Study on the effect of photostimulation in the methotrexate therapy of Walker 256 tumour

Studiu privind efectul fotostimulării în terapia cu metotrexat a tumorii Walker 256

Valentin Budascu[1,3], Radu Fumarel[2], Ana M. Coman[2], Maria Crivineanu[3,*], Diana M. Alexandru[3]

1. National Agency for Zootechnics 'Prof. Dr. G. K. Constantinescu', Baloteşti, Romania
2. The Institute of Oncology 'Professor Doctor Alexandru Trestioreanu', Bucharest, Romania
3. University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine, Bucharest, Romania

*Corresponding author: maria_crivineanu@yahoo.com

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Rezumat

Scopul acestui studiu este acela de a evidenţia efectul favorabil al fotostimularii obţinut în terapia cu metotrexat asupra tumourii Walker 256. Metoda fotostimulării tumourale selective urmareşte creşterea concentraţiei în ţesutul malign a oricărei substanţe cu specificitate pentru celula neoplazică administrabilă "in bolus" în vederea tratamentului citostatic. Obţinerea acestui deziderat se poate realiza prin expunerea ţesutului tumoural în câmpul optic emis de către un echipament special conceput în acest sens. Pe de altă parte, în cazul chimioterapiei, există anumite tipuri de citostatice ce prezintă un aşa-zis "potential fotodinamic pozitiv", astfel încât, în urma interacţiei dintre moleculele acestora si radiaţia luminoasă cu parametrii strict determinaţi, iau naştere radicali liberi ce distrug ţintele infracelulare de fixare ale citostaticului. Acest fenomen poartă numele de activare fotodinamică a citostaticului, iar metoda se numeşte Chimioterapie Fotostimulată (PSChT), iar medicamentul utilizat în acest studiu este Metotrexatul (MTX).

Abstract

The aim of this study is to highlight the favorable effect of photostimulation obtained in the methotrexate therapy on Walker 256 tumour. The method of selective tumour photostimulation follows the increase, in the malignant tissue, of the concentration of any specific substance for the neoplastic cell that can be administered "in bolus" for cytostatic treatment. This goal is achieved by exposing the tumour tissue to the optical field emitted by a specially designed equipment. On the other hand, in the case of chemotherapy, there are certain types of cytostatics that have a "positive photodynamic potential", thus, following the interaction between their molecules and the light radiation with the strictly determined parameters, free radicals are born that destroy the intracellular cytostatic fixation targets. This phenomenon is called the photodynamic activation of the cytostatic, the method is called Photostimulated Chemotherapy (PSChT), and the drug used in this study is Methotrexate (MTX).

Introduction

Every year, hundreds of thousands of patients around the world are diagnosed with cancer and a significant percentage of them lose their fight with this disease. Thus, in this study, the authors aim to take a small step forward on this front, developing an innovative therapeutic protocol by combining classical chemotherapy with selective photostimulation of neoplastic tissue, with the purpose of increasing the concentration of cytostatic in the malignant cell.

Photostimulation is a physical method that uses a light source that has the property of artificially activating biological components, cells, tissues and even microorganisms. Given this fact, we selected a cytostatic agent, namely Methotrexate (MTX), which has the ability to photoinduce DNA chain alterations (9), to test the efficacy of this treatment scheme on Walker carcinoma 256 (6). Methotrexate is a cytostatic antineoplastic of the antifolate type that acts by a mechanism of competitive inhibition of its

reductase, a mechanism by which it interferes with the de novo synthesis of nucleic acids and with cell replication in the tissues with active proliferation (7, 8).

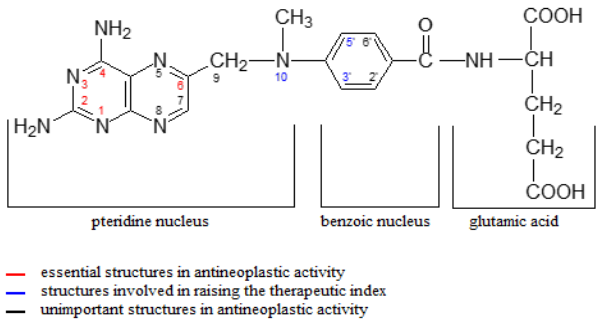

Figure 1. Structure of the Methotrexate molecule (10)

As shown in Figure 1, the Methotrexate structure has a pteridine nucleus in the composition, which gives the cytostatic molecule the ability to get involved in various photochemical processes. The highlighting of the photodissociation mechanisms, according to the literature data, was performed using the spectral absorption and emission (fluorescence) analyzes of some saline solutions (pH = 8.5 adjusted with NaOH) of methotrexate with concentrations between 10^{-4} - 10^{-5} M. These solutions were subjected to the optical irradiation generated by the field of conventional generators (mercury vapor lamp) for various time intervals between 1 and 20 minutes (3, 4, 5).

