

***In vivo* Effects of CortiNon+ on the Emergence and Progression of Experimental Graffi Tumor in Hamsters**

Reneta Toshkova¹, Ignat Ignatov^{2*}, Elisaveta Zvetkova³, Georgi Gluhchev⁴ and Georgi Dinkov⁵

¹*Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences (BAS), Sofia, Bulgaria.*

²*Scientific Research Center of Medical Biophysics, Sofia, Bulgaria.*

³*Bulgarian Association of Bioreology, Institute of Mechanics, Bulgarian Academy of Sciences (BAS), Sofia, Bulgaria.*

⁴*Institute of Information and Communication Technologies, Bulgarian Academy of Sciences, Sofia, Bulgaria.*

⁵*IDEALABS, Limited Liability Company (LLC), Washington 20036, USA.*

Authors' contributions

This work was carried out in collaboration among all authors. Authors RT designed the study. Author GG performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors RT and II managed the analyses of the study. Authors RT and EZ managed the literature searches. All authors read and approved the final.

Article Information

Editor(s):

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Complete Peer review History: <http://www.sdiarticle4.com/review-history/56203>

Original Research Article

Received 02 March 2020

Accepted 07 May 2020

Published 11 May 2020

ABSTRACT

Results from a three arms pilot study on the preventive and therapeutic effects of the food supplement CortiNon+ on the development of Graffi myeloid tumor in hamsters have been presented. Biometric parameters of tumor growth and peripheral blood count were evaluated. Two schemes of experimental oral formulation of antitumor therapy were applied ne started 7 days

*Corresponding author: E-mail: mbioph@abv.bg, mbioph@dir.bg;

before tumor transplantation and a second one that started simultaneously with the tumor transplantation. The control 3rd group did not receive any oral supplementations. The results demonstrated protective antitumor effect of CortiNon+, expressed by decrease of transplantability and lowering lethality, inhibition of tumor growth and increase of survival rate of the treated animals compared to the untreated ones. The efficacy of the experimental therapy was more pronounced when it was started 7 days before transplantation of the tumour cells. Also, differences in hematological parameters were registered between the groups. Presented results suggest that CortiNon+ is a promising drug candidate for treatment of haematological malignancies

Keywords: Graffi myeloid tumor; cortinon+ therapy; tumor growth parameters.

1. INTRODUCTION

The spread of the cancer diseases permanently increases in the world irrespectively of the significant advancement of the medicine and the obtained achievements in tumor genesis understanding. Curiously enough, the most developed countries like USA and Canada are in the front line. The major cancer therapies as radiation and chemotherapy usually lead to a significant increase in the survival time but together with this, they may seriously harm the patient. This is the reason to pay attention to the products that may delay or even stop the tumor's development. Having in mind that the initiation and development of tumors are mainly provoked from oxidative stress, the investigations are aimed at the development of bioproducts acting as immunomodulators and/or tumor suppressors. The electrochemically activated water is one of the leaders in this direction Ignatov, Gluhchev, [1], Komatsu et al., [2], Shirahata, Hamasaki, Teruya, [3], Toshkova et al., [4,5]. The suggestions of the oncologists for specific diets are usually based on their common understanding about the food properties [2]. Progesterone (Pr) and dehydroepiandrosterone (DHEA), the major constituent of supplement CortiNon+ have a number of desirable properties such as anticancer Blagosklonny, Neckers, [6] Check et al., [7,8], anti-inflammatory Ignatov et al., [9,10], Matsuzaki, Honda, [12] antigluco-corticoid, antimicrobial, antiviral, anti-proliferative and chemopreventive immunomodulatory and etc. Jiang et al., [11], McNelis et al., [12], Schwartz, Pashko, [13], Yahya et al., [14], Yoshida [15].

To our best knowledge there are no reports about the antitumor action of the food supplement CortiNon+ offered in the market. The purpose of this study is to examine the influence of the supplement CortiNon+ on the initiation, growth and development of experimental Graffi myeloid tumors in hamsters.

