C-kit expression and potential role of iNOS, COX-2, and ICAM-3 in Giardiasis

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**Abstract**

**Objective:** Giardiasis is an intestinal infection and inflammatory mediators take place in host defense mechanisms. The aim of this study was to examine the consistency and usefulness of c-kit in diagnosis, to establish the roles of iNOS, COX-2 and ICAM-3 in the pathogenesis of Giardia Lamblia infection.

**Materials and methods:** The study was performed on tissue samples obtained from 59 patients. Sections of duodenal biopsies were stained for iNOS, COX-2, ICAM-3, and c-kit immunohistochemically. The tissue samples were processed and evaluated by authors at the Department of Pathology.

**Results:** In our study iNOS and COX-2 expression in the duodenal epithelium of the giardiasis group showed statistically significant difference as compared to the control group. Similarly, ICAM-3 expression in the inflammatory cells showed statistically significant difference. None of the cases showed positivity with c-kit.

**Conclusion:** Our results indicate that these mediators play a role in the pathogenesis of Giardia infection and immunohistochemistry is not a useful method for the diagnosis of giardiasis.

**Keywords:** Giardiasis; iNOS; ICAM-3; COX-2; C-kit

**Statement of significance:** The prior aim of the study was to examine the expression of C-kit on giardia lamblia protozoas. We obtained that C-kit is not a useful method for the diagnose of giardiasis.

**Introduction**

Giardiasis is an intestinal infection caused by the worldwide protozoan parasite, *Giardia lamblia* (*G. lamblia*), which is also known as *Giardia intestinalis* or *Giardia duodenalis*. The parasite was first described by Leeuwenhoek in 1681, who observed the parasite in his own stool [1-3]. It spreads by means of contaminated food or water or by direct fecal-oral transmission [3,4]. There is a bimodal age distribution of clinical presentation, with peaks at ages 0 to 5 years and 31 to 40 years. It has a high prevalence in the developing nations [5]. In giardiasis, reinfections are common because acquired immunity against *G. lamblia* is not complete either due to insufficient immune defences or antigenic variation of the parasite [4].

The diagnosis of giardiasis can be made by a number of modalities, some of which are facilitated by endoscopy [6]. Microscopic examination of the stool is the most established diagnostic technique. Techniques using the Polymerase Chain Reaction (PCR), Enzyme Immuno Assay (EIA) and direct fluorescence are commercially available for the diagnosis of giardiasis. Duodenal aspirate, biopsy, brush cytology, and the string test are methods of identifying Giardia trophozoites. Of these methods, duodenal biopsy specimen appears to be the most sensitive, with studies showing sensitivity rates of 82.5% to 100% [3]. In asymptomatic infected persons, histologic study of the duodenal and jejunal mucosa usually shows no abnormality. In symptomatic persons, findings may include villous atrophy, crypt hyperplasia, epithelial cell damage, and extensive infiltration of the lamina propria by plasma cells, lymphocytes, and polymorphonuclear leukocytes. Trophozoites are able to attach to the microvillous brush border of enterocytes and penetrate crypts but do not invade mucosa, whereas high invasiveness of *G. lamblia* has been shown to occur in the case of IgG and IgA deficiency [3,7].

In Hematoxylin and Eosin (H&E)-stained duodenal biopsies, *G. lamblia* trophozoites are not easily seen, especially under low-power examination, and can be either missed or interpreted as fragments of the cytoplasm of epithelial cells, mucus, or artifacts. According to the English literature there is only one report which suggests c-kit immunostaining to be superior to the currently available methods for the diagnosis of *G. lamblia* in duodenal mucosal biopsies [8].

In majority of cases giardiasis is a self-limiting process, indicating existence of a host defense against the parasite. The released inflammatory mediators lead to parasite damage, accelerate intestinal peristalsis, and facilitate parasite eradication by the production of mucus in the intestine, which is indispensable for parasite removal [9]. One of them is Nitric Oxide (NO), which is generated in the reaction initiated by NO synthase with hydrobiopterin [7].

