

EXPERIMENTAL STUDIES

ЭКСПЕРИМЕНТАЛЬНЫЕ ИССЛЕДОВАНИЯ

The Therapeutic Effect of Antitumor Drugs Erufosine and Doxorubicin on the Metastatic Process in the Testes of Hamsters with Graffi Myeloid Tumor. Morphometric and Histological Studies

Влияние противоопухолевых препаратов эруфозина и доксорубицина на развитие метастазов в яичках хомяков с миелоидной опухолью Graffi. Морфометрическое и гистологическое исследования

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ABSTRACT

Aim. The goal of the current study is directed to investigate the therapeutic effect of erufosine (EPC3) alone, as well as in combination with doxorubicin (DOX) on the development of metastases in the testes in an experimental model of transplantable Graffi myeloid tumor (GMT) in hamsters.

Materials & Methods. Experimental *in vivo* model of GMT in Golden Syrian hamsters was used. Animals were inoculated subcutaneously with live virus-transformed malignant cells. Testes from male tumor-bearing hamsters (TBHs), treated and non-treated with the antitumor agent erufosine alone or in combination with DOX, as well as untreated healthy controls, were subjected to morphometric and histological assays on the testicular architecture to evaluate potential antitumor/antimetastatic effects in *in vivo* conditions.

Results. The results showed no changes in blood vessels and morphology (impaired spermatogenesis and/or metastatic changes) of the testicular tissue in TBHs treated with antitumor agents (alone or in combination) compared to control healthy animals. In contrast, in the testes of TBHs without therapy, impaired spermatogenesis and foci of metastases, as well as significant changes in testicular blood vessels were found.

Conclusion. The data obtained demonstrated an antitumor/antimetastatic effect of EPC3 in the testes of hamsters in the experimental GMT model.

Keywords: metastases, antitumor activity, erufosine, doxorubicin, Graffi myeloid tumor, morphology of testicular tissue, spermatogenesis, acute myeloid leukemia.

РЕФЕРАТ

Цель. Исследование терапевтических эффектов эруфозина (ERS3) в виде монотерапии или в комбинации с доксорубицином (DOX) в отношении развития поражений яичек на экспериментальной модели трансплантируемой миелоидной опухоли Graffi.

Материалы и методы. Использовали экспериментальную модель миелоидной опухоли Graffi у сирийских золотистых хомяков. Животным подкожно вводили взвесь живых вирус-трансформированных опухолевых клеток. Часть животных получала терапию ERS3 в комбинации или без DOX. Контрольная группа включала здоровых животных без лечения. На материале яичек животных, у которых развились опухоли, а также контрольных животных проводили гистологическое и морфометрическое исследования.

Результаты. В сравнении с животными контрольной группы различия в структуре яичка и характеристиках сосудов у животных, получавших лечение, не обнаружены. Напротив, в яичках хомяков с опухолью, но без лечения выявлены скопления опухолевых клеток, нарушения сперматогенеза, а также значительные изменения диаметра сосудов.

Заключение. Полученные данные продемонстрировали противоопухолевый и противометастатический эффекты ERS3 в экспериментальной модели миелоидной опухоли Graffi.

Ключевые слова: метастазы, противоопухолевый эффект, эруфозин, доксорубицин, миелоидная опухоль Graffi, морфология ткани яичек, сперматогенез, острый миелоидный лейкоз.

Received: June 15, 2023

Accepted: November 20, 2023

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For citation: Ilieva IN, Sainova IV, Toshkova RA, et al. The Therapeutic Effect of Antitumor Drugs Erufosine and Doxorubicin on the Metastatic Process in the Testes of Hamsters with Graffi Myeloid Tumor. Morphometric and Histological Studies. Clinical oncohematology. 2024;17(1):18–26.

DOI: 10.21320/2500-2139-2024-17-1-18-26

Получено: 15 июня 2023 г.

Принято в печать: 20 ноября 2023 г.

