CULTURA DE TECIDOS DE RINS DE RATOS PARA A AVALIAÇÃO DA TOXICIDADE DE ALGUNS CITOSTÁTICOS**TISSUE CULTURE OF MICE KIDNEYS FOR THE TOXICITY EVALUATION OF SOME CYTOSTATICS****ИСПОЛЬЗОВАНИЕ ТКАНЕВЫХ КУЛЬТУР ПОЧЕК МЫШЕЙ ДЛЯ ОЦЕНКИ ТОКСИЧНОСТИ НЕКОТОРЫХ ЦИТОСТАТИКОВ**

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RESUMO

A justificativa para o presente estudo é explicada pelo alto risco de desenvolvimento de efeitos colaterais, incluindo nefrotóxicos, em pacientes que recebem o tratamento com citostáticos bleomicina, lastet e cisplatina para doenças oncológicas. O presente artigo enfoca a avaliação da toxicidade dos medicamentos mencionados. Foi utilizado o método de culturas de tecidos renais sem plasma em camundongos nos filtros perfurados de milípede. A identificação da toxicidade de diferentes doses dos fármacos foi realizada pela comparação das áreas das colônias cultivadas. Os autores apresentaram os dados de que a inibição do crescimento de culturas de tecidos renais (em 50%) exigiu um aumento de 4,3 vezes na dose de lastet em comparação com a bleomicina. A inibição do crescimento de colônias da cultura de tecidos com cisplatina em 85% foi registrada na dose do medicamento que correspondia a uma solução a 3%. Os materiais do artigo têm valor prático significativo, porque uma indicação racional dos citostáticos bleomicina, lastet e cisplatina em relação ao seu efeito nefrotóxico permitirá que os especialistas melhorem o resultado do tratamento e contribuam para a melhoria da qualidade de vida dos pacientes.

Palavras-chave: *cultura de tecidos, rim, rato, Lastet, Bleocina, Cisplatina, toxicidade.*

ABSTRACT

The rationale for the present study is explained by a high risk of the development of side effects, including nephrotoxic, in patients that receive the treatment with cytostatics bleocin, lastet, and cisplatin for oncologic diseases. The present article focuses on the evaluation of the toxicity of the mentioned drugs. The method of plasma-free renal tissue cultures in mice on the perforated millipore filters was used. The identification of the toxicity of different doses of the drugs was performed by the comparison of the areas of the grown colonies. The authors presented the data that the inhibition of the growth of renal tissue cultures (by 50%) required a 4.3-fold increase in the dose of lastet in comparison with bleocin. The inhibition of the tissue culture colony growth with cisplatin by 85% was registered in the dose of the drug that corresponded to a 3%

solution. The materials of the article have significant practical value because a rational indication of cytostatics bleocin, lastet, and cisplatin with regard to their nephrotoxic effect will allow the specialists to improve the treatment outcome and will contribute to the improvement of the quality of life of patients.

Keywords: *tissue culture, kidney, mouse, Lastet, Bleocin, Cisplatin, toxicity.*

АННОТАЦИЯ

Актуальность исследования обусловлена большим риском развития побочных эффектов в том числе нефротоксического действия при применении цитостатиков блеоцина, ластета и цисплатина у пациентов, страдающих онкологическими заболеваниями. В связи с этим, данная статья направлена на выявление токсичности данных препаратов. Одним из методов для исследования данной проблемы является метод бесплазменных тканевых культур почек мыши на поверхности перфорированных миллипоровых фильтров. Определение токсичности разных доз препаратов проводилось путём сопоставления площади выросших колоний. В статье представлены достоверные результаты, свидетельствующие, что ингибирование роста клеточной культуры почек (на 50%) требовало 4,3 кратного увеличения дозы ластета по сравнению с блеоцином. Угнетение численности тканевой культуры на 85% цисплатином регистрировалось в дозе препарата, соответствующего 3% раствору. Материалы статьи представляют важную практическую ценность, поскольку рациональное назначение цитостатиков блеоцина, ластета и цисплатина с учётом их нефротоксического действия позволят повысить результаты лечения и будут способствовать улучшению качества жизни пациентов.

