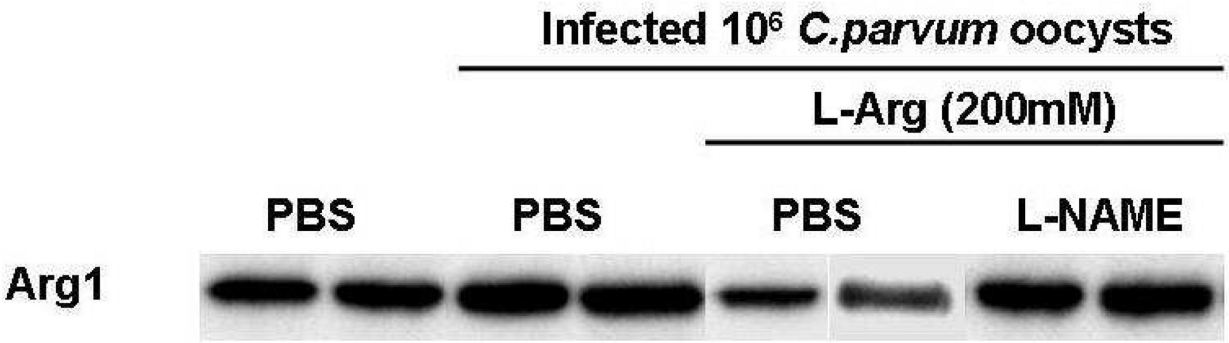


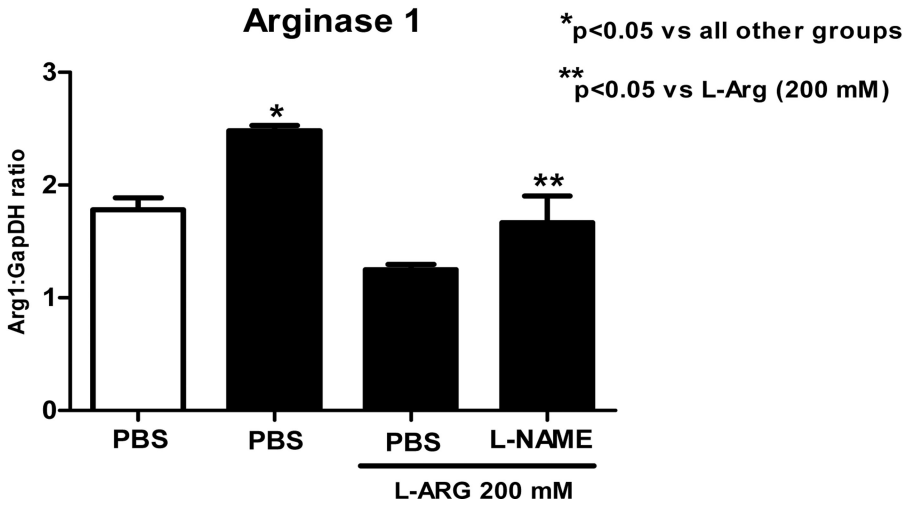
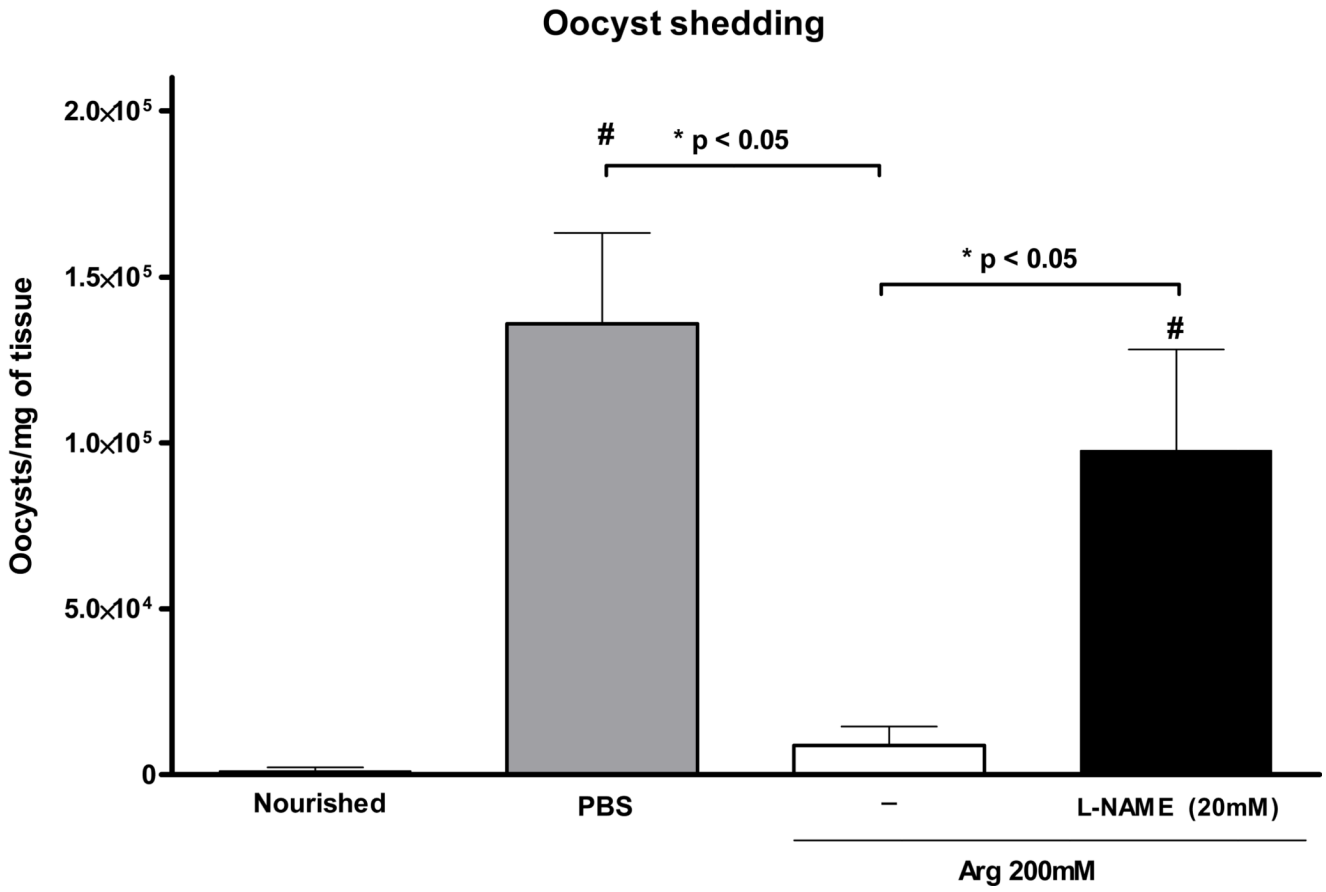