For this study we evaluated comparatively two therapeutic schemes and their efficiency on Walker 256 tumour, which is an important experimental model, widely used in the development of new therapies, due to the aggressive biological behavior, the capacity of loco-regional invasion and the increased metastatic potential (1, 2).

Material and Methods

Research into cancer biology in general and experimental oncology in particular could not have been realized without the main biological work material, namely the animals used for experimental purposes. Thus, the experiment was performed on a number of 30 male Wistar rats, whose body weight ranged between 90-120 g. These animals were transplanted, subcutaneously, in the dorsal region, a tumour graft from a Walker 256 tumour donor, ascitic form. The donor comes from the same species, line and sex as the recipient. The donor animal was anesthetized, and we collected ascitic cells, which we suspended in physiological serum with an antibiotic (Gentamicin). Then, the solution obtained was centrifuged for 4 minutes at 2500 rpm. We collected the obtained sediment and suspended it in the physiological serum to obtain a concentration of 5 X 10^6 viable tumour cells / 1 ml solution. The solution obtained was centrifuged and we inoculated the sediment subcutaneously into the recipient animal. In order to determine the viability of the tumour cells, vital staining with trypan blue (0.2 in TFS) was performed. The living cells remained uncolored, and the dead cells appeared colored in violet blue. To determine the number of cells in a known amount of fluid, we used the Turk hemocytometer. It should be noted that in the tumour suspension to be inoculated to the recipient animal, cell mortality should not exceed 10% of the total number of cells in the suspension. The inoculated animals were followed daily to capture the macroscopic progression of the tumour. Thus, for 70% of the inoculated rats, we detected this phase around the interval of 12-16 days after inoculation, the rest of 30% rejected the graft.

The tumor on which this experiment was performed comes from the biobank of experimental tumors of the Oncological Institute, Bucharest. This tumor is an important experimental model, widely used in the development of various therapies due to its biological behavior similar to human carcinoma tumors. Walker 256 tumor was discovered by Walker in 1928 in the mammary gland of a pregnant female rat and it continued to be transplanted over time, both in ascitic and solid form, subcutaneously. After the tumour was

detected, the animals were divided into 3 uniform lots, respectively:

- Group I - 10 animals treated with MTX,
- Group II - 10 animals treated with MTX + photostimulation,
- Group III - 10 animals for control.

The treatment schedule for group I consisted of intratumoural inoculation of an amount of 0.5 mg / 100g MTX, at an interval of 14/30/60 / days.

For group II, the treatment consisted of intratumoural inoculation of an amount of 0.5 mg /100g MTX, and after 60 minutes, followed by irradiation of the tumour, at a distance of 10 cm, for 10 minutes, at an interval of 14/30/60/ days. The equipment used for irradiation of the tumour is based on the generation of ultraviolet radiation. The power supply is made at a mains voltage of 220 v 50 HZ with a current absorbed by A. The lamp starts after a build-up time necessary for the filament to reach the optimum temperature for emitting ultraviolet radiation.

The third Group (control group), represents a standard against which we report the results obtained in the other lots, and includes animals that are not given any treatment scheme.

The animals were evaluated daily in terms of temperature, weight and tumour volume, until their exitus. During the experiment, animals from all three groups were sacrificed to track the effectiveness of the therapy on tumour evolution. From these, representative tissue fragments were collected for the oncological pathology and were histologically examined within the Pathological Anatomy discipline of the Faculty of Veterinary Medicine Cluj-Napoca. For this purpose, the fragments of tumours, lungs, liver, spleen and lymph nodes were fixed for 24 hours in 10% formalin buffered solution, embedded in paraffin and sectioned to a thickness of 2-3 µm, with a RM 2125 RT manual microtome (Leica Biosystems). The sections obtained from each sample were automatically stained with Hematoxylin-Eosin (H&E).