Ключевые слова: *Тканевая культура, почки, мышь, ластет, блеоцин, цисплатин, токсичность.*

1. INTRODUCTION

One of the leading methods in the treatment of oncological diseases is chemotherapy. The drugs used for chemotherapy of tumors, which are represented by different classes of chemical compounds, exert an antiproliferative effect. Bleocin (bleomycin) is an antitumor antibiotic that is produced by *Streptomyces verticillus* fungi (Mashkovskiy, 2013). Primarily, it is effective in patients with squamous cancer of the skin, tumors of head and neck, esophagus, lungs, uterine cervix, vulva, kidney, testicle, and Hodgkin disease. According to the data on the pharmacokinetics of the drug, around 60-70% of the injected bleocin is excreted unchanged in the urine due to glomerular filtration. The concentration of the drug in plasma increases after the injection of high doses of bleocin to patients with impaired renal function. In clinical practice, the drug is indicated by different treatment schemes, primarily in the dose of 30-60 mg twice a week. Published studies indicate that there was experience of bleocin application in synchronous combined therapy, wherein along with the injection of antitumor drugs, in the doses that did not exceed single daily dose, directly in the body of the primary tumor or metastasis, radiographic fat-soluble contrast medium was used as a transporter and radiotherapy was performed along with it. This method sharply enhances the toxicity of antitumor drugs due to an increase in the concentration of free-radical

compounds in the tumor that develops as a result of radiotherapy, which reduces the tumor size (Blank and Blank, 2018).

Cisplatin belongs to the pharmacological group of alkylating compounds (*cis*-diamminedichloridoplatinum) containing heavy metal (Mashkovskiy, 2013). It is often used in the combined chemotherapy for ovarian cancer, cancer of the uterine cervix, bladder, testicle, esophagus, squamous cancer of the head and neck, osteosarcoma, and neuroblastoma (Andreev *et al.*, 2009; Smirnova *et al.*; 2018; Trushina *et al.*, 2018). The drug is characterized by a high rate of distribution in biological fluids and tissues with the highest concentration in kidneys, liver, and prostate. The nephrotoxicity of the drug has a cumulative character. A single injection dose can be 100 mg/m².

Lastet (etoposide) is a semi-synthetic derivative of podophyllotoxin isolated from the root of perennial medicinal drug wild jalap *Podophyllum peltatum* L (Berberidaceae) (Gammerman *et al.*, 1983; Yakovleva and Blinkova; 2002). The plant is poisonous and along with podophyllotoxin, contains tropane alkaloids: hyoscyamine and scopolamine. The plant's common name is American mandragora that growth in Northern America.

It is indicated as a monotherapy and in combination with other antitumor drugs. It is effective in the treatment of small-cell lung cancer, testicle cancer, Hodgkin disease, lymphogranulomatosis, etc. The contraindications

to this drug include expressed impairments of renal and hepatic function. Lastet is indicated in 100 mg/m² doses, and the course duration is 4-5 days (Mashkovskiy, 2013).

The antitumor effect of bleocin, cisplatin, and lastet is primarily realized due to the inhibition of DNA synthesis of a tumor and healthy cells, including the cells of the excretory organs. The toxic effect of cytostatics is described in a number of scientific publications (Bezborodova *et al.*, 2013; Kolina and Bobkova, 2014; Hansen, 1990; Ozben *et al.*, 2007). Renal pathology varies from the mechanical impact of the tumor or metastasis on kidneys or urinary tract to paraneoplastic manifestations, such as nephritis, amyloidosis, and nephropathy induced by pharmaceutical agents. This pathology will be manifested by the increase in the concentration of creatinine, urea, uric acid, and other serum parameters. The enhancement of nephrotoxicity in cancer patients who receive combined chemotherapy with cisplatin is described in the study performed by Gozhenko *et al.* (2010). The authors performed a retrospective analysis of the medical histories of 100 patients (70 women and 30 men) aged 32 to 73 and revealed the increase in the incidence rate of renal pathologies, such as proteinuria, leukocyturia, erythrocyturia, etc. The successful outcome of chemotherapy was primarily determined by choice of the drugs that did not exert the nephrotoxic effect. One of the factors that reduce nephrotoxicity can be dose correction in regards to the level of creatinine in the serum (Dzhumabaeva and Birykova, 2015) or parallel indication of detoxicants. One of the promising examples of such therapy with the maintenance of expressed chemotherapeutic effect of lastet can be experimental study on Remaxol (succinic acid, N-methylglucamine, riboxinum, methionine, nicotinamide) (Bezborodova *et al.*, 2013). The authors used mice tumor model of lymphatic leukemia P388 to show a decrease in the toxicity of lastet monotherapy that was injected at the dose of 100 mg/kg along with Remaxol, which was associated with an increase in the lifespan by 27.2%. At the same time, the effectiveness of the treatment depends on the localization of the tumor, stage of the tumor growth, development of metastasis, associated pathologies, etc. The correct evaluation of these parameters provides satisfactory long-term results of the indicated chemotherapy (Barsukov *et al.*, 2015; Bychkov *et al.*, 2016; Zelenikhin *et al.*, 2016). However, in spite of the evident success of chemotherapy, an increase in the incidence rate of malignant neoplasms is observed, especially those that are

associated with dangerous damage of kidneys.