2. MATERIALS AND METHODS

2.1 Experimental Animals

Breed "Golden Syrian" hamsters, aged 2 - 4 months, male and female, weighing around 100 g, grown in individual plastic cages with free access to food and water, bred in the vivarium of the Institute of Experimental Morphology, Pathology and Anthropology with Museum (IEMPAM) at the Bulgarian Academy of Sciences (BAS) were used in the trials. All experiments were conducted in accordance to the ethical standards of the institutional guidelines for care and use of laboratory animals.

2.2 Experimental Tumor Model

This study used an animal cancer model based on the Graffi solid tumor, a type of murine myeloid tumor. The experimental Graffi solid tumor is maintained in vivo in hamsters on a monthly basis from the research team at IEMPAM-BAS [4] via subcutaneous (s.c.) transplantation of live tumor cells (1-2.10⁶) in the area of the back. Between days 7 and 15 after transplantation tumors appeared at the spot of injection, growing progressively, and hamsters died 30 - 35 days after the injection. For this tumor model 100% transplantability of tumor and 100% mortality rate have been observed. Spontaneous regression, i.e. spontaneous shrinking and disappearance of the tumor has been not reported [4].

2.3 Chemical Composition of CortiNon+

CortiNon+ was kindly provided by Dinkov (IdeaLabs, LLC, Washington, USA). CortiNon is a combination of the steroids progesterone (Pr) (200 mg/ml concentration) and dehydroepiandrosterone (DHEA) (25 mg/ml concentration) in solvent (ratio was 1:1), containing vitamin E, triglycerides and saturated fatty acids.

2.4 Experimental Groups

Hamsters were randomly assigned to 3 experimental groups as follows:

- Gr1: Hamsters, subcutaneously injected with 5×10^4 Graffi tumor cells receiving 18 doses totally (x^2 drops of CortiNon+ / per hamster), once a day - 7 doses before and 11 doses after tumor cell transplantation.
- Gr2: Hamsters, subcutaneously injected with 5×10^4 Graffi tumor cells receiving 12 doses totally (x^2 drops of CortiNon+ / per hamster), once a day, concomitantly starting with tumor transplantation.
- Gr3: Tumor carrier hamsters subcutaneously injected with 5×10^4 Graffi tumor cells without treatment (control group).

2.5 Experimental Design

The pre-treatment of hamsters from Gr1 with CortiNon+ started 7 days before tumor transplantation. On the 7th day of the pre-treatment, 5×10^4 Graffi tumor cells were injected subcutaneously into the hamsters of Gr1, Gr2, and Gr3. Following peripheral blood count and tumor parameters were measured and analyzed.

- Hematologic parameters were reported on the blood cell counter BC-2800 Vet (Mindray, China) on the 10th and 20th days after tumor transplantation.
- Biometric parameters of tumor growth: tumor appearance/transplantability (%), tumor size (mm), duration of survival and average survival (days), and mortality (%) were measured. Transplantability % was defined as number of hamsters which developed tumor versus total number of tumor cells transplanted hamsters. Tumor size (mm) determined by measuring two mutually perpendicular diameters of the tumor (A-width and B-length) with a caliper at specified intervals after transplantation of tumor cells and their averaging. Mortality / death rate - Percentage of dead hamsters relative to the total number of hamsters in the group. Average Survival Time (MST) - calculated in days for the respective group and individual group survival schedule.
- Photographic material: pictures of necropsied animals taken on the 10th day after tumor transplantation were presented.

2.6 Statistical Analysis

All the data were expressed as mean \pm standard deviation (SD). The statistical significance

between the treatments was evaluated by one-way ANOVA and with Bonferroni's post hoc test using GraphPAD InStat, Software, USA. Values of * $P < 0.05$ were considered significant.

3. RESULTS

Two hamsters from every group were euthanized on the 10th and 20th day after the tumor cell transplantation. The obtained peripheral blood was used for the complete blood count analysis by BC-2800 Vet apparatus (Table 1) [5]. The remaining hamsters from all three experimental groups were monitored over time for biometric parameters of tumor growth.

3.1 Macroscopical Evaluation

Photos of hamsters presenting macroscopic evaluation before and after skin removal, obtained from two experimental groups (Gr1 and Gr2), as well as from the control group (Gr3), were demonstrated on the Fig. 1.

