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Hemozoin is a product of heme detoxification in the gut of the most medically important species of the family Opisthorchiidae

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ABSTRACT

Many species of trematodes such as *Schistosoma* spp., *Fasciola hepatica* and *Echinostoma trivolvis* are blood-feeding parasites. Nevertheless, there is no consensus on the feeding habits of the family Opisthorchiidae (*Opisthorchis felineus, Opisthorchis viverrini* and *Clonorchis sinensis*). Previously, histological studies of *O. felineus* and *C. sinensis* revealed some dark stained material in their gut lumen. In this study we conducted a comprehensive analysis of the gut contents of three members of the family Opisthorchiidae (*O. felineus*, *O. viverrini* and *C. sinensis*). Using transmission electron microscopy, we demonstrated for the first known time the presence of disintegrating blood cells in the gut of *O. felineus* as well as electron-dense crystals in the gut of *O. felineus* and *C. sinensis*. Electron energy loss spectroscopy revealed iron atoms in these crystals, and mass spectrometry of the purified pigment demonstrated the presence of heme. Fourier-transform infrared spectroscopy identified the signature peaks of the common iron–carboxylate bond characteristic in crystals isolated from *O. felineus* and *C. sinensis*. Scanning electron microscopy showed layered ovoid crystals of various sizes from 50 nm to 2 μm. Morphological, chemical and paramagnetic properties of these crystals were similar to those of hemozoin from *Schistosoma mansoni*. Crystal formation occurs on the surface of lipid droplets in *O. felineus* and *C. sinensis* guts. Our results suggest that the diet of *O. felineus* and *C. sinensis* includes blood. Detoxification of the free heme produced during the digestion proceeds via formation of insoluble crystals that contain iron and heme dimers, i.e. crystals of hemozoin. Furthermore, we believe that biocrystallisation of hemozoin takes place on the surface of the lipid droplets, similar to *S. mansoni*. Hemozoin was not detected in the closely related species *O. viverrini*.

1. Introduction

Infections caused by trematodes of the family Opisthorchiidae—*Opisthorchis felineus*, *Opisthorchis viverrini* and *Clonorchis sinensis*—remain a major public health problem in many parts of Asia and Europe. It has been established that more than 40 million people are infected and approximately 600 million are at risk of being infected by these parasites; furthermore, there has been an increase in the incidence of these diseases in non-endemic regions such as Israel (Yossepowitch et al., 2004; Keiser and Utzinger, 2009). Infection is acquired through ingestion of raw or undercooked freshwater Cyprinoid fish carrying infective metacercariae. Mature flukes inhabit the large intrahepatic and extrahepatic bile ducts, the gallbladder and, occasionally, pancreatic ducts. In humans, the infection that is caused by these liver flukes is characterised by long duration (decades) and development of severe complications such as cholangitis, cholecystitis, obstructive jaundice, liver abscesses and pancreatitis including cholangiocarcinoma, a bile duct cancer (Al'perovich and Brazhnikova, 1989; Sripa, 2003; Hong and Fang, 2012).

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The parasites of the family Opisthorchiidae coexist with the host for a long time. Thus, understanding of their physiology, and the feeding habits in particular, is essential to identify the mechanism through which complications develop in the definitive host. This, in turn, may also help improve disease diagnosis and treatment.

The peculiarities of the digestive tract structure in trematodes, e.g., the oral sucker, participating both in attachment and ingestion of food, as well as the well-developed muscle layer around the initial segments of the digestive tract, ensure that these parasites can feed only via active suction of liquid or semiliquid food (Smyth and Halton, 1983). To date, there is no consensus on the feeding habits of species of the Opisthorchiidae. It is believed that they ingest bile, mucin or suck tissue fluid from the naked connective tissue stroma and possibly blood (Hou, 1955; Sripa, 2003). A large number of parasitic species, including trematodes (*Schistosoma* spp., *Fasciola hepatica*, *Echinostoma trivolvis*, and others) consume the host's blood (Smyth and Halton, 1983; Oliveira et al., 2000; Pisciotta et al., 2005). According to Dalton et al. (2004), hematophagy is the predominant feeding strategy of most trematodes.

