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Original Paper

Cytostatic Therapy on Tumor Bearing Mice: Biochemical and Hematological Aspects

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REZUMAT

Terapia cu citostatice la șoareci putători de tumoră: aspecte biochimice și hematologice

În acest studiu am folosit un model experimental alografă cu carcinom și melanom pe șoareci C57BL/6. Am administrat trei substanțe citostatice (ifosfamida, carboplatina, clorhidrat de epirubicină) în scopul observării modificărilor parametrilor biochimici și hematologici. De asemenea, am măsurat evoluția masei tumorale în timpul experimentului. Studiul a fost realizat pe un numar de 77 de șoareci, C57BL/6, cu greutatea de 20 – 22 g, timp de două luni.

Cuvinte cheie: model murin de tip alografă, citostatic, carcinom, melanom, parametrii biochimici și hematologici

ABSTRACT

In this study we used a carcinoma and melanoma allograft experimental model on C57BL/6 mice. We administrated three chemotherapy substances (ifosfamide, carboplatin, epirubicin hydrochloride), in order to observe changes of the biochemical and hematological parameters. Additionally, the evolution of tumor mass was measured during the experiment. The study was conducted on a total of 77 mice with a weigh between 20 – 22 g, during two month.

Key words: allograft murine model, cytostatic, carcinoma, melanoma, biochemical and hematological parameters

INTRODUCTION

The animals used in research as experimental models are selected for genetic, anatomical and physiological similarities. Rodents are the most representative experimental model; to emphasize this statement mice are used due to human DNA similarity (According to The Jackson Laboratory over 95% of the mouse genome is similar to our own, making mouse genetic research particularly applicable to human disease), simplicity in procurement, handling and short lifespan [1-3].

The purpose of this information is to help investiga-

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tors who pursue an economical and efficient research that still allows the achievement of the study goals [4-7].

In terms of experimental models for such studies (carcinogenesis, xenograft and allograft models), each comes with benefits and limitations. Some chemical agents may have broad-spectrum effects in mouse models. These may offer many leads on the general mechanisms of cellular alterations as well as aspects of specific tissue features.

For clinical tumors the xenograft model should be used because this involves the transplantation of tumor tissue to another immunosuppressant individual. The advantage is that the same disorders are reproduced and also the mutation occurs, the deficit is the lack of an immune response, which would not recreate the actual situation of the donor.

We used the allograft transplantation model, which allows us to do the tumor tissue transplantation between individuals with similar genome, like the strains of mice. The tumor tissue is accepted by the immune system due to genetic similarities and allows us to monitor the changes that occur in tumor mass, metastasis and survival rate. The advantage is the presence of the immune system, which reflects better the real-tumor microclimate. The disadvantage is that transplanted tumor tissue is not entirely representative for the complexity encountered in clinical tumors [8-10].

The tumors of our study are carcinoma and melanoma, maintained by periodic passage.

Basal cell carcinoma grows from the basal cells of epidermis. The tumor has the appearance of single or multiple well defined nodules, 1 – 10 cm in diameter, ulcerated without healing, who continue with skin. Basal cells penetrate into dermis; the cells are small, uniform, with oval tahichromatic nuclei and with an intense basophilic cytoplasm; numerous mitotic figures are captured. Several histological types have been described: cells arranged in cords, the solid type, the medusoid type, the adenoid type and the cystic type. The tumor grows slowly, producing local invasion; metastases do not occur.

Melanoma grows from dendritic cells derived from neuroectodermal melanoblasts localized in the skin, fingers, eyes, lips and oral cavity. Pigmentation is not a specific feature as other tumor lesions may be pigmented and some melanomas are amelanotic. Other melanomas may be dome-shaped, smooth, sessile mode, polypoid, looking like a lobe mass. The large tumors frequently ulcerate.