Для переписки: Assist. Prof. Iliana Najdenova Ilieva, PhD in Biology, Acad. G. Bonchev str., bld. 23, 113 Sofia, Bulgaria; e-mail: ilieva@abv.bg

Для цитирования: Илиева И.Н., Сайнова И.В., Тошкова Р.А. и др. Влияние противоопухолевых препаратов эруфозина и доксорубина на развитие метастазов в яичках хомяков с миелоидной опухолью Graffi. Морфометрическое и гистологическое исследования. Клиническая онкогематология. 2024;17(1):18–26. (на англ.)

DOI: 10.21320/2500-2139-2024-17-1-18-26

INTRODUCTION

In modern medicine, the idea of personalized application of the holistic approach in therapy and the fight against oncological diseases is becoming more and more important. The preparation of a specific (individual) therapeutic program suitable for the individual needs to take into account not only the type and area in which the primary malignant process develops but also the whole physical body (general health and normal body functions), including mental health and overall quality of life. In this complex of health measures, modern methods of rapid and accurate diagnosis should not be excluded, as well as referral of patients to post-healing activities related to the quality of the healing process (clinical follow-up during remission and/or relapse), but also physiotherapy and/or balneotherapy and rehabilitation.

Conventional therapy for the treatment of cancer usually includes surgical intervention, chemotherapy (including adjuvant chemotherapy), and/or radiotherapy, but also endocrine therapy or antihormonal treatment, which, however, often leads to slight, or unsatisfactory results without an effective cure [1]. The attention of anti-cancer therapy is primarily directed to two main features in the development of tumor formations: the prolonged process of proliferation and the exceptional ability of cancer cells to avoid apoptosis [2]. Another main goal of the therapeutic strategies is to overcome the resistance mechanisms and to reduce the toxicity of the administered drugs and their consequences by improving the efficacy (survival rate) of the treatment. It is equally important to decipher the underlying mechanisms that lead to recurrences and/or metastases (why and how they occur), which may pre-determine new treatment approaches [3].

Chemotherapy is a systemic approach that targets rapidly growing and dividing cancer cells, aiming to slow the growth of blast cells or eliminate (damage/kill) them in the affected tissue/organ, but also destroy those of them, which have metastasized (spread) to the rest of the body, not only the original/primary tumor cells. Sometimes chemotherapy is the once-necessary treatment strategy, but it often causes side effects and discomfort. In most cases, chemotherapeutic agents are found to be toxic to normal healthy cells and tissues, while at the tumor site, the antitumor substance does not reach a sufficiently high concentration to obtain a positive therapeutic effect.

Another major drawback of conventional chemotherapy is that cancer cells often develop multidrug resistance, which necessitates the search and development of new, more effective, and more tolerable chemotherapeutic agents for treatment.

In this aspect, alkylphosphocholines (APCs) are a new class of lipophilic drug compounds with a different pathway of cytostatic action compared to conventional anticancer agents. They are synthetic phospholipid analogs with a simplified structure of lysophosphatidylcholine, in which the glycerol skeleton is replaced by a simple alkyl chain [4]. APCs localize to the plasmalemma and intracellular membranes, disrupting the structure and metabolism of membrane lipids, and thus inhibit related to them intracellular signal transduction pathways of mitosis and induce apoptosis in cancer cells [5]. APC analogs are believed to be more effective in cancer treatment than other drugs because they do not cause myelotoxicity and DNA damage [6]. APCs has also been found to be effective in various tumor cell lines, including acute leukemia, lymphoma [7, 8], colorectal cancer [9], breast cancer [10], multiple myeloma [11], brain tumors [12], prostate cancer [13], etc.

Erufosine (EPC3, erucylphosphocholine) belongs to a new generation of APCs analogs that readily incorporate into the plasma membrane and distribute between intracellular membrane compartments of tumor cells. EPC3 inhibits the synthesis of key phospholipids such as phosphatidylcholine, sphingomyelin, and other secondary lipid intermediates involved in cell cycle progression, growth regulation, and tumor cell survival [14, 15]. Thus, by interfering with lipid metabolism and suppressing lipid-dependent signal transduction, EPC3 induces strong stress and apoptotic changes in proliferating metabolically active cancer cells without affecting healthy (normal) resting cells [16]. Erufosine is the first substance, belonging to the class of the alkylphosphocholines that can be applied intravenously because it does not cause hemolytic and myelotoxic effects and even stimulates normal hematopoiesis [17].

