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Protective and Antitumor Effects of Oxidal and Pyrucet in Hamsters with Experimental *Graffi* Tumor

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Authors' contributions

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

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ABSTRACT

The use of combination drug therapy instead of monotherapy is a new positive and promising approach in the treatment of cancer. Oxidal® and Pyrucet® are food supplements containing Methylene blue (USP), Caffeine (USP), Salicylic acid (USP) and Ethyl Acetoacetate and Ethyl Pyruvate respectively. The therapeutic significance of each of these compounds (their derivatives) is well established, but no data are available on their combined action in *in vivo* experimental tumor models.

The aim of the present study was to investigate the *in vivo* protective effect of mono- and combined experimental therapy with dietary supplements Oxidal® and Pyrucet® in the *Graffi* myeloid tumor model in hamsters. For this purpose, the two drugs were administered alone or in combination in two treatment regimens - prophylactic (before) and therapeutic (simultaneously) with the transplantation of *Graffi* tumor cells. The protective effect was determined by the biometric parameters of tumor growth (transplantability, tumor size, mortality, mean survival time, survival

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rate) recorded during the experiment. The results showed a favorable effect of both drugs, administered alone or in combination, before and simultaneously with the transplantation of tumor cells on the appearance and development of *Graffi* tumor in hamsters. About 2-fold lower transplantability, prolongation of the latency period by 7 days, inhibition of tumor growth till 30thday of experiments, reduced mortality and increased individual and overall survival time between 7 to 10 days were observed in the *Graffi* tumor-bearing hamsters with experimental therapy compared to control-*Graffi* tumor-bearing hamsters, without therapy. The obtained data revealed that Oxidal® and Pyrucet® could be a promising candidate for the treatment of tumor diseases.

Keywords: Oxidal®; Pyrucet®; Graffi myeloid tumor; experimental therapy; parameters of tumor growth.

1. INTRODUCTION

Globally, cancer is a major health problem associated with an annual increase in morbidity and mortality [1-3]. Despite advances in the early detection of tumors and in the use of chemotherapy, radiation and surgery to treat cancer, there are two main problems with therapy of neoplasms - (multiple) drug resistance and adverse side effects, some of which are life-threatening or dose-limiting [4]. The search for alternative cancer treatments, as well as the repurposing of conventional drugs to new applications in oncology, is one of the most promising modern strategies to combat cancer. Attention is focused on long-established in clinical practice preparations with a well-known pharmacokinetic/pharmacodynamic profile and toxicity. Oxidal® and Pyrucet® are food supplements. Oxidal® includes Methylene blue (USP), Caffeine (USP) and Salicylic acid (USP), while Pyrucet® includes Ethyl Acetoacetate and Ethyl Pyruvate. The therapeutic potential of each of these compounds (their derivatives) is well established.

Methylene blue (MB) is apolyaromatic cationic dye of the phenothiazine group widely used as a diagnostic agent/histological dye with established clinical applications, including oncological diagnosis by tissue staining and for treatment of methemoglobinemia [5]. It has been extensively used as photosensitizing agent for photodynamic inactivation of RNA viruses, including human immunodeficiency virus (HIV), hepatitis B virus, and hepatitis C virus in plasma [6,7]. (MB) of cases with SARS-CoV-2 [8]. There is preventative role of Methylene blue] and inhibition of replication [9,10]. MB is also a promising photosensitizer for photodynamic therapy (PDT) against microbial cells, viruses, as well as cancer cells [11-14]. In *in vivo* trials in female BALB/c mice,the combined photodynamic/photothermal (PDT/PTT) therapies promoted complete tumor ablation and metastasis prevention while only PDT or PTT were unable to stop tumor development [15].

According to several separate studies, caffeine inhibited the growth of different cancer cell lines in vitro [16-20]. Okano et al. [16] found that caffeine inhibited the proliferation of hepato cellular carcinoma (HCC) cells. Liu et al. [17] showed significantly suppressed growth and viability of human gastric cancer cells and induction of apoptosis. Caffeine was cytotoxic to the human breast MCF-7 and MDA-MB-231 cancer cell lines. It induced oxidative stress and predominantly apoptosis in both cell lines [18]. The antitumor action of caffeine against human colorectal cancer (HT-29 and RKO) is linked in part to its anti-inflammatory properties [19]. Research of Wang et al. [20] indicated that treatment with acombination of caffeine and atorvastatin maybe an effective strategy forinhibiting the growth of human prostate cancer cells *in vitro*.

Salicylic acid (SA) is the major metabolite and active component of aspirin. Besides analgesic, antithrombotic and anti-inflammatory effects, use of aspirin is associated with a reduced risk of developing different cancers such as colon and rectal, pancreatic, breast, head and neck cancer, and etc. [21-25]. Vejselova and Kutlu [25] reported on the antitumor (apoptotic, cytotoxic, and growth-inhibiting effects) activity of the salicylic acid on A549 human lung adenocarcinoma cells. Acetylsalicylic acid and salicylic acid at 10 mM to 20 mM concentrations inhibit the viability and induce apoptosis of cervical cancer cells line (HeLa) [26]. The pathways by which aspirin and salicylic acid exert their anti-cancer effects are still not fully elucidated, although multiple mechanisms affecting enzyme activity (both COX-dependent and independent), transcription factors, cellular signalling and mitochondrial functions have been proposed [23,27-32].

Ethyl pyruvate (EP) was recently identified as a stable lipophilic ester of pyruvic acid, with notoxicity and with significant pharmacological benefits and antineoplastic activities. Ethylpyruvate (EP) suppressednon-smallcell lung cancer (NSCLC) cell growth, invasion and migration *in vitro*, and promotedapoptosis [33]. Blockade of HMGB1-mediated signaling pathway by EP effective lyinhibited diffuse large B-cell lymphoma (DLBCL) tumorigenesis *in vitro* and disease progression in mice *in vivo* [34]. Several reports demonstrated that EP treatment suppressed tumor progression and increased the overall surivial of animals in various *in vivo* tumor models, including hepatic, gastric, gall bladder cancer, lung and mesothelioma [35-40].

The combination therapy is one of new and interesting methods in cancer therapy researches. Combinations of multiple drugs in cancer treatments offer the potential for inhibiting multipletar gets and path ways simultaneous lytomore effective lykill cancer cells and prevent or delay the emergence of drug resistance [32,41,42].

The aim of the present study was to evaluate the antitumor influence of the dietary supplements Oxidal® and Pyrucet®, and their combination against *Graffi* tumor in hamsters. We report the results of two surveys in which prophylactic (before tumor implantation) and therapeutic (at time of tumor implantation) treatments were applied.

2. MATERIALSAND METHODS

2.1 Test Substances

Oxidal® - aqueous solution of methylene blue, with ink color. Each drop contains Methylene blue (USP) 0.4 mg; Caffeine (USP) - 0.4 mg and Salicylic acid (USP) 0.4 mg in purified water. Pyrucet® - slightly viscous (oleose), colorless solution of Ethyl Acetoacetate and Ethyl Pyruvate in medium chain triglyceride. Each drop contains 6 mg Ethyl Acetoacetate and 6 mg Ethyl Pyruvate.

2.2 Experimental Animals

Hamsters 2-4 months aged, from both sexes of the race "Golden Syrian", weighing about 100 g were used in the experiments. They were bred in individual plastic cages with free access to food and water, in the vivarium of the Institute of Experimental Morphology, Pathology and Anthropology with Museum (IEMPAM), Bulgarian Academy of Sciences (BAS).

2.3 Experimental Tumor Model

The experimental *Graffi* solid tumor was maintained monthly *in vivo* in hamsters from the research team at IEMPAM-BAS [43] via subcutaneous transplantation of live tumor cells ($1\text{-}2.10^6$) in the back area. Between days 7 and 15, tumors appeared at the injection site and grew progressively. Hamsters died around 30-35 days after the injection of tumor cells. In this tumor model, 100% appearance of tumor (= transplantability) and 100% mortality rate were observed. No spontaneous regression was observed (= spontaneous shrinking till tumor disappearance).