Tumor cells might take epithelioid or fusiform shape. Tumors with junctional activity manifest as cell cultures made up from 3 – 20 ovoid cells and highly pigmented skin, located in the vicinity of the dermal-epidermal junction. From these groups of melanocytes come loose clumps which extend at various distances into dermis and become elongated, fusiform. Tumor has clear demarcation from healthy tissue. Histological pattern varies with the amount of pigment in the cytoplasm of tumor cells. Large nucleoli appear and cytoplasm is transparent and abundant. Melanoma grows rapidly and produces invasion, relapses and generates metastases in regional lymph nodes, lung and other organs. Inflammatory reaction overlaps the tumor [11].

Related to the cytostatic drugs used, ifosfamide and carboplatin are in the alkylating agents group, organic substances that work by releasing electrophilic compounds which bind covalently to DNA bases, determining crosslink between two strands of DNA or chain points that block DNA replication process. Alkylating agents work in all phases of the cell cycle [12].

Platinum salts have the mechanism of action similar to alkylating agents, acting on cells only in G0 [13].

Antitumor antibiotics derived from microorganisms, anthracyclines such as epirubicin hydrochloride, which act by intercalation between DNA base pairs, stopping the DNA replications and/or transcription.

Experimental studies in cell cultures have shown that epirubicin hydrochloride penetrates quickly the cells and is found in the nucleus where it inhibits nucleic acid synthesis in mitosis [14].

The biochemical parameters analyzed are: GGT – gamma-glutamyltransferase, ALB – albumin, TP – total protein, CHOL – cholesterol, ALT – alanine-aminotransferase, AST – aspartate-aminotransferase, ALP – alkaline phosphatase, CREAT – creatinine, DBILI – direct bilirubin, TRI – triglycerides, GLUPAP – glucose, Ca – calcium, Mg – magnesium.

The hematological parameters: WBC – white blood cells, Ne – neutrophils, Ly – lymphocytes, Mo – monocytes, RBC – erythrocytes, Hb – hemoglobin, HTC – hematocrit, PLT – thrombocytes [15].

MATERIALS AND METHOD

We used 77 C57Black/6 conventional mice (Mus – musculus), males and females, weighting between 20 – 22 g and with age between 3 – 4 months, from Animal Husbandry Department of National Institute of Research – Development in the Pathology Domain and Biomedical Sciences "Victor Babeş" Bucharest.

Experiments were approved by the internal Bioethical Committee. Animals handling and care followed the protocol and the institutional guidelines.

The mice were kept in the following conditions: temperature 22 ± 2 C°, humidity 55 ± 10%, artificial ventilation, lighting 12/12 – day/night, feed and watering ad libidum with special pelleted for mice (produced at Cantacuzino National Institute), filtered and sterilized water. All mice were kept under a strict cleaning and sanitation.

The tumor transplant was carried out from mice bearing tumor, regular maintained in our laboratory by in vivo passage.

Subcutaneous inoculation of 0.5 mL per mouse of these cell suspensions:

- melanoma cells suspended in saline solution had a cell concentration of 3.36 x 10^8 cells;
- carcinoma cells suspended in saline solution had a cell concentration of 2.26 x 10^8 cells.

A pilot study was conducted in order to find the work doses for: epirubicin hydrochloride (LD50=64 mg/kg), Carboplatin (LD50=150 mg/kg) and ifosfamide (LD50 = 397 mg/kg) and consisted in administrating the lethal dose (LD50) to obtain our work doses (therapeutic latitude). For each dose we developed a lot of mice. For epirubicin hydrochloride we used a first dose of 32 mg/kg and a second one of 16 mg/kg, for carboplatin the first dose was 100 mg/kg and the second dose was 50 mg/kg and for ifosfamide we used 360 mg/kg and 180 mg/kg.

The anticancer drugs for carcinoma and melanoma were administrated intraperitoneally in unique dose. We also measured the tumor mass longitudinally and transversally. Regarding the control group, we use control for substances (lots 01 – 03), control for tumors (lots 1 and 2) and mice control (lot C) (**Table 1**).

The analysis probe for hematology was about 100 μL of blood (on EDTA), collected three times for each individual from the retro-orbital venous plexus after a short sedation with acepromazine and performed at the automatic hematology analyzer Hemavet 950 FS. The probe for biochemical analysis was obtain after the mice were euthanized, about 200 μL of blood (without EDTA), and performed at ACE Automated Chemistry Analyzer Systems (Alfa Wassermann).