FIGURE 3.

(A) Ileal arginase I immunohistochemistry from experimental groups, 400X (a) Undernourished uninfected control, (b) undernourished infected control; (c) undernourished infected mice treated with L-arginine 200 mM; (d) undernourished infected mice treated with L-arginine 200 mM and the NOS inhibitor NG-nitroarginine methyl ester (L-NAME), at the dose of 20 mM; arrowhead indicates *C.parvum* oocysts. Brown color represents arginase 1 immunostaining. Counterstaining with Harris' hematoxylin.

(B) Ileal immunoblots of the experimental groups for arginase I (ARG1). Band densitometry was plotted after normalizing for the housekeeping protein GADPH. Statistical analyses were done using *Student T* test. The results are shown mean ± SEM. *p< 0.05, significant increase versus uninfected control and **p<0.05, significant decrease versus infected control. N=4 for each group.

A:



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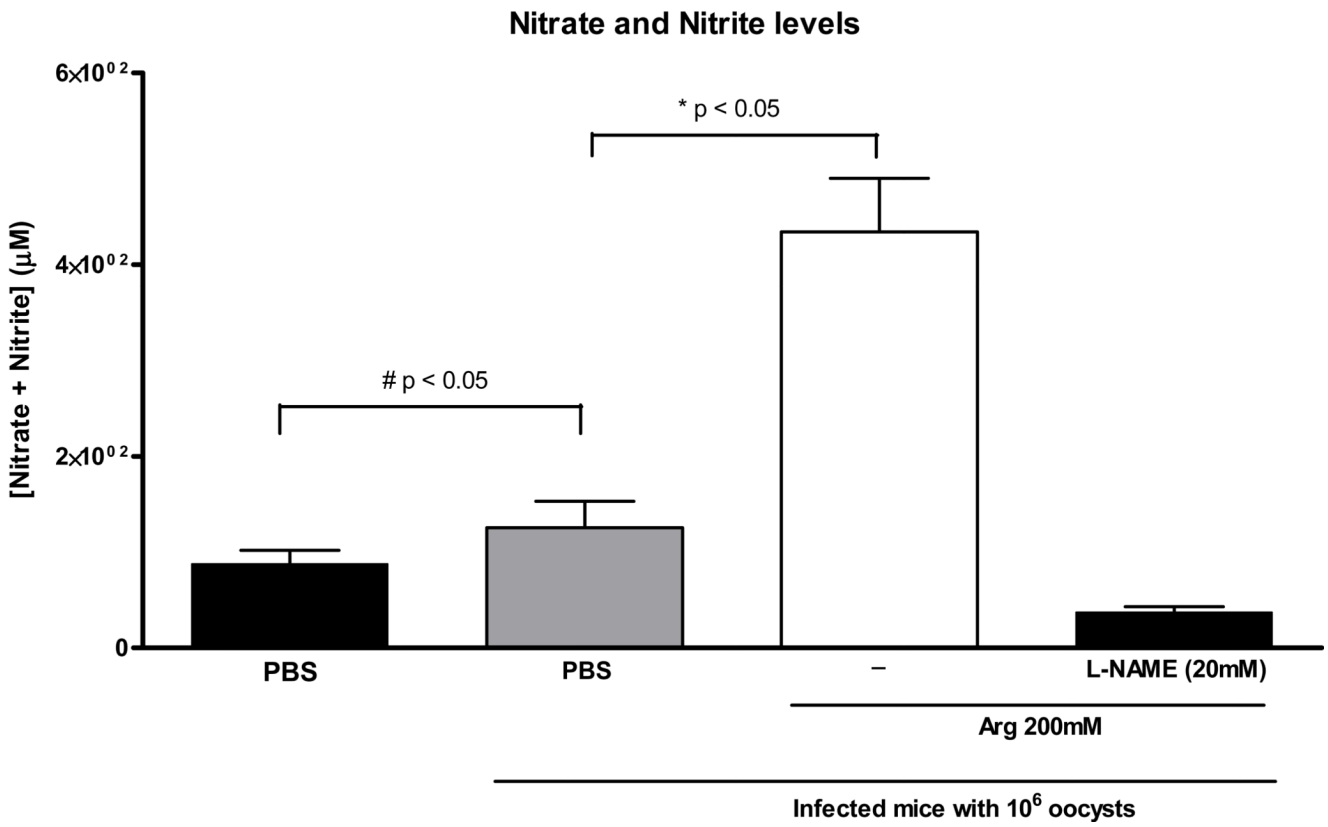

FIGURE 4.

(A) Quantification of fecal shedding from the harvested ileal tissue by real-time PCR at day 14 of age (day 8 of infection). Nourished infected controls (N=5); undernourished infected controls (N=7); undernourished infected mice treated with L-arginine 200 mM (N=6); undernourished infected mice treated with L-arginine 200 mM and the NOS inhibitor NG-nitroarginine methyl ester (L-NAME), at the dose of 20 mM (N=5). Statistical analyses were done using *Student T* test. The results are shown mean ± SEM and represent the average oocyst shedding from each mg of ileal tissue. (**B**) Nitrate and Nitrite colorimetric assay for ELISA absorbance at 550nm using an ELISA plate reader. Undernourished uninfected controls (N=4); undernourished infected controls (N=6); undernourished infected mice treated with L-arginine 200 mM (N=6); undernourished infected mice treated with L-arginine 200 mM and the NOS inhibitor NG-nitroarginine methyl ester (L-NAME), at the dose of 20 mM (N=5). Statistical analyses were done using *Student T* test. The results are shown mean ± SEM. Meaningful statistical difference was set at $P < 0.05$.

Table 1

Morphometry of the ileal villus and crypt from experimental mice at fourteen days old, eight days post-inoculum.

Groups Variables	Nourished control	N	Nourished infected	N	Uninfected Control	N	Infected control	N	L-Arg (200 mM)	N	L-NAME (20 mM)	N
Villus height (µm)	193.69 ± 23.58$^{\#}$	30	102.88 ± 16.38**	30	147.5 ± 34.4$^{\#}$	43	83.18 ± 13.21	23	92.0 ± 38.97**	39	67.81 ± 15	19
Crypt length (µm)	29.70 ± 3.22$^{\Psi}$	30	55.296 ± 5.85$^{\delta}$	30	25.27 ± 5.04$^{\Psi}$	43	74.72 ± 15.71	59	59.27 ± 12.58***	46	67.5 ± 20.57	42

$^{\#}$p<0.05 vs all groups

*p<0.05 vs infected control.

**p<0.05 vs L-NAME (20mM)

$^{\Psi}$p<0.05 vs nourished infected, infected control, L-Arg 200 and L-NAME groups

$^{\delta}$p<0.05 vs nourished control, uninfected control, infected control and L-NAME groups

Comparisons were done by *Student Unpaired T test*. Data are presented as Mean ± SD. N= number of measured ileal villi and crypts from H&E stained sections in at least four animals per group. Villi and crypts were only measured when their full longitudinal axis was found. Groups: Nourished uninfected control; nourished infected control; undernourished uninfected control; undernourished infected mice treated with L-arginine 200 mM; undernourished infected mice treated with L-arginine 200 mM and the NOS inhibitor NG-nitroarginine methyl ester (L-NAME), at the dose of 20 mM. Infection inoculum: 10^{6} *C. parvum* oocysts.