2.4 Experimental Design

The therapy in hamsters with experimental tumors was applied in two variants: prophylactic and therapeutic. In the prophylactic variant of therapy, the individual (Oxidal®, Pyrucet®) or combined (Oxidal® + Pyrucet®) treatment started on day 1 of the experiment and continued until day 20, after the transplantation of the tumor cells (a total of 27 days daily treatment of each hamster were performed). In the therapeutic variant, the administration of single (Oxidal®, Pyrucet®,) or combined (Oxidal® + Pyrucet®,) treatment began on the day of injection of hamsters with tumor cells and lasted 20 days (a total of 20 days daily treatment for hamsters were performed). For control, *Graffi* tumor bearing hamsters without therapy were used (Control group).

The experimental hamsters were randomly assigned to a total of 7 experimental groups as follows:

Group 1 - hamsters treated with Oxidal® (1 drop per os) 7 days before and 20 days after the subcutaneous injection of $5x10^4$ *Graffi* tumor cells.
Group 2 - hamsters treated with Oxidal® (1 drop per os) 20 days after the subcutaneous injection of $5x10^4$ *Graffi* tumor cells. Therapy started at the same time with the injection of *Graffi* tumor cells
Group 3 - hamsters treated with Pyrucet® (1 drop per os) 7 days before and 20 days after the subcutaneous injection of $5x10^4$ *Graffi* tumor cells.
Group 4 - hamsters treated with Pyrucet® (1 drop per os) 20 days after the subcutaneous injection of $5x10^4$ *Graffi* tumor cells. Therapy

started at the same time with the injection of *Graffi* tumor cells

Group 5 - hamsters treated with a combination of Oxidal® + Pyrucet® (1 drop of each preparation per os) 7 days before and 20 days after the subcutaneous injection of $5x10^4$ *Graffi* tumor cells.

Group 6 - hamsters treated with a combination of Oxidal® + Pyrucet® (1 drop of each preparation per os) 20 days after the subcutaneous injection of $5x10^4$ *Graffi* tumor cells. Therapy started at the same time with the injection of *Graffi* tumor cells.

Group 7 - Control group without treatment (*Graffi* tumor-bearing hamsters, subcutaneously injected with $5x10^4$*Graffi* tumor cells).

2.5 Biometric Parameters

The effect of the applied two variants of therapy with the studied dietary supplements Oxidal® and Pyrucet®, was evaluated by determining the biometric indicators of tumor growth for hamsters from all experimental groups. Transplantability (%)-number of hamsters that developed a tumor from all injected hamsters in the group. Tumor size (mm)-represents the average size of two mutually perpendicular diameters of the tumor (A-width and B-length), measured with a caliper. Mortality/Lethality (%) -number of dead hamsters compared to the total number of hamsters in the group. Average survival (days)-average number of days survival of all hamsters in the group. Individual survival rate was also measured in hamsters with experimental therapy.

2.6 Statistics

The statistical significance of the differences between the control and treatment groups was evaluated by using one-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test using the GraphPad Prism software package. Significance was defined at $P<0.05$.

3. RESULTS AND DISCUSSION

In this research the biometric tumor parameters – transplantability (%), tumor size (mm), lethality (%), mean and individual survival (days) in all experimental groups were analyzed.

3.1 Transplantability (%)

Transplantabilityi indicates in % the number of hamsters that developed tumors compared to the total number of injected hamsters in the group.On a daily basis following the 7^{th} day after injecting of *Graffi* tumor cells in the trial animals, via palpation of the skin at the injection site, the appearance of a tumor was reported. Transplantability data (%) in hamsters with therapy with Oxidal® (A), Pyrucet® (B) and Oxidal® + Pyrucet®(C) were presented in Fig. 1A, B&C.

From Fig. 1 (A, B &C) it could be seen that the transplantability percent in the control group (Gr. 7-TBH) without therapy was 20% on day 9 and 100% on day 12 after tumor cell transplantation.

In the hamsters from the experimental groups with applied single or combined therapy, the transplantability was reduced (the graphs were shifted to the right of those of the control (Fig. 1A, B &C).

For the hamsters from group 1 it was observed 22% transplantability on the 10^{th}, 44% on the 12^{th}, and 100% on the 16^{th} day. For group 2 the transplantability was 12.5% on the 9^{th} day, 25% on the 10^{th} day and 100% on the 19^{th} day (Fig. 1A).

Decreased transplantability was observed in hamsters from group 3 and group 4. The transplantability by groups was as follows: For group 3 - on the 9^{th} day - 12.5%, on the 12^{th} day - 25% and on the 16^{th} day - 100% and for group 4 - on the 10^{th} day. 16.6%, on the 12^{th} day - 50% and on the 17^{th} day - 100% (Fig. 1B).

For hamsters from group 5 - on the 10^{th} day tumors appeared in 12.5%, on the 12^{th} day - in 25%, on the 13^{th} day - in 50%, on the 14^{th} day - in 62.5% and on 16^{th} day - in 100% of hamsters. For group 6 the data were as follows: on the 10^{th} day tumors appeared in 20%, on the 13^{th} day - in 60%, on the 17^{th} day - in 80% and on the 19^{th} day - in 100% of hamsters (Fig. 1C).

The chart showed that in control untreated *Graffi* tumor bearing hamsters (Gr. 7-TBH), the tumor appearance time (latent period) was within the interval of 9-12 days. In the other groups this interval was extended - in the range of 9-16 days for Gr. 1, Gr. 3 and Gr. 5, in the range of 9-19 days for Gr. 2 and Gr. 6, and in the range of 9-17 days for Gr. 4 (Fig. 1A, B &C). The positive effect in extended time for tumor appearance and the reduced percentage of transplantability in the drug-treated groups can be explained with the applied therapy.

3.2 Tumor Size (mm)

Tumor size in hamsters from experimental (Oxidal®, Pyrucet® and Oxidal® + Pyrucet® therapy) and control groups was measured regularly with a caliper for 30 days, after tumor injection. The data are presented in Fig. 2A, B& C.

In the hamsters from the control group (Gr.7-TBH), the tumor size increased progressively. The reported values were: 5.4 ± 2.5 mm on the 13th day, 9.6 ± 3.91 mm on the 15th day, 16.0 ± 3.16 mm on the 18th day, 17.2 ± 3.34 mm on the 21st day, 23.2 ± 4.14 mm on day the 25th and 28.5 ± 3.41 mm on the 30th day of the study (Fig. 2A, B&C).

In the hamsters from the groups with applied single or combined therapy, the size of the tumors was smaller than that of the control (the graphs were shifted down from those of the control (Fig. 2A, B& C).

For hamsters from group 1 (Oxi + Tu) (Fig. 2A) a delay in tumor size was seen compared to the control group (Gr.7-TBH). The reported dimensions for hamsters of Gr. 1 were: 1.6 ± 2.3 mm, 4.87 ± 2.23 mm, 8.87 ± 2.85 mm, 11.12 ± 2.85 mm, 18.12 ± 4.82 mm and 20.62 ± 3.02 mm on the 13th, 15th, 18th , 21st, 25th and 30th day respectively. The following dimensions were reported for hamsters of Gr. 2 (Tu + Oxi): 1.85 ± 2.4 mm, 6.71 ± 5.1 mm, 9.28 ± 6.6 mm, 12.71 ± 6.39 mm, 19.71 ± 7.0 mm and 21.71 ± 5.80 mm on the 13th, 15th, 18th, 21st, 25th and 30th day respectively.

In the groups with Pyrucet® therapy (Fig. 2B) there was a decrease in the tumor size in comparison with the control group (Gr.7-TBH). The reported values for hamsters of group 3 (Pyr + Tu) were: 1.8 ± 2.1 mm, 4.0 ± 2.68 mm, 8.0 ± 4.09 mm, 10.83 ± 3.25 mm, 17.83 ± 5.15 mm and 23.0 ± 4.53 mm on the 13th, 15th, 18th, 21st, 25th and 30th day accordingly. For the hamsters of group 4 (Tu + Pyr) the following dimensions were reported: 1.2 ± 1.64 mm, 4.2 ± 2.94 mm, 6.0 ± 2.73 mm, 13.0 ± 6.0 mm, 14.1 ± 4.63 mm and 18.6 ± 3.28 mm at the same time intervals - 13th, 15th, 18th, 21st, 25th and 30th day(Fig. 2B).