For the evaluation of toxic peculiarities of pharmaceutical agents and the viability of cells and tissues, different methods are widely used, including the method of the evaluation of acute and chronic toxicity at the preclinical stage of pharmacological studies (Khabriev, 2005). Besides, the methods that involve cellular and tissue cultures are used (Pinaev and Bagdanova, 2008; Pinaev, 2008; Paschenko *et al.*, 2010; Alieva *et al.*, 2008a). In the studies on the evaluation of the toxicity of herbal composition "Phytomorozko" (Nazarenko *et al.*, 2008b) and "herbal drugs" (Nazarenko *et al.*, 2008a), that exert frigoprotective effect and used for the treatment and prevention of freezing injuries, mice renal tissue culture was used as a study model (Alieva *et al.*, 2008b). The method of tissue culture was used for the evaluation of the safety of sodium fluoride in food products (Gromova *et al.*, 2007, 2008) and drinking water (Paschenko, 2003). The utilization of tissue cultures for the evaluation of the toxicity of pharmaceutical agents is more feasible than cell cultures because the former preserve cellular associations that are observed in the organism (Paschenko, 2006; 2016). The processes that develop in the tissue culture have a lot in common with physiological mechanisms of tissue regeneration (Paschenko *et al.*, 2019). The growth of tissue culture (*in vitro*) is an integrative indicator of the condition of the whole organism (*in vitro*), its capacity to adapt to unfavorable environmental factors (Zaalkind and Tsyppkin, 1971; Onischenko, 2004), including influence of toxic agents.

The aim of the present study was to evaluate the toxicity of cytostatics bleocin, lastet, and cisplatin that exerts the nephrotoxic effect on the renal tissue culture of mice.

The relevance of the study is explained by the fact that insufficient attention to the possible development of side effects in patients that receive antitumor therapy can reduce the favorable effect of the therapy and worsen the quality of life of patients.

2. MATERIALS AND METHODS

The tests were performed on white outbred male mice (18-20 g) that were kept in standard conditions of the vivarium (standard conditions according European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (msu.ru, 2019); confirming Protocol #01/04-19 date 24 April 2019 by Ethics committee of

Northern State Medical University of the Ministry of Healthcare of the Russian Federation). Renal tissue sampling was conducted during the decapitation of animals that were exposed to ether anesthesia. For the evaluation of the toxicity of antitumor drugs, the authors used the method of multiple plasma-free renal tissue cultures on the surface of perforated millipore filters, which allowed them to perform quantitative estimation of the degree of the toxic influence of the drugs on the cells of renal tissue of the laboratory animals (Paschenko, 1999; Alieva *et al.*, 2008a; Alper, 1980). The cultivation of the fragments of renal tissue was performed in test tubes in 1 ml of medium 199 with 5% embryonal serum. Preliminary, the kidneys taken from mice were separated from the capsule and divided into small fragments of tissue that were placed on the surface of perforated millipore filters (Millipore Corporation Belford MA 01730). Standard large filters were used for the preparation of small filters 7 mm in diameter. On the surface of each filter, 64 fragments of tissue ($0.2 \pm 0.1 \text{ mm}^2$) were arranged randomly. Each test tube contained one filter with fragments of tissues. The studied drugs were diluted (several series of dilutions) and introduced directly to the nutrient medium in the test tube with filter for the period of cultivation (6 days). The incubation was performed in a microbiological water-cooled thermostat "SZ-1125 M" (ngpedia.ru, 2019) at 38°C. The stands with tubes were placed to a thermostat at 2-3° inclination. The cultures that grew on the surface of the filters were cleaned in a saline solution, fixed in Bouin's fixative, and hematoxylin-eosin stained (Gromova *et al.*, 2008; Paschenko *et al.*, 2010). The filters were placed on microscope slides and covered with balsam and cover slides for further microscope investigation ("Biomed", (Biomed.ru, 2019) at x100 magnification.

