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Pomegranate (*Punica granatum*) peel is effective in a murine model of experimental *Cryptosporidium parvum* ultrastructural studies of the ileum

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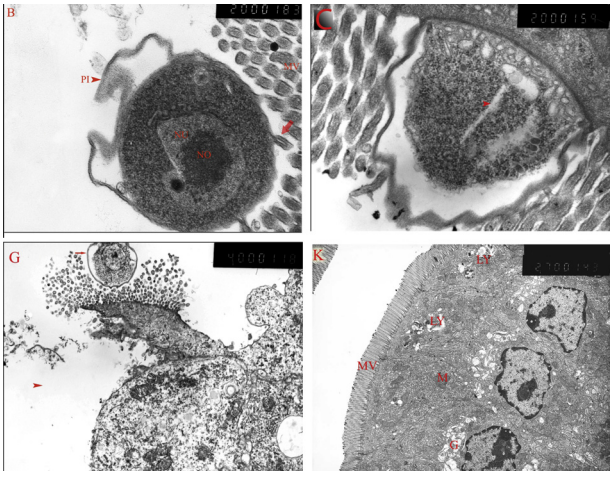
HIGHLIGHTS

- *C. parvum*-infected mice were treated with pomegranate peel suspension.
- Suspension-treated mice showed substantial parasite decomposition and death.
- Suspension treatment restored normal villi structures and eliminated acute symptoms.
- Suspension treatment directly affected *C. parvum* at various development stages.

GRAPHICAL ABSTRACT

G: Photomicrographs of transverse sections of the ileums of infected/untreated mice *Showing*: *C. parvum* trophozoite between degenerated microvilli and evidence of complete degeneration of columnar epithelial cells with their intracellular contents spilled out into the lumen (arrow head)
K: Photomicrographs of transverse sections of the ileums of infected/*P. granatum*-treated mice *Showing*: Improvement in epithelial cell structure, including an increase in the number of Golgi apparatus elements, the normal distribution of mitochondria, and many lysosomes.
B: Photomicrographs of *C. parvum* in transverse sections of the ileums of infected/untreated mice *Showing*: Merozoite with a large nucleus that has penetrated the host cell and has lateral processes that extend towards the upper surface of the epithelial cell.
C: Photomicrographs of *C. parvum* in transverse sections of the ileums of infected/*P. granatum*-treated mice *Showing*: Degenerated nucleus and cytoplasm and malformation of the FO and parasitophoric envelope (PI).



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ABSTRACT

The current treatments for cryptosporidiosis are ineffective, and there is an urgent need to search for more effective and safer alternatives. One such alternative may be treatments derived from natural resources. The pomegranate peel has been used effectively in traditional medicine to cure diarrhea and dysentery. The purpose of this study was to examine the effectiveness of a *Punica granatum* (pomegran-

Abbreviations: pi, post-inoculation; TEM, transmission electron microscope; IBD, inflammatory bowel disease.

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ate) peel suspension as a treatment for *Cryptosporidium parvum* infection. In this study, the effects of this treatment on the ultrastructure of both the intestinal epithelial layer of infected nursling mice and the parasite were observed with a transmission electron microscope. The histological study focused on the examination of the microvilli, columnar epithelium, goblet cells, lamina propria, and crypts of Lieberkuhn. Examination of the ileums of infected mice that received the pomegranate peel suspension demonstrated that the general structure of the ileal tissue of these mice was similar to that of the control group. In the infected mice treated with the suspension, but not the infected/untreated mice, there was an improvement in all ultrastructure aspects at 28 days post-inoculation. The study of the ultrastructure of the parasite (*C. parvum*) in mice treated with the suspension showed that there was decomposition in the parasite to the extent that in some cases we were unable to identify the stage of the parasite due to the severe degeneration. Significant decomposition of the nutrition organ was also observed. Additionally, microgamonte and macrogamonte were not observed in the suspension-treated group, explaining the disappearance of the sexual phases of the parasite in the lumens of this group. In all, this examination demonstrated the restoration of the normal structures of villi and the disappearance of acute symptoms in the suspension-treated mice and showed that the suspension directly affected the parasite at various stages of its development and led to its decomposition and death.