In the hamsters with combination therapy from Gr.5 (Fig. 2C) a reduction in the size of the tumors was seen in comparison with the control (Gr.7-TBH). The reported dimensions for the Gr. 5 were: 2.4 ± 2.0 mm, 4.87 ± 2.5 mm, 9.87 ± 2.85 mm, 14.12 ± 3.85, 20.14 ± 4.86 and 24.62 ± 3.7 mm on the 13th, 15th, 18th, 21st, 25th and 30th day day respectively. In the hamsters with combination therapy from Gr. 6 (Fig. 2C) the reported dimensions were: 1.85 ± 2.4 mm, 2.71 ± 4.1 mm, 4.28 ± 5.6 mm, 10.8 ± 6.39, 16.7 ± 8.0 and 24.30 ± 5.8 mm on 13th, 15th, 18th, 21st, 25th and 30th day day respectively. Impressive was the slower rate (horizontal course of the graph in the interval 13-18th day) of tumor growth for hamsters from Gr. 6 with a therapeutic regimen of both drugs.

3.3 Mortality (%)

The mortality presents the percentage of dead animals in comparison with the total number of hamsters in the group, reported at certain intervals (30th, 35th, 40th and 50th day) after the transplantation of tumor cells. The data are presented in Fig. 3.

In the control group (Group 7-TBH), without therapy, the reported mortality values showed 40%, 60% and 100% mortality occurred on the 30th, 35th and 40th day (Fig. 3A, B &C). In hamsters with experimental therapy, mortality was reduced. The recorded rates for hamsters with Oxidal® therapy were the following: 0, 12.5%, 50%, 87.5%, 87.5% and 100%, respectively, at 30th, 35th, 40th, 45th, 50th and 55th day for Gr. 1 (Oxi + Tu) and 0, 25%, 75% and 100% on the same time for group 2 (Tu + Oxi) (Fig. 3A).

There was a lower mortality in hamsters with applied Pyrucet® therapy (group 3 and group 4), on the 30th, 35thand 40thday compared to the control group (Group 7, TBH - without therapy). The data show 100% mortality in the experimental groups – on the 45th day for group 3 (Pyr + Tu) and 55th day for group 4 (Tu + Pyr) (Fig.3B).

Lower mortality was also established in hamsters with Oxidal® + Pyrucet® combination therapy in both approaches (prophylactic – Gr. 5 and therapeutic – Gr. 6), on the 30th, 35th, 40th and 50th day compared to the control (Gr.7, TBH) (Fig.3B).The reported values for lethality were respectively: 50%, 62.5% and 100% on the 40th, 45th and 50th day for Gr. 5 and 20%, 80% and 100% on the 40th, 45th and 50th day for Gr. 6. On the 30th and 35th day no mortality was recorded for both experimental groups with combination therapy.

Applied single or combination therapy with Oxidal® and Pyrucet® reduces the mortality rate in hamsters with *Graffi* tumor.

3.4 The Median Survival Time (Days)

The data in fig. 4 presented the median survival time of hamsters with experimental therapy with Oxidal® and Pyrucet®. For Gr. 1 (Oxi + Tu) - 40.75 ± 6.36 days, for Gr. 2 (Tu + Oxi) - 36.29 ± 3.35 days, for Gr. 3 (Pyr + Tu) - 39.67 ± 4.88 days, for Gr. 4 (Tu + Pyr) - 38.20 ± 8.46 days, for Gr. 5 (Oxi + Pyr + Tu) - 41.25 ± 4.95 days, for Gr. 6 (Tu + Oxi + Pyr) - 43.60 ± 2.7 and for Gr. 7-TBH (Control) - 33.2 ± 3.27 days. Higher and statistically significant prolongation of the median survival time was observed in the hamsters from Gr.1, Gr. 5 and Gr. 6 compared to the control group (Gr. 7-TBH) (Fig. 4).

3.5 The Individual Survival Rate (Days)

The individual survival in the control group (Gr. 7 - TBH) reached 37 days (Fig. 5, A, B, C). Higher individual survival rate compared to the control was reported in hamsters with experimental therapy. For Gr. 1 (Oxi + Tu) and Gr. 2 (Tu + Oxi), the survival was 53 and 44 days, respectively (Fig. 5, A), for Gr. 3 (Pyr + Tu) and Gr. 4 (Tu + Pyr) was respectively 52 and 47 days (Fig. 5, B) and for Gr.5 and Gr.6 was 48 and 47 days (fig. 5, B).

3.6 Illustrative Images of Hamsters from the Trial Groups

In support of the metrics data in Fig. 2A, B&C, we also present photographic material (Fig. 6-9 and Fig. 10-12) of hamsters from experimental groups treated with Oxidal® (Gr. 1, Gr. 2), Pyrucet® (Gr. 3, Gr. 4) or combination of Oxidal® + Pyrucet® (Gr. 5, Gr. 6) and control *Graffi* tumor bearing hamsters without therapy (Gr. 7). Hamsters from all groups were randomly selected and, after deep anesthesia, were euthanized.

Via palpation of the skin on the back of hamster, from Gr. 1 firmness with a size of 3-6 mm was felt, and after skin dissection it was observed a hyperemic zone with a tumor formation with the same size (Fig. 6A). In the case of a hamster from Gr. 2, a diffuse firmness with a size of 3-5 mm was palpated, and after skin dissection it was observed a hyperemic zone with a diameter approximately 1 cm and 2-3 single tumor formations with a size of 1-2 mm (Fig.6B).

In a hamster from Gr.3, no firmness was felt, and after dissection of the skin, no hyperemia was observed at the tumor injection site (Fig. 7A). When palpating the skin on the back of a hamster from Gr.4, firmness with a size 3-4 mm was established and after the skin dissection there was observed a clearly defined zone of hyperemia with centrally located formation of the same dimension (Fig.7B).

No firmness was felt when palpated the skin of hamster N1 from Gr.5 (Fig. 8A, left panel), but after dissection of the skin there was seen a hyperemic area with initial tumor formation. In hamster N2 from Gr.5, a seal with a size of 1-2 mm was palpated and after skin dissection an intensely colored hyperemic zone with a single tumor with a size of about 1-2 mm was observed (Fig. 8A, right panel).

Via palpation, it was established a firmness with a size of about 2-3 mm in hamster 1 from Gr.6 and after skin dissection a hyperemic zone and a single tumor nodule gray-whitish-colored with a size of about 2 mm was seen (Fig. 8B, left panel). At the other hamster N2 from Gr.6 we found out a seal with an elongated shape, and after dissection of the skin, there was a pronounced elongated hyperemic zone with elongated in shape tumor, whitish-gray colored and 2-4 mm in size (Fig.8B, right panel).

In a hamster from the control group (Gr. 7), a tumor with a size of 10-12 mm was palpated on the back, and after skin dissection, a large hyperemic area with a well-formed solid tumor with a size of 10-12 mm was observed (Fig.9A).

Graffi-TBH without drug treatment on 30th day after tumor cell transplantation was presented (Fig.9B). The macroscopic finding showed an oval in shape, subcutaneous solid tumor with a size of 2.5 /4.5 cm that covered half of the dorsal surface of the hamster. After skin dissection it was visible a grey to off-white in color tumor formation with uneven surface and hemorrhagic areas. A large tumor metastasis was seen in the right axillary region (Fig. 9B).

The results obtained from the current research (hamsters randomly selected from 7 experimental groups) indicate:

- In hamsters from Gr. 1 and Gr. 2 (Oxidal® therapy) via palpation a compact or diffuse skin firmness was detected, accompanied

by developing of a solitary or diffuse tumor formation.