Results and Discussions

Following the daily examination of the Wistar rats from the 3 experimental groups, we found that the body weight of the animals had an upward curve in the animals in group II until day 80, compared with the group I whose curve was ascending until on day 60, then it started to descend. Also, in group III, it was found that by day 30 post inoculations, the curve was ascending, and after this day, there was a sharp decrease, most likely due to the evolution of tumours, the appearance of metastases and the installation of the cachexia. The tumour volume (Vt), evaluated in this study, was calculated based on the formula $Vt = D \times d^2 \times 0.4$, where "D" is the large diameter of the tumour and "d" is the small diameter. If at the beginning of the experiment Vt was, on average 0.5-1 cm^3, in all three groups, after the first therapeutic session we found a slowing of the tumour growth evolution in the two experimental groups compared to the control. We also found differences in the mean tumour volume between the two experimental groups, in the sens that group II had a lower mean Vt compared with group I, which indicates that MTX + photostimulation therapy has a much more intense cytostatic effect (as shown in table 1).

Table 1.
Tumour volume and weight reported during the evolution period
(Vt – tumour volume, G – weight, E - exitus)

Group	No.	Tumoral volume (Vt) and weight (G)							
		14 day after inoculation		30 day after inoculation		60 day after inoculation		80 day after inoculation	
		VT (CM)	G(G)	VT (CM)	G(G)	VT (CM)	G(G)	VT (CM)	G(G)
	1	0.5	160	0.57	215	0.16	190	E	E
	2	0.7	155	1.58	210	0.12	195	E	E
	3	0.9	165	7.2	250	E	E	E	E
	4	0.8	170	6.58	240	3.29	210	5.83	185

I	5	0.7	180	7.84	250	8.78	E	E	E
	6	1.0	178	10.092	240	5	199	14.04	150
	7	0.7	95	3.4	145	22.4	130	11.88	E
	8	0.8	100	7.26	157	10.09	160	72	110
	9	0.5	96	3.03	150	10.09	110	E	E
	10	1.0	100	13.23	150	E	E	E	E
II	1	0.5	185	1.76	200	0.19	210	0.0036	290
	2	1	190	9.8	230	2.4	260	0.09	280
	3	1	180	5.11	219	0.61	250	0.07	300
	4	1	190	8.28	230	0.01	270	E	E
	5	1	185	5.25	240	2.30	300	E	E
	6	1	170	4.65	210	0.32	250	0.098	E
	7	0.7	104	3.17	140	1.17	142	0.15	300
	8	0.5	100	1.33	135	0.58	140	0.018	230
	9	0.9	90	2.75	139	0.55	142	0.036	170
	10	1.0	100	13.23	150	E	E	E	E
III	1	0.6	100	7.3	169	E	E	E	E
	2	1.0	110	9.41	187	E	E	E	E
	3	1.0	120	9.41	170	E	E	E	E
	4	0.7	150	8.10	200	E	E	E	E
	5	0.8	150	8.07	150	E	E	E	E
	6	0.5	155	9.08	170	E	E	E	E
	7	0.5	150	0.74	150	15.19	130	E	E
	8	0.8	140	1.81	145	E	E	E	E
	9	0.7	150	5.75	155	48.02	125	E	E
	10	0.9	150	4.28	165	129.47	145	E	E

The histopathological examination complements and supports the aforementioned observations. Thus, the histological preparations from group I attest that the neoplastic formation is well delimited, unencapsulated, partially infiltrative and intensely cellular (fig. 2). The neoplastic lesion is made up of polymorphic cells, arranged in the form of nests and small islands, separated by a fine connective-vascular stroma. The neoplastic cells are round, oval and polygonal, with a weak acidophilic cytoplasm, vacuolar at times and with moderate nucleus / cytoplasm ratio. The nuclei are round-oval, centrally arranged, with fine granular chromatin and 1-3 conspicuous nucleoli. Anisocytosis and anisocaryosis are moderate, with aspects of cariomegaly. Occasionally, some cells have a fusiform appearance. The number of mitotic divisions ranges from 2 to 7 / 40x field, some with atypical (bizarre) appearance. Multifocal, intratumoural, numerous foci of necrosis were present (fig. 3), associated with hemorrhage, edema and discrete neutrophilic infiltrate. No neoplastic lesions were identified in the examined lymph nodes (fig. 4) nor in the visceral organs studied.

