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ORIGINAL ARTICLE

The role of L-arginine-nitric oxide pathway in bacterial translocation

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Abstract This study investigated the nitric oxide (NO) role as a mediator of arginine on bacterial translocation (BT) and gut damage in mice after intestinal obstruction (IO). The effects of pretreatment with arginine with or without NO inhibition on the systemic and local immunological response were also assessed. Mice were categorized into four groups. Group ARG received chow containing 2 % arginine, while group ARG + L-NAME received the same diet plus L-NAME (*N*-nitro-L-arginine methyl ester) by gavage. The IO and Sham groups were fed standard chow. After 7 days, animals were gavaged with radiolabeled *Escherichia coli*, anesthetized and subjected to IO, except the Sham group. Animals were euthanized after 18 h, and BT was evaluated in the mesenteric lymph nodes, blood, liver, spleen and lungs. In another experiment, the intestinal injury was assessed regarding intestinal permeability and ileum histological analyses. Intestinal secretory immunoglobulin A (sIgA) levels, serum IFN-γ and IL-10 cytokines were assessed. Arginine reduced BT, but NO inhibition enhanced BT compared with the ARG group ($p < 0.05$). Intestinal permeability in the ARG and ARG + L-NAME groups was similar but decreased when compared with the IO group ($p < 0.05$). Histological preservation was observed. Arginine treatment increased IL-10 and sIgA levels when compared with the Sham and IO groups ($p < 0.05$). The cytokines and sIgA concentrations were similar in the ARG + L-NAME and Sham groups. Arginine appeared to reduce BT and its effects on the modulation of cytokines and secretory IgA in mice after IO are mediated by NO production.

Keywords Arginine · Nitric oxide · Bacterial translocation · Immune modulation · Intestinal permeability

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Introduction

Localized and systemic disorders such as ischemia, intestinal obstruction (IO), shock or sepsis can damage the intestinal barrier by increasing mucosa permeability and allowing for bacterial translocation (BT). These disorders worsen the primary pathological event and may induce multiple organ failure and death (Ding et al. 2004; Samel et al. 2003).

Previous research conducted by our group has demonstrated that arginine preserves intestinal barrier integrity and prevents BT in mice and rats after IO (Quirino et al. 2007; Viana et al. 2010). However, the pathway involved in this process is not clear.

Experimental evidence has demonstrated that arginine has an important role in improving the immune response of animals under stress, primarily due to the increase of cytotoxic macrophage effects stimulated by nitric oxide (NO). In addition, studies suggest that dietary arginine can increase the number of CD4+ T lymphocytes, which have a role in cytokine synthesis (Kobayashi et al. 1998; Kung et al. 2001; Ochoa et al. 2001).

However, arginine use has been controversial because it is the only substrate for NO synthesis, the primary non-adrenergic and non-cholinergic body vasodilator (Rhoads and Wu 2009). NO has been reported to have a double role in the intestine; it exerts potent bactericidal action against several microorganisms, including those found in the intestinal microbiota, and has also been shown to provide direct benefits to the intestinal barrier, demonstrating its protective role (Nadler and Ford 2000; Samel et al. 2003). Conversely, the overproduction of NO due to inducible nitric oxide synthase (iNOS) expression does not positively impact the antimicrobial response. Rather, it appears to act as an autotoxic agent in the enterocytes, eventually resulting in the loss of tight junction integrity, thereby increasing intestinal permeability and BT (Demirkiran et al. 2006; Samel et al. 2003).

In this study, animals underwent IO, an extremely destructive condition that induces BT, in order to evaluate NO function as an arginine mediator preventing intestinal damage and as well as its possible effects on the local and systemic immune responses.

Methods

Animals and treatment

Swiss adult mice (5 weeks) with weights of 25–30 g were studied. The animals were randomly assigned to four groups for each experiment (intestinal permeability, BT determination, histological examination, and immunological analyses).