- In the hamsters of Gr.3 and Gr.4 (Pyrucet® therapy), one of the hamsters (Gr.3) at the time of research had no evidence of tumor formation, while the hamster of Gr. 4 formed a tumor with 3-4 mm of size.
- In hamsters from Gr. 5 and Gr. 6 (combination therapy with Oxidal® + Pyrucet®) skin thickening was found, accompanied by the formation of a single tumor of different size in individual hamsters.
- The tumors of hamsters that received experimental mono- or combined therapy with Oxidal® and Pyrucet® however were 2-3 times smaller in size than that of the control Gr. 7. This fact gives us reason to draw a conclusion with regards to the positive effect over the occurrence and development of *Graffi* tumor in hamsters with Oxidal® and Pyrucet® therapy.

In support of the data stated above for tumor size in Fig. 2A, B&C photo material from the relevant experimental groups were presented.

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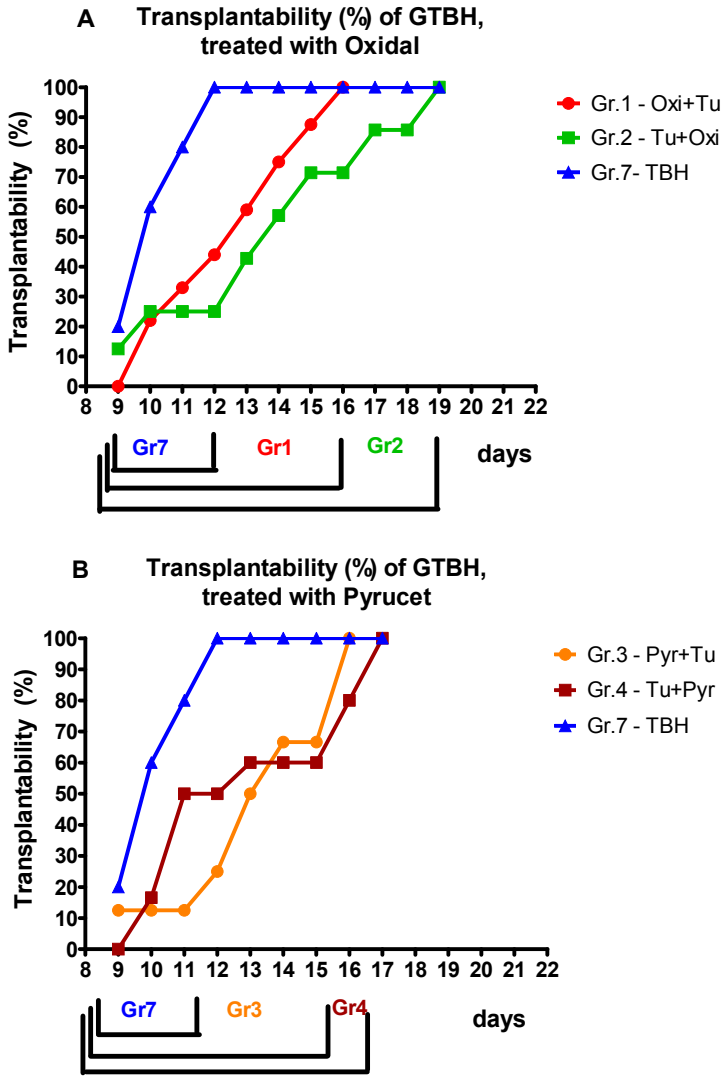


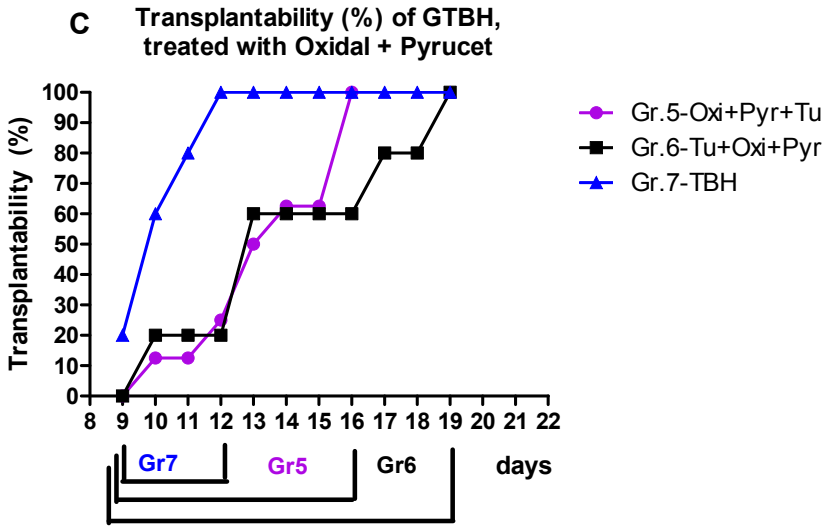
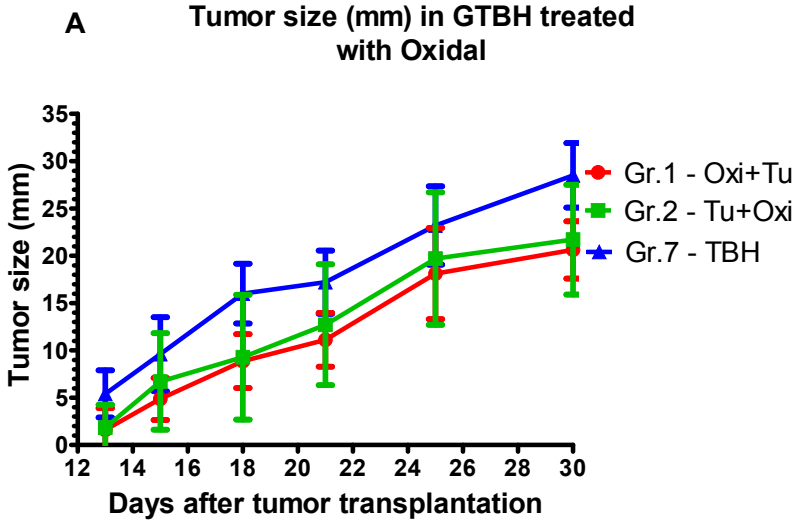


Fig. 1. Transplantability (%) in *Graffi* tumor bearing hamsters with Oxidal® (A), Pyrucet® (B) and Oxidal® + Pyrucet® (C) therapy. Legend: Group 1 - hamsters treated daily with 1 drop of Oxidal® for 27 days (7 days before and 20 days after transplantation of tumor cells.). Group 2 - hamsters treated daily with 1 drop of Oxidal® for 20 days, starting on the day of injection of tumor cells. Group 3 - hamsters treated daily with 1 drop of Pyrucet® for 27 days (7 days before and 20 days after transplantation of tumor cells.). Group 4 - hamsters treated daily with 1 drop of Pyrucet® for 20 days, started on the day of injection of tumor cells. Group 5. - hamsters treated daily with 1 drop of Oxidal® + 1 drop of Pyrucet® per os for 27 days (7 days before and 20 days after tumor cell transplantation. Group 6. hamsters treated daily with 1 drop of Oxidal® + 1 drop of Pyrucet® per osos for 20 days, started on the day of injection of tumor cells Group 7 - control group of hamsters with *Graffi* tumor, untreated (without therapy).