Doxorubicin (DOX) is a mighty anthracycline antibiotic, a DNA intercalator, which is related to conventional chemotherapeutic agents for the treatment of various types of cancers, including hematological malignancies. One of the main mechanisms of the cytotoxic action of doxorubicin on malignant, but also on normal cells, involves interaction with DNA (by intercalation of DOX molecules in the nu-

cleotide bases), binding to the lipid component of the cell membrane, and disruption of topoisomerase II (an enzyme which relaxes supercoils in DNA for transcription), which leads to halting of nucleotide replication (by inhibition the activity of DNA/RNA polymerases), and the cancer cell growth/proliferation, respectively [18]. Prolonged replication arrest then leads to molecular programs that initiate cell death [19]. Doxorubicin is often given together with other chemotherapy agents. The combined application of the anti-tumor agents erufosine and doxorubicin (which have non-overlapping toxic effects) enables a simultaneous attack on cancer cells and, accordingly, doubly effective inhibition of tumor growth. On the other hand, EPC3 has been suggested to reduce significantly the side effects of DOX, but at the same time, to enhance its anti-tumor effect when administered at lower doses. In this context, APCs may be suitable as combination partners in the treatment of leukemias because they do not cause myelosuppression, the main side effect of most anti-leukemic agents, and thus limit the overall toxicity to the host within acceptable limits [20].

Metastatic (blast cell) foci in the testes are most often a complication of lymphoma or leukemic malignancies, especially in cases of acute myeloid leukemia (AML; monoblastic and myelomonocytic subtypes of AML), but also primary tumors with non-hematological origin (most often of the prostate, but also lung, melanoma, seminal vesicles, etc.) [21, 22]. Blast cell infiltration usually occurs in the interstitial spaces, but it can also invade the seminiferous tubules of the testis. The way, by which tumor cells reach the male gonads, is through vasodilation of blood vessels (capillaries) with impaired permeability, as a result of neoangiogenesis associated with the neoplastic process [22]. According to literature data, several main mechanisms of secondary malignant decimation in the testicular tissue are described: by lymphatics, *vas deferens*, epididymis, the spermatic (testicular) veins, and by the arterial (hematogeneous) pathways [22, 23].

In the present, but also in our previous studies [24, 25] was hypothesized that the blood-testis barrier (BTB) is one of the main factors for the secondary spread and invasion of tumor cells in the male gonads in an experimental transplantable model of Graffi myeloid tumor (GMT) in hamsters. The main reason for the application of this model in our experimental work is that a tumor of Graffi is analogous and has cellular characteristics to AML in humans [26], and enables possible, through histological studies, to trace and demonstrate the early and later pathological changes in the testes (induced by Graffi myeloid cells) and the occurrence of metastatic foci in the testicular tissue of hamsters. Similar malignant changes have also been described in other extramedullary locations, such as the brain, lymph nodes, skin, etc. [27, 28].

The objective of the present study was to determine the therapeutic effect of erufosine applied alone or in combination with doxorubicin on the development of metastases in the testes of hamsters with GMT (as an experimental model).

MATERIALS AND METHODS

Experimental Graffi myeloid tumor model

Male Golden Syrian hamsters, 2–3 months old, weighing approximately 100 g were used as experimental

