Significance of Nitric Oxide Level in Giardiasis

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SUMMARY

**Background:** Giardiasis is one of the most prevalent intestinal protozoa infections in humans. Nowadays, nitric oxide (NO) is known to be involved in the immune system against *Giardia intestinalis*. Therefore, the aim of the present study was to evaluate the level of NO in individuals with giardiasis in comparison to normal subjects.

**Methods:** This descriptive study was conducted among 49 Giardia positive and 39 age and gender matched healthy volunteers. Examination of stool samples was done by wet mount technique and formol-ether concentration method. Serum samples were obtained for laboratory examination. NO production was quantified by measuring nitrite, a stable end product of NO, using the Griess reaction based on ELISA method. By using the standard curve in Excel program, the concentration of NO₂⁻ in samples was obtained. Finally, all data were analyzed using SPSS version 17.

**Results:** Values obtained from NO assays were placed into 4 groups: ≤ 10 (decline), 10.01 - 15 (normal), 15.01 - 25 (increase), and more than 25 µM (sharp increase). The mean level of NO in patients with *G. intestinalis* was 32.19 ± 2.15 µM and in people without *G. intestinalis* was 17.1 ± 1.33 µM. Eight point two percent of patients with Giardiasis were in normal range, but 2%, 20.4%, and 69.4% were in decline, increase, and sharp increase ranges, respectively. In group 2 (without infection), 17.9% were in normal range, and 20.5%, 51.3%, and 10.3% were in decline, increase, and sharp increase ranges, respectively. There was a statistical difference in nitric oxide levels between positive and negative groups with a 95% confidence interval. (p-value = 0.001).

**Conclusions:** In our study, the number of people who showed a sharp increase in NO levels was significantly higher in individuals with giardiasis as compared to the control group, and patients infected with giardiasis showed significant increase in NO levels. Therefore, we suggest that further studies are required to understand the exact function of NO in the immune system against giardiasis in humans. It will be important to offer a new therapeutic target for eliminating *G. intestinalis*.


**KEY WORDS**

Giardiasis, nitric oxide, immune system

**INTRODUCTION**

*Giardia intestinalis* (synonyms: *G. lamblia* and *G. duodenalis*) is a microscopic unicellular flagellant protozoa which can cause giardiasis [1-3]. Giardiasis is one of the most prevalent intestinal protozoa infections in human beings [4]. Intestinal parasitic infections have an extensive global distribution with a high occurrence in people with poor living conditions and in most crowded areas.
with poor public health, unsafe water, and improper disposal of garbage [5]. About 200 million people in Africa, Asia, and Latin America have giardiasis and about 500,000 new cases reported [2]. *Giardia intestinalis* has two stages in its life cycle: the cyst and the trophozoite. The infectious, resistant cyst is responsible for transmission and can survive in cold water and humid environment. People can become infected by swallowing the cysts found in contaminated water or food. The cyst is activated while crossing through the stomach and ex-cystation occurs in the duodenum. Finally, the motile trophozoite comes out and inhabits in the upper small intestine and it is responsible for disease symptoms [3, 6,7]. Clinical symptoms of giardiasis are different and can range from asymptomatic to acute and chronic symptoms. Early symptoms include diarrhea, abdominal pain, nausea, and vomiting. But in some cases such as undernourished children and immune compromised patients, the infections become chronic with diarrhea, malabsorption, and growth failure [8]. The host immune system can control and eliminate *Giardia intestinalis* by using B-cell and T-cell functions, so giardiasis is a self-limiting process [9,10]. Also, B-cell independent mechanisms exist and can eradicate the parasitic infection. However, their identity and physiological performances are poorly understood [11]. One of the B-cell independent mechanisms that defends against parasitic infection is NO (nitric oxide). NO is a free radical that is produced by NOS (nitric oxide synthase) from L-arginine in an oxygen dependent reaction [11,12]. NOS has three forms: endothelial (eNOS) and neuronal (nNOS) that are essentially expressed in vascular endothelial cells and nervous system. The third form is inducible (iNOS) that is produced in macrophages and in a variety of cells when stimulated by cytokines and pathogens [11,13]. In the gastrointestinal tract NO has many functions including action of sphincters, peristaltic movement, enlargement of blood vessels, inhibition of platelet and leukocyte aggregation [14]. One of the most outstanding roles of NO is its performance in the innate immune system against various intracellular pathogens like protozoa, viruses, and bacteria [11,15]. NO is known to be cytotoxic and cytostatic to *G. intestinalis* [15,16]. Since the trophozoite remains in close contact with the epithelial cells and NO is detected at the apical side of the cells, NO may be a potential host defense against *G. intestinalis* [14].

Therefore, the aim of this present study was to investigate the NO level in patients infected with *Giardia intestinalis* in comparison to normal subjects and understanding whether nitric oxide plays a role in patients with giardiasis.

**MATERIALS AND METHODS**

This descriptive study was performed to evaluate the serum nitric oxide in patients with giardiasis. Questionnaires were obtained from all volunteers. Stool samples were gathered from all individuals in sterile clean stool cups. Giardiasis was diagnosed based on the parasitological examinations of stool in two ways: direct wet smear technique and formol-ether concentration method [17].

The inclusion criteria were positive detection of *Giardia intestinalis* in stools, not infected with other intestinal parasites, and absence of other diseases. A total of 49 individuals met the inclusion criteria and were involved in the present study, in group 1. Also the control group (group 2) was set up of 39 healthy subjects according to the criteria described in the questionnaire. Their stool examinations were negative for all intestinal parasites, and they did not have any disease. Informed written consent was obtained from all enrolled patients and healthy subjects prior to the study. The blood samples were collected and serum was prepared and stored at -70°C in the School of Public Health in Tehran University of Medical Science for laboratory examinations. NO production was quantified by measuring nitrite, a stable end products of NO, using the Griess reaction following the manufacturer's protocol (Griess reagent system PROMEGA G2930), which uses sulfanilamide and N-1 naphthylethylene diaminedihydrochloride (NED) under acidic conditions. A nitrite (NO$_2^-$) standard reference curve must be ready for each assay for accurate quantitation of NO$_2^-$ levels in samples. So 6 standards with concentrations 100, 50, 25, 12.5, 6.25, 3.125, and 1.56 μM were prepared. Biological samples like serum have high content of protein so a deproteinization step is necessary in the Griess reaction. ZnSO$_4$ removes 50% of the protein from biological samples. Per 200 μl of serum, 30 μl saturated ammonium sulfate was added and was kept at room temperature (RT) for 15 minutes, protected from light, then centrifuged at 5000 rpm for 5 minutes. The supernatant was then transferred to another micro tube and again centrifuged at 5000 rpm for 5 minutes. This supernatant was used for testing.

First 50 μl of standards and blank were added into the wells of ELISA plates. Then 50 μl of deproteinized sera were added to the remaining wells. For all of the wells, 50 μl sulfanilamide were dispensed and incubated for 5-10 minutes at room temperature, protected from light. At the end, 50 μl vanadium chloride were dispensed into each well (vanadium chloride reduces nitrate to nitrite) and incubated at 37°C for 45 minutes. Finally, by the ELISA reader, the absorbance was determined at 540 nm. By using the standard curves in Excel program, the concentrations of NO$_2^-$ in samples were obtained. All data were analyzed using SPSS version 17, and the percentages of increasing, decreasing, and normal levels of NO and significant or not significant association of mean NO levels in the case and control groups were obtained.
Table 1. The percentage of people in group 1 (with Giardiasis) and group 2 (without Giardiasis) in different ranges of nitric oxide.

<table>
<thead>
<tr>
<th>Ranges of NO (µM)</th>
<th>Group 1 (Infected)</th>
<th>Group 2 (control group)</th>
</tr>
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<tbody>
<tr>
<td>≤ 10</td>
<td>2%</td>
<td>20.5%</td>
</tr>
<tr>
<td>10.01 - 15</td>
<td>8.2%</td>
<td>17.9%</td>
</tr>
<tr>
<td>15.01 - 25</td>
<td>20.4%</td>
<td>51.3%</td>
</tr>
<tr>
<td>&gt; 25</td>
<td>69.4%</td>
<td>10.3%</td>
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</table>

RESULTS

Values obtained from NO assays were placed into 4 groups: ≤ 10 (decline), 10.01 - 15 (normal), 15.01 - 25 (increase), and more than 25 µM (sharp increase) (Table 1). The mean level of NO in patients with G. intestinalis was 32.19 ± 2.15 µM and in people without G. intestinalis was 17.1 ± 1.33 µM. 8.2% of patients with giardiasis were in normal range, but 2%, 20.4%, and 69.4% were in decline, increase, and sharp increase ranges, respectively. In group 2 (without infection), 17.9% were in normal range, and 20.5%, 51.3% and 10.3% were in decline, increase, and sharp increase ranges, respectively. The mean level of NO revealed significant association between group 1 and group 2 with a 95% confidence interval (p-value = 0.001) (Figure 1, 2).

DISCUSSION

Giardiasis is one of the most prevalent intestinal protozoa infections in human being [4]. In parasitic infections with Giardia intestinalis the immune system produces antibodies and releases inflammation factors that cause damage to the parasite. IgA is important and is a major mechanism as it contributes to the elimination of
**Giardia intestinalis.** Non-specific inflammatory mediators such as IL-1 and TNF-α lead to stimulation of goblet cells which produce mucosa to facilitate elimination of dead parasites [9,18,19]. Besides the production of antibodies, another important mechanism that causes inactivation and death of the parasite is production of NO (nitric oxide). NO is produced by NOS (nitric oxide synthase) from L-arginine in an oxygen dependent reaction [11,12]. NOS has three isoforms: neuronal NOS (nNOS/NOS1), endothelial NOS (eNOS/NOS3), and inducible NOS (iNOS/NOS2). In a variety of cells, cytokines and microbial products induce NOS expression. iNOS is a main isoform expressed by intestinal cells [20]. Expression of iNOS can be inducible during intestinal inflammation by cytokines such as Interferon-γ in infection conditions [21]. NO has many roles in the human body. One of the important roles of NO is an antimicrobial property against bacterial and parasitic infections [22]. NO can affect the biology of **Giardia** either by direct toxicity or through influencing essential metabolites or even by increasing the immune system response against the parasite [11]. In addition to intestinal epithelial cells, macrophage and fibroblast can also produce NO against **Giardia** [13,14]. Matowicka-Karna et al. demonstrated that giardiasis stimulated NO production, which was not decreased even when antiparasitic infection treatment was applied [23]. In our study, a higher number of people showed a sharp increase in NO levels with giardiasis compared to the control group, and the patients infected with giardiasis have significantly increased NO levels. In this field our results are in concordance with several studies, and this shows that maybe NO plays an important role in the immune system against giardiasis [21,24,25]. Nitric oxide and sustainable end products of NO, nitrate, and nitrite exist in the apical side of intestinal epithelial cells [22]. On the other hand, trophozoites are in close contact with enterocytes, suggesting that parasite could be a related target for NO produced by epithelial cells [14]. The mechanism of how NO is released on the apical side is not yet known. But maybe it depends on iNOS localization at the apical side of epithelial cells, under the cell membrane [21]. Moreover, the effects of NO on pathogen biology are not clear, but it seems that the oxidative capacity of NO and its end-products work in synergy with other reactive oxidant species (H₂O₂). For instance, peroxynitrite (ONOO⁻) causes restructuring of target molecules in the parasite and is a main mechanism for the direct influence of NO [26]. Also, increased gastrointestinal motility is an important mechanism for defending against parasitic infections [25]. A study was conducted in mice shows that nNOS increased the motility of gastrointestinal motility and contributes to clearance of **Giardia** infections. So neuronal NOS (NOS1) participates in the removal of parasitic infection [25], and it was the first report of NOS1 being involved in elimination of an infection. The results of another study demonstrated that NO inhibits the trophozoite growth and proliferation *in vitro*, but does not kill them. Therefore, NO was rather cytostatic than cytotoxic in this study [27]. Also, NO inhibits encystations of trophozoite and excystation of cysts. Inhibitory effects of NO on excystation may limit the number of parasites in the intestine. Inhibition of encystations could reduce
the formation of cysts, so transmission to others may be reduced [22].

Despite the antiparasitic effect of NO, the parasite has mechanisms to escape from host defense. Arginine is an important energy source for *Giardia intestinalis*. *Giardia*, through taking up and consumption of arginine, inhibits NO production in epithelial cells [21,27]. As a product of this reaction, ornithine is produced that competitively inhibits arginine uptake by the enterocytes. Both mechanisms inhibit NO production by intestinal cells [14]. As NO and arginine are components of the immune system in intestinal epithelium, the balance between NO production by enterocytes and immune system in intestinal epithelium, the balance between NO production by enterocytes and immune system in intestinal epithelium, the balance between NO production by enterocytes and immune system in intestinal epithelium, the balance between NO production by enterocytes and immune system in intestinal epithelium, the balance between NO production by enterocytes and immune system in intestinal epithelium, the balance between NO production by enterocytes and immune system in intestinal epithelium, the balance between NO production by enterocytes and immune system in intestinal epithelium, the balance between NO production by enterocytes and immune system in intestinal epithelium, the balance between NO production by enterocytes and immune system in intestinal epithelium, the balance between NO production by enterocytes and immune system in intestinal epithelium, the balance between NO production by enterocytes and immune system in intestinal epithelium, the balance between NO production by enterocytes and immune system in intestinal epithelium, the balance between NO production by enterocytes and immune system in intestinal epithelium, the balance between NO production by enterocytes and immune system in intestinal epithelium, the balance between NO production by enterocytes and immune system in intestinal epithelium, the balance between NO production by enterocytes and immune system in intestinal epithelium, the balance between NO production by enterocytes and immune system in intestinal epithelium, the balance between NO production by enterocytes and immune system in intestinal epithelium, the balance between NO production by enterocytes and immune system in intestinal epithelium, the balance between NO production by enterocytes and immune system in intestinal epithelium, the balance between NO production by enterocytes and immune system in intestinal epithelium, the balance between NO production by enterocytes and immune system in intestinal epithelium, the balance between NO production by enterocytes and immune system in intestinal epithelium, the balance between NO production by enterocytes and immune system in intestinal epithelium, the balance between NO production by enterocytes and immune system in intestinal epithelium, the balance between NO production by enterocytes and immune system in intestinal epithelium. As NO and arginine are components of the immune system response to giardiasis. But it is not very clear how it affects *Giardia intestinalis*, whether by cytotoxicity or cytostatic property or what concentrations of NO are required to affect *Giardia intestinalis* is needs to be determined. Therefore, we suggest that further studies are required to understand the exact function of NO in the immune system against giardiasis in humans. It will be important to offer a new therapeutic target for eliminating *Giardia intestinalis*. A number of different mechanisms have been proposed which might control *Giardia* infections. These include anti-microbial peptides, NO, and mast cell products [28,29]. In most humans, infected with *Giardia lamblia*, the parasites are effectively eliminated by the immune system [18,19]. NO has been recognized as playing an important role in the control of infections with numerous microbes. Nitric oxide (NO), which is produced when NOS consumes arginine, has demonstrated that NO can have cytotoxic or cytostatic effects on the parasite. NO can inhibit parasite replication and differentiation. Furthermore, data indicate through signals mediated by the enzyme that NOS plays a key role in elimination of infection [25]. In our study, the number of people who showed a sharp increase in NO levels, individuals with giardiasis, is much higher than the control group, and patients infected with giardiasis have significantly increased NO levels. These results suggest that NO and its derivatives are important components of the immune system response to giardiasis. Therefore, we suggest that further studies are required to understand the exact function of NO in the immune system against giardiasis in humans. It will be important to offer a new therapeutic target for eliminating *Giardia intestinalis*.

**Declaration of Interest:**
We have no financial interests related to the material in the manuscript and the authors declare that there is no conflict of interest.

**References:**


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