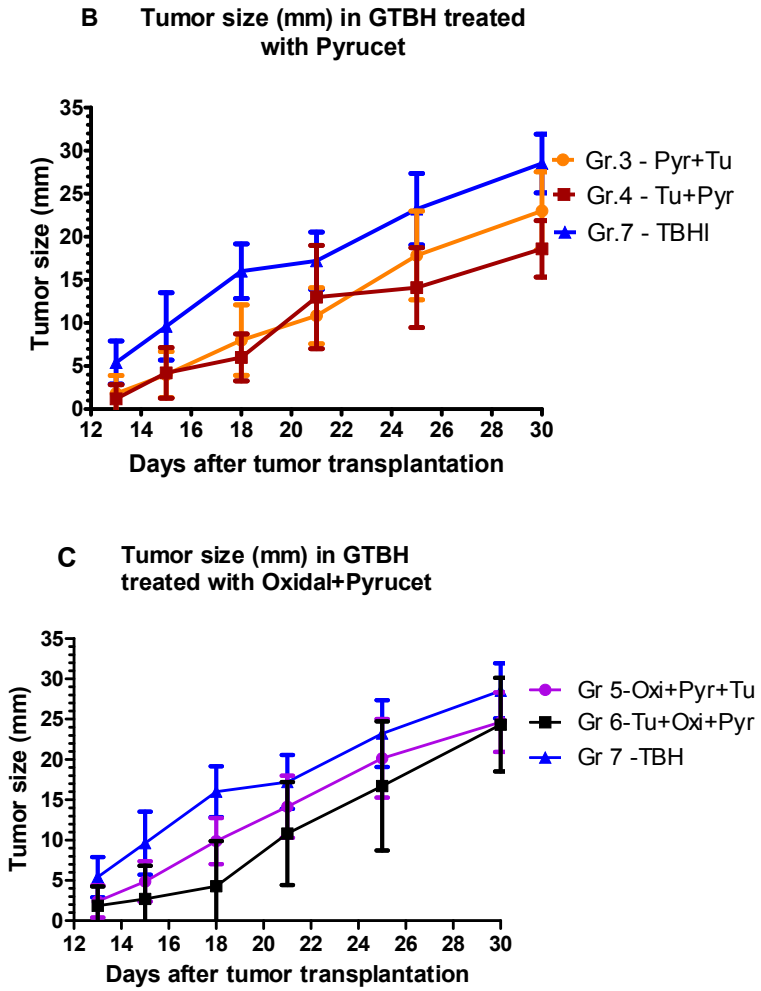
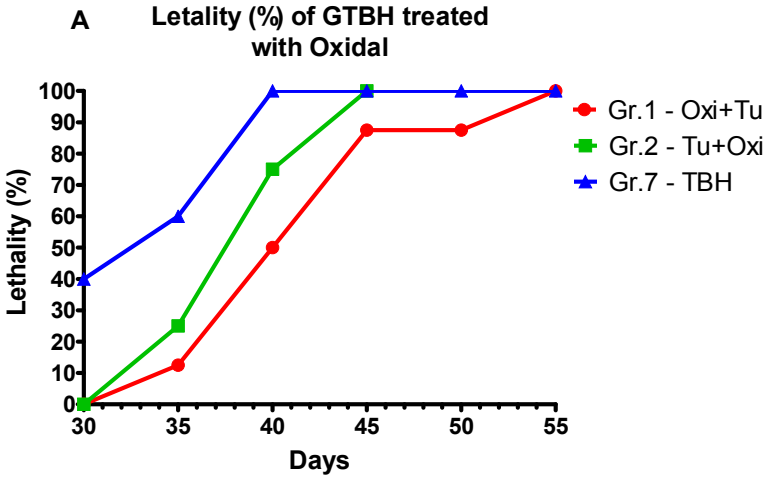


Fig. 2. Tumor size (mm) in *Graffi* tumor bearing hamsters with Oxidal® (A), Pyrucet® (B) and Oxidal® + Pyrucet® (C) therapy. Experimental groups - as shown in Fig. 1.



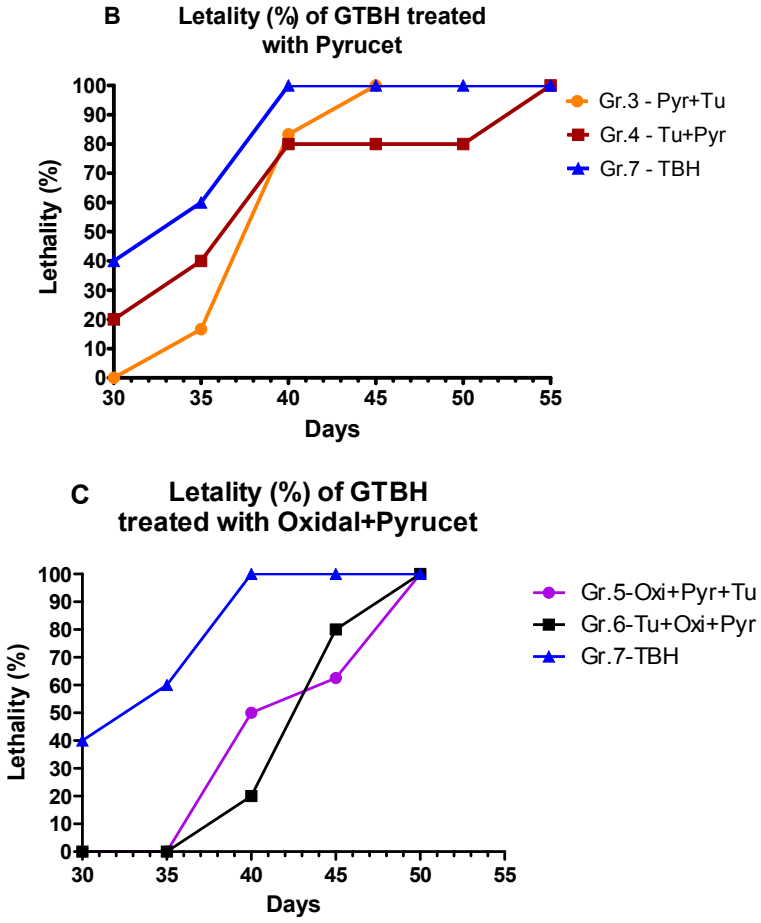


Fig. 3. Mortality rate (%) in *Graffi* tumor bearing hamsters with Oxidal®(A), Pyrucet®(B) and Oxidal® + Pyrucet®(C) therapy. Experimental groups - as shown in Fig. 1.

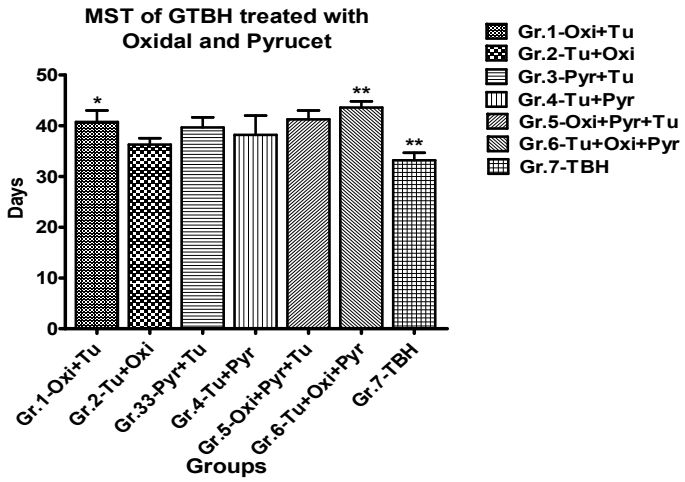


Fig. 4. Mean survival time (days) in *Graffi* tumor bearing hamsters with Oxidal®(A), Pyrucet®(B) and Oxidal® + Pyrucet®(C) therapy. Experimental groups - as shown in Fig. 1.