Another mediator Cyclooxygenase (COX) shares a number of similarities with NOS [14]. COX enzymes catalyse the conversion of arachidonic acid to prostaglandins and exist in 2 genetically different isoforms, constitutive COX-1 and inducible COX-2 [15]. Sustained inhibition of both COX isoforms by Non-Steroidal Anti-Inflammatory Drugs (NSAIDS) can cause intestinal ulceration in humans and laboratory animals [17]. COX-1 is constitutively expressed in all tissues and is involved in many physiological functions, while COX-2 is a rate-limiting enzyme which is associated with inflammation and tumorigenesis [16]. In the small intestine (duodenum, jejunum, and ileum) COX-2 positive cells were observed in the epithelium and also within the lamina propria, predominantly at the apex of the villi [17]. In several systems, NO increases COX-2 activity. The finding that, especially in cerebral ischemia, COX-2 and iNOS are induced at the same time raises the possibility that NO produced by iNOS activates COX-2 in the ischemic brain. Therefore, COX-2-derived pro-inflammatory prostanooids and reactive oxygen species could contribute to the toxic effect of NO. Nogawa et al indicated that the iNOS inhibitor aminoguanidine attenuates postischemic accumulation of the COX-2 reaction product prostaglandin E2 only in regions of the ischemic hemisphere wherein both iNOS and COX-2 are expressed [18]. All these show that the signal pathway of cytokine-induced iNOS and COX-2 coexpression is tightly associated.

Intercellular adhesion molecule-3 (ICAM-3) (ICAM-R, CD50) is a 124-kDa glycoprotein which mediates a plethora of immunologically relevant homotypic and heterotypic intercellular interactions, such as leukocyte recruitment during migration, removal of apoptotic cells and lymphocyte interactions with antigen-presenting cells [19]. As ICAM-3 is more expressed than the other LFA-1 ligands on cell types implicated in antigen presentation, it could be pivotally important in the genesis of immune response [20].

The aim of this study was to examine the consistency and usefulness of c-kit immunostaining to aid the diagnosis of *G. lamblia* and to establish the roles of iNOS, COX-2 and ICAM-3 in the pathogenesis of *G. lamblia* infection.

**Materials and methods**

The study was performed on tissue samples obtained from 59 patients with intestinal symptoms who underwent endoscopic duodenal biopsies at Mersin University Research and Application Hospital between 2007 and 2011. The study group (n=31) consisted of duodenal biopsies diagnosed as giardiasis. The patients were admitted to hospital due to chronic abdominal pain and/or chronic diarrhea. Giardiasis was diagnosed on the basis of positive stool tests for the Giardia antigen or by microscopic detection of trophozoites in duodenal fluid, obtained by aspiration from a naso-duodenal tube. In all children several tests were performed including routine serum and urine biochemical tests, stool culture, stool sample examination for Giardia antigen and ova of parasites and abdominal ultrasound. Gastroscopy with duodenal biopsy was also performed to exclude esophagitis, ulcer disease or celiac disease. The control group (n=28) consisted of duodenal biopsies with no evidence of giardiasis on H&E sections with symptoms like abdominal pain, diare, gastric complaints The tissue samples were processed and evaluated.
Results

The patient group consisted of 7 adults and 24 children. In this group 13 were females and 18 were males. The age at presentation ranged from 3 to 57 years (median age 14 y). The control group consisted of 14 adults and 14 children where 15 were females and 13 were males. The median age was 27. There is no statistically significant difference between the median age of the groups and INOS, COX-2 and c-kit expressions. Is the median age difference between groups significant or relevant to outcomes?