Statistical analysis

The results were processed using Microsoft Excel and have been expressed as mean ± standard error of the mean. Comparison between groups was made using T test with unequal variances. We considered that differences between groups were statistically significant if $p < 0.05$. Processing and statistical analysis was performed using the software package Excel Analysis Tool Pack (Microsoft Office 2007).

Establishing tumor diameters was performed with a caliper between second and third sampling and represented an estimation of tumor mass (we measured the tumor mass longitudinally – d_L and transversally – d_T). The measured value was expressed in millimeters and was used to calculate the average diameter ($d_M = d_L + d_T/2$), radius ($d_M/2$) and the tumors volume ($4/3\,\pi r^3$). The results were processed using the software package Excel Analysis Tool Pack (Microsoft Office 2007).

RESULTS

Lot 3 – Dose 1 carboplatin for carcinoma was

Table 1. The structure of mice lots in the experiment

Lots	Purpose	No. mice/ identification	Tumor type	Procedures performed
C	Control	8 / 1,2,3,4,5,6,7,8	----	H1*, H2*, H3*
01	Carboplatin control	3 / 011, 012,013	----	H1, C adm*, H2, H3
02	epirubicin hydrochloride control	3 / 021, 022,023	----	H1, C adm, H2, H3
03	ifosfamide control	3/ 031, 032, 033	----	H1, C adm, H2, H3
1	Carcinoma control	6/ 11, 12, 13, 14, 15,16	Carcinoma	H1, Tr*, H2, H3
2	Melanoma control	6/ 21, 22, 23, 24, 25, 26	Melanoma	H1, Tr, H2, H3
3	Dose 1, carboplatin	4/ 31, 32, 33, 34	Carcinoma	H1, Tr, C adm, H2, H3
4	Dose 2, carboplatin	4/ 41, 42, 43, 44	Carcinoma	H1, Tr, C adm, H2, H3
5	Dose 1, epirubicin hydrochloride	4/ 51, 52, 53, 54	Carcinoma	H1, Tr, C adm, H2, H3
6	Dose 2, epirubicin hydrochloride	4/ 61, 62, 63, 64	Carcinoma	H1, Tr, C adm, H2, H3
7	Dose 1, ifosfamide	4/ 71, 72, 73, 74	Carcinoma	H1, Tr, C adm, H2, H3
8	Dose 2, ifosfamide	4/ 81, 82, 83, 84	Carcinoma	H1, Tr, C adm, H2, H3
9	Dose 1, carboplatin	4/ 91, 92, 93, 94	Melanoma	H1, Tr, C adm, H2, H3
10	Dose 2, carboplatin	4/ 101, 102, 103, 104	Melanoma	H1, Tr, C adm, H2, H3
11	Dose 1, epirubicin hydrochloride	4/ 111, 112, 113, 114	Melanoma	H1, Tr, C adm, H2, H3
12	Dose 2, epirubicin hydrochloride	4/ 121, 122, 123, 124	Melanoma	H1, Tr, C adm, H2, H3
13	Dose 1, ifosfamide	4/ 131, 132, 133, 134	Melanoma	H1, Tr, C adm, H2, H3
14	Dose 2, ifosfamide	4/ 141, 142, 143, 144	Melanoma	H1, Tr, C adm, H2, H3

Order of procedures:
H1* = harvesting no.1 for hematology analyses
Tr.* = tumor tissue transplantation
C adm* = cytostatic administration in a single dose after 10 days
H2* = harvesting no.2 for hematology analyses, 24 hours after cytostatic administration
H3* = harvesting no.3 for hematology and biochemistry analysis, 15 days after administration

administered in dose of 100 mg/kg and caused increases in: WBC, Mo, Ca, TP, ALT, ALP, GLUPAP and decreases in: RBC, Hb, HTC, PLT, ALB, DBILI, TRI. In the first 24 hours after administration we observed a temporary Ne increase. Tumor mass reduction was observable (**Chart 1**).