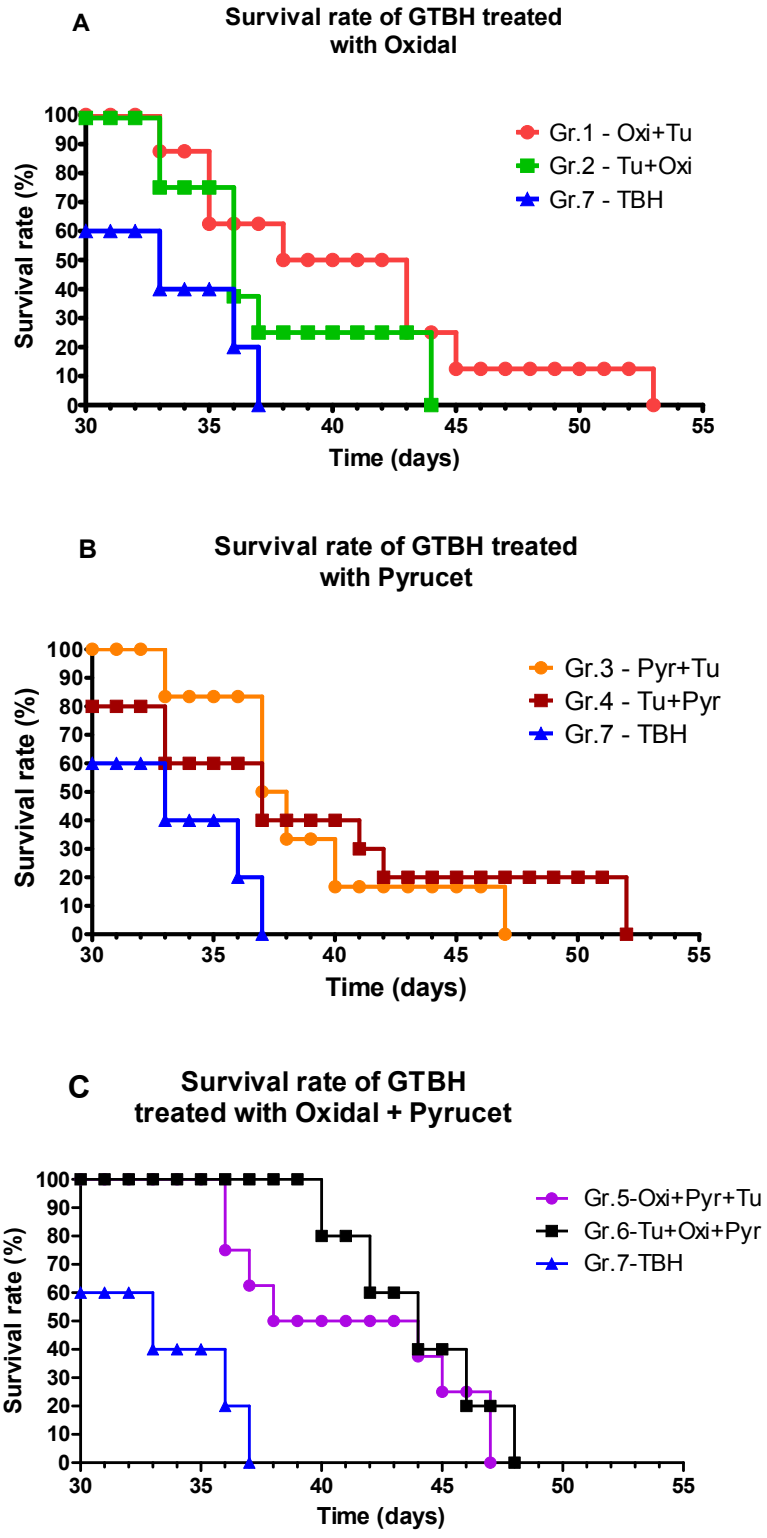
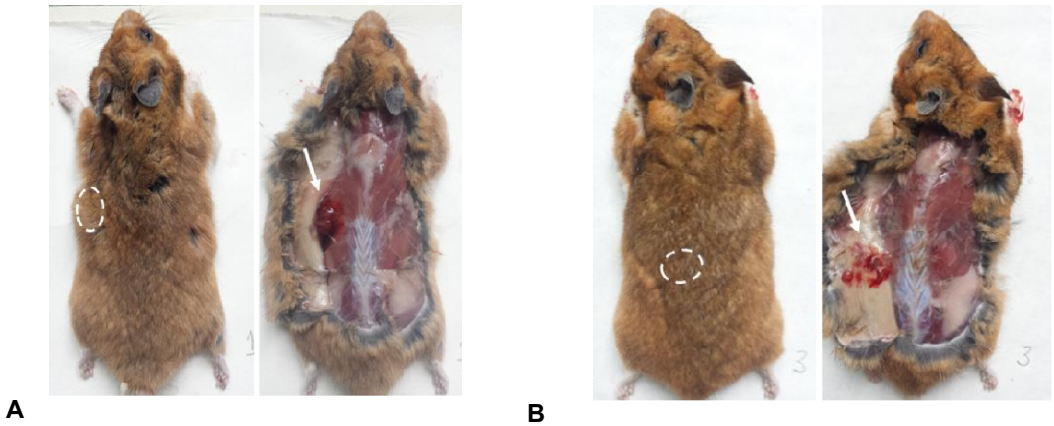
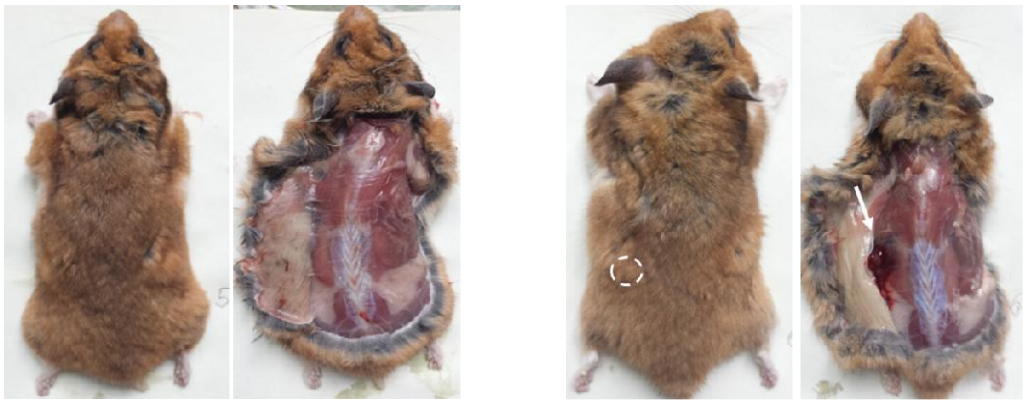


Fig. 5. Individual survival (days) in *Graffi* tumor bearing hamsters with Oxidal®(A), Pyrucet®(B) and Oxidal® + Pyrucet®(C) therapy. Experimental groups - as shown in Fig. 1.



A B

Fig. 6. Hamsters from Gr. 1 (Oxi + tu) (12 days after injection of *Graffi* tumor cells and 19-day daily treatment with Oxidal per os) (A) and Gr. 2 (tu + Oxi) (12 days after injection of *Graffi* tumor cells and 12-day daily treatment with Oxidal® per os started on the day of tumor cell injection) (B)



A B

Fig. 7. Hamsters of Gr. 3 (Pyr + tu) (12 days after injection of *Graffi* tumor cells and 19 days of daily treatment with Pyrucet per os) (A) and Gr. 4 (tu + Pyr) (12 days after injection of *Graffi* tumor cells and 12-day daily treatment with Pyrucet® per os, started on the day of injection of the tumor cells) (B)

The photograph material in Fig. 10 (Oxidal® therapy), 11 (Pyrucet® therapy) and 12 (Oxidal® + Pyrucet® combination therapy) supported the results presented in Fig. 2A, B &C, in particular, in the groups with prophylactic or therapeutic, single or combination therapy with Oxidal® and Pyrucet®, the tumor size was smaller compared to the untreated control group – *Graffi* tumor bearing hamsters (comparative consideration of line above and middle line towards line below in Figs. 10, 11 & 12).