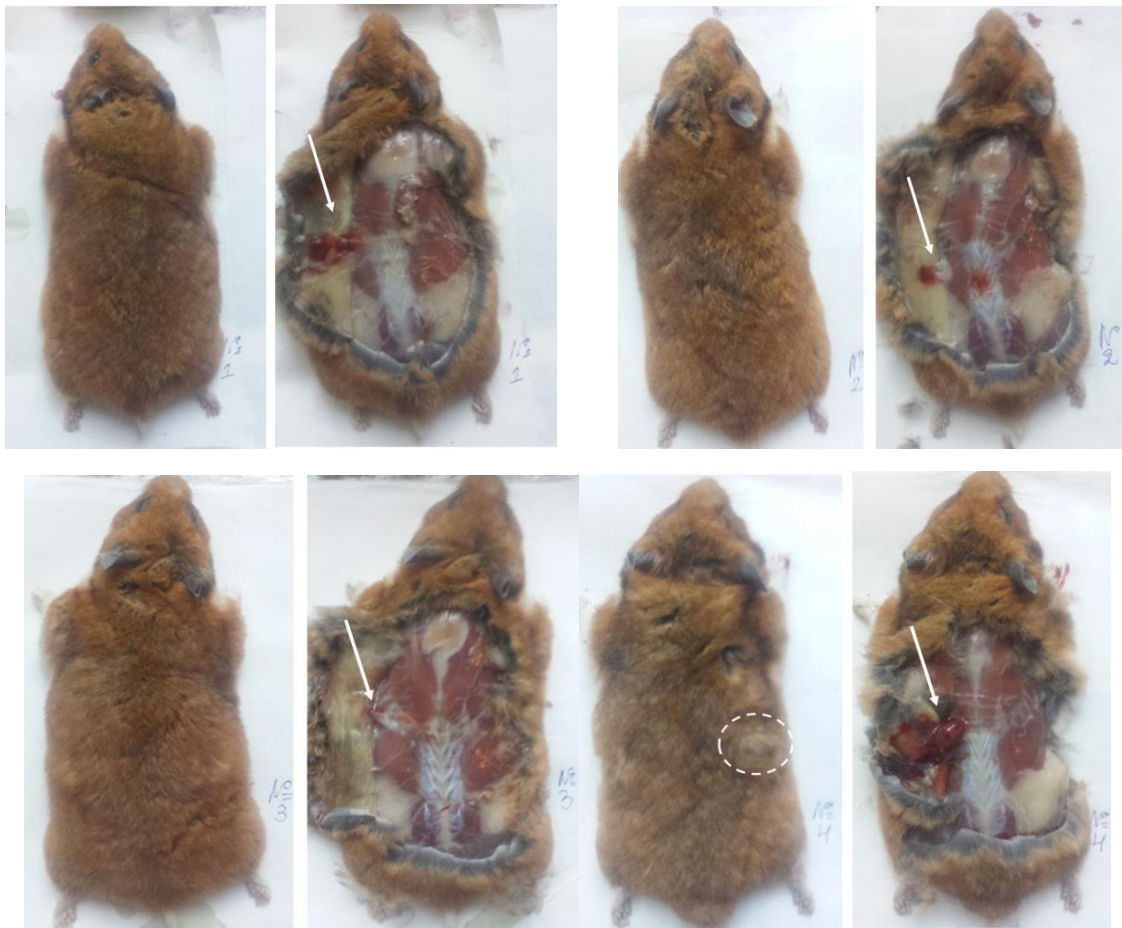
Fig. 1 images of hamsters from Gr1 (top row), Gr2 (middle row) and Gr3 (bottom row) on the 10th day after tumor cells transplantation. Left images from each pair were prepared before skin removal; right ones were taken after skin removal in the appearing tumor area on the back. Animals from Gr1 and Gr2 were treated with CortiNon+ as pointed in section 2.4. The white arrows indicate the site of tumor formation after skin removal. The white circles indicate the site of palpable tumor formation before the skin is removed.