In our study, of the 31 patients diagnosed with giardiasis 16 (52%) showed no expression of iNOS in epithelial cells, whereas 6 (19%) showed 1+ staining, 7 (23%) showed 2+ staining, and 2 (6%) showed 3+ staining. On the other hand, all patients in the control group showed varying degrees of iNOS expression in the epithelial cells: 14 (50%) patients showed 3+ staining, 9 (32%) showed 2+ staining, and 5 (18%) showed 1+ staining (Figure 2A, 2B). According to these results iNOS expression in the epithelium of the giardiasis group showed statistically significant difference as compared to the control group (p<0.001). The iNOS expression in the inflammatory cells was evaluated as well. In the giardiasis group 3 patients (9.7%) showed no expression of iNOS in the inflammatory cells, whereas 6 (19%) showed 1+ staining, 6 (19%) showed 2+ staining, and 16 (52%) showed 3+ staining. In the control group, however, all patients showed varying degrees of iNOS expression in inflammatory cells; 17 (61%) patients showed 3+ staining, 5 (18%) showed 2+ staining, and 6 (21%) showed 1+ staining. According to these findings, iNOS expression in inflammatory cells did not show statistically significant difference between the two groups (p=0.248). Twenty patients (71%) in the control group showed 2+ immunostaining for ICAM-3, whereas 10 patients (32%) in the giardiasis group showed 2+ staining (Figure 2C, 2D). This led to the interpretation that the ICAM-3 expression in the inflammatory cells was decreased in the giardiasis group as compared to the control group which was statistically significant (p=0.011). All patients in control group expressed COX-2 in epithelial cells. The expression rates were as follows: 1+ in 3 patients (11%), 2+ in 2 patients (7%), 3+ in 22 patients (81%). In the giardiasis group 6 (19%) patients scored 0, 4 (13%) scored 1+, 11 (35%) scored 2+, and 10 (32%) scored 3+ (Figure 2E, 2F). According to these results COX-2 expression of epithelial cells in giardiasis group showed statistically significant difference as compared to the control group (p=0.001).

In the giardiasis group 6 (19%) patients showed no staining in the inflammatory cells with COX-2, whereas 14 (45%) showed 1+ staining, and 11 (35%) showed 2+ staining. In the control group 4 (14%) patients showed no staining whereas 9 (31%) showed 1+ staining, and 16 (55%) showed 2+ staining. Hence COX-2 expression in the inflammatory cells in the giardiasis group did not showed statistically significant difference as compared to the control group (p=0.307). Finally none of the patients in both groups showed immunoreactivity with c-kit (Figure 3).

is adherent to the mucosal surface or located within the cytoplasm of the epithelial cells, its appearance is subtle. Special histochemical staining procedures such as Giemsa or trichrome stains may aid in the demonstration of the parasite in duodenal biopsies, but it still appears inconspicuous and can be easily overlooked. Sinelnikov et al. suggested that c-kit immunostaining is superior to other currently available methods for the diagnosis of G. lamblia in duodenal mucosal biopsies and recommended performing c-kit immunostaining in every duodenal biopsy with a clinical or pathologic suspicion of giardiasis [8]. However, in the current study we did not find positive staining with c-kit in G. lamblia trophozoites. Few articles have reported an inappropriate immunostaining of different antibodies in many pathologic settings, speculating on the diagnostic value of these aberrant expressions [21]. Therefore caution must be taken when using aberrant expression of immunostains with diagnostic intent.

Both humoral and cellular mechanisms are involved in combating parasitic invasions. The infections are normally self-limiting, as immunocompetent hosts can control and typically eradicate G. lamblia, a process that involves T and B lymphocytes. Based on previous reports, secretory IgA antibodies have a central role in antigiardial defense. While the immunological processes of the antigiardial host response has already been intensely investigated, little is known about non-immune defenses, mainly in human models [4]. Hence, the elucidation of key antigiardial effector mechanisms will be important to understand mucosal immune defense against this parasite and suggest new pharmacological targets for drug therapy [22].

In the past decades, the immune response against G. lamblia has been extensively investigated in terms of the parasite’s ability to continuously change its surface antigen coat. A further investigation addressed the putative function of epithelial NO as an antigiardial effector. NO is produced enzymatically and in intestinal epithelial cells the most important pathway mediating this enzymatic reaction involves the iNOS. The evidence presented in previous reports support the conclusion that in the majority of parasitic infections, NO plays an important role. Whether this role is beneficial or detrimental depends on a number of factors including the quality of the host immune response, the production of other cytokines and immune mediators, and the target organ of infection. The existence of such an antigiardial effector mechanism was assumed because NO was revealed to inhibit in vitro growth of the parasite. However, the effectiveness of NO against a giardial infection was questioned by the observation that co-cultivation of G. lamblia trophozoites with human epithelial cells led to a remarkable suppression of the epithelial NO production. Eckmann et al [12] found that this suppression was the consequence of a depletion of arginine (a substrate for cellular NO synthesis) in the culture medium, which was caused by a high affinity uptake of the compound by the parasite to defend itself. According to the authors, it is feasible that this competitive effect represents a survival strategy, which enables G. lamblia to counteract host antiparasitic NO production within the intestinal habitat of the parasite [4]. Therefore studies define NO and arginine as central components in a novel cross talk between a pathogen and immune response in intestinal epithelium, but the balance between giardial arginine consumption and epithelial NO production could contribute to the variability in the duration and severity of infections by this ubiquitous parasite [22].

Human infection with G. lamblia often results in severe abdominal cramps and malabsorptive diarrhea. Smooth muscle contraction is the result of cholinergic stimulation of smooth muscle, whereas relaxation is mediated in part through inhibitory signaling via NO. Andersen et al. reported that intestinal hypermotility is an important host defense against Giardia as well as other enteric parasites, particularly helminthes [23]. Li et al. proposed that immune responses during infection increase motility, and that inhibition of NOS activity results in reduced gastrointestinal motility by interfering with muscle relaxation [24].

In addition consistent with a role of epithelial cell-produced NO as a potential anti-giardial effector molecule, NO was found to inhibit proliferation of G. lamblia trophozoites in vitro, but not to kill them. Thus, NO was cytostatic rather than cytotoxic.

**Discussion**

G. lamblia is a common intestinal dwelling protozoa and causes diarrhea in humans and animals worldwide [4]. The diagnosis of giardiasis can be established by several methods. In H&E stained duodenal biopsies, when G. lamblia is adherent to the mucosal surface or located within the cytoplasm of the epithelial cells, its appearance is subtle. Special histochemical staining procedures such as Giemsa or trichrome stains may aid in the demonstration of the parasite in duodenal biopsies, but it still appears inconspicuous and can be easily overlooked. Sinelnikov et al. suggested that c-kit immunostaining is superior to other currently available methods for the diagnosis of G. lamblia in duodenal mucosal biopsies and recommended performing c-kit immunostaining in every duodenal biopsy with a clinical or pathologic suspicion of giardiasis [8]. However, in the current study we did not find positive staining with c-kit in G. lamblia trophozoites. Few articles have reported an inappropriate immunostaining of different antibodies in many pathologic settings, speculating on the diagnostic value of these aberrant expressions [21]. Therefore caution must be taken when using aberrant expression of immunostains with diagnostic intent.

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**Figure 2:** A: Intense staining of the epithelium and inflammatory cells in the control group (iNOS, x400). B: Decreased intensity of staining of the epithelium in the giardiasis group (iNOS, x200). C: Intense staining of inflammatory cells in the control group (ICAM-3, x200). D: Decreased intensity of staining in the inflammatory cells in the giardiasis group (ICAM-3, x200). E: Intense staining of epithelium in the control group (COX-2, x100). F: Decreased intensity of staining in the giardiasis group (COX-2, x200).

**Figure 3:** There is no staining in G. Lamblia trophozoites with c-kit whereas immunoreactive mast cells were observed in the lamina propria (c-kit, x200).
for trophozoites [12]. NO inhibits encystation of trophozoites and excystation of *G. Lamblia* cysts [4]. Inhibition of growth and excystation would be expected to reduce the number of trophozoites in the intestinal lumen, whereas inhibition of encystation might have the opposite effect but this could reduce the formation and passing of infectious cysts and, thereby, transmission to other potential hosts [12]. Growth inhibition may be important for the infected host, because local trophozoite growth is probably crucial for the ability of *G. lamblia* to establish and maintain infection of the proximal small intestine.

For the host benefit, a balance between NO and cytokines produced in response to the invading parasites needs to be struck. Increased NO production may successfully limit parasite numbers, but at the same time, depending on the cytokine milieu, this increase may affect normal physiological functions of NO or may be responsible for immune mediated pathology. In summary, it is the balance between NO and anti- and pro-inflammatory cytokines which determines the severity of morbidity and the efficiency of the immune response. Our results are compatible with the literature since we observed an intense iNOS expression in the epithelium of control group whereas the expression was less in group with giardiasis.

NO plays a key role in the pathophysiology of chronic inflammation thus the generation of proinflammatory PGE2 is almost entirely driven by NO. Endogenous NO has been found to switch on/off the COX pathway, and might induce expression of COXs [26]. Several studies suggested an interaction of NO on the COX-2 pathway [26]. It has been shown that COX-2 is an immediate early response gene, that can be induced by hypoxia and by a variety of proinflammatory factors [25].

