The role of artemether as a possible drug for treatment of Blastocystis hominis infection: *In vivo* and *in vitro* studies

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The study is carried out to evaluate the *in vitro* and *in vivo* effect of treatment of artemether against *Blastocystis hominis* infection. In addition, transmission electron microscopic examination (TEM) and histopathological studies of *B. hominis* were carried out. The effect of artemether on *Blastocystis* viability showed reduction in viability 7 and 15% after 1 h in the group treated with 500 and 700 µg/ml, respectively. There are reductions in living cell count (LCC) and living cell rate (LCR) activity of artemether on *B. hominis* isolates in 500 and 700 µg/ml, respectively in both counts. The highest concentration (1000 µg/ml) caused rapid death of the parasite. The combination of artemether + metronidazole (MTZ) showed remarkable improvement in histopathological picture. By TEM examination, *Blastocystis* cells treated with metronidazole (120 µg/ml) showed shrinking of the cytoplasm and appetites change. Blastocystis cells after exposure to 500 µg/ml artemether showed large cytoplasmic vacuoles which appeared empty with rupture of the cell wall. Finally, our studies showed that artemether can be useful as herbal candidate for *B. hominis* infection therapy.

**Key words:** Artemether, TEM examination, *Blastocystis* cells, metronidazole, histopathological picture.

**INTRODUCTION**

*Blastocystis* hominis is one of the most important protozoan parasite that affects the human intestinal tract. Various epidemiological surveys have recorded a prevalence of up to 10% of the population in developed countries and as much as 50 to 60% in developing countries. Tan (2008) and El-Shewy et al. (2002) reported the prevalence of *Blastocystis hominis* among school children in Egypt as 10%. In Dakahlia governorate (Egypt), the prevalence rate of *B. hominis* cysts reached 22% (El-Shazly et al., 2006). The parasite is commonly associated with gastro intestinal tract (GIT) symptoms such as watery and mucus diarrhea, vomiting, abdominal cramps and bloating (Kaya et al., 2007). Epidemiological and parasitological studies also suggested its role in irritable bowel syndrome (Ustun and Turgay, 2006; Stark et al., 2007; Eida and Edia, 2008).

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Blastocystis is not only found in patients with enteric symptoms, but also in healthy and asymptomatic subjects (Tan, 2004, 2008). Blastocystis in the human intestine shows pathogenic potential which is controversial due to foundation of organism in both symptomatic and asymptomatic individuals (Chen et al., 2003). Pathogenicity of B. Hominis has been proven experimentally on guinea-pigs, which were infected either by natural way (orally) or by rectal infection of the parasite into the cecum (Boeva-Bangyozova et al., 2008).

The most commonly recommended drugs for treatment of B. hominis and other pathogenic intestinal protozoa is metronidazole (MTZ), which was reported to cause undesirable side effects and failures in treatment (Lemee et al., 2000). The combination of trimethoprim/sulfamethoxazole with metronidazole is effective in some individuals (Moghaddam et al., 2005). The use of metronidazole as an effective drug is not recommended in intervention. The cystic form has been shown to be resistant (up to 5 mg/ml) to the cytotoxic effect of the drug. These observations may often explain the failure of treatment (Rossignol et al., 2005).

Rossignol et al. (2005) reported the effective drug against Blastocystis were Nitazoxanide, a 5-nitrothiazazole broad-spectrum antiparasitic agent, and cotrimoxazole. Garavelli (1991) stated that, metronidazole, iodoquinol, or co-trimoxazole are effective in Blastocystis infections.

Introduction of a natural antimicrobials, such as standardized oil of oregano, black walnut, artemesinin, berberine sulphate, citrus seed extract, can be of most importance (Garavelli, 1991).