animals. They were obtained from the animal facility in Slivnitsa of the Bulgarian Academy of Sciences, Sofia, and were maintained under standard laboratory conditions (temperature: 26 ± 2 °C; 12 h dark/light cycle; food pellets and water: *ad libitum*). The GMT, primary induced with Graffi murine leukemia virus (MuLV) in new-born hamsters was created and maintained monthly *in vivo* by subcutaneous (s.c.) implantation of live tumor cells ($2 \times 10^6/\text{mL}^{-1}$ in phosphate-buffered saline, PBS) in the inter-scapular area of animals [29]. The tumor appeared subcutaneously between Days 7 and 15 after tumor transplantation as a solid formation in the area of injection. It is 100 % reproducible in hamsters, and grew progressively to 4–5 cm in diameter, and caused the death (100 %) of the experimental animals on Days 28–30 after tumor cell transplantation. Spontaneous regression was not observed in this tumor model. For the experiments, hamsters were inoculated (s.c.) with 2×10^4 viable Graffi tumor cells obtained aseptically from tumor fragments (without necrosis) by disaggregation. The tumor cell suspension was filtered through a 100-mesh grid, washed three times by centrifugation (at 4 °C) with PBS, and cell viability was determined with 0.2 % Tr. blue. All animals were kept under standard conditions in individual plastic cages with free access to food and water.

All studies were performed in accordance with the Guide for Care and Use of Laboratory Animals, as proposed by the Committee on Care Laboratory Animal Resources, Commission on Life Sciences, and National Research Council, and a work permit No. 11130006.

Antitumor agents (doses and schedule of application)

Erufosine (EPC3; MG 504.7) is the (N,N,N-trimethyl) — propylammoniumester of erucyl-phosphoric acid. It was synthesized in the Max Planck Institute for Biophysical Chemistry, Göttingen, Germany, and was kindly provided by Prof. Martin R. Berger. EPC3 was dissolved in a PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 , and 2 mM KH_2PO_4 , pH 7.4) in 10 mM stock solution and kept at 4 °C before use. EPC3 was administered in a dose of 1.5 mg/100 g BM, s.c. in a volume of 0.5 ml PBS per hamster, twice a week, for 4 weeks, alone or in combination with DOX. Therapy begins on the day of tumor cell transplantation.

Doxorubicin (DOX) — an anthracycline antitumor antibiotic. Administered at a dose of 0.00058 mg/100 g BM, s.c. in a volume of 0.5 ml PBS in combination with EPC3 per hamster. Therapy begins on the day of tumor cell transplantation.

Experimental design

The experimental male animals were separated into four groups ($n = 3$ in each group) and treated as follows: Gr-1 — Graffi tumor-bearing hamsters without any treatment (Day 30 after tumor transplantation); Gr-2 — Graffi tumor-bearing hamsters treated only with erufosine (Day 28 of therapy); Gr-3 — Graffi tumor-bearing hamsters treated with erufosine in combination with DOX (Day 28 of therapy) and Gr-4 — control healthy hamsters.

In experimental groups, 2 and 3, treatment with EPC3 and EPC3 + DOX respectively started simultaneously with Graffi tumor cell engraftment and continued for 28 days (twice a week, for 4 weeks) after transplantation.

Table 1. Morphometric data of blood vessels in the testes of untreated TBHs, TBHs treated with erufosine, TBHs treated with EPC3 + DOX, and healthy (control) hamsters

Luminal diameter, μm	Blood vessels diameter, μm			
	6.0–15.0	15.5–30.0	30.5–50.0	> 50.0
Control	9.19 \pm 2.05	23.25 \pm 2.36	34.62 \pm 1.22	60.95 \pm 6.11
TBH-EPC3	9.90 \pm 3.49*	23.95 \pm 3.98	37.75 \pm 5.76*	63.20 \pm 8.90**
TBH-EPC3 + DOX	9.99 \pm 2.83*	23.31 \pm 4.06*	37.01 \pm 5.41*	62.93 \pm 7.41***
TBHs (untreated)	12.93 \pm 1.66	25.72 \pm 3.26	43.33 \pm 4.95	82.65 \pm 7.77

* $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$.

Morphometric and histopathological examination

Samples of testes from control (healthy) and tumor-bearing hamsters (TBHs) were taken, fixed, and embedded in paraffin using routine histological practice. Tissue sections (5–7 μm) were stained by hematoxylin-eosin and examined under a light microscope Leica DM5000B. The morphometric data were obtained at 400 \times magnification using an eyepiece micrometer. The luminal diameter was measured as the perpendicular distance across the maximum chord axis of each vessel in testicular tissue.