1. Introduction

Cryptosporidium parvum is a parasitic protozoan that develops in the intestinal tract of humans and other mammals. The parasite develops within the microvillus membrane of enterocytes causing the loss of villous enterocytes and villous atrophy that lead to severe diarrhea. Cryptosporidiosis is most common in young calves and may lead to weight loss and significant morbidity (Fayer and Ungar, 1986). The intracellular but extracytoplasmic nature of *Cryptosporidium* presents a unique challenge in terms of chemotherapeutic control due to its unusual parasitological niche (Armson et al., 2003). Over the last several years, many anticoccidial drugs that have been examined for their efficacy as anti-cryptosporidiosis treatments, including tultrazuril, have shown little effect against *Cryptosporidium* (Armson et al., 1999). Many drugs have been tried as treatments against cryptosporidiosis but have shown at best limited effectiveness in treating the disease in ruminants (Waters et al., 2000; Guitard et al., 2006; Castro-Hermida and Ares-Mazás, 2003; Castro-Hermida et al., 2004; Smith and Corcoran, 2004). Nita zoxanide, paromomycin, and halofuginone lactate are the most important treatments and have shown some efficacy in reducing parasite effectiveness, but cannot completely eliminate the parasite in lambs and calves (Viu et al., 2000; Schnyder et al., 2009; Silverlas et al., 2009; De Waele et al., 2010). In fact, several studies suggest the lack of an effective treatment for cryptosporidiosis (Theodos et al., 1998; Kayser et al., 2002; Del Coco et al., 2009).

New and active cures for cryptosporidiosis are urgently needed. Cures derived from alternative local medicines may lead to new effective compounds with useful activities. Pomegranate (*Punica granatum* L., family: Punicaceae) is a promising alternative treatment of plant origin that has antibacterial (Braga et al., 2005; Naz et al., 2007; Choi et al., 2009), antimalarial (Dell'Agli et al., 2009), and antiheminthic (Prakash et al., 1980; Akhtar and Riffat, 1985; Pradhan et al., 1992; Korayem et al., 1993; Fernandes et al., 2004) effects. Moreover, pomegranate is used frequently in local medicine for curing diarrhea (Sudheesh and Vijayalakshmi, 2005) and ulcers (Caceres et al., 1987) and as an anti-parasitic agent (Nagvi et al., 1991).

Although there are some limited electron microscopy studies on the structure and pathology of *Cryptosporidium* (in vitro), there are very few studies on the acute impact of therapeutic materials on the ultrastructure of parasites in the lumens of infected animals (in vivo). This may be because of the difficulty and high cost of such a study. Such an ultra structural study, however, provides invaluable information about how therapeutic material affects cells of both the parasite and the host. Our last study tested the effectiveness of an aqueous suspension of pomegranate peel against cryptosporidiosis (Al-Mathal and Alsalem, 2012), the presence of diarrhea, oocyst shedding, and weight gain/loss, and the histopathology of ileal sections were examined. Infected mice treated with the *P. granatum* peel suspension showed improvement in all parameters examined. Additionally, these mice did not exhibit any clinical symptoms and no deaths occurred. This study continues that work by testing the impact of pomegranate peel on the ultrastructure of both villous enterocytes and parasites in mice that are experimentally infected with *C. parvum*.

2. Materials and methods

2.1. Preparation of oocysts

We collected *C. parvum* oocysts from naturally-infected calves. Oocysts were concentrated according to Heelan and Ingersoll (2002); identified with the modified Zeihl-Neelson technique (Henriksen and Pohlenz, 1981) and ELISA (*Cryptosporidium* bovine ELISA kit; Cypress Diagnostics, Langdrop, Belgium); and confirmed as *C. parvum* with polymerase chain reaction of the polythreonine gene, using *C. parvum* specific primers (cry 44: CTCTTAATCCAATCATTACAAC and cry 39: GAGTETAA TAATAA ACC ACTG) and according to Wu et al., 2000 (data not shown). Sedimented oocysts were collected and stored in a 2.5% potassium dichromate solution at 4 °C. Prior to experimentation, we concentrated oocysts (Heelan and Ingersoll, 2002) in a phosphate-buffered saline solution, where they were enumerated with a hemocytometer.

2.2. Plant materials

We obtained *P. granatum* peels from fruit purchased at a local market. The Botany Department at the University of Dammam authenticated samples. Peels were cold-dried under ambient conditions, pulverized, and stored at 4 °C.

2.3. Animals

We obtained pregnant, white, albino mice (Laurent et al., 1999; Sherwood et al., 1982) no more than 3 months old from the Arabian Gulf University animal home. We tested mice for infection over 10 consecutive days and housed each litter with its mother in separate cages under hygienic conditions. The mothers remained with their nurslings to feed them as needed throughout the course of the experiment. Animal fodder (General Organization of Grain Silos and Flour Mills, Dammam, Saudi Arabia) and water