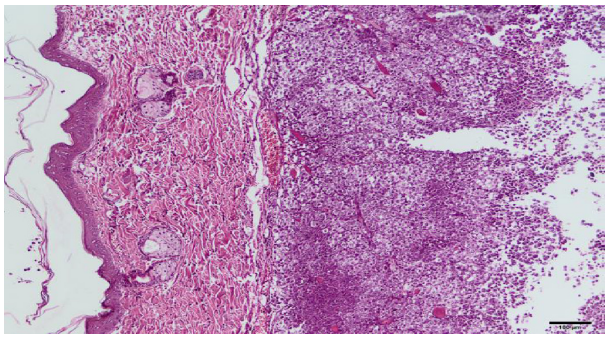

Figure 2. Group I - rat, subcutaneous Walker 256 tumour, well delimited, unencapsulated, partially infiltrative and intensely cellularized. HE Col.,Bar=100µm

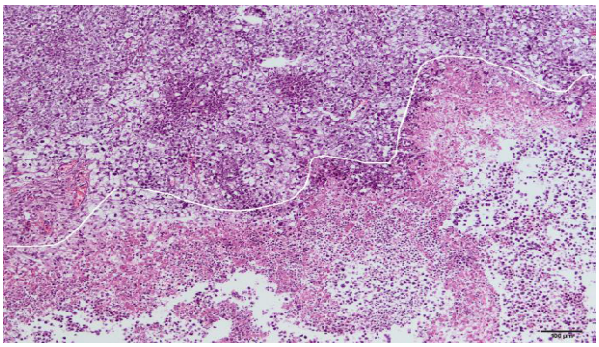

Figure 3. Group I - rat, Walker 256 tumour, numerous foci necrosis that occupy about 40-50% of the tumour surface (red, acidophilic areas). The solid white line indicates the junction between the normal tissue (upper area of the image) and the area of necrosis (lower part of the image). HE Col., Bar = 100 µm

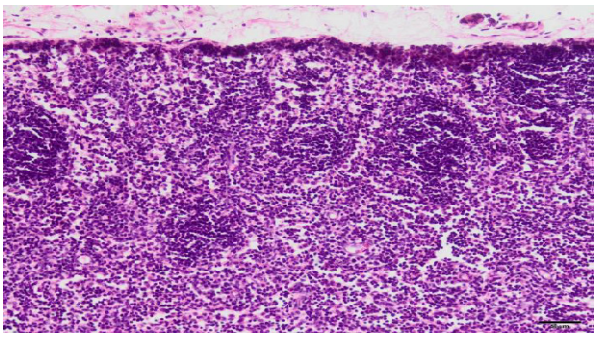

Figure 4. Group I, rat, axillary lymph node, specific cellular appearance, without the presence of tumour metastases. HE Col., Bar = 100 µm

The histopathological picture of the tumour, in the case of group II has the following characteristics is much more suggestive and attests a significantly higher efficiency of the therapy used. Thus, at the subcutaneous level, was observed the presence of a neoplastic formation, well delimited, not encapsulated, partially infiltrating in the muscular and intensely cellular tissue. The morphological characteristics of the tumour formation are similar to those described previously. Multifocal, intratumoural, numerous foci of necrosis (fig. 5) and dystrophic mineralization were present. In the examined lymph nodes, as well as in the visceral organs (fig. 7, fig. 8), no neoplastic structures were observed (fig. 6).

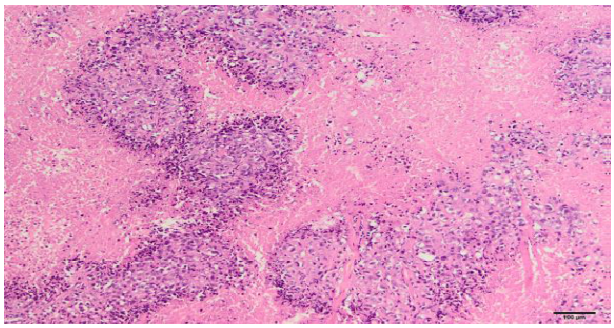

Figure 5. Group II, rat, Walker 256 tumour, numerous foci of necrosis occupying approximately 60-70% of the tumour surface (red, acidophilic areas) Col HE, Bar = 100 µm.

In group III, compared to the other groups, at the same interval of time after the inoculation, was observed the appearance of tumour metastases at the pulmonary level (fig. 9), and at the tumour level, a massive infiltration capacity of the tumour on the surrounding tissues was observed (fig. 10).

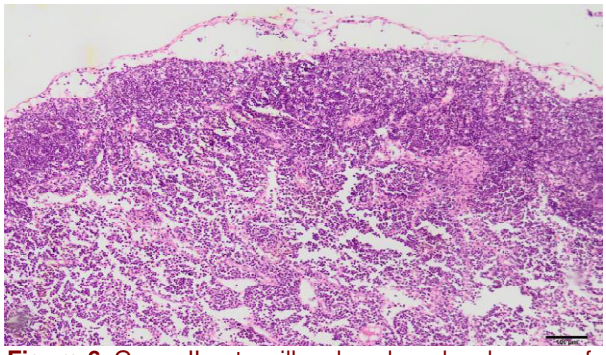

Figure 6. Group II, rat, axillary lymph node, absence of neoplastic lesions, Col. HE, Bar = 100 µm.

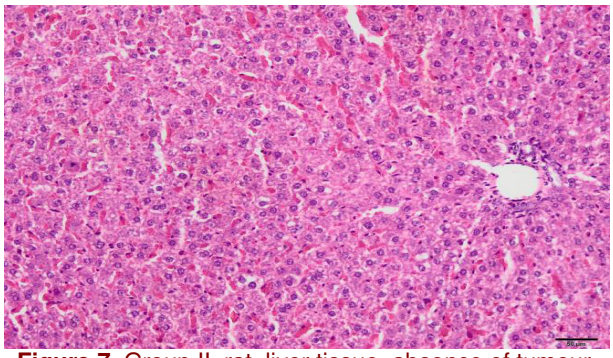

Figure 7. Group II, rat, liver tissue, absence of tumour cells in parenchyma, Col. HE, Bar = 100 µm.

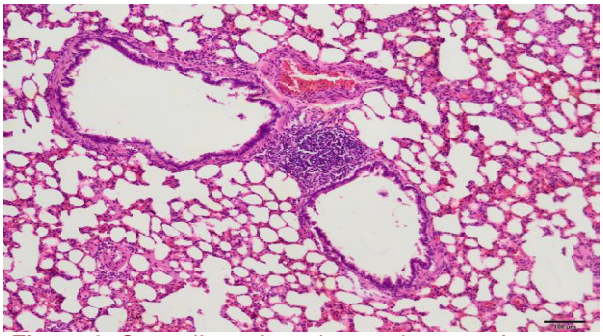

Figure 8. Group II, rat, lung tissue, absence of tumour cells in parenchyma, Col. HE, Bar = 100 µm.

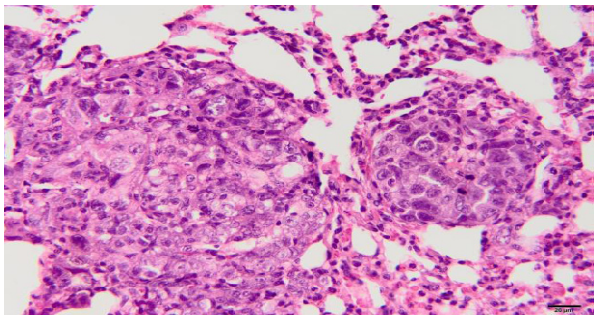

Figure 9. Group III, rat, lung tissue, numerous nodular structures composed of polymorphic neoplastic cells compatible with Walker tumour, Col HE, Bar = 20 µm

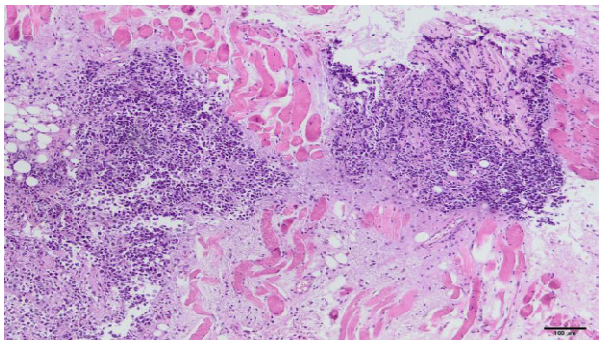

Figure 10. Group III, rat, tumour tissue infiltrated into the adjacent muscle tissue, col. HE. Bar = 100 µm

Conclusions

Using cytostatics combined with photostimulation with ultraviolet radiation in tumour therapy, a reduction in their size was observed.

Following the experiment, an increase in the effectiveness of anticancer chemotherapy was observed by combining it with photostimulation even in the advanced stages of tumors.

This could help reduce the dose of cytostatics and thus its side effects. The costs of therapy could be reduced given that a cheap chemotherapeutic has been associated with a simple method of photostimulation.

Both the quality of life and the survival time of the animals subject to the mentioned protocol were superior to the classical treatments.

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