Statistical analysis

The results were reported as mean values \pm standard error of the mean (SEM). Significance was evaluated by one-way ANOVA, followed by Student's *t*-test. Differences were considered statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

The current investigations are a continuance of our previous studies, related to the development of metastases in the testes of Graffi-TBH, as an appropriate experimental model [24, 25]. In the present study, we followed the morphological/morphometric changes in the testes, in the four experimental groups: non-treated tumor-bearing hamsters (untreated TBHs), TBHs treated with erufosine (TBH-EPC3), TBHs treated with EPC3 in combination with DOX (TBH-EPC3 + DOX), and control group (healthy) hamsters. The efficacy of antitumor therapy was evaluated in a terminal stage of malignant disease development by comparing the morphometric and histopathological changes in the testes of Graffi-TBH, undergoing therapy, and Graffi-TBH without therapy. Morphometric values of the luminal diameter of the testes vessels, divided into four subgroups in control (healthy) and TBHs, were determined.

Significant changes in the mean luminal diameter, with dilatation of capillaries, as well as the larger blood vessels (arterioles and venules) and large vessels of > 50.0 μm of the testes of all TBHs (Gr-1) were found in comparison with the control group (healthy hamsters). The luminal diameter of the large vessels > 30.0 μm and > 50.0 μm , was the most significantly increased in TBHs without therapy (43.33 \pm 4.95 μm and 82.65 \pm 7.77 μm , respectively). No significant changes in blood vessel diameter (mean lumen diameter) were found in the testes of EPC3- and EPC3 + DOX-treated TBHs compared to the healthy control group (Table 1). However, we observed statistically significant changes in the mean lumen diameter of both capillaries (vessels < 15.0 μm and vessels with a diameter of

15.5–30.0 μm) and large blood vessels (vessels with 30.5–50.0 μm and > 50.0 μm), in testes between the group of untreated TBHs and TBHs treated with EPC3 alone or with the combination of EPC3 + DOX ($p < 0.01$, $p < 0.001$). These changes were most pronounced in blood vessels > 50.0 μm in diameter between the TBH-EPC3 + DOX and untreated TBHs ($p < 0.0001$) (Table 1).

Furthermore, no significant changes were found in the percentage distribution of blood vessels in the testes of hamsters from Gr-2 (TBH-EPC3) and Gr-3 (TBH-EPC3 + DOX) compared to healthy animals, but a significant difference was observed in comparing them with untreated TBHs (Gr-1) (Fig. 1).

The percentage distribution of blood vessels of different sizes in Gr-1 showed an increase in the percentage of large blood vessels — venules, arterioles, and/or dilated capillaries (vessels of 30.5–50.0 μm and > 50.0 μm in diameter) in the testes of these animals (9.35 % and 5.14 %, respectively), compared to those of Gr-2 (2.5 % vs. 1.45 %) and Gr-3 (2.1 % vs. 1.2 %) and the control (3.43 % vs. 1.00 %). These results indicated an interesting correlation in the percent distribution of the smaller vessels (capillaries with < 15.0 μm and vessels with a diameter of 15.5–30.0 μm) in the untreated TBHs. The number of blood vessels with lumen diameter 15.5–30.0 μm in Gr-1 (41.77 %) was significantly increased, while the percentage of those with the smallest size (< 15.0 μm) was reduced (43.74 %), compared to Gr-2 and Gr-3 hamsters with therapy, as well as the control group (healthy). In the same group (untreated TBHs), however, the ratio between these blood vessels was almost equalized (Fig. 1). In the hamsters with therapy (Gr-2 and Gr-3), no changes in the luminal diameter of capillaries and other blood vessels were observed and the values were similar to those of the control healthy hamsters. The results presented showed the positive effect of the antitumor agents EPC3 and EPC3 + DOX in tumor-bearing animals with a progressively developing malignant process (Gr-1). The established changes in the testicular vessels of hamsters from Gr-1 (TBHs) are a “signal sign” for the initiation of the neoangiogenesis process, which is associated with the development of secondary tumors — metastases (terminal stage).