Lot 4 – Dose 2 carboplatin for carcinoma was administered in dose of 50 mg/kg and, in the first 24 hours after administration, we observed increases in: WBC, Ne, Ly, Mo, RBC, Hb, HTC, PLT; these parameters decreased then to the end of the experiment. Biochemical parameters presented higher levels for: ALT, AST, GLUPAP, and lower levels for: Ca, Mg, ALB, TP, CREAT, DBILI, TRI, CHOL. The tumor mass reduction was considerable (Chart 1).

Lot 5 – Dose 1 epirubicin hydrochloride for carcinoma was administered in dose of 32 mg/kg and caused higher levels of: WBC, Ne, Mo, GGT, ALT, AST, GLUPAP and TRI. In the first 24 hours after administration, Ly and PLT had lower levels but gone up until the end of the experiment. Lower levels had been observed for: RBC, Hb, HCT, Mg, Ca, ALB, TP, ALP, CREAT, DBILI, CHOL. In addition, the reduction of the tumor mass was considerable (**Chart 3**).

Lot 6 – Dose 2 epirubicin hydrochloride for carcinoma was administrated in dose of 16 mg/kg and caused in the first 24 hours after administration lower levels for WBC and Ly. Ne increased during the experiment. Decreases had been observed for: RBC, Hb, HCT, PLT. Biochemical parameters presented higher levels for: GGT, ALT, AST, ALP, GLUPAP,TRI and lower levels for: Mg, Ca, TP, ALB, CREAT, DBILI, CHOL. The tumor reduction was considerable, too (**Chart 3**).

Lot 7- Dose 1 ifosfamide for carcinoma was administrated in dose of 360 mg/kg and decreased in the first 24 hours WBC and PLT levels; these parameters increased for the rest of the experiment. A slight increase of Mo and Ne was noticed. Ly levels increased at the beginning but expressed a constant level afterwards. Biochemical parameters with low activity are: Mg, ALB, TP, DBILI, TRI, CHOL, and high activity on GGT, ALT, AST, GLUPAP, also the tumor mass reduction was significant (**Chart 5**).

Lot 8 – Dose 2 ifosfamide for carcinoma was administrated in dose of 180 mg/kg and caused in the first 24 hours initially a short increase of WBC and PLT, then lower levels. Ly decreased at the first determination and after that expressed a tendency to increase. Decreases had been observe for: RBC, Hb, HCT. Biochemical parameters increased for: GGT, ALT, AST, ALP, GLUPAP, TRI and decreased for: Mg, ALB, TP, CREAT, DBILI, CHOL; we noticed a considerable tumor reduction (**Chart 5**).

Lot 9 – Dose 1 carboplatin for melanoma was administrated in dose of 100 mg/kg and caused in the first 24 hours increases for: WBC, Ne and Ly, followed by their decrease for the rest of the experiment. RBC, Hb, HCT and PLT

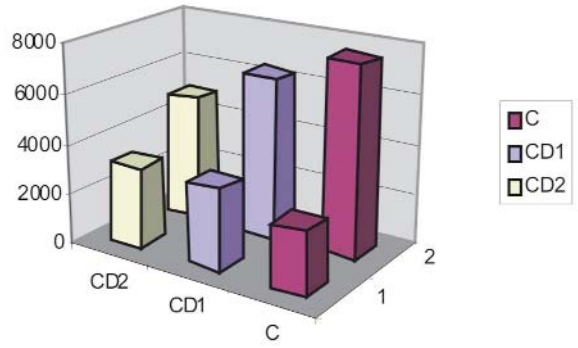

Chart 1. Carcinoma evolution after carboplatin administration. Tumor mass reduction is more significant for the smaller dose.
C – control carcinoma; CD1 – 100 mg/kg; CD2 – 50 mg/kg; 1 – first measurement; 2 – second measurement.