I. Sham group, received standard chow (Labina®) and no IO ($n = 23$);
II. IO group, treated with standard chow (Labina®) and IO ($n = 23$);
III. ARG group, fed a specially prepared chow with arginine addition (2 % of daily caloric intake) according to the criteria proposed by Quirino et al. (2007) and IO ($n = 23$);
IV. ARG + L-NAME group, with IO, fed the arginine-supplemented chow and gavaged with 0.3 mg/kg/day of the non-selective NOS inhibitor, *N*-nitro-L-arginine methyl ester (L-NAME) ($n = 23$).

The animals were treated for 7 days before the surgical procedure for IO with isocaloric and isonitrogenous diets cited above. The mice had free access to water throughout the experimental period. The animals of groups I, II and III also received gavage with 0.1 mL of saline to equalize the stress due to gavage.

This study was approved by the Ethics Committee of the Universidade Federal de Minas Gerais (CETEA/UFMG—Protocol number: 109/2007) and followed the rules proposed by CONCEA, the National Council for the Control of Animal Experimentation.

Surgical procedure

The animals were anesthetized using a xylazine and ketamine combined solution (8 and 60 mg/kg, respectively) intraperitoneally. The abdomen was opened through a midline incision, and the terminal ileum was isolated and ligated, without inducing necrosis. The abdominal wall was closed in two layers. Only the Sham group underwent a laparotomy with intestinal manipulation.

Radiolabeling of *Escherichia coli*

A sample of *E. coli* ATCC-10536 culture grown overnight in tryptic casein agar was transferred to 10 mL of sterile saline solution. The bacterial concentration was adjusted to 31 % of transmittance in a spectrophotometer at 580 nm, which corresponds to approximately 10^8 colony-forming units/mL. An aliquot of bacterial suspension (2 mL) was incubated in tubes containing 1 mL of stannous chloride solution (580 mM, pH 7.0) at 37 °C for 10 min. After incubation, 37.0–55.5 MBq of technetium-99m (^{99m}Tc) obtained by elution from the sterile ^{99}Mo/^{99m}Tc generator (IPEN/Brazil) was added and the preparation was maintained at 37 °C for another 10 min. The tubes were then centrifuged at 3,000×*g* for 25 min. This procedure was repeated three times. After the last centrifugation, the radioactivity of the supernatant and precipitate was measured in a dose calibrator (CRC®-25R Dose Calibrator,

Capintec, Ramsey, NJ, USA), and the percent ^{99m}Tc incorporated into the bacterial cells was determined using the following equation (Diniz et al. 1999):

$$\text{Labeling yield} = \frac{\text{cpm of precipitate}}{\text{cpm of precipitate} + \text{cpm of supernatant}} \times 100,$$

where cpm is counts per minute.

Bacterial translocation

To investigate BT, 32 mice were categorized into four groups of 8 animals. After 7 days of treatment, 0.1 mL of a suspension containing 1.8 MBq of the ^{99m}Tc-*E. coli*, which corresponds to 10^7 CFU/mL, was administered by gavage to all animals. After 90 min, the mice underwent operations as described before, and 18 h after the surgical procedure, the animals were euthanized and blood, mesenteric lymph nodes (MLN), liver, spleen and lungs were collected, weighed, and placed in tubes to measure the incorporated radioactivity. The samples were counted in a counter with a NaI (Tl) crystal (ANSR-Abbott, Chicago, IL, USA). The results are expressed as count/min/g of tissue.

Intestinal permeability

To evaluate intestinal permeability, 60 mice were categorized into four groups of 15 animals and were treated as described above. After 7 days, all mice received 0.1 mL of diethylenetriamine pentaacetic acid (DTPA) solution labeled with 18.5 MBq of ^{99m}Tc by gavage. After 90 min, all mice underwent IO as previously described. At 4, 8, and 18 h after the IO procedure, five animals per period per group were anesthetized and 500 μL of blood was collected and placed in the appropriate tubes for radioactivity determination. Data were expressed as % dose, using the following equation:

$$\%\ \text{of injected dose in blood} = \frac{\text{cpm in the blood}}{\text{cpm of the administrated dose}} \times 100,$$

where cpm is counts per minute.