Similar data have been received in our previous studies, related to the investigation of the presence of malignant changes (metastases) in the testes of Graffi-TBH, in dynamics (Days 10, 25, and 30 after tumor transplantation). No significant changes in the blood vessel diameter in the testes of hamsters from Day 10 were found, in contrast to Days 25 and 30, where measurements showed significantly higher mean values of the lumen of the testicular blood vessels, as in capillaries (vessels < 15.0 μm and vessels with 15.5–30.0 μm

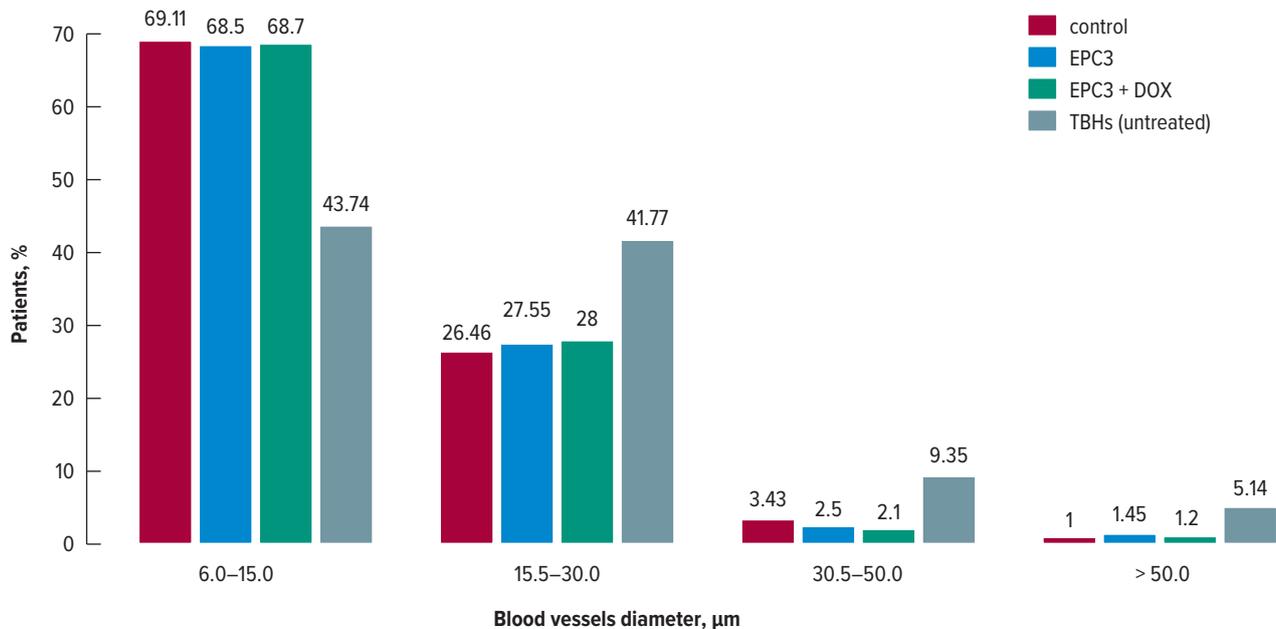


Fig. 1. Comparison of morphometric data in the distribution of testis blood vessels in treated and untreated TBHs (proportion in %)