Erythrocytes or red blood cells (RBCs) are the most common type of blood cell. Hemoglobin constitutes approximately 90% of the total protein in RBCs, and its degradation by proteolytic enzymes results in the release of large amounts of the iron-containing prosthetic group, heme. Exogenous heme has to be metabolised quickly due to its toxicity, so heme degradation is one of the key physiological processes for blood-feeding organisms. It has been established that free heme strongly catalyses the generation of reactive oxygen species, which in turn lead to oxidation of lipids, proteins and DNA (Kumar and Bandyopadhyay, 2005). Due to its lipophilic nature, heme can also associate with phospholipid membranes, disrupting their physical integrity (Jeney et al., 2002). Blood-feeding parasites have several strategies for detoxification of heme derived from the catabolism of host RBCs (Toh et al., 2010). The most prevalent pathway involves formation of an iron-containing black or dark brown crystalline material, which was initially found in the digestive vacuole of the malaria parasite and therefore bears the name "malaria pigment" or hemozoin (Pagola et al., 2000). Blood-feeding trematodes such as *Schistosoma* spp. and *E. trivolvis* use a similar pathway for heme detoxification (Oliveira et al., 2000; Pisciotta et al., 2005). Hemozoin consists of centrosymmetric dimers of heme that are linked via coordinate iron–carboxylate bonds between Fe^{3+} of one heme and propionate side chains of the other; these dimers form crystals through hydrogen bonds (Slater et al., 1991; Pagola et al., 2000). In contrast to monomeric heme, hemozoin crystals are insoluble in water, some detergents or bicarbonate buffer and have paramagnetic properties (Fitch and Kanjananggulpan, 1987; Butykai et al., 2013). Hemozoin formation is specific to parasites; therefore, it is an attractive target for the development of new diagnostic and treatment methods for opisthorchiasis and clonorchiasis. For instance, detection of hemozoin as heme hyper-accumulated crystals in RBCs by laser desorption mass spectrometry (MS) is one of the most sensitive and specific methods for diagnosis of malaria (Demirev et al., 2002).

Our comparative histopathological studies of the hepatobiliary system during experimental opisthorchiasis led us to hypothesise that formation of hemozoin is likely a part of the nutrition process of *O. felineus* but not of *O. viverrini* (Lvova et al., 2012). We showed that starting from week 3 after the infection by *O. felineus* and until the end of the experiment (6 months after the infection); there were brown-black pigments in the liver tissue of the hamsters and in the gut lumen of *O. felineus*, but not in the gut of *O. viverrini*. However, experiments with differential histological staining failed to identify the nature of these pigments (Lvova et al., 2012). Similar results were obtained with *C. sinensis* where the dark granules and blood cells were found in the gut lumen of this liver fluke (Chu et al., 1982). It is logical to assume that *O. felineus* and *C. sinensis* consume blood and the lumen of their gut contains hemozoin. Thus, a comprehensive analysis of the gut contents in some members of the family Opisthorchiidae (*O. felineus*, *O. viverrini* and *C. sinensis*) was the main aim of our study. To this end we used a complex analytical approach combining electron microscopy, spectroscopy, MS and Fourier-transformed infrared (FTIR) spectroscopy to reveal the nature and the origin of granules observed in the gut of *O. felineus* and *C. sinensis*.

2. Materials and methods

2.1. Ethics statement

Animal experiments were approved by the Committee on the Ethics of Animal Experiments of the Institute of Cytology and Genetics, The Siberian Branch of the Russian Academy of Sciences, Russia (permit number 7 of 19 December 2011). All of the procedures were in compliance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for animal experiments http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm.

2.2. Parasites

Metacercariae of *O. viverrini*, *O. felineus* and *C. sinensis* were obtained from naturally infected fishes (family Cyprinidae) captured from fresh water reservoirs in the endemic areas of Khon Kaen province, northeastern Thailand; Novosibirsk, Russia and the Far East, Russia, respectively. The fish meat was digested by pepsin – HCl overnight at 37 °C followed by filtration. After being washed several times with normal saline, the metacercariae were collected and identified under a light microscope. Three-month old hamsters (*Mesocricetus auratus*) were infected per os with 50 viable active metacercariae. Adult worms of *O. viverrini*, *O. felineus* and *C. sinensis* were obtained from bile ducts of euthanised hamsters within 3 months after the infection. The fresh worms were washed several times in sterile normal saline containing penicillin (100 U/mL) and streptomycin (100 μg/mL) to remove any debris or residual blood and to prevent bacterial contamination. After thorough washing, the viable worms were used to prepare specimens for electron microscopy and obtain crystals.

2.3. Transmission electron microscopy (TEM)

For TEM, we used 3–5 adult worms of each species (*O. felineus*, *C. sinensis* and *O. viverrini*). They were dissected into small parts that were fixed with 2.5% glutaraldehyde solution in 0.1 sodium cacodylate buffer (pH 7.2) for 2.5 h. After that, the samples were washed and incubated for 1 h in a 1% osmium tetroxide solution in the same buffer. The samples were washed with water and placed in 1% uranyl acetate aqueous solution for 12 h at 4 °C. The samples were dehydrated through a graded series of ethanol and acetone, and embedded in Agar 100 Resin (Agar Scientific Ltd., United Kingdom). Ultrathin sections were double stained with uranyl acetate and lead citrate according to Reynolds (1963) and examined using a JEOL 100 SX electron microscope.

2.4. Electron energy loss spectroscopy (EELS) and electron spectroscopic imaging (ESI)

For parallel EELS and ESI, 70–80 nm thick unstained sections were used. The latter were mounted on unfilmed copper grids and then coated with carbon. The resulting samples were analysed at 120 kV in an EFTEM Libra 120 microscope with an in-column

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