Macroscopical evaluation. Palpation of the skin on the back of hamster from Gr1 reveals a small area (about 1-2 mm) of thickened skin (a sign of tumor formation) and after the skin removal a hyperemic area, with an initial tumor formation (nodule with the same size) has been observed. No thickened skin area was felt in second hamster, but after skin removal, a hyperemia was observed at the tumor cell injection site (Fig. 1, top row).

Palpating the skin on the back of hamster from Gr2 no thickened skin area was observed, but after skin removal an initial stage of skin hyperemia was established. In the other hamster a thickened skin area (tumor formation) of about 5 mm in size was registered, after removal of the skin in a zone of hyperemia, a grayish-white tumor of about 5 - 6 mm in size with well-developed blood vessels on its surface, could be visualized (Fig. 1, middle row).

Table 1. Blood count measurement on the 10th day and on the 20th day (red numbers) for all groups

Parameters	Units	Gr1	Gr1	Gr2	Gr2	Gr3	Gr3
		10 th day	20 th day	10 th day	20 th day	10 th day	20 th day
WBC	x10 ⁹ /L	3.8/2.4	8.7/9.6	4.2/3.1	10.2/15.2	2.8/1.9	2.7/12
Lymph	x10 ⁹ /L	2.9/1.5	3.8/3.6	1.6/2.0	5.6/8.4	1.0/1.4	1.6/5.4
Mon	x10 ⁹ /L	0.2/0.1	0.8/1.1	0.2/0.2	0.5/1.0	0.2/0.0	0.1/0.9
Gran	x10 ⁹ /L	0.8/0.8	4.1/4.9	2.4/0.9	4.1/5.8	1.6/0.5	1.0/5.7
Lymph	%	73.4/62.8	44/38	38.4/64.9	54.1/54	36.4/71.3	58.6/44.8
Mon	%	4.8/4.9	8.7/11	4.9/4.9	5.3/6.9	6.4/4.9	5.1/7.7
Gran	%	21.8/32.3	47.3/51	56.7/30.2	40.6/38.4	57.2/23.8	36.3/47.5
RBC	x10 ¹² /L	3.98/4.72	3.58/4.63	3.52/4.99	4.90/4.53	4.33/4.71	2.49/4.66
HGB	g/L	80/88	77/84	63/83	87/89	66/78	99/80
HCT	L/L	0.221/0.262	0.203/0.257	0.196/0.268	0.256/0.236	0.218/0.256	0.139/0.25
MCV	fl	55.6/55.6	56.8/55.6	55.7/53.9	52.3/52.1	59.5/54.4	56.1/53.7
MCH	pg	20.1/18.6	21.5/18.1	17.8/16.6	17.7/19.6	15.2/16.5	39.7/17.1
MCHC	g/L	361/335	379/326	321/309	339/377	302/304	712/320
RDW	%	12.4/11.2	14.2/13.9	13.6/13.1	13.7/15.2	12.2/12.9	11.5/12.6
PLT	x10 ⁹ /L4.5	168/293	158/208	366/247	412/188	245/66	132/347
MPV	f/L17.6	4.6/4.2	6.1/5.7	5.4/5.1	5.4/6.1	5.6/5.3	4.5/6.6
PDW	%	18.3/17.3	19.2/19.4	18.7/18.3	18.4/19.3	17.8/19.1	17.9/19.6
PCT	%	0.077/0.123	0.096/0.118	0.197/0.125	0.222/0.114	0.137/0.034	0.059/0.229