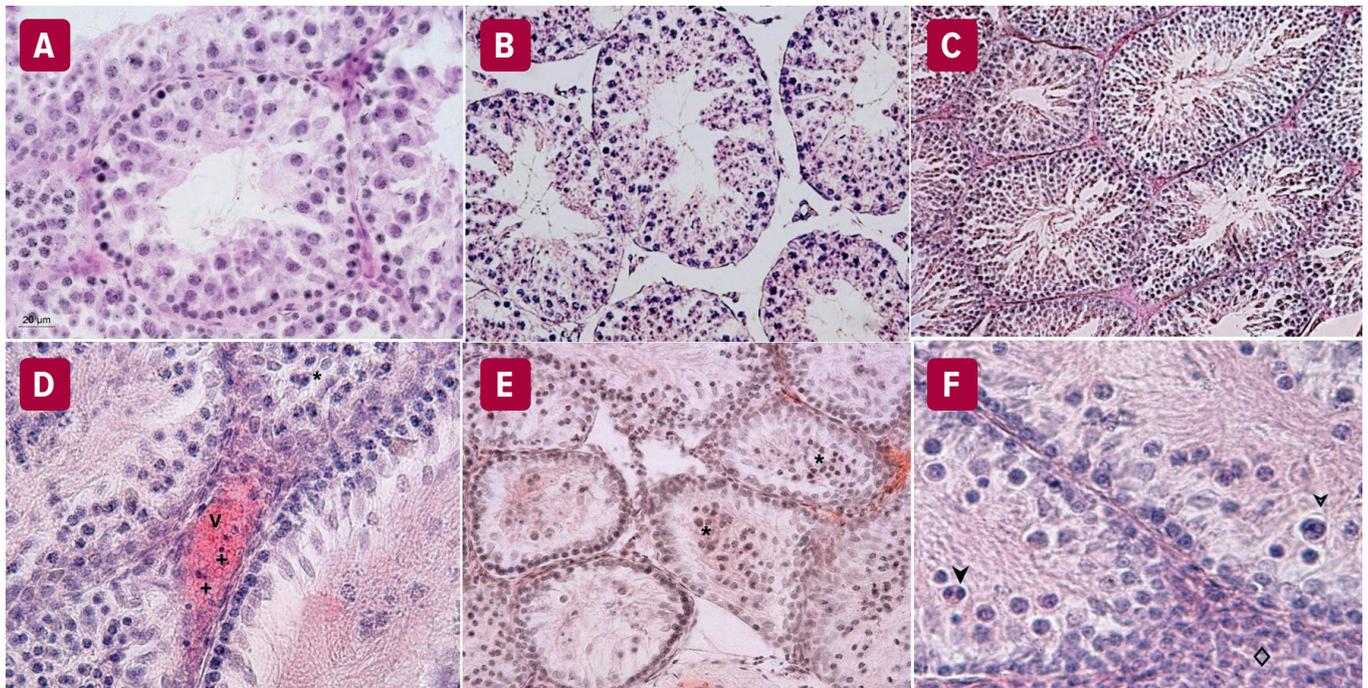


Fig. 2. Testicular cross-sections of untreated TBHs, treated with EPC3 and EPC3 + DOX TBHs, and control groups. (A) Cross-section of TBHs EPC3-treated. (B) Cross-section of EPC3 + DOX-treated TBHs. (C) Healthy hamsters (controls). Normal morphology of the seminiferous tubules and preserved spermatogenesis. (D, E, F) Untreated TBHs. Seminiferous tubules with damaged basal membranes and impaired/suppressed spermatogenesis. The germinal epithelium is disorganized, showing depletion of germ cells, degenerating primary spermatocytes (St) with abnormal chromatin condensation/fragmentation (apoptotic cells), immature spermatids (Sd), giant multinuclear cells, and atypical GMT blast cells, (D) disrupted vascular wall and effusion of blood elements in the intratesticular space. H&E, ×200, ×400. Arrowheads — giant multinuclear cells; v — microvessels; + — atypical tumor/GMT cells; * — abnormal germ cells/St; ◇ — interstitium.

in diameter; $p < 0.01$) but also in the larger blood vessels (with 30.5–50.0 µm, $p < 0.001$, and > 50.0 µm in diameter; $p < 0.0001$) of TBHs compared to the healthy animals [24, 25]. In this case, the data showed that the processes of neoangiogenesis started after Day 10 of Graffi-tumor transplantation, and were closely related to the progressive development of the primary malignant process. In TBHs (Gr-2 and Gr-3) treated with the antitumor agents, no deviations in the size and

distribution of testicