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A new approach to assessing the efficacy of anthelmintic agents *in vitro*

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ABSTRACT

Aim. To develop a new method to determine the viability of *Opisthorchis felineus in vitro* using the MTS reagent and to evaluate its applicability for analyzing the efficacy of anthelmintic agents in the treatment of opisthorchiasis.

Materials and methods. Golden hamsters were used to create a model of *O. felineus* infection. The animals were infected with metacercariae obtained from fish of the Cyprinidae family. Three months after infection, adult parasites were extracted from the hepatobiliary system. Their viability was assessed using the motility scale and a new method based on the modified MTS test protocol. To account for differences between the size and number of adult parasite cells, the results were normalized with respect to protein content. To evaluate the feasibility of the new approach in the study of pharmacological activity against opisthorchiasis, the viability of adult parasites in the presence of praziquantel was tested.

Results. During incubation of adult flukes in a medium with the addition of the MTS reagent, colored water-soluble formazan was accumulated. Thermal inactivation of parasites significantly decreased the production of this compound. Since the studied adult parasites differed in size and number of cells, the obtained data on their viability were normalized to protein content. The results correlated with the data on parasite viability obtained by the traditional method using the motility scale. Evaluation of praziquantel efficacy at different concentrations using two independent methods (the MTS test and the motility scale) showed that the results of the MTS test were consistent with literature data and comparable with the results obtained using the motility scale.

Conclusion. A new method for *in vitro* evaluation of anti-opisthorchiasis activity of drugs was developed. It is based on the assessment of water-soluble formazan production by adult *O. felineus* flukes in the culture medium using the MTS reagent for screening anti-opisthorchiasis activity of new anthelmintic drugs.

Keywords: trematode infection, *O. felineus*, viability, MTS test, praziquantel, anthelmintic agents, motility

Conflict of interest. The authors declare the absence of obvious or potential conflicts of interest related to the publication of this article.

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Conformity with the principles of ethics. The study was approved by the Commission for the control over the maintenance and use of laboratory animals of the Center for Preclinical Research of the Central Research Laboratory, Siberian State Medical University (Conclusion No. 1 of 02.09.2024).

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Новый подход к оценке эффективности антигельминтных средств *in vitro*

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РЕЗЮМЕ

Цель. Разработать новый метод определения жизнеспособности описторхов in vitro с использованием MTS-реактива и оценить его применимость для анализа эффективности антигельминтных средств при лечении описторхоза.

Материалы и методы. Для создания модели инвазии *O. felineus* использовались золотистые сирийские хомяки. Животные инфицировались метацеркариями, полученными из рыб семейства карповых. Спустя 3 мес после заражения взрослые особи паразитов (мариты) извлеклись из гепатобилиарной системы. Жизнеспособность марит оценивалась с помощью шкалы подвижности и нового метода, основанного на модификации протокола MTS-теста. Для учета различий между размерами и количеством клеток марит результаты были нормализованы относительно содержания белка. Для оценки возможности применения нового подхода в изучении противоописторхозной фармакологической активности проверена жизнеспособность марит в присутствии празиквантела.

Результаты. При инкубации марит описторхов в среде с добавлением MTS-реактива происходит накопление окрашенного водорастворимого формазана. Термическое инактивирование паразитов значительно снижает продукцию этого соединения. Так как исследуемые мариты различались по размеру и количеству клеток, полученные данные об их жизнеспособности были нормированы на содержание белка. Результаты коррелируют с данными о жизнеспособности паразитов, полученными традиционным методом с использованием шкалы подвижности. Оценка эффективности празиквантела в разных концентрациях с помощью двух независимых методов (MTS-тест и шкала подвижности) показала, что результаты MTS-теста согласуются с литературными данными и сопоставимы с результатами, полученными с использованием шкалы подвижности.

Заключение. Разработан новый метод оценки противоописторхозной активности лекарственных препаратов *in vitro*, основанный на оценке продукции водорастворимого формазана маритами *O. felineus* в среде культивирования с использованием MTS-реагента для скрининга противоописторхозной активности новых антигельминтных препаратов.

Ключевые слова: трематодоз, *O. felineus*, жизнеспособность, MTS-тест, празиквантел, антигельминтные средства, двигательная активность

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Исследование выполнено в рамках реализации государственного задания на оказание государственных услуг (выполнение работ) № 056-03-2024-063 от 24.01.2024, дополнительное соглашение № 056-03-2024-063/3 от 29.08.2024 «Разработка нового лекарственного средства на основе природного комплекса фенолгликозидов и арабиногалактанов для терапии трематодозов».