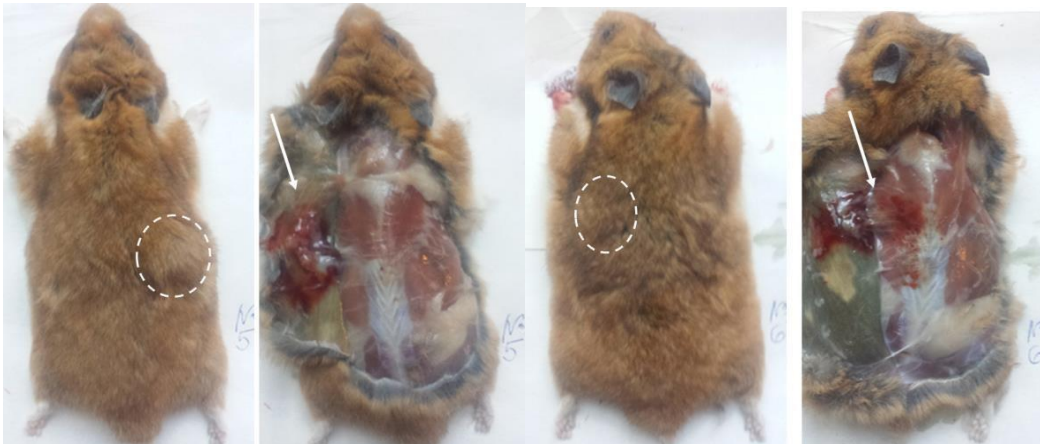


Fig. 1. Images of hamsters from Gr1 (top row), Gr2 (middle row) and Gr3 (bottom row) before and after the skin removal

Palpation of the skin on the back of hamsters from Gr3 reveals a tumor of size 8 - 12 mm, and after removal of the skin, large hyperemic areas with well-shaped tumor-nodules with grayish-white color were observed (Fig. 1, bottom row).

The results of the present pathological material (randomly selected hamsters from the three groups) show: In hamsters of Gr1 a tumor initiation phase was observed; in Gr2 -both initiation and onset of tumor development were observed, whereas in control hamsters of Gr3 the well-formed tumors were in advanced stages of development.

To follow the tumor development, the same analysis was also carried out on the 20th day after tumor cells injection. The macroscopic finding from the experimental (Gr1, Gr2) and control (GR3) groups on the 20th day from tumor cells transplantation showed the presence of subcutaneous solid tumors formation with no significant differences in size in all randomly selected animals from the three experimental groups.

3.2 Biometric Parameters of Tumor Growth

The in vivo antitumor effects of CortiNon+ on the Graffi myeloid tumor development in hamster model has been investigated by determining the main tumor growth parameters: Transplantability (%), tumor size (mm), mortality, average and individual survival time. Experimental and control hamsters were monitored daily.

Transplantability. The appearance of tumors was reported daily by palpation of the skin on the back of the hamsters from day 7 to day 20 after injection of tumor cells (each skin thickening area of at least of 2 – 3 mm size was reported as tumor appearance). The results are presented in Fig. 2.

The results show that between 40% and 100% of hamsters from Gr3, responded with the appearance of tumors in the 7th - 12th day interval. In hamsters of Gr2 tumors occurred in 30% to 100% of the animals, in the 8th to 14th day interval. The appearance of tumors in the hamsters of Gr1 was reported at 50% on the 11th and 100% on the 19th day, consequently the interval included the 11th to 19th day.

The latency period (onset of tumors) in hamsters of Gr3 is in the 7th-12th day range, in hamsters of Gr2 it was extended by 2 days, and in hamsters of Gr1 it was extended by 7 days. The conclusion of these observations was that the application of CortiNon+ delayed the appearance of tumors and extended the latency period to 19 days.

Tumor size (mm) it was defined as the average diameter between the longest diameter and its perpendicular. Values were measured with a caliper at regular intervals. The results were presented in the Fig. 3.

Tumor size in the treated and control groups were monitored regularly for 30 days after tumor cell injection. In hamsters from the control group the tumor size increased progressively (Fig. 3,

red line). The reported values are: 1.88 ± 1.9 mm at day 7, 4.38 ± 1.8 mm at day 12, 12.42 ± 6.2 mm at day 15, 19 ± 3.74 mm at day 20, 24.5 ± 3.41 mm at day 25, 27.33 ± 3.0 on day 30 of the study. The hamsters of Gr1 and Gr2 show a lag in the rate of tumor growth compared to Gr3. Tumor size values for Gr1 were lower than those reported for Gr2. No subcutaneous skin thickening area was palpated in the hamsters of Gr1 and Gr2 on the 7th day.