Ileum histology

Samples of small bowels were taken for histological analysis 18 h after surgery. A 1-cm ring of distal ileum adjacent to the IO was resected, fixed in a 4 % buffered formalin solution, dehydrated, cleared, embedded in paraffin, cut into sections 4–5 mm thick, stained by hematoxylin and eosin (H&E) and coded and analyzed by optical microscopy performed by a single pathologist who was unaware of the experimental conditions of each group.

Cytokines analyses

Eighteen hours after the operation, the animals ($n = 5$) from the four study groups underwent euthanasia. Blood was collected from the axillary plexus and centrifuged at $1{,}000 \times g$ for 10 min to achieve serum separation. The levels of IL-10 (regulatory cytokine) and IFN-γ (pro-inflammatory cytokine) in the serum were evaluated using ELISA commercial kits (Biosource International, Camarillo, CA, USA). The results are expressed as pg/mL.

Intestinal secretory immunoglobulin A (sIgA)

The small intestines of mice from all groups were removed after euthanasia, and the contents were withdrawn, weighed, and suspended in PBS using 500 mg of intestinal contents per 2.0 mL PBS supplemented with an anti-protease cocktail. After centrifugation at $2{,}000 \times g$ for 30 min at 4 °C, the supernatant was collected and kept frozen at −70 °C until use. Immunoglobulin levels in the intestinal fluid were evaluated using ELISA with goat anti-mouse IgA (Sigma Chemical Co., St. Louis, USA) and horseradish peroxidase-conjugated goat anti-mouse IgA (Sigma). Color was developed with *o*-phenylene-diamine (OPD, Sigma), and absorbance at 492 nm was determined using an ELISA plate reader. The concentrations of the immunoglobulin were determined using a purified mouse IgA standard (Southern Biotechnology Associates Inc., Birmingham, USA) (Rodrigues et al. 2000). The results are expressed as mg/mL.

Statistical analysis

Intestinal permeability, cytokine and sIgA results were compared using Student's *t* test. BT data were subjected to a Kruskal–Wallis analysis of variance and a post hoc analysis using Dunn's test. The differences were considered statistically significant at $p < 0.05$. All analyses were performed using the program BioEstat Version 3.0 (Mamiraua Civil Society/MCT-CNPq).

Results

Nitric oxide influence on bacterial translocation

Food intake and weight gain were similar among the four groups throughout the 7 days of experimental treatment. IO promoted an increase in BT as demonstrated by a significantly higher uptake of ^{99m}Tc-*E. coli* in the blood, MLN,

Table 1 Effect of nitric oxide synthase (NOS) on bacterial translocation

Organs/blood	Groups (cpm/g)			
	Sham	IO	ARG	ARG + L-NAME
MLN	166.67[a]	660.00[b]	100.00[a]	1,366.66[b]
Blood	41.30[a]	175.79[b]	60.60[a]	583.67[c]
Liver	204.80[a]	1,154.49[b]	556.52[a]	2,494.05[c]
Lung	14.29[a]	794.12[b]	110.52[a]	636.84[b]
Spleen	109.09[a]	1,022.22[b]	390.00[a]	912.50[b]

a, *b*, *c* different letters on the same line indicate statistically significant differences ($p < 0.05$) by Kruskal–Wallis analysis of variance and post hoc analysis using Dunn's test

Data are expressed as medians

$n = 7$

liver, spleen, and lungs of the animals in the IO group when compared with the Sham group ($p < 0.05$) (Table 1). Treatment with arginine reduced BT to the blood and to all the organs investigated when compared with the IO group ($p < 0.05$), in levels similar to those found in the Sham group. However, in the animals fed with arginine and gavaged with L-NAME, BT levels were high when compared with ARG group and similar to the IO group ($p > 0.05$) in the organs, except to the blood and the liver, which revealed a significantly higher uptake of ^{99m}Tc-*E. coli* ($p < 0.05$) than observed in the IO group.

Nitric oxide and intestinal permeability

The data indicated that intestinal permeability was greater in the IO group than in the Sham group ($p < 0.05$) (Fig. 1). Conversely, pretreatment with arginine and arginine + L-NAME reduced intestinal permeability to similar values observed in the Sham group ($p > 0.05$).

Influence of nitric oxide in ileum histology

The IO group (Fig. 2b) presented marked histological damage in ileum due to IO. There were classical alterations with ischemic changes such as edema in the connective tissue, cells and veins of the lamina propria, which are associated with degenerative changes and necrosis in all the intestinal layers. In the Sham group (Fig. 2a), normal ileal architecture was observed. The arginine group (Fig. 2c) exhibited partially preserved mucosal structure and integrity; the continuity of the epithelium and the calceiform cells was preserved; however, there was short villous and lamina propria edema (Fig. 2a). In the NOS blockage group, ARG + L-NAME, the ileal mucosa and the overall structure were preserved, with no relevant

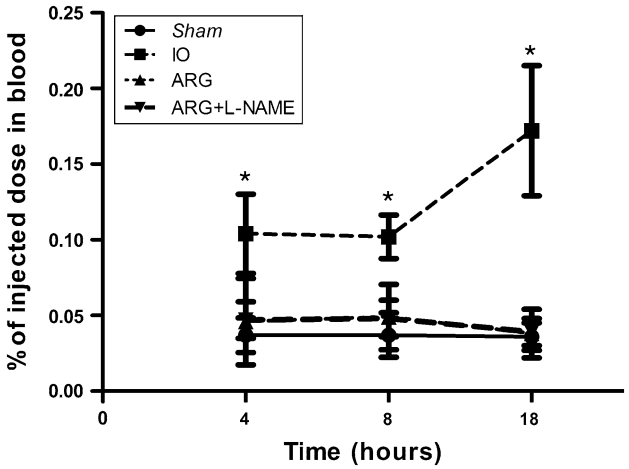

Fig. 1 Effects of nitric oxide inhibition on intestinal permeability. % dose = (counts per minute of blood/counts per minute of administrated dose) × 100. *Errors bars* show the SD. *Asterisk* indicates statistically significant differences ($p < 0.05$) by Student's *t* test between the IO group and the other groups throughout all investigated times. $n = 5$ for each time

changes to the tissue structure. Instead, intestinal preservation and no ischemia, with only discrete edema in the lamina propria, were observed (Fig. 2d).

Influence of nitric oxide in the cytokines

Serum IFN-γ and IL-10 levels in the IO group were 120.1 ± 21.3 and 50.7 ± 5.8 pg/mL, respectively, which were higher than that found in the Sham group (64.9 ± 7.0 and 29.6 ± 8.6 pg/mL, respectively) ($p < 0.05$). The animals receiving arginine-enriched diets demonstrated similar IFN-γ levels (156.4 ± 38.1 pg/mL) ($p > 0.05$) and significantly higher IL-10 levels (88.47 ± 21.9) compared to the IO group ($p < 0.05$). In the group treated with L-NAME in addition to the arginine, the levels of IFN-γ and IL-10 (30.7 ± 8.2, 64.6 ± 12.7 pg/mL, respectively) were low and comparable with those observed in the Sham group ($p > 0.05$) (Fig. 3).

Effects of nitric oxide in the local immune response

Levels of sIgA in the intestinal fluid were significantly greater in the IO group (769.36 ± 171.47 mg/mL) than in the Sham group (474.24 ± 125.42 mg/mL) ($p < 0.05$). The animals fed arginine presented a significant increase in sIgA concentrations (1,049.00 ± 170.09 mg/mL) compared to the Sham and IO animals ($p < 0.05$). However, in the group with NOS inhibition, IgA levels (434.15 ± 187.07 mg/mL) were similar to the Sham group ($p > 0.05$) (Fig. 4).