Artemisia annua (sweet annee or qinghao) was naturally occurring substances having anti-protozoan activity, a plant that yields the lactone artemisinin (qinghaosu) which is the basis for a new class of anti-malarial compounds widely used in Asia and Africa (Hien and White, 1993). The anti-protozoan effects of artemisinin to its content of endoperoxides kills parasites through oxidation. The low toxicity of artemisinin and its antibiotic activity are stimulatedmacrophages, which an important componentich are important component of the immune response to protozoan infection (Tang, 1992). Armentia et al. (1993) studied that the eradication which depend on the successful restoration of mucosal immune will function and result in continued non exposure to repeat infection.

Different concentrations (10, 100, 500 microg/ml) of Nigella aqueous extract and metronidazole are an active standard drug for B. hominis (El-Wakil, 2007). Hence, the need to develop a safe and effective alternative antiprotozoal agent is required. Artemether is active in vitro against the parasitic protozoa Entamoema histolytica and Giardia lambia (Sanmi, 2012). The present study was carried out to evaluate the in vitro and in vivo effect of treatment of artemether against B. hominis infection. In addition, transmission electron microscopic examination (TEM) and histopathological studies of Blastocystis hominis were used.

**MATERIALS AND METHODS**

**Stool samples were subjected to:**

1. Direct microscopy: Stool samples were obtained from diarrheic patients attending parasitology lathe outpatient clinic of TBRI, examined by wet smear (both unstained and iodine stained) from fresh stool (Melvin and Brooke, 1974) and methylene iodine formaldehyde concentration technique (MiF) (Blagg et al., 1955).

2. In-Vitro cultivation of stool samples positive for B. hominis: Approximately 50 mg of each faecal specimen was inoculated into 2 ml of Jones’ medium (0.01% yeast extract in buffer saline) (Jones, 1946), supplemented with 20% horse serum in a screw cap tube. The tubes were, then, incubated at 37°C for 48 h and a drop of the cultured solution was examined under light microscope at 10× and 40× magnifications. B. hominis vascular forms were found in culture specimens and were variable in size. Culture is regarded as negative when there is failure to detect B. hominis after 72 h (Zierdt, 1988). Stool culture was undertaken to exclude bacterial pathogens.

3. All tubes of the different group were incubated in a humidified CO2 at 37°C for ascending time intervals (30 min and up to 2 h in some assays).

(a) Parasites were counted using a Neubauer haemocytometer (Weber, England) after treatment. Total trophozoite numbers per ml in each group were calculated from the mean of at least three haemocytometer counts.

(b) Trophozoite viability was assessed using Eosin brilliant cresyl blue stain. The possible effect of artemether on B. hominis living cell count (LCC) was assessed 30 min, 1 and 2 h and the counts were compared with those of metronidazole for the same time intervals.

**Experimental animals**

Twenty laboratory bred male hamsters each weighting 100 to 110 g were used. Experimental animals were kept for 3 weeks in air conditioned rooms at 21°C, receiving food containing 24% protein. The animals were supplied and housed throughout the study in Schistosome Biological Supply Center (SBSP) at Theodor Bilharz Research Institute (TBRI), an Institution responsible in animal ethics.

**Infection**

B. hominis cells were obtained from diarrheic patients attending parasitology laboratory, in the outpatient clinic of TBRI. Each hamster was administered B. hominis orally using stainless steel esophageal in a dose of 10,000 B. hominis cell.

**Drugs**

Artemether was obtained in tablet form (Kunming Pharmaceutical Cooperation, PR China) with a documented purity of 99.6%. Artemether was suspended in 7% Tween-80 and 60% (v/v) DMSO to get a final stock solution of 1 mg/ml. Serial dilutions of both drugs were performed using RPMI-1640 medium to get the desired test concentrations. Metronidazole was used as a standard drug supplied by Rhone Poulence Rorer in dose of 120 µg/ml.

**Treatment**

Experimental animals were divided into four groups, each
Table 1. Effect of artemether on Blastocystis viability using eosin-brilliant cresyl blue supravital stain.