The dimensions of the remaining time intervals were as follows: 02.5 ± 1.8 mm for the 12th day, 8.3 ± 4.6 mm for the 15th day, 12.4 ± 5.48 mm for the 20th day, 14.87 ± 5.0 mm for the 25th day and 23 ± 4.53 mm on the 30th day for Gr1 and 3.6 ± 1.7 mm for the 12th day, 9.37 ± 3.37 mm for the 15th day, 14.75 ± 5.03 mm for the 20th

day, 18.6 ± 5.3 mm on the 25th day and 24.2 ± 3.2 mm on the 30th day for Gr2.

Upon examination of the back of the hamsters on the 10th day after tumor cell transplantation, well-developed subcutaneous tumors were discovered in all hamsters of Gr3, in 4 hamsters in Gr2 and in 2 hamsters of Gr1.

Upon examination of the hamster backs on the 20th day after tumor cell transplantation, well-developed subcutaneous tumors were discovered in all hamsters of the three experimental groups. However, it was evaluated that the average size of the tumors in hamsters from Gr1 was smaller in comparison with Gr2 and Gr3. This substantiates the expectation that CortiNon+ therapy can delay the tumor's growth.

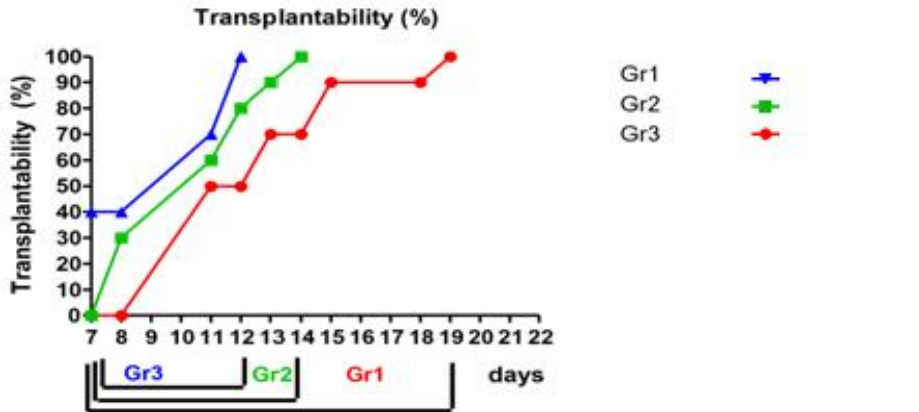


Fig. 2. Transplantability of tumors in hamsters from Gr1, Gr2 occurred after experimental treatment with CortiNon+. and from Gr3 (controls)

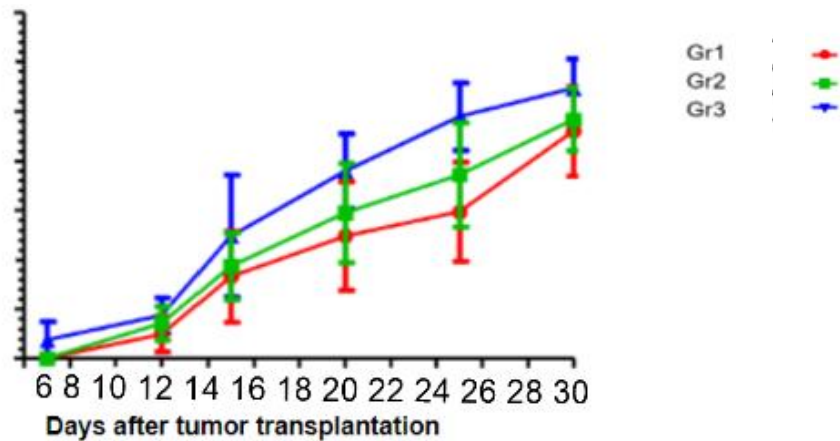


Fig. 3. Size (mm) of tumors in hamsters from Gr1, Gr2 after treatment with CortiNon+. and Gr3 (controls)