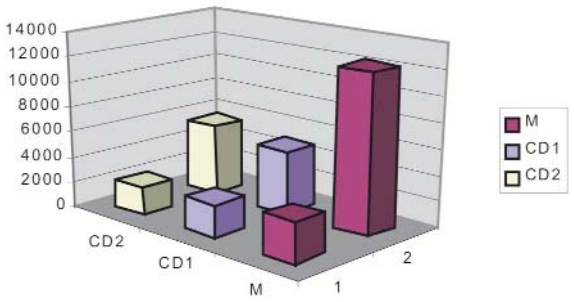

Chart 2. Melanoma evolution after carboplatin administration. Tumor mass reduction is more significant for the higher dose.
M – control melanoma; CD1 – 100 mg/kg; CD2 – 50 mg/kg; 1 – first measurement; 2 – second measurement.

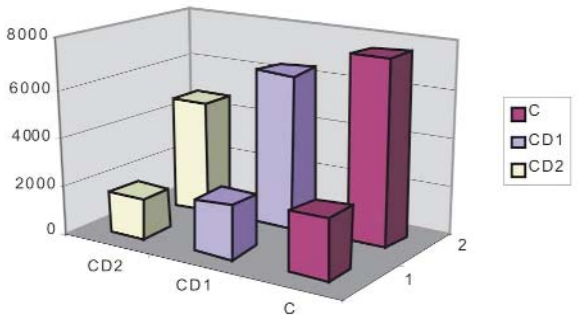

Chart 3. Carcinoma evolution after epirubicin hydrochloride administration. Tumor mass reduction is more significant for the smaller dose. C – control carcinoma; CD1 – 32 mg/kg; CD2 – 16 mg/kg; 1 – first measurement; 2 – second measurement.

presented lower levels. Biochemical parameters had been increased for: GGT, ALT, AST, ALP and decreased for: Mg, Ca, ALB, TP, CREAT, DBILI, GLUPAP and TRI. The tumor reduction was considerable. (**Chart 2**).

Lot 10 – Dose 2 carboplatin for melanoma was administrated in dose of 50 mg/kg and caused in the first 24 hours increased levels of: WBC, Ne, Ly, Mo; but the levels

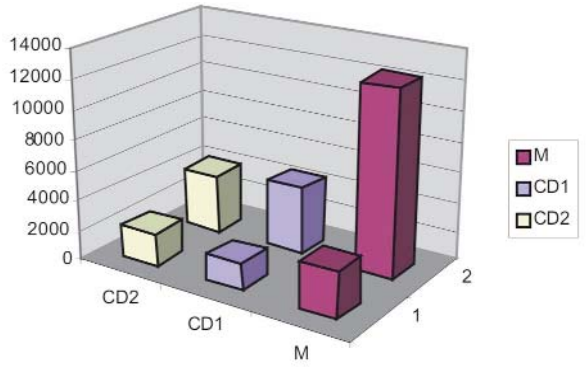

Chart 4. Melanoma evolution after epirubicin hydrochloride administration. Tumor mass reduction is more significant for the smaller dose. M – control melanoma; CD1 – 32 mg/kg; CD2 – 16 mg/kg; 1 – first measurement; 2 – second measurement.

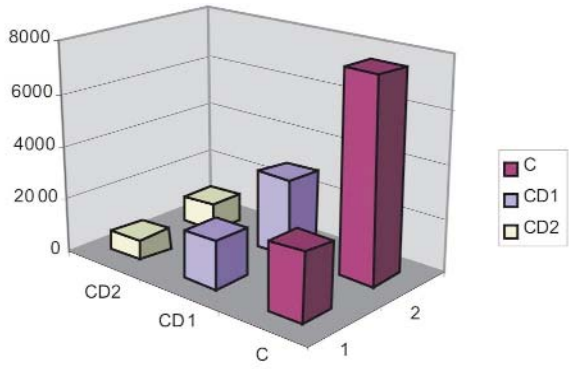

Chart 5. Carcinoma evolution after ifosfamide administration. Tumor mass reduction is more significant for the smaller dose. C – control carcinoma; CD1 – 360 mg/kg; CD2 – 180 mg/kg; 1 – first measurement; 2 – second measurement.

dropped for the rest of the experiment. Modifications appeared in RBC, Hb, HCT and PLT, too, which presented lower levels. Biochemical parameters: GGT, ALT, AST, GLUPAP and TRI increased. Lower levels had been reported for: Mg, Ca, ALB, TP, CREAT, DBILI, CHOL and also a considerable tumor reduction (**Chart 2**).

Lot 11 – Dose 1 epirubicin hydrochloride for melanoma was administrated in dose of 32 mg/kg and caused in the first 24 hours decreases for: WBC, Ne, Mo, Ly, with an increase tendency for the rest of the experiment. The RBC, Hb, HCT and PLT levels were low. Biochemical parameters had higher levels for: ALT, AST, ALP, GGT, TRI and lower levels for: Mg, Ca, ALB, TP, CREAT, DBILI, GLUPAP, CHOL. Tumor reduction was observed (**Chart 4**).

Lot 12 – Dose 2 epirubicin hydrochloride for melanoma was administrated in dose of 16 mg/kg and caused in the first 24 hours after administration Ly decrease and increases for the rest of the experiment for: WBC, Ne, Mo. Lower levels had been observed for: RBC, Hb, HCT, PLT. Biochemical parameters decreased for: Mg, Ca, ALB, TP, ALP, CREAT, DBILI, GLUPAP, CHOL and increased for: GGT, ALT, AST, TRI. Tumor mass reduction was significant (**Chart 4**).

Lot 13 – Dose 1 ifosfamide for melanoma was administrated in dose of 360 mg/kg and caused in the first 24 hours an increased value of WBC and decrease of Mo; for the rest of the experiment WBC decreased and Mo increased. Ne presented high levels during the hole experiment. The rest of the hematological parameters were decreased all the time: Ly, RBC, Hb, HCT, PLT. Biochemical parameters had higher levels for: GGT, ALT, AST, DBILI, GLUPAP and lower levels for: Mg. Ca, ALB, TP, ALP, CREAT, TRI, CHOL. In this lot we observed the most obvious tumor reduction (**Chart 6**).

Lot 14 - Dose 2 ifosfamide for melanoma was administrated in dose of 180 mg/kg and generated in the first 24 hours level reductions for WBC and PLT; in the next analysis the two parameters grew along with Ne and Mo. The rest of the parameters decreased: Ly, RBC, Hb, HCT. The biochemical parameters with increased activity were: GGT, ALT, AST, DBILI, GLUPAP, TRI, the rest of them presented decreased activity: Mg, Ca, ALB, TP, ALP, CREAT, CHOL. Significant tumor mass reduction (**Chart 6**).

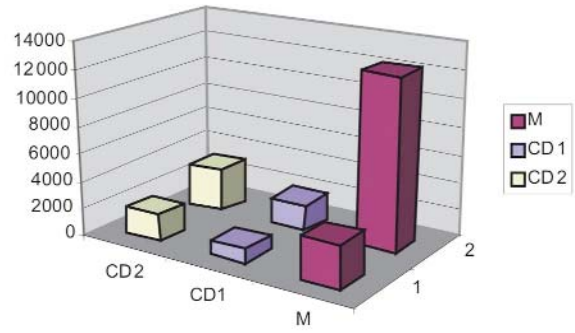

Chart 6. Melanoma evolution after ifosfamide administration. Tumor mass reduction is more significant for the higher dose. M – control melanoma; CD1 – 360 mg/kg; CD2 – 180 mg/kg; 1 – first measurement; 2 – second measurement.

DISCUSSIONS

Carboplatin administrated to conventional mice determine decreased levels of albumin, total protein, alkaline phosphatase, direct bilirubin and creatinine, reported to the control group (control mice) and increased levels of aspartate-aminotransferase and glucose.

For hematology parameters we observe decreases of hemoglobin, hematocrit and thrombocytes.

Comparing mice with tumor without chemotherapy

with mice treated with carboplatin, we observe the following:

- significant reductions for aspartate-aminotransferase and alkaline phosphatase;
- glucose – reduction.

The type of tumor did not influence the biochemical parameters. The increase or decrease direction of parameter values were the same for both types of tumors treated with carboplatin.

Epirubicin hydrochloride administered to conventional mice registered relevant decreases for albumin, alanine-aminotransferase, direct bilirubin and creatinine, compared to the control mice, and increased gamma-glutamyltransferase and aspartate-aminotransferase. For hematology we notice an increase of the white blood cells, neutrophils, monocytes (in case of melanoma), and decrease for hemoglobin and hematocrit. We also observe that epirubicin hydrochloride is the most aggressive agent against erythrocytes compared with ifosfamide and carboplatin, who also cause anemia.

Comparing mice with tumor without chemotherapy with administration of epirubicin in mice with tumor, we observed the following:

- alkaline phosphatase and glucose decreases in both types of tumors;
- significant increases only in carcinoma for gamma-glutamyltransferase, aspartate- aminotransferase and alanine-aminotransferase.

Ifosfamide administered to conventional mice registered relevant decreases for alkaline phosphatase and glucose reported to the control group and increased albumin and direct bilirubin; white blood cells, lymphocytes and monocytes were suppressed. Also we observed an increase for thrombocytes.

Comparing mice with tumors not receiving chemotherapy with mice with tumors treated with ifosfamide, we observed as follows:

- increase activity for direct bilirubin and gamma-glutamyltransferase;
- decreased activity for alanine-aminotransferase, aspartate-aminotransferase and alkaline phosphatase.

Despite the progress in development of new therapeutic methods for the treatment of tumor diseases, specifically, target therapy, making use of monoclonal antibodies, the treatment of cancer patients remains a serious problem. The main causes of difficulties are immunogenic activity of drugs, heterogeneity of the tumors, and stimulation of autoimmune reactions. That is why the classical chemotherapy is going to remain the base of drug therapy of this patient population for a long time. However, cytostatic therapy is not a guarantee of cure and does not rule out the development of relapses and metastases. The administration of maximally tolerable doses of antitumor drugs for as complete as possible suppression of tumor growth, leads to the development of cytostatic disease and damage to the rapidly regenerating cell systems [16].

The establishment of anticancer therapy is accompanied by alterations of the digestive system. The effect of the digestive system in the mentioned conditions has important implications upon the patients' nutritional status, as it limits the ability to provide the required nutritional support, the absorbtion of nutritional principles and their metabolism.

The objectives of the cancerous disease's nutrition are represented by the ability to provide the caloric and nutritional requirements which are able to improve the patient's clinical status, to increase the effort capacity, to extend the survival duration and to amplify the capacity to tolerate chemotherapy [17].

Among other approaches for cancer therapy, the new concept of theranostics offers different strategies in terms of cancer diagnosis and local, personalized therapy.

The targeted therapy of tumors will provide a significant decrease of the therapeutic agents in terms of side effects due to smaller doses that will be used, resulting a well bearing of the treatment by the patient. The new approach is currently under preclinical studies with promising results but still under developing [18,19].

CONCLUSIONS

We observe that melanoma cells are more resistant to cytostatic than carcinoma cells due to higher dose that cause a more significant tumor mass reduction. The carcinoma cells respond better to the smaller dose. The exception is epirubicin hydrochloride, who proved us that is efficient for both tumors, in the smaller dose.

Carboplatin behaves closed to epirubicin hydrochloride regarding tumor mass reduction but with doses variation like ifosfamide. However, ifosfamide is the most efficient cytostatic from the perspective of tumor mass reduction and for the impact on the organism.

The use of cytostatic agents is well known that has a great impact on the organism in terms of anemia, immunosuppression, hepatotoxicity, nephrotoxicity as the results has showed. Additional therapies should be taken into account for sustaining the altered organism functions.

It is important to take into account the current state of the patient and to decide which cytostatic agent suit him the best, with a better tumoral response to therapy and reduce adverse effects. The present work enriches the information needed to optimize the therapy in relationship with the current state of the patient.

Conflict of interests

The authors declare that there are no conflict of interests.

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