<table>
<thead>
<tr>
<th>Drug/contraction (µg/ml)</th>
<th>% Viable cells (1/2 h)</th>
<th>% Viable cells (1 h)</th>
<th>% Viable cells (2 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% DMSO</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Metronidazole 120</td>
<td>63.0</td>
<td>63.0</td>
<td>63.0</td>
</tr>
<tr>
<td>200</td>
<td>70.1</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>500</td>
<td>30</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>700</td>
<td>14</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>1000</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2. Effect of artemether (Art) on Blastocystis cell concentration in culture.

<table>
<thead>
<tr>
<th>Drug/contraction (µg/ml)</th>
<th>Cells/mL±SEM (1/2 h)</th>
<th>Cells/mL±SEM (1 h)</th>
<th>Cells/mL±SEM (2 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100×10^4±4.06</td>
<td>100×10^4±4.06</td>
<td>100×10^4±4.06</td>
</tr>
<tr>
<td>1% DMSO</td>
<td>72.55×10^4±1.55</td>
<td>72.66×10^4±1.65</td>
<td>74.65×10^4±1.55</td>
</tr>
<tr>
<td>Metronidazole 120</td>
<td>60.72×10^4±1.23*</td>
<td>65.75×10^4±1.70*</td>
<td>52.75×10^4±1.90*</td>
</tr>
<tr>
<td>200</td>
<td>52.52×10^4±0.86**</td>
<td>35.35×10^4±1.41*</td>
<td>32.35×10^4±1.27**</td>
</tr>
<tr>
<td>500</td>
<td>40.5×10^4±2.02*</td>
<td>30.25×10^4±1.25*</td>
<td>21.25×10^4±1.20**</td>
</tr>
<tr>
<td>700</td>
<td>25.5×10^4±0.44*</td>
<td>13.25×10^4±2.35*</td>
<td>0</td>
</tr>
<tr>
<td>1000</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Significant difference between treated group and control groups (P < 0.05). **Significant difference between treated group (Contraction 200) and control groups (P < 0.05)

Experimental design

Group 1: infected untreated group (control group).
Group II: hamsters receiving metronidazole (supplied by Rhone Poulence Rorer) in a dose of 400 µg/kg body weight.
Group III: hamsters were given Artemether in a dose of 400 mg/kg/day for 3 consecutive days.
Group VI: Infected-treated with a combination of 1/3 dose of metronidazole that is, 100 µg/kg body weight) plus artemether a dose of 300 mg/kg/day for 3 consecutive days.

Parasitological analysis

Three weeks following infection, treatment was given. Two weeks later stool analysis was performed by the merthiolate iodine formaldehyde concentration (MIFC) technique (Blagg et al., 1955) Blastocystis cell which is vacuolated from cells/ml were counted.

Transmission electron microscopic examination (TEM)

From culture sediment after receiving different drugs was done as a trial to discuss the mode of action of these drugs. Samples from different groups were fixed in 4% glutaraldehyde in 0.1 sodium cacodylate buffer to be studied by TEM according to Grimaud et al. (1980). TEM examination was performed in the electron microscopy lab at TBRI.

Histopathological studies

After scarification of hamsters, the small bowel was removed. A 1 cm segment was excised 5, 15 and 25 cm from the gastroduodenal junction. The excised segment was opened longitudinally and finally embedded in paraffin wax. Transverse sections of paraffin blocks were cut by microtome and mounted on glass slides. Thickness 5 µm deparaffinization sections were performed by dipping slides in 100% xylene and descending grades (100, 95, 80 and 70%) of ethanol for rehydration. Finally, sections were stained with Enrich's hematoxylin and counter stained with eosin. Five slides/animal and 3 sections/slide in each group were prepared. Histological section (3 to 5 µm) were stained with hematoxylin-eosin lymph nodes (Peyer's patches) and spleens were also examined (Bancroft and Stevens, 1975).

Statistical analysis

Statistical analysis of the results was carried out using one-way analysis of variance (ANOVA) according to Campbell (1989). Comparison between two groups was done by the Student' test. The data were considered significant if P < 0.05.