In the small intestine (duodenum, jejunum, and ileum) COX-2 positive cells were observed in the epithelium and also within the lamina propria, predominantly at the apex of the villi. Intraintestinal positive cells were distributed at an incidence of approximately 1 positive cell per villus. Higher levels of expression were present in the jejunum and ileum compared to the duodenum. COX-2 protein can be expressed in a wide range of cell types, including monocytes, macrophages, fibroblasts, and endothelial cells in ulcerated gastric mucosa [19].

The regulation of COX-2 expression by NO signaling is yet not well understood. Two major mechanisms proposed involve either the stimulation of guanylyl cyclase by direct binding of NO to iron in heme at the active site of guanylyl cyclase, or S-nitrosylation of protein targets on appropriate cysteines. Since COX-2 has heme at its active site and contains 13 cysteines, it was proposed that iNOS and activates COX-2 via S-nitrosylation [18].

Furthermore both of these enzymes are known to be regulated by NF-kB. Kobayashi et al. indicated that the COX-2 protein itself or one of its eicosanoid products decreases iNOS expression by suppressing transactivation of NF-kB. As a result, COX-2 overexpression downregulates iNOS induction. Therefore, the ability of COX-2 to reduce iNOS induction may have clinical relevance in humans, where COX-2 overexpression may play a cytoprotective role in inflammatory diseases by reducing iNOS expression or preventing the transactivation of NF-kB [27].

Thus, NO has a dual (potentiating in small amounts and inhibiting in large amounts) effect on the activity of COX-2 and an inhibitory effect on the expression of COX-2 protein. Mechanistically, this inhibition of COX-2 activity can be explained by the ability of NO to reduce the ferric-active form of COX to the ferrous-inactive form, or to nitrosylate tyrosine groups within COX-2. Conversely, there is not a good mechanistic explanation for the suggestion that NO directly stimulates the activity of COX [28].

Our results suggested that in intestinal epithelium in the giardiasis group COX-2 and iNOS expression was reduced significantly. These results were consistent with the literature and to our knowledge this is the first report which evaluates the manner of COX-2 expression in giardiasis [27].

Mahmoud et al. demonstrated that there is a relationship between the expression of adhesion molecules: the ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1) act as mediators in development of skin allergy caused by giardiasis [29]. El shalzy et al. found no significant difference in giardiasis while they determined highly significant increase in serum levels of ICAM-1 in toxoplasmosis and amoebiasis [30]. Unlike ICAM-1, the cell surface expression of ICAM-3 is not dependent on the state of cellular activation, although higher ICAM-3 levels are seen in memory than in naive T lymphocytes. In addition to its role in leukocyte adhesion, ICAM-3 also contributes to leukocyte migration by virtue of its relocation to the trailing edge upon leukocyte polarization. Consequently, ICAM-3 is not only a cell surface adhesion molecule but functions as a co-stimulatory molecule with intracellular signaling capability. More specifically, ICAM-3 is found on the surface of most leukocytes and is highly expressed on peripheral blood granulocytes, monocytes and lymphocytes [19]. Importantly, ICAM-3 is involved in the interactions that take place during the early stages of the immunological synapse establishment.

In our study we determined a significant difference in ICAM-3 expression between the two groups so that ICAM-3 expression in the inflammatory cells was decreased in the giardiasis group as compared to the control group. This result indicates that ICAM-3 may not play a role in the pathogenesis of Giardia infection or takes part in inflammation by decraseation. (We have interpreted ICAM 3 results incorrectly) This result is observational and not mechanistic thus the observed IHG differences does not indicate the conclusions above. The observation may be an effect of infection and not causative. To the best of our knowledge, this is the first report that evaluated the role of ICAM-3 in giardiasis and our findings should be investigated by additional studies.

Accordingly, approaches investigating Giardia growth in an inflammatory intestinal environment will provide novel information on the immunological and physiological functions that are involved in pathogenicity which promote either resistance or susceptibility to the parasite infection. However, additional studies are required to further understand the multiple roles played by this cytokine in giardiasis.

**References**


