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Effect of *Opisthorchis felineus* infection and dimethylnitrosamine administration on the induction of cholangiocarcinoma in Syrian hamsters

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ABSTRACT

The food-borne liver trematode *Opisthorchis felineus* is an emerging source of biliary tract diseases on the territory of the former Soviet Union and Eastern Europe. This parasite along with trematodes *Opisthorchis viverrini* and *Clonorchis sinensis* belong to the triad of epidemiologically important liver flukes of the Opisthorchiidae family. It is known that *O. viverrini* and *C. sinensis* are the main risk factors of cholangiocarcinoma (CCA) in the endemic regions. The carcinogenic potential of *O. felineus* has not been well researched because of the absence of systematic pathomorphological, clinical, and epidemiological studies on *O. felineus* opisthorchiasis.

In the present study, we show the results of detailed histopathological analysis and comprehensive evaluation of inflammation, bile duct dysplasia, periductal fibrosis, bile duct hyperplasia, bile duct proliferation, egg granuloma, cysts, cholangiofibrosis, and CCA from 10 to 30 weeks following infection of Syrian hamsters with *O. felineus* accompanied by oral administration of dimethylnitrosamine (DMN). The results revealed that *O. felineus* contributes to bile duct cancer development in the hamster model. During the combined action of *O. felineus* and DMN, morphological features of the liver underwent dramatic changes at the cellular and organ levels. Already in the early stages of the experiment, we observed extensive periductal fibrosis, active inflammation, proliferation of the bile duct, bile duct dysplasia and egg granulomas. Later, against the background of all these changes, cholangiofibrosis and CCA were found.

Our work is the first step in the study of carcinogenic potential of *O. felineus*. Obtained data indicate the risk of CCA of patients having chronic *O. felineus* opisthorchiasis, and underscore the need for the development of programs for control of this helminthiasis.

1. Introduction

Opisthorchis felineus (Rivolta, 1884), *Opisthorchis viverrini* (Poirier, 1886) and *Clonorchis sinensis* (Loos, 1907) are three epidemiologically important species of the Opisthorchiidae family (class Trematoda). Each of these three species has discrete, though occasionally overlapping, geographical distribution: *O. felineus* is endemic in Europe and Russia [1,2]; *C. sinensis* in China, the Republic of Korea, and northern Vietnam; and *O. viverrini* in Southeast Asia. Together they affect more than 45 million people worldwide [3].

The area where *O. felineus* occurs includes vast expanses of North Eurasia as well as parts of Southern and Western Europe. Recent studies assessed the prevalence of *O. felineus* in Europe (reviewed in [3]), and these flukes were found on the Iberian Peninsula, the Balkan Peninsula, in Germany [5], and in Italy [6]. The Ob-Irtysh basin in Western Siberia is thought to be the world's largest endemic centre of opisthorchiasis caused by *O. felineus* [1,4].

Human infection with *O. felineus* results from eating raw or undercooked freshwater Cyprinoid fish carrying the metacercariae of the parasite [1]. According to preliminary estimates, at least 1.6 million people in the world are infected with *O. felineus* [7]. The prevalence of *O. felineus* infection in the population of the endemic regions of Western Siberia is 10–45% according to various estimates [8–10].

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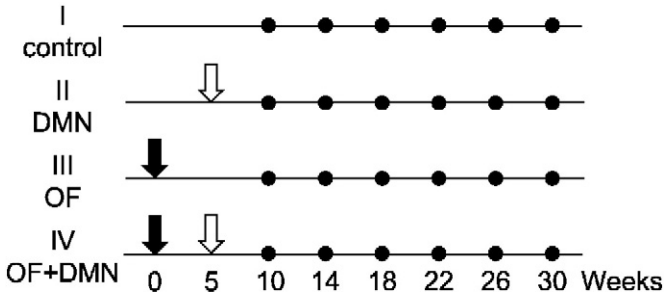

Fig. 1. The experimental procedure for induction of cholangiocarcinoma (CCA) in hamsters. The black arrow denotes infection with *Opisthorchis felineus*, the white arrow the start of dimethylnitrosamine (DMN) administration, and the black dot corresponds to euthanasia.

The International Agency for Research on Cancer (IARC) classifies the trematodes *O. viverrini* and *C. sinensis* as group 1 carcinogens [11] and as the main factors for the development of cholangiocarcinoma (CCA) in endemic areas [12,13]. In contrast to its closest relatives, *O. felineus* at present is classified by IARC as a group 3 carcinogen: potentially dangerous for humans [11].

Because of the absence of systematic epidemiological and clinical studies on opisthorchiasis in Russia, the role of *O. felineus* in the development of CCA in humans is unknown. Nevertheless, there are some data from several research groups showing that in the endemic regions of Western Siberia, the prevalence of liver cancer is several times higher than in Russia on average [9,14,15]. It is noteworthy that the liver cancer in *O. felineus*-infected patients is largely diagnosed as CCA [15,16]. Certainly, the similarity of disease manifestations between the diseases caused by *O. felineus* and *O. viverrini* [10,17–19] as well as the results of studies on the pathogenesis of these helminthiases in experimental animal models [20,21], point to fairly high probability of the involvement of *O. felineus* in the development of CCA. Nonetheless, to date, the biology of *O. felineus* and its epidemiology and carcinogenic potential have not been studied well, even worse than those of *O. viverrini* and *C. sinensis*. The research into the role of *O. felineus* in the development of liver cancer in the experimental opisthorchiasis model based on hamsters is one of the first stages in assessment of the carcinogenic potential of this parasite.

The aims of this study were to examine the carcinogenic potential of *O. felineus* infection in Syrian hamsters during administration of DMN and to conduct a detailed histopathological analysis and comprehensive evaluation of inflammation, bile duct dysplasia, periductal fibrosis, bile duct hyperplasia, bile duct proliferation, egg granuloma, cysts, cholangiofibrosis, and CCA during 30 weeks after *O. felineus* infection.

2. Materials and methods

2.1. The hamsters and parasites

Syrian hamsters (*Mesocricetus auratus*) were purchased from the stock of the Puschino Animal Facility (Russia). 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. The animals were kept and treated according to protocols approved by the Committee on the Ethics of Animal Experiments of the Institute of Cytology and Genetics (Permit Number: 7 of 19.12.2011). Euthanasia was performed by decapitation, and all efforts were made to minimize suffering.

O. felineus metacercariae were collected from naturally infected fish (*Leuciscus idus*) caught in the Ob River near the city of Novosibirsk (Western Siberia) and extracted accordingly [22,23]. Territories where sample collection (fishing) took place were neither conservation areas nor private, nor otherwise protected; hence, no fishing permits were required. The fish species collected are not considered endangered or rare, and the fishing methods were in full compliance with the Federal Law N166-F3 of 20.12.2004 (ed. 18.07.2011) "Fishing and conservation of water bio-resources".

2.2. Experimental design

One hundred and fifty five male Syrian golden hamsters, aged 6 to 8 weeks, were used. The hamsters were distributed into four groups: (I) the untreated control, (II) 12.5 ppm DMN intake (with water), (III) infection with 50 metacercariae of *O. felineus*, and (IV) infection with 50 metacercariae of *O. felineus* and 12.5 ppm DMN intake (with water). The hamsters were housed at five per cage under conventional conditions and received a stock diet and water ad libitum.

The hamsters of groups III and IV were infected with 50 metacercariae per os. After one month, we verified the infection in the hamsters by the coproovoscopy [24]. The dose of DMN was selected according to the previously published data [25–27]. After confirmation of the infection, in groups II and IV, we replaced the drinking water with a 12.5 ppm DMN solution in distilled water (ad libitum consumption). A fresh DMN solution was prepared daily and placed in nontransparent bottles. Our experimental procedure for induction of CCA in the hamsters is presented in Fig. 1. The hamsters received DMN at 12.5 ppm until the day of euthanasia. The hamsters that received DMN were kept in a separate room. The total duration of the experiment was 30 weeks. Four or five hamsters from the control group and six hamsters from each of the other groups were euthanized and necropsied on weeks (Wk) 10, 14, 18, 22, 26, and 30 post-infection (p.i.).

2.3. Sample collection and pathological study

After each collection of biological samples, we measured the body weight and liver weight. The spleen weight was measured only after 26 and 30 weeks p.i. The liver was carefully dissected and placed in 10% buffered formalin (Biovitrum, Russia). After fixation overnight at 4 °C, the specimens were dehydrated in a graded series of ethanol solutions and then absolute ethanol, cleared in xylene, and soaked in melted paraffin. Then we embedded the specimens in paraffin using Microm (Microm, UK). Four-μm-thick slices were prepared by means of a microtome.

For histopathological analysis, the tissue slices were stained with hematoxylin and eosin by the standard method. Pathological changes were graded semiquantitatively according to previously described methods [20,27,28]. The finished slides were examined under a light microscope (Axioskop 2 Plus; Zeiss, Germany).

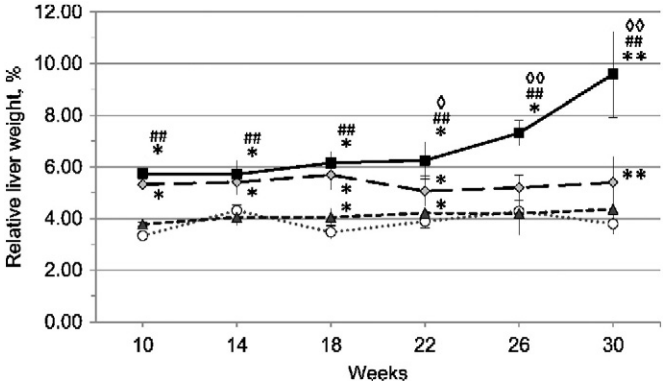

Fig. 2. The liver-to-body weight ratio (in percentage points, mean ± standard deviation). Treatment groups: I(○), control; II(▲), dimethylnitrosamine (DMN); III(◊), *Opisthorchis felineus*; IV(■), *O. felineus* + DMN. *Compared to group I, #compared to group II, ◊compared to group III; *, #, ◊ correspond to $P < 0.05$; **, ##, ◊◊ correspond to $P < 0.01$..