4. DISCUSSION

In the present study, an antitumor effect of Oxidal®, Pyrucet® and Oxidal® + Pyrucet® combined therapy was found in hamsters with experimental *Graffi* tumors by measuring tumor parameters. Both supplements, as well as their combination, present similar positive effects - decreased tumor transplantability, lethality and tumor size and increased mean and individual survival of experimental *Graffi* tumor bearing animals. Oxidal® and Pyrucet® are food supplements containing Methylene blue (USP), Caffeine (USP), Salicylic acid (USP) and Ethyl Acetoacetate and Ethyl Pyruvate respectively. Their therapeutic properties are well established. In manyreports there is evidence that these substances exhibit antitumor effects in *in vitro* and *in vivo* human and animal model systems [16-20,21-25,35-40]. A different target

mechanisms were observed for the different compounds - they act by influencing (activate/inhibit) cellular signaling pathways, regulatory proteins, transcription factors, mitochondrial functions, interfering with the cellular metabolism and with lysosomal function, etc. [16-20,28-33,40]. For example caffeine works in hepatocellular carcinoma (HCC) cells - via activation of MEK/ERK/EGFR signalling pathway (cell cycle arrest independent of apoptosis) [16]; in human gastric cancer cells by activating the caspase-9/caspase-3 signalling pathway [17]; in human breast MCF-7 and MDA-MB-231 cancer cell lines by interfering with the cellular metabolism and with lysosomal function [18]; in human colorectal cancer (HT-29 and RKO) lines is linked in part to its anti-inflammatory properties [19]. In human prostatecancer cells, the combinationtherapy ofcaffeineandatorvastatindownregulatedphospho -Akt, phospho-Erk1/2, anti-apoptotic Bcl-2 and Survivin protein [20]. To exert their chemopreventive effects aspirin and salicylic acid may target cell cycle regulatory proteins, both COX-dependent and independent enzyme activity, mitochondrial functions, etc. [28,29,30,31]. Aspirin's anti-cancer effect may occur through down regulation of c-Myc gene expression [28]. Published evidence demonstrates that salicylic acid exerted anti-tumor effects (significantly inhibited cell proliferation accompanied by apoptosis induction) in liver cancer cells in part mediated by the nitric oxide (NO) pathway [29]. The cell structure damage and ultrastructural changes, concretely indicating apoptosis in A549 cells by using transmission electron (TEM) and confocal microscopy were also reported [25]. It was demonstrated that combination photodynamic therapy of salicylic acid and methylene blue can improve the cell killing effectiveness of MB on the human breast cancer cells MDA-MB-23 [32].

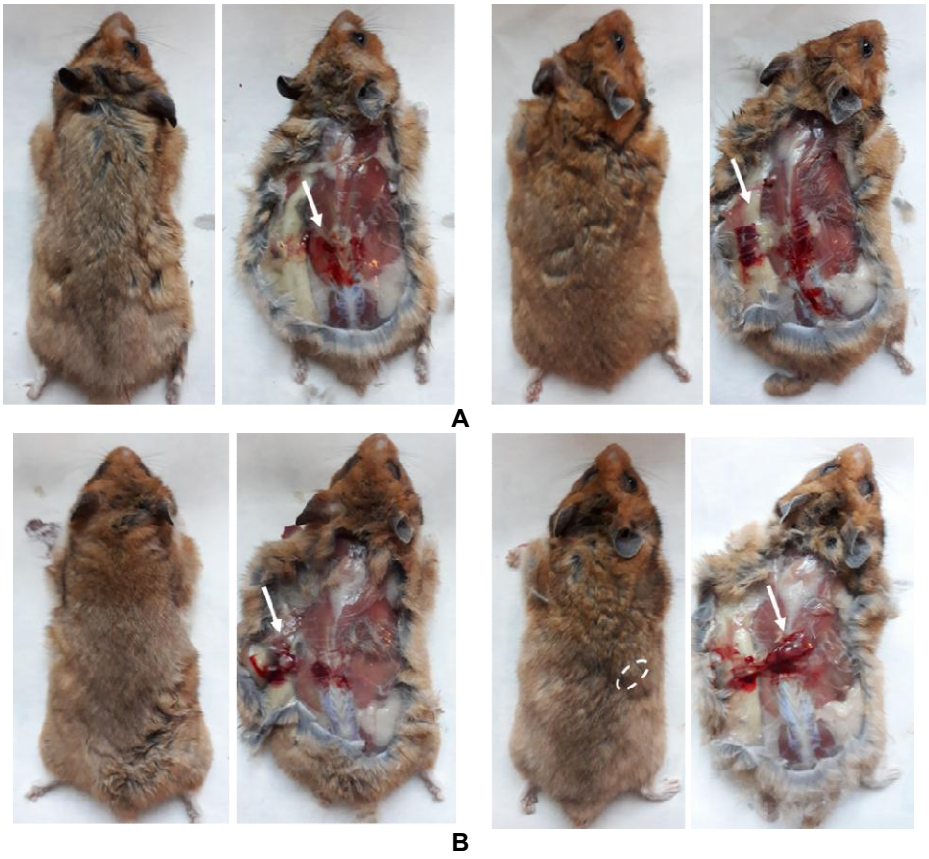


Fig. 8. Hamsters of Gr. 5 (Oxi + Pyr + tu) (12 days after injection of *Graffi* tumor cells and 19 days of daily combined treatment per os) (A) and Gr. 6 (tu + Oxi + Pyr) (12 days after injection) of *Graffi*tumor cells and 12-day daily combination treatment per os started on the day of tumor cell injection) (B)

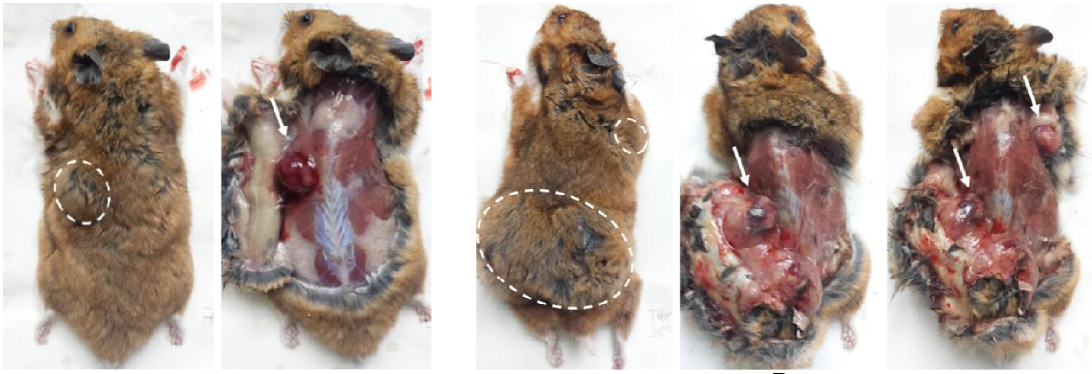


Fig. 9. Hamster from the control group with *Graffi*tumor without treatment (Gr. 7 of the graphs) - 12 days after injection of *Graffi* tumor cells (A) and 30 days after injection of Graffi tumor cells (B) - photo before and after skin preparation on the back in the area of the tumor