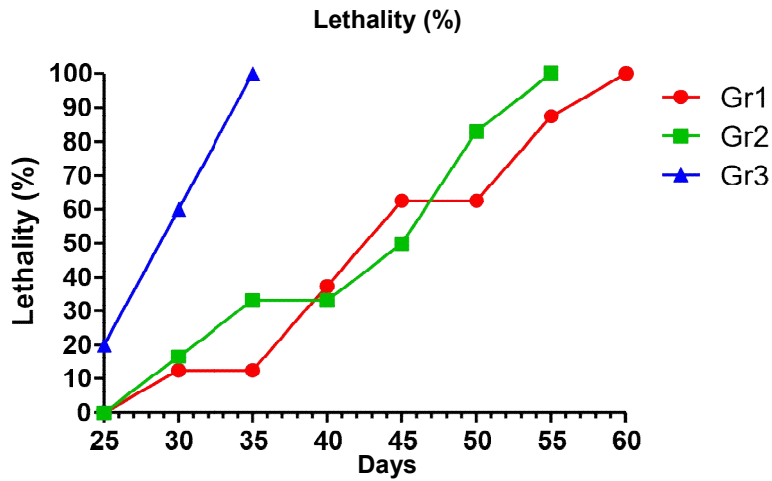


Fig. 4. Lethality (%) for Graffi tumor bearing hamsters after experimental CortiNon+ therapy.

Lethality (%) The lethality data for hamsters with experimentally induced Graffi myeloid tumor, after experimental CortiNon+ therapy have been presented in Fig. 4.

With respect to the lethality parameter, 100% mortality was reported on day 35, for the control group (Gr3). Such percent of mortality was achieved on the 51st day for Gr2, and on the 58th day for Gr1, respectively. A decreased rate of mortality was observed between the 25th and 60th days after tumor cell transplantation for the groups Gr1 and Gr2 compared to the control Gr3. The Gr1 and Gr2 mortality values are varying in a close range. The conclusion is that the administration of oral CortiNon+ therapy reduces mortality in tumor bearing hamsters.

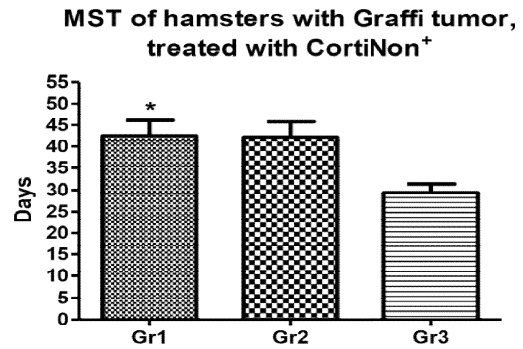


Fig. 5. Average survival (in days) of tumor bearing hamsters from three experimental groups (Gr1,Gr2 and Gr3). The star indicates statistically significant difference. * P<0.05

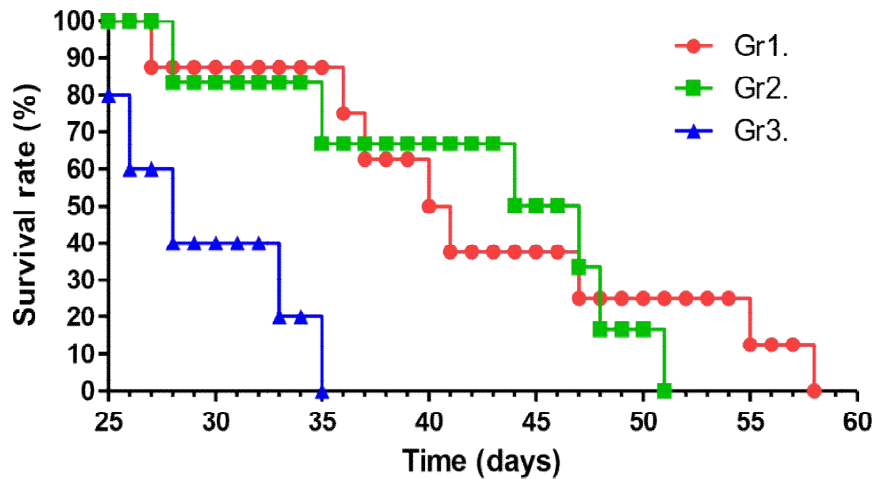


Fig. 6. Individual survival (in days) of tumor bearing hamsters was drawn with a Graph Pad Prism program, based on daily reported mortality of hamsters

The average survival and individual survival of tumor bearing hamsters (in days) were other parameters in close correlation with the above one (see Fig. 5 and Fig. 6).

The evaluated average survival for Gr1 was 42.5 ± 10.10 days, for Gr2 - 42.17 ± 8.84 days, and for Gr3 - 29.4 ± 4.39 days. A statistically significant difference ($p * < 0.05$) was obtained between the mean values of Gr1 and Gr3 using one-way analysis of variables (ANOVA), followed by Bonferroni's test using GraphPAD PRISM software, Version 5 (GraphPad Software Inc., San Diego, USA). In conclusion the median survival of the tumor bearing hamsters treated with CortiNon+ (Gr1 and Gr2) was prolonged, compared to the median survival of the untreated animals (Gr3). The graphs in Fig. 6 depicting the individual survival of the animals from the three groups support this conclusion.

Hamsters were identified as being alive or dead every day. Survival time (in days) of hamsters from Gr1 was longer than that of Gr2 and of control-Gr3. The increased survival of the hamsters from Gr1 and Gr2 reached the 58th day and the 51st day accordingly, while all animals from Gr3 lived no more than 35 days.

4. DISCUSSION

The results obtained in the current *in vivo* experiments show that the oral administration of CortiNon+ in hamsters with experimental Graffi myeloid tumor demonstrates a positive effect on the biometric parameters of tumor growth, namely increasing the tumor latency period up to 19 days, slowing-down the tumor growth, delaying mortality and increasing the median and individual survival in the treated groups compared to the untreated one. Also, the blood parameters have been influenced showing differences between the two experimental groups and control one. The results also give information that CortiNon+ acts as an immunomodulatory agent, raising the production of lymphocyte blood count on the 10th and 20th days for Gr1 and Gr2 compared to control-Gr3. Nevertheless, all the animals died. Thus, the antitumor effect of CortiNon+ needed explanation. We presumed that it might be associated with different levels of activation - non-selective immunomodulating activity (unpublished data), optimization of the oxidant-antioxidant balance, cytotoxic action on tumor cells by an apoptotic way [9,14] and etc. Recent evidence has also demonstrated that membrane progesterone receptors (mPRs)

mediate most of the non-classical progesterone actions Valadez-Cosmes [16].

The food supplement CortiNon+ is a combination of the steroids progesterone (Pr) and dehydroepiandrosterone (DHEA). A series of studies have shown that sex steroids exert a wide spectrum of pharmacological effects [6,7,8,17,11,13,16,18,19]. In the skin tumor model in mice, DHEA treatment inhibits tumor initiation, as well as tumor promoter-induced epidermal hyperplasia and promotion of papillomas [13]. Pharmacological doses of DHEA exhibit chemopreventive and anti-proliferative effects on malignant human cancer cell lines and some tumors in experimental animals. The growth of HepG2 and HT-29 cancer cells was significantly inhibited by DHEA, in a dose- and time-dependent manner [8,17,20]. Progesterone (Pr) containing compounds have been extensively used in the treatment of patients with early, advanced or recurrent endometrial cancer Francis et al. [18], Montz et al., [20]. Cytostatic and cytotoxic activities of sex steroids were also evaluated *in vitro* against ten human leukemia/lymphoma cell lines [6]. The influence of Pr on the autoimmune, infectious and malignant diseases via Pr-receptors (adaptive and innate immune effects) is examined in human leukemia [7,8].

We first in the scientific literature examined the influence of CortiNon+, a progesterone and dehydroepiandrosterone containing compound on the model of experimentally induced myeloid Graffi tumor in hamsters, evaluating that the treatment has a positive effect on tumor appearance and growth, and improves survival rate of tumor-bearing animals.

5. CONCLUSION

The *in vivo* evaluation of CortiNon+ provides a unique opportunity for the discovery of novel therapeutic agent that exhibit beneficial immunomodulatory and anticancer properties. The general conclusion that we can draw is that the use of specific food supplement may support the conventional therapies hampering the tumor development and increasing their efficacy. However, to prove this additional investigations are required.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our

area of research and country. There is no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All experiments were carried out in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (OJ L 222) and approved by the National Veterinary Office in Bulgaria. (Regulation 20/01.11.2012 regarding laboratory animals and animal welfare).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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