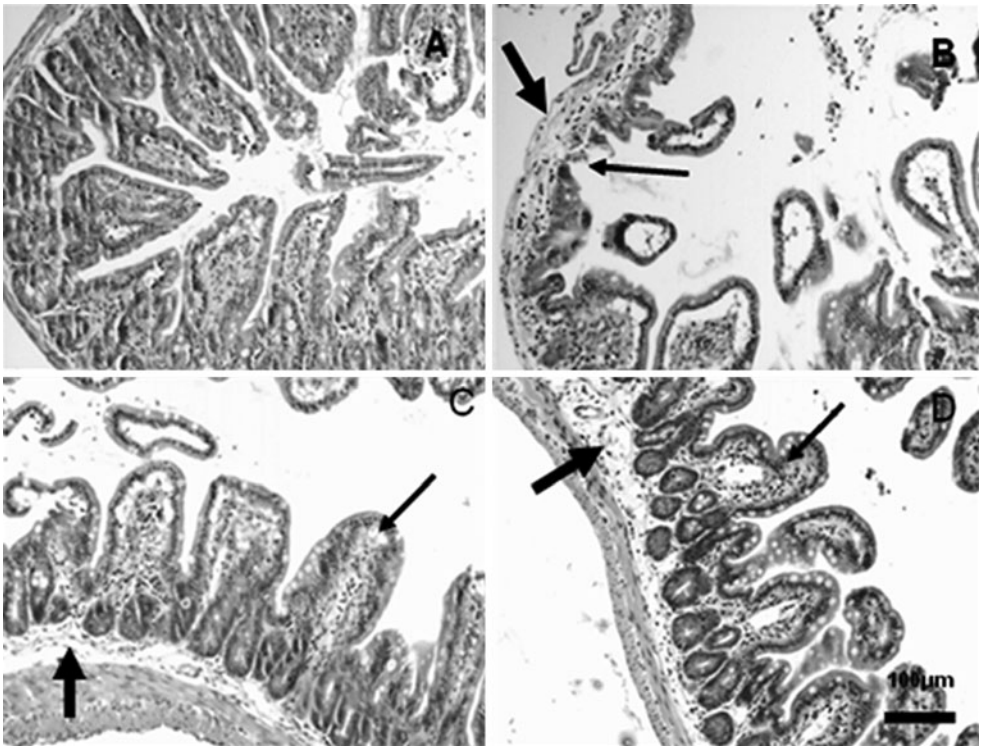

Fig. 2 Ileum histopathology from ARG + L-NAME animals. Representative histological aspects of Sham (**a**), IO (**b**), ARG (**c**) and ARG + L-NAME (**d**) groups. Intense ischemic changes in the ileum wall (*large arrow*, **b**), short villus and mucosal erosions (*thin arrow*, **b**). Partial keeping of mucosa integrity (*thin arrow*) and lamina propria edema (*large arrow*) in (**c**) and (**d**). *H&E* 10× objective, *bars* 100 μm

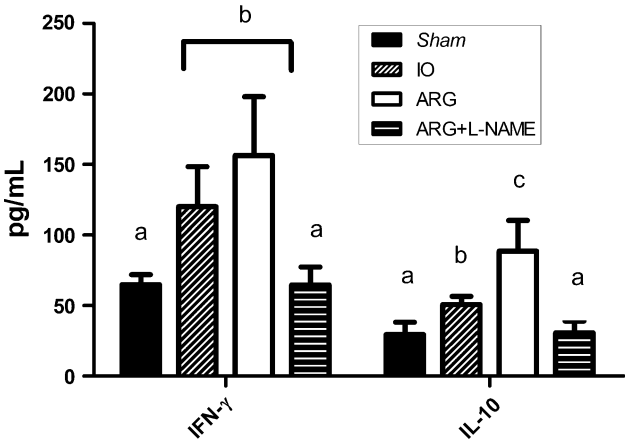

Fig. 3 Levels of IFN-γ and IL-10 in the animals' serum. Results are expressed as medians ± SD ($n = 5$). *Different letters* indicate statistical differences, considering each cytokine separately ($p < 0.05$)

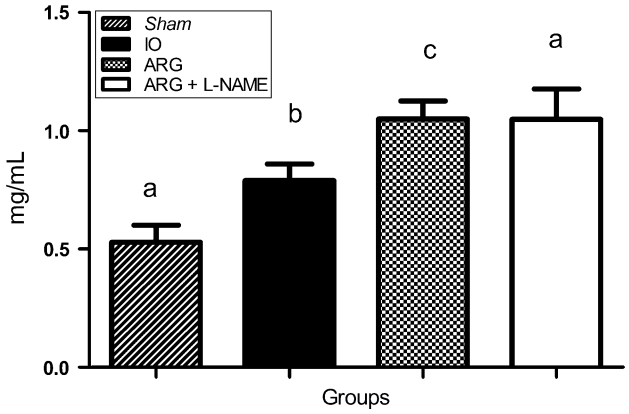

Fig. 4 sIgA levels in the intestinal fluid. Results are expressed as medians ± SD ($n = 5$). *Different letters* indicate statistical differences ($p < 0.05$)

Discussion

The role of arginine in the so-called "immune enhancing diets" and their impact on clinical outcomes in critical illness has been the subject of continuing controversy (Manzanares and Heyland 2012). Some studies have demonstrated a beneficial effect of this type of diet in critical illnesses (Zulfikaroglu et al. 2003), but other authors have suggested that arginine may be responsible for harmful effects (Heyland et al. 2001). The Canadian Clinical Practice Guidelines for Nutrition Support recommend avoiding diets supplemented with arginine for critically ill patients (Moinard et al. 2012).

An excessive supply of arginine could lead to an overproduction of NO and could be responsible for septic shock

and multiple organ failure (Moinard et al. 2012). However, previous studies have demonstrated that pretreatment with arginine reduces BT, which may lead to reduced infection (Quirino et al. 2007; Viana et al. 2010).

In the present study, the potential mechanisms and pathways for the protective effects of arginine in BT, which have been investigated in a previous paper (Viana et al. 2010), were assessed. In this context, NO production was investigated in an attempt to clarify its effects on intestinal mucosa integrity and its role in the local and systemic immune response in mice.

Ours results showed that arginine significantly reduced BT and this effect appears to be due to NO production once animals treated with arginine and L-NAME [a non-selective inhibitor of the enzyme NO synthase (NOS)] presented increased levels of BT than the ARG group ($p < 0.05$).

Arginine is critical to maintain the mucosal integrity of the intestine in various intestinal disorders. The mechanisms of these positive effects are still unclear; however, a stimulating effect of NO on enterocyte proliferation and its vasodilator property, providing normal blood flow to intestine, may be some of them (Koppelmann et al. 2012). It is well established that arginine can enhance morphometric aspects, the enterocytes proliferation, the number and villus height in trauma, ischemia, intestinal resection and obstruction (Ersin et al. 2000; Shang et al. 2004; Sukhotnik et al. 2004; Osowska et al. 2008; Viana et al. 2010). In the present study, the arginine presented a satisfactory protection against histological alterations in the intestinal mucosa. Despite that these animals groups have undergone IO, both ARG and ARG + L-NAME groups showed only a minor edema in the lamina propria. No alterations in intestinal permeability were found after NOS blockage.

Tanaka et al. (2001) found that NO blockage by L-NAME enhanced BT and decreased intestinal mucus and fluid production, suggesting that the physical barrier may be one of the key components in BT. However, the primary finding of our study is that the inhibition of the NOS, using L-NAME, induced BT but not intestinal permeability or intestinal lesions.

In fact, the intestinal barrier is a semi-permeable structure allowing active transcellular and passive paracellular absorption, but preventing BT. However, to transit the intestinal mucosal barrier, bacteria can either be actively internalized by dendritic cells or macrophages that lie within the lamina propria with the capacity to extend cellular processes between the intestinal epithelial cells that can internalize the bacteria and deliver them to the lymph nodes and circulation, so that bacterial products pass independently of intestinal barrier disruption, mainly in immunodeficiency states (Brayden et al. 2005; Du Plessis et al. 2013; Wu et al. 2011). NO is a component of the nonspecific host defense effective against intracellular and extracellular parasites that may play a crucial role in BT (Wiley 2007). The NO produced by iNOS exerts antimicrobial actions. NO can inhibit bacterial replication by binding to DNA, causing deamination and breakage by disrupting zinc metalloproteins involved in DNA synthesis. NO can also disrupt heme-containing bacterial enzymes and oxidize bacterial lipids, impairing bacterial function. (Ibiza and Serrador 2008; Samel et al. 2003).

Situations of intestinal barrier disruption lead to intestinal inflammation by increasing the pro-inflammatory stimuli in contact with the immune system. The increase in the circulating blood of pro-inflammatory cytokines triggers immune cells to release anti-inflammatory cytokines with the aim of downregulating, through complex feedback mechanisms, the pro-inflammatory process so as to maintain homeostasis (Biancofiore et al. 2013; Izcue et al. 2009).

In this study, IFN-γ and IL-10 cytokines were evaluated in order to elucidate the arginine effects in the pro- and anti-inflammatory responses in the IO model. Our results demonstrated higher IFN-γ levels in IO group than in Sham group ($p > 0.05$) and that the arginine treatment elevated the IFN-γ levels. On other hand, the IL-10 blood concentrations in the IO group were higher than that in the Sham group ($p > 0.05$). However, in the ARG group, the serum IL-10 levels were higher than that in the IO group. These results show that arginine increased IFN-γ while further enhancing IL-10, which likely contributed to the beneficial effects of this immunonutrient on BT and intestinal permeability.

The administration of L-NAME suppressed the effects of arginine in immune response, suggesting the importance of NO in arginine action. The levels of IFN-γ and IL-10 cytokines in L-NAME group were significantly lower when compared to ARG and IO groups. According to Van Der Veen (2001), NO inhibits T-cell proliferation without inhibiting cytokine production. There is also evidence indicating that NO can promote a decline in pro-inflammatory mediators, including IFN-γ and IL-2, accompanied by a decline in anti-inflammatory mediators like IL-4 and IL-10, causing an immune depression in the host (Benbernou et al. 1997; Miki et al. 2005; Roozendaal et al. 1999).

sIgA is in the first immunological line of defense on intestine, acting as a barrier against the adhesion of pathogens to the intestinal mucosa (Hajishengallis et al. 1992). In the present study, the stimulation of intestinal sIgA production after treatment with arginine-enriched diets was confirmed, once the levels of IgA in intestinal fluid were significantly increased compared with IO ($p < 0.05$), but this effect was not observed in animals that received L-NAME that showed similar to the Sham group

($p > 0.05$). Fan et al. (2010) conducted a study using severely burned mice and found that IL-10 levels were strongly correlated with intestinal IgA levels. It appears that this balance between pro-inflammatory and anti-inflammatory cytokines plays a critical role in IgA control, as IL-10 predominance can have an important role in sIgA secretion, thus leading to intestinal mucosal surface protection (Clayburgh et al. 2004; Fan et al. 2010; Shanahan 2002; Shang et al. 2004).

In conclusion, our results indicate that the role of arginine in preventing BT in mice undergoing IO is related to NO production, and this mediator impacts pro-inflammatory and anti-inflammatory cytokine modulation and gut sIgA production.

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Conflict of interest The authors declare that they have no conflict of interest.

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