Соответствие принципам этики. Исследование одобрено комиссией по контролю содержания и использования лабораторных животных центра доклинических исследований центральной научно-исследовательской лаборатории СибГМУ (заключение № 1 от 02.09.2024).

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INTRODUCTION

The liver fluke *Opisthorchis felineus* (*O. felineus*) is a member of the triad of epidemiologically important liver fluke species of the Opisthorchiidae family and is the main causative agent of opisthorchiasis in a vast territory, including Russia, Kazakhstan, and a number of European countries [1]. A recent study conducted by O.S. Fedorova et al. (2023) showed that the spread of *O. felineus* infection in the population is closely associated with the development of cholangiocarcinoma [2]. In addition, opisthorchiasis can provoke the development of liver abscesses and pancreatitis [1] Thus, trematode infections are a group of dangerous infectious diseases that pose a significant public health threat [3].

The main approach recommended by WHO to reduce the incidence of opisthorchiasis is to increase the availability of safe and effective drug therapy. To date, praziquantel (PZQ) is the only recommended therapy for opisthorchiasis. The development of new drugs for the treatment of trematode infections is a high priority because of the possibility of liver fluke developing resistance to PZQ [4]. Indeed, *in vitro* studies have confirmed that adult helminth strains exposed continuously to PZQ show increased resistance to the drug [5]. In addition, PZQ has no preventive effect and is ineffective against parasite larvae, which represents a serious gap in the development of mass chemoprophylaxis strategies in regions with high morbidity [6].

Thus, given the limitations of existing preventive and therapeutic approaches, it is necessary to create new therapeutic agents capable of effectively inhibiting the activity of the parasite at all stages of its life cycle.

The assessment of trematode viability plays a key role in the development of new methods for combating these parasites. Currently, the method of assessing parasite motility by visual inspection during microscopy is widely used [7, 8], while scanning electron microscopy to study ultrastructural integrity is less common [8]. The former is a semi-quantitative method requiring a high level of specialist training; the latter requires more effort and involves significant costs.

Currently, a wide range of reagents are used in *in vitro* pharmacological studies to analyze the viability of cultured cells. Among the most common techniques are methods of intravital or postmortem staining followed by spectrophotometry or fluorometry and luminometric analysis [9]. Some articles mention that viability of *Schistosoma mansoni* sporocysts may be assessed by staining with the fluorescent dye propidium iodide [10] or trypan blue [11]. Using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), widely used in cell culture, is of particular interest as a method to assess parasite viability [7, 12].

However, all the references found refer exclusively to the study of the viability of schistosomiasis pathogens (*Schistosoma mansoni*). There are no data on the application of a protocol using the MTT reagent for opisthorchiasis in the available scientific literature. It is important to note that formazan, formed as a result of MTT molecule reduction by mitochondrial enzymes of living cells, is insoluble in water and forms purple crystals in cells [9]. This means that organic solvents, such as dimethyl sulfoxide (DMSO) or isopropanol, are required to quantify the formed formazan before optical density determination, which complicates the application of this method to multicellular organisms, including Opisthorchis maritas.

The MTT test is a standard method for assessing cell viability and determining cytotoxicity of compounds. When performing it, it is necessary to take into account the duration of incubation and cell seeding density to ensure the accuracy and reproducibility of the results [9]. However, a significant limitation of the MTT test for determining the viability of multicellular organisms is the inability to correctly interpret differences in the size and number of cells, which can lead to distortion of the final data and misinterpretation of the results.

The MTS test based on the use of a modified MTT molecule is a simpler and more reliable assay. This test allows living cells to produce water-soluble stained formazan directly in the culture medium, which simplifies the analysis [9].

The development of a simple and affordable protocol to assess the viability of liver fluke *O. felineus* maritas will facilitate screening of potential therapeutic agents with activity against opisthorchiasis.

The aim of the study was to develop a new method to determine the viability of opisthorchiasis *in vitro* using the MTS reagent and to evaluate its applicability for analyzing the efficacy of anthelmintic agents in the treatment of opisthorchiasis.