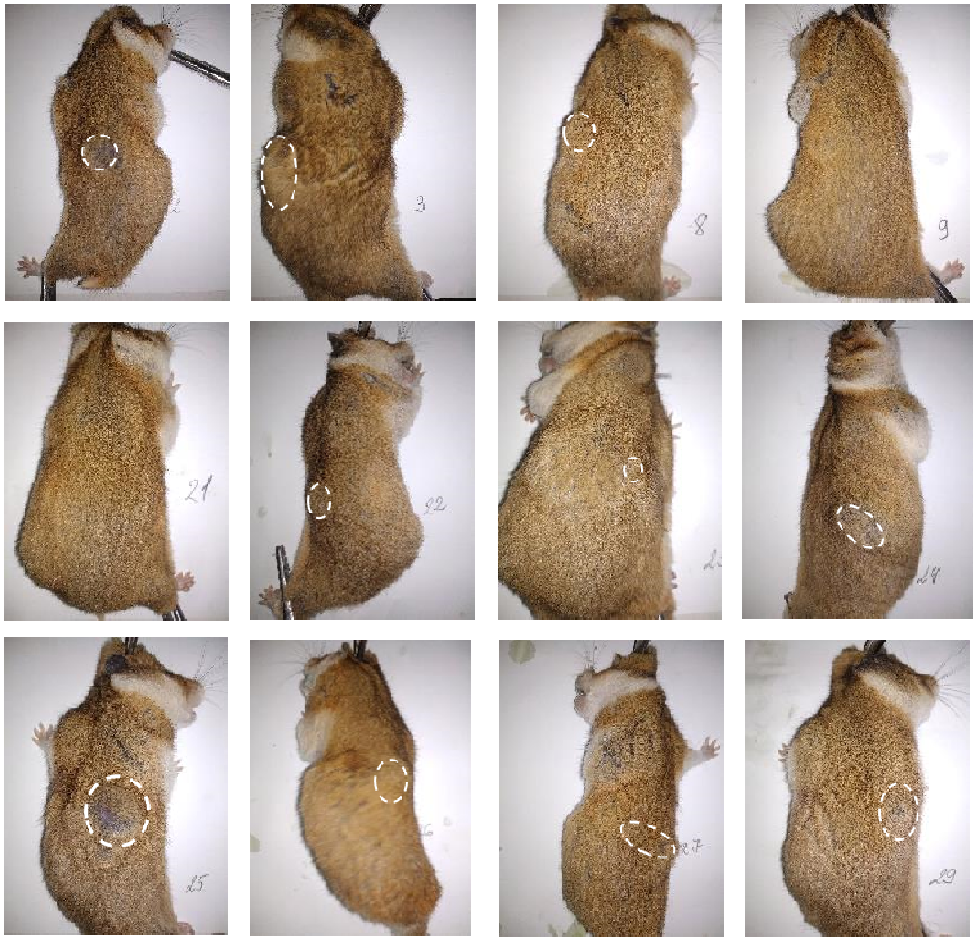


Fig. 10. *Graffi*tumor bearing hamsters treated with Oxidal®
Legend: Top row – Gr. 1 - hamsters treated daily with 1 drop of Oxidal® for 22 days (7 days before and 15 days after tumor cell transplantation). Medium row – Gr. 2 - hamsters treated daily with 1 drop of Oxidal® for 15 days, started on the day of injection of tumor cells. Bottom row – Gr. 3 - control group - hamsters with *Graffi*tumor, untreated

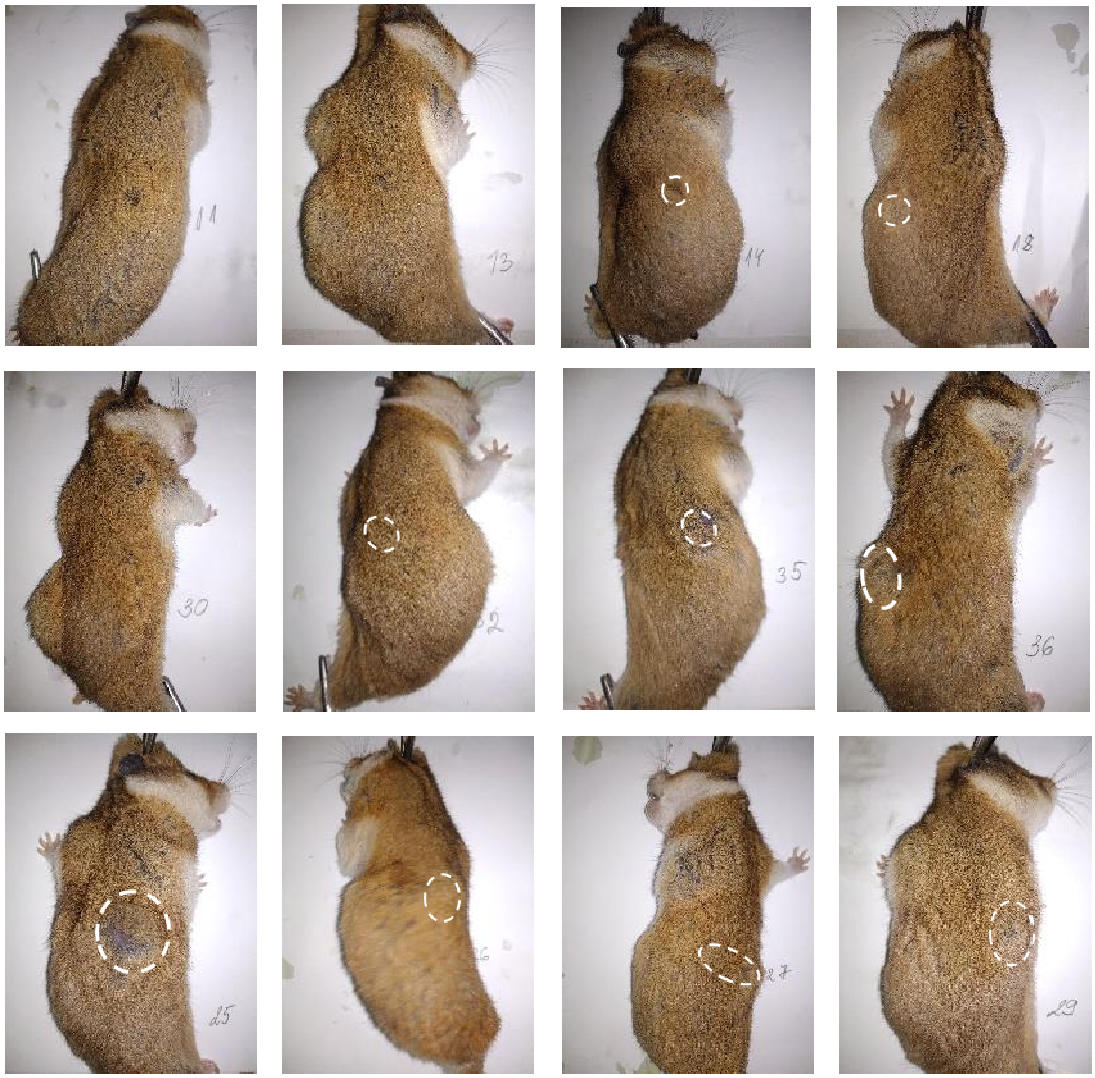


Fig. 11.*Graffi* tumor bearing hamsters treated with Pyrucet®
Legend: Top row – Gr. 1 - hamsters treated daily with 1 drop of Pyrucet® for 22 days (7 days before and 15 days after tumor cell transplantation). Medium row – Gr. 2 - hamsters treated daily with 1 drop of Pyrucet® for 15 days, started on the day of injection of tumor cells. Bottom row – Gr. 7 - control group - hamsters with *Graffi*tumor, untreated

Ethyl pyruvate (EP) mediated suppression of lung cancer cell growth via inhibition of the HMGB1-RAGE signaling pathway sand the NFκB/STAT3 pathway [33]. Necrosis-to-apoptosiss witch activity of EP may contribute to its anti-inflammatory action and suppression of tumor development [39]. EP may suppress tumor growth in the liver through its anti-inflammatory and apoptotic-inducing properties. In *in vivo* experiments, in trahepatic visible tumors were generated with in 2 weeks after direct portal injection of MC38 colorectal cancer cells. The obtained results showed that pretreatment with EP 30 min prior to infusion of tumor cells and continuing daily for 9 days inhibited tumor growth significantly in a dose-depend entmanner when compared with untreated mice [40].

The combination therapy is one of new and interesting methods in cancer therapy researches. It is believed that the multitarget approach in the treatment of tumors is important for the realization of successful antitumor therapy [32,41,42,44,45].

Fig. 12. Hamsters with *Graffi* tumor, with combination therapy Oxidal® + Pyrucet®. Legend: Top row – Gr. 1 - hamsters treated daily with 1 drop of Oxidal® + 1 drop of Pyrucet® per os for 27 days (7 days before and 20 days after transplantation of tumor cells.). Middle row - Gr.2 - hamsters treated daily with 1 drop of Oxidal® + 1 drop of Pyrucet® per os for 20 days, started on the day of injection of tumor cells. Bottom row - Gr.3 - control - hamsters with *Graffi* tumor, untreated

5. CONCLUSION

In the present study the *in vivo* protective effect of dietary supplements Oxidal® and Pyrucet®, administered alone or in combination, in two treatment regimens in the *Graffi* tumor in hamsters were assessed. The results obtained by us demonstrate a protective antitumor effect of both food supplements, reported on the values of biometric parameters in all schemes and combinations of applied therapy. We observed inhibition of tumor transplantation and prolongation of the latency period, slow tumor growth during the experiment, as well as reduced mortality. In addition, the mean and individual survival of hamsters with therapy was significantly increased compared to control-untreated hamsters. The obtained results are supported by rich illustrative material.This positive effect has been attributed to the multi target mechanism of action of Oxidal® and Pyrucet® on the tumor and to defense mechanisms of experimental animals after experimental therapy. Our data support the idea that dietary supplements Oxidal® and Pyrucet® may be promising candidates for the treatment of tumors.

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 and approved by the National Veterinary Office in Bulgaria, regarding laboratory animals and animal welfare.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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