For the evaluation of the areas of the grown cells colonies, the authors used statistical software ImageJ (Schneider *et al.*, 2012). The average size of the colonies around the tissue fragments varied within 0.30-0.05 mm². The graphs were drawn based on the conventional units of the size of cellular colonies defined by the software ImageJ (n=64. Conventional unit of 350,000 corresponded to 0.27 mm²; 300,000 – 0.238 mm²; 250 000 – 0.198 mm²; 200,000 – 0.159 mm²; 150,000 – 0.12 mm²; 100,000 – 0.079 mm²; 50,000 – 0.039 mm²).

Statistical processing of the results was performed with Microsoft Excel and the software package Statistica 6. The parameters were compared with Student's t-test, the level of significance was $p < 0.05$.

The evaluation of the toxicity of different doses of the drugs (inhibiting effect) was performed by the comparison of the areas of grown colonies and the areas of the colonies from the control test tubes that contained the same amount of nutrient medium but did not contain the studied drugs. Totally, 3 series of tests were performed.

The 1st series of tests included bleocin (15 mg flasks; "Hunnon Kayaku Co." Ltd, Japan). The drug was 2, 4, 8, and 16 times diluted in the Hanks' solution to obtain 50%; 25%; 12% and 6% solutions, respectively. The control probes were introduced the same volume of Hanks' solution. Thus, the content of bleocin in 1 ml of medium contained 0.0037 mg/ml; 0.00187 mg/ml; 0.00097 mg/ml and 0.00047 mg/ml, respectively.

The 2nd series of tests included lastet (5 ml flask with 100 mg of the active ingredient; "Hunnon Kayaku Co." Ltd, Japan). The test design and conditions of lastet cultivation were the same as for bleocin. The drug was 2, 4, 8 and 16 times diluted (50%; 25%; 12% and 6% solutions, respectively). Final content of the drug in 1 ml of medium corresponded to 0.0083 mg/ml; 0.0042 mg/ml; 0.0021 mg/ml and 0.00104 mg/ml.

The study of the toxicity of cisplatin (10 ml ampules with 10 mg of the active ingredient; "LENS-Pharm", Russia) showed that it had higher toxicity than bleocin and lastet. For this reason, the dynamics of the influence of cisplatin on renal cells was evaluated with more significant dilutions of the drug. The 3rd series of tests included 4, 6, 8, and 32 times dilutions of cisplatin in Hanks' solution, which corresponded to 25%; 12%; 6% and 3% solutions, respectively.

3. RESULTS AND DISCUSSION

The obtained results were presented in graphs (Fig. 1; 2). The tests showed that cytostatics bleocin and lastet cause dose-dependent inhibition of the quantity of renal tissue culture in mice (Fig. 1). The presented data show that the maximum statistically significant inhibition of the growth of renal tissue culture was observed after two-fold dilution (50% solution) of bleocin and lastet. A direct impact of lastet on the renal tissue culture was less toxic than bleocin. A similar effect of inhibition (without statistical significance) of the amount of cell culture was obtained with a higher dose of lastet (4.3 times) than bleocin, in particular, 0.0042 mg/ml of lastet and 0.00097 mg/ml of bleocin. It should be noted that a decrease in the concentration of these drugs in the medium led to the differences in their toxicity, except for 6% solutions of lastet and

bleocin, where the amount of cell culture remained similar (without statistical significance). In this dilution, the area of cells colonies exposed to bleocin was 279,120 relative conventional units (CU), which was lower than the control values by 12.8% (312,480 CU); the area of colonies exposed to lastet was equal to 270,500 CU, which was lower than the control values by 15.5%.

Figure 1. The influence of bleocin* and lastet** on the growth of renal tissue in the dilution: 1-2 control; 3-4-(6%); 5-6-(12%); 7-8 (25%); 9-10 (50%) solutions. X – rows of studies; Y – quantitative content of cells in relative units
 *(Alieva *et al.*, 2008b; Alper, 1980; Bezborodova *et al.*, 2013; Gozhenko *et al.*, 2010; Gromova *et al.*, 2007)
 ** (Alieva *et al.*, 2008a; Barsukov *et al.*, 2015; Bychkov *et al.*, 2016; Gromova *et al.*, 2008; Dzhumabaeva and Birykova, 2015)

Statistically significant inhibition of the renal cells population size in comparison with the control values in the series with bleocin was also registered in the samples №5 (12% solution) and №7 (25% solution), and in the series with lastet, it was registered only in the samples №8 (25% solution) and №10 (50% solution). There were no statistically significant differences registered in the samples №6 (12% solution) and №4 (6% solution) in comparison with the control samples.