MATERIALS AND METHODS

Golden hamsters (*Mesocricetus auratus*) were purchased from the vivarium of the Research Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences (Novosibirsk, Russia). The use of animals in this study was approved by

the Committee for the Care and Use of Laboratory Animals of the Center for Preclinical Research of the Central Research Laboratory of Siberian State Medical University (Conclusion No. 1 of 02.09.2024).

Cyprinidae (carp) fish caught in the rivers of the Ob basin in the Tomsk region were used as a source of primary biological material containing Opisthorchis metacercariae. In order to isolate Opisthorchis metacercariae from infected fish, we subjected them to digestion in artificial gastric juice [13, 14].

Three months after infection, the animals were euthanized, and *O. felineus* maritas were extracted from the hepatobiliary tract. After washing in buffer (200 U / ml penicillin – streptomycin in 1×PBS), the maritas were placed in wells of culture plate filled with the culture medium (RPMI 1640, 100 U / ml penicillin – streptomycin, 10 g / l glucose, 2 g / l sodium bicarbonate) at 37 °C in an atmosphere of 5% CO_2 [15]. The viability of Opisthorchis maritas was assessed immediately after the extraction from the bile ducts of infected hamsters and after 24 h of incubation in complete culture medium.

The new proposed method for assessing the viability of adult forms of *O. felineus* includes the use of the MTS reagent and, at the first stages, coincides with previously proposed protocols that include the use of the MTT test [7, 12]. Maritas were placed in eppendorf tubes (1.5 ml) containing 200 µl of phosphate-buffered saline (PBS) and 40 µl of the MTS reagent (Promega, USA) and incubated at 37 °C for 2 hours. The incubation medium was collected and centrifuged at 5,000 rpm for 10 minutes to precipitate eggs of the liver fluke and excretory-secretory products. The supernatant was put into wells of a 96-well plate, and then the optical density of the medium was measured at 492 nm using the Tecan 200 Infinite Pro multifunctional microplate reader (Tecan, Austria). As a negative control, maritas were subjected to heat treatment at 65 °C for 30 min before the test, which is known to cause their death [14].

To compensate for differences in the size and cellularity of the maritas, the optical density values were normalized to the amount of protein. The protein content in adult liver flukes was determined by the bicinchoninic acid assay method using the BCA Protein Assay Kit (Sigma Aldrich, USA) [16]. For this purpose, maritas were homogenized in lysis buffer (0.1% SDS, 0.1M NaOH) followed by ultrasound treatment (Bandelin Sonoplus 2070, Germany). After centrifugation (3,000g for 10 minutes), the protein content was determined in the collected supernatant.

The final viability value was determined as the difference between the optical density of the medium in the experimental wells after the MTS test and the average optical density of the heat-treated samples. The obtained value was normalized to the protein content in each liver fluke.

To evaluate the prospects of using a new approach to study the specific pharmacological activity of anti-opisthorchiasis agents, isolated liver flukes were cultured in the complete medium for 24 h in the presence of PZQ at final concentrations of 0.1, 0.5, 1, and 10 µg / ml. After incubation with PZQ, the viability of Opisthorchis maritas was assessed by two independent methods: the standard semi-quantitative method for assessing parasite motility and a newly developed quantitative method based on the production of water-soluble formazan by live marita cells in the incubation medium using the MTS reagent, taking into account the protein assay content.

Motility of *O. felineus* maritas was assessed visually using a generally accepted motility scale [14, 17]. Qualitative characteristics of motility were described using absolute frequency and the percentage value. Ten maritas were used for each concentration of PZQ. The motility of viable liver flukes was graded on a scale from 0 to 3 ([+++] very active (movement similar to the control flukes); [++] active (reduced motility compared to the control; however, body movements were preserved), [+] reduced viability (only oral sucker movements), and [–] immobility) [17]. Mean parasite motility was calculated for each PZQ concentration and expressed as a percentage relative to the control values.

The experimental data were processed using the GraphPad Prism 8 software (GraphPad Software, USA). All results were presented as the mean and the standard deviation ($M \pm SD$). The nonparametric Kruskal – Wallis and Shapiro – Wilk tests with the Benjamini – Hochberg procedure were used to test the significance of differences between the studied groups. Differences were considered statistically significant at $p < 0.05$ and $p < 0.001$. Normalized non-linear regression analysis was used to calculate the PZQ concentration at which fluke viability decreased by 50% (IC50) after 24 h of incubation.

RESULTS

The results of the experiments showed that incubation of freshly extracted Opisthorchis maritas from the liver of infected hamsters with the addition of the MTS reagent in the culture medium produced

colored water-soluble formazan, and the average value of optical density in the wells was 1.018 ± 0.109 (n = 10) (Fig. 1). It is known that elevated temperature (65 °C for 30 minutes) leads to death of maritas [14]. Indeed, after heat inactivation of maritas, no significant formazan production was observed in the incubation medium under these conditions (Fig. 1). Therefore, in the future, when calculating the viability of maritas using the MTS reagent, this well in the plate was taken as a control sample (blank), to which all the measurements were compared.

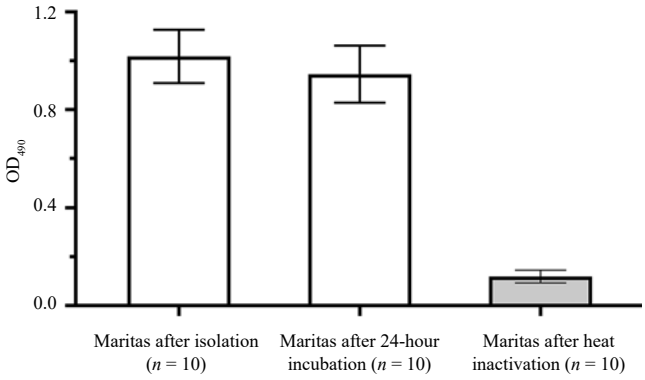

Fig. 1. Production of water-soluble formazan in the marita incubation medium immediately after isolation from the liver bile ducts of infected hamsters, after 24 hours of incubation in a complete culture medium, and after heat inactivation (65 °C, 30 min)

After 24-hour incubation of maritas in the complete culture medium in a CO_2 incubator at 37 °C, the amount of formazan in the medium determined by the MTS test did not differ from the values obtained for freshly incubated mature forms of *O. felineus* (Fig. 1). Firstly, these results confirm the preservation of the viability of Opisthorchis maritas under the indicated culture conditions. Secondly, they indicate that the accumulation of water-soluble formazan in the incubation medium during the MTS test does reflect the viability of maritas.

The process of tetrazolium reduction occurs during oxidative metabolism in the cell mitochondria [9], and the liver flukes isolated from the liver of hamsters differ in size and cellularity. Therefore, the normalization of the formazan amount to the protein content in a marita is a more objective indicator for assessing the viability of flukes.

Indeed, the amount of formazan product formed, expressed in units of optical density after the MTS test, positively correlates with the protein content in *O. felineus* maritas (Fig. 2). Therefore, in further experiments, the obtained data on parasite viability were normalized to the amount of protein.

PZQ is currently one of the most common antiparasitic drugs used to treat diseases, such as opisthorchiasis, clonorchiasis, schistosomiasis, and other trematode infections. In some endemic regions, it is actively used as part of preventive chemotherapy programs against parasitic infections [18, 19]. We studied the effect of PZQ at concentrations of 0.1, 0.5, 1, and 10 μg / ml on the viability of *O. felineus* maritas in the incubation medium using an *in vitro* model. Incubation of maritas for 24 hours with increasing PZQ concentrations in the culture medium (0.1, 0.5, 1, and 10 μg / ml) leads to a dose-dependent decrease in fluke motility up to complete immobilization, as well as a partial change in their color (Table).

Table

Motility of *O. felineus* maritas 24 hours after adding different concentrations of praziquantel (PZQ) to the medium *in vitro*

PZQ concentration, μg / ml	Number of *O. felineus* maritas, % according to the motility scale			
	0	1	2	3
0	0 0	0 0	0 0	10 100
0,1	0 0	9 90	1 10	0 0
0,5	6 60	4 40	0 0	0 0
1	8 80	2 20	0 0	0 0
10	10 100	0 0	0 0	0 0

Note. Motility scale of *O. felineus* maritas: 3 – normal motility; 2 – reduced motility; 1 – very weak motility visible only at microscope magnification of x 20; 0 – absence of motility or death, which is registered when no movement is observed for 2 minutes only at microscope magnification of x 20.

Application of PZQ at a concentration of 0.1 μg / ml in the parasite culture medium leads to paralysis of *O. felineus* muscle tissue, minimizing their motility but preserving their viability during 24 hours of exposure. At a PZQ concentration of 0.5 μg / ml, 40% of *O. felineus* maritas were characterized by very weak motility (1 point on the motility scale), while 60% had no motility. When the PZQ concentration in the incubation medium was increased to 1 μg / ml, 80% of *O. felineus* maritas showed no motility (0 points on the motility scale). At a PZQ concentration of 10 μg / ml, no O. felineus maritas showed motility (Table).

At the same time, a dose-dependent decrease in the production of water-soluble formazan in the incubation medium was observed under the influence of the drug. The percentage of viability of cultured maritas was 63.7, 38.9, 26.5, and 6.4% at PZQ concentrations of 0.1, 0.5, 1, and 10 μg / ml, respectively (the values of formazan production in the incubation medium without the drug were taken as 100%) (Fig. 3). The results correlate with the data obtained by an independent method for assessing the viability of maritas using the motility scale (Table).

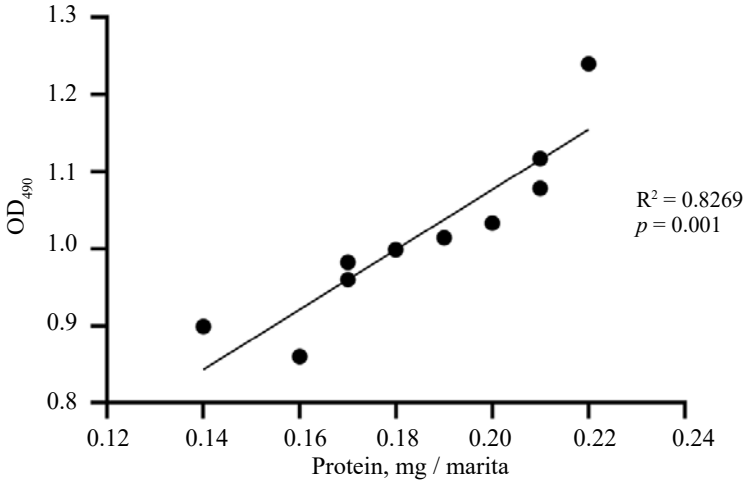

Fig. 2. The relationship between the accumulation of formazan in the marita incubation medium during the MTS test and the protein content in them, $n = 10$

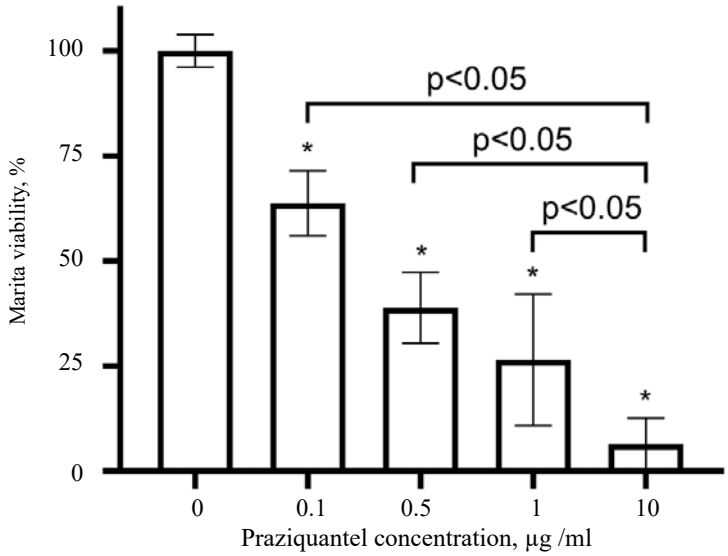

Fig. 3. The effect of different concentrations of praziquantel (μg / ml) on the viability of Opisthorchis maritas during their culture for 24 hours, $n = 10$. * $p < 0.05$ compared to the controls

The IC50 value (concentration causing 50% cell death of marita cells after 24 h of incubation with the drug) was used as an integral index characterizing the anti-opisthorchiasis activity of PZQ.

The nonlinear regression analysis showed that the IC50 value for *O. felineus* maritas was 0.24 μg / ml after 24 hours of incubation with PZQ. Thus, the proposed MTS test approach, which takes into account the protein content in the samples, represents an objective quantitative method for analyzing the viability of *O. felineus* maritas and can be used to evaluate the efficacy of new anthelmintic agents *in vitro*.