RESULTS

Table 1 shows the effect of artemether on Blastocystis viability using eosin-brilliant cresyl blue supravital stain. It shows reduction in viability 7 and 15% after 1 h in the group treated with 500 and 700 µg/ml, respectively. Table 2 shows living cell count (LCC) and living cell ratio (LCR) activity of artemether showing reduction in LCC of B.
Figure 1. Intestine of control infected group, lined by columnar mucin secreting cells with partial loss of villous pattern showing erosions and ulcers. Lamina and core of villi are infiltrated by a large number of chronic inflammatory cells (H&E ×100).

Figure 2. Intestine of treated group with Metronidazole lined by columnar mucin secreting cells with presentation of villous pattern showing focal minute ulceration of the lining epithelium. Lamina and core of villi connective tissue are infiltrated by mild number of chronic inflammatory cells (H&E ×100).

Figure 3. Intestine of treated group with Artmether lined by columnar mucin secreting cells with presentation of normal villous pattern showing focal superficial erosions (short arrow). The lamina and core of villi are infiltrated with moderate number of chronic inflammatory cells (H&E ×200).

Figure 4. Artmether + Metronidazole: Intestinal mucosa showed, normal architecture. The villi are lined by mucin secreting cells with no erosions & ulcers. The lamina and villi cores are infiltrated by mild number of chronic inflammatory cells (H&E, ×200).

*hominis* isolates in 500 and 700 µg/ml, respectively. The highest concentration (1000 µg/ml) caused rapid death of the parasite. Metronidazole produced less reduction in mean number of count 52.75 cells/ml of *Blastocystis* cells in culture which was after 2 h.

**Histopathological study**

Figure 1 shows intestine of control infected group, lined by columnar mucin secreting cells with partial loss of villous pattern showing erosions and ulcers. Lamina and core of villi are infiltrated by a large number of chronic inflammatory cells (H&E ×100). Figure 2 shows intestine of the treated group with metronidazole lined by columnar mucin secreting cells with presentation of villous pattern showing focal minute ulceration of the lining epithelium. Lamina and core of villi connective tissue are infiltrated by mild number of chronic inflammatory cells, these lead to partial hilling of intestine (H&E ×100). Figure 3 shows intestine of the treated group with artmether lined by columnar mucin secreting cells with presentation of normal villous pattern showing focal superficial erosions. The lamina and core of villi are infiltrated with moderate number of chronic inflammatory cells, these lead to partial hilling of intestine (H&E ×200). Figure 4 shows
artmether + metronidazole. Intestinal mucosa showed normal architecture. The villi is lined by mucin secreting cells with no erosions and ulcers. The lamina and villi cores are infiltrated by mild number of chronic inflammatory cells (H&E ×200). It shows remarkable improvement of histopathological study.

**Transmission electron microscopic (TEM) studies**

Figure 5 shows TEM *B. hominis* cell displaying normal morphology and DNA chromatin seen as a crescentic mass (×14000) control. Figure 6 shows TEM *Blastocystis* cell treated with metronidazole (120 μg/ml) showing
concentration (200, 500, 700 and 1000 µg/ml) at different time intervals 1/2, 1 and 2 h) were studied. In this study, artemether at 500 and 700 µg/ml induced highly significant reduction in the number of concentrations of Blastocystis cells after 2 h incubation (p < 0.01). Higher concentration (1000 µg/ml) caused rapid death of the parasite and no viable cells were detected after 30 min. Our results agreed with El-Wakil (2007) who proved that N. sativa aqueous extract could be useful in the treatment of B. hominis. This study is an arrangement with Hassan et al. (2010) who stated that, dodecanoic acid (monolaurin) induced highly significant reduction of viability of B. hominis cells, 30 and 20% in culture after 2 h incubation at 500 and 700 µg/ml concentration, respectively at (p < 0.01). The highest concentration (1000 µg/ml) caused rapid death of the parasite and no viable cells were detected after 30 min incubation. Shrinking of B. hominis cells were observed using direct microscopy ×100 following incubation with monolaurin. TEM studies of B. hominis cells displayed key morphological features of programmed cell death, viz., nuclear condensation and DNA in nucleus showed reduced cytoplasmic volume with maintenance of plasma membrane integrity.

El-Gayar and Soliman (2011) studied the effect of Quillaja saponaria (QS) against cultures of B. hominis and demonstrated its implementation on ultrastructure changes. They found that the QS (1000 µg/ml) produced a high significant reduction in both living cell counts (LCC) and living cell rate (LCR) on day 1 compared to the control. Zierdt (1991) stated that an active drug (metronidazole) concentration should inhibit the growth of B. hominis. Sawangjaroen and Sawangjaroen (2005) recorded that at a concentration of 2000 µg/ml the three extracts Acacia catechu (Fabaceae) resin, Amaranthus spinosus (Amaranthaceae) and Brucea javanica (simaroubaceae) killed 82, 75 and 67% of B. hominis parasites tested, respectively and was used as ant diarrheic agents against B. hominis.

Mahmod et al. (2006) studied the effect of the antimalarial drug artesunate, in intestinal giardiasis. He found that, the double dose of artesunate (100 mg/kg) gives the lowest mean number of Giardia (0.7 ± 1.1) and completed regeneration of endothelial cells in intestinal villi. This could be of utmost help in endemic areas like Egypt, where Giardia lamblia intestinalis is a common parasitic infection and resistance to the commonly used antigiardial drugs commonly known as supervenes. In our histopathological study, best results were obtained in the group receiving Artemether + MTZ. In Figure 4, intestinal mucosa showed normal architecture. The villa is lived by rows of early epithelial cell with no erosions and alces. The lamina and villi cones are unfiltered by needle under number of chronic inflammatory cells. This means remarkable improvement in histopathological picture. El-Gayar and Soliman (2011) found a remarkable improvement in the intestinal histopathological findings, the mucosa was infiltrated with few acute inflammatory cells, the lining columnar epithelial cells were intact and the submucosa was free from inflammation, in infected rats receiving 500 µg/ml Quillaja saponaria (QS) was observed in comparison with those receiving MTZ.

Zhang et al. (2006) showed that when metronidazole was used (120 µg/ml), TEM observation indicated a reduction of microvilli on the surface of absorptive cells. Mitochondrial edema, rough endoplasmic reticulum dilatation and degranulation were found on absorptive cells and goblet cells. Lymphocyte infiltration and eosinophilia were found in intercellular stroma. These results agree with our results which indicated that Blastocystis cell treated with metronidazole (120 µg/ml) showed shrinking of the cytoplasm and apoptotic change (Figure 6). In this study, blastocystis cell after exposure to 500 µg/ml artemether showed large cytoplasmic vacuoles that appeared empty and a ruptured cell wall (Figure 7).

This agreed with El-Gayar and Soliman (2011), in which their study on QS proved that B. hominis programmed cell death with apoptic-like features and concluded that QS is a promising new herbal therapeutic agent against B. hominis infection.

In this study, histopathological studies showed significant improvement in the experimental hamster receiving artemether comparable to MTZ. The effect of artemether on Blastocystis viability showed reduction in viability 7 and 15% after 1 h in the group treated with 500 and700 µg/ml, respectively. There is reduction in living cell count (LCC) and living cell rate (LCR) activity of artemether on B. hominis isolates in 500 and 700 µg/ml, respectively. The highest concentration (1000 µg/ml) caused rapid death of the parasite. The combination of artemether + MTZ showed remarkable improvement in histopathological picture. By TEM examination, Blastocystis cell treated with metronidazole (120 µg/ml) showed shrinking of the cytoplasm and apoptotic change.

Blastocystis cell after exposure to 500 µg/ml artemether showed large cytoplasmic vacuoles which appeared empty in ruptured cell wall.

**Conclusion**

Artemether can be useful as herbal candidate therapy for B. hominis infection therapy.

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**Conflict of interest**

Authors have none to declare.
REFERENCES