The results of the test series with cisplatin showed that the studied drug exerts a high toxic effect (Fig. 2). In this test series, the area of the grown colonies in the control probe was equal to 230,000 CU. The drug inhibited the growth of cell culture in all the solutions with bleocin and lastet that were tested. In tests with the minimum dilution of the initial dose of cisplatin to 3% solution (32 times), the authors registered an expressed statistically significant decrease (by

85%) in the size of the renal cells population in comparison with the control values. In this sample, the area of the grown colonies corresponded to the size equal to 65,000 CU. In the sample with a 6% solution, the area of the grown cells colonies was even lower and was registered at the level of -10000 CU. Further, when the dose of the studied drug was raised to 12% and 25% solutions, the cell colonies count was not performed due to their absence.

Figure 2. The influence of cisplatin on the growth of renal tissues in the dilutions: 1 – control; 3- (3%); 5- (6%); 7-(12%); 9- (25%); 11- (50%) solutions. X – rows of studies; Y – quantitative content of cells in relative units

The tests on the toxicity of bleocin and lastet by the method of renal tissue culture in mice allowed the authors to register the nephrotoxic effect of the mentioned cytostatics after direct contact with the culture in the respective doses. The extrapolation of the obtained results from animals to humans allows the authors to predict the development of a wider range of side effects in patients that receive bleocin than in those that receive lastet, which requires a more precise individual dosing in clinical practice. Especially, this is true in cases when bleocin or lastet are used in chemotherapies with high doses. Taking into account the factor of nephrotoxic effect, for the treatment of tumors of the same form and localization and a lack of contraindications, the therapy of choice would likely be lastet than bleocin. This conclusion fully agrees with a wide-known statement that plant-derived drugs (in this particular case, lastet) are less toxic for the organism than the synthesized ones. Although lastet is a synthesized derivative of natural podophylotoxin that exerts an antiproliferative effect on cancerous cells, it is toxic for the surrounding tissues only in high doses.

The analysis of the results of the test series with cisplatin showed its expressed toxicity. The only sample that allowed the authors

to perform a statistically significant comparative analysis of the results of the inhibition of the cell culture for all the tree drugs was the sample that was introduced the drugs as 6% solutions. In the samples with lower dilutions (12% and 25%), there was no technical possibility to evaluate the area of the grown population because of their complete absence. In the sample with the studied drugs in the doses that corresponded to 6% solution in the test series with bleocin, the area of the grown colonies was equal to 279,120 CU; lastet – 270,000 CU, and cisplatin - only 10,000 CU. A registered acute decrease in the size of mice renal cells culture colonies revealed a high toxic effect of cisplatin on renal tissue. The obtained result is highly predictable because cisplatin is a platinum-containing compound. This element exerts not only antiproliferative effect used in the treatment of oncologic diseases but also a high general toxic effect on the organism. As a rule, metal-containing compounds circulate in blood plasma for a long time, they are excreted via kidneys, get accumulated there, and harm renal function. This fact should be taken into account before the indication of metal-containing compounds to patients with renal pathology.

4. CONCLUSION

It can be concluded that the method of tissue culture with quantitative evaluation of the growth of tissues is a highly-sensitive method of the evaluation of the toxicity of antitumor drugs and provides more precise individual dosing of drugs used in chemotherapy in clinical practice. The use of mice renal tissues, that preserve intercellular associations, as a study model for the evaluation of the toxicity allowed the authors to provide a precise interpretation of the obtained study results in respect to the human organism and to confirm nephrotoxic effect in bleocin, lastet, and cisplatin, expressed in different degrees. The comparison of the areas of the grown colonies in the control samples and in the test series with different doses of the drugs (bleocin and lastet – 50%, 25%, 12% and 6% solutions; cisplatin – 25%, 12%, 6% and 3% solutions) allowed the authors to reveal the differences in the inhibiting influence of cytostatics. The test results did not reveal any difference in the renal cells colonies size after direct contact of the culture with bleocin and lastet in low doses (6% solutions). However, as the concentration of both drugs increased, the authors registered a statistically significant difference in the size of the grown colonies. Bleocin exerted a more expressed inhibiting

effect than lastet on the renal culture. The highest nephrotoxicity was registered in cisplatin in all the possible dilutions. The obtained results agreed with the available published data and allowed the authors to provide additional proof of the nephrotoxicity in the studied cytostatics when they get in direct contact with the renal tissue.

1. For the inhibition of the growth of cells renal tissue culture in mice by 50%, the dose of lastet should be 4.3 times higher than the dose of bleocin.

2. The reduction of the size of the grown mice renal tissue colonies by 85% is registered after the dose of cisplatin that corresponds to 3% solution.

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