DISCUSSION

To date, PZQ remains the only drug of choice for the treatment of opisthorchiasis. Given the potential risk of resistance to PZQ in liver flukes, the development of new drugs for the treatment of trematode infections remains an urgent task and has a high priority [4].

When evaluating the anthelmintic properties of new compounds, it is often necessary to determine the viability of the parasite *in vitro* when it is incubated with different concentrations of the test substance. Currently, the parasite motility scale from 0 to 3 is used to determine the viability of *O. felineus* [14]. This method is not standardized, requires much effort, and depends on the subjective assessment of the researcher.

A more objective approach to assessing trematode viability was first demonstrated in experiments with *Schistosoma mansoni* using the MTT test [19]. This method is most commonly used to estimate the number of viable cells in culture and is based on the ability of mitochondrial and cytoplasmic dehydrogenases of live and metabolically active cells to reduce tetrazolium derivatives, MTT reagent, into water-insoluble formazan, which requires DMSO dye extraction to determine its amount [20]. However, this study does not take into account the differences in cellularity of mature forms of *S. mansoni*, which is an important limitation in interpreting the results.

Recently, a second-generation tetrazolium dye, MTS, has appeared as part of laboratory methods for assessing cell viability [21], which, with the participation of phenazine methasulphate, is reduced in the culture medium to stained water-soluble formazan, which makes it possible to significantly accelerate the analysis and obtain more objective results.

In this study, an attempt was made to develop a screening method to evaluate the *in vitro* anti-opisthorchiasis activity of drugs using the MTS test.

When analyzing the results of the experiments, it was found that during incubation of Opisthorchis maritas in the medium with the addition of the MTS reagent, colored water-soluble formazan accumulates. Heat inactivation of the parasites results in several times lower production of the colored compound. This confirms that the MTS test, traditionally used to assess cell viability in *in vitro* cell culture [22], indeed allows to assess the mitochondrial metabolic activity of cells of multicellular organisms, such as maritas, and thus determine their viability.

Since the investigated maritas differed in size and, consequently, in the number of cells, the data on their viability were normalized to their protein content. Formazan production by the maritas, expressed through their protein content, was consistent with the parasite viability results obtained according to the traditional method using the motility scale [13].

The effect of different PZQ concentrations, the drug of choice for the treatment of opisthorchiasis, on the viability of maritas *in vitro* was also assessed by two independent methods (the MTS test and the use of the motility scale). The data obtained on the viability of maritas using the MTS test were consistent with those obtained using the motility scale. For PZQ, the IC50, defined as the concentration required to kill 50% of the marita cells after 24 hours of incubation with the drug, was calculated to be 0.24 μg / ml. The results obtained are consistent with literature data that the IC50 for PZQ determined using other methods is 0.16 μg / ml for *O. viverrini* 23] and 0.14 μg / ml of *O. felineus* [14].

Thus, we have developed a quantitative method to examine the anti-opisthorchiasis activity of drugs *in vitro*, based on the evaluation of water-soluble formazan production by *O. felineus* maritas in the culture medium using the MTS test, taking into account the protein content in the sample.

CONCLUSION

A new method for *in vitro* evaluation of anti-opisthorchiasis activity of drugs has been developed, based on the assessment of water-soluble formazan production by *O. felineus* maritas in the culture medium using the MTS reagent. The results obtained coincide with the parasite viability indices determined according to the traditional methodology using the motility scale. The normalization of the optical density of the formed formazan to the amount of protein in the parasite is a more objective indicator for expressing the viability of *O. felineus* maritas.

The results of evaluating the efficacy of the drug of choice for the treatment of opisthorchiasis – PZQ – using the MTS test *in vitro* correlate with the published research data, which allows to consider the developed approach as a simple and affordable method for the study of anti-opisthorchiasis activity of new anthelmintic agents.

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Authors' contribution

Perina E.A. – literature analysis, culture of mature forms of liver fluke, determination of viability in vitro. Buyko E.E. – literature analysis, acquisition and interpretation of the experimental data, drafting of the manuscript. Kaminskiy I.P. – reproduction of opisthorchiasis invasion. Sobakin D.S. – acquisition of the experimental data. Ufandeev A.A., Kaidash O.A. – literature analysis, drafting of the manuscript. Ivanov V.V. – conception and design, coordination of the research, drafting of the manuscript, final approval of the manuscript for publication. Udut E.V. – coordination of the research, final approval of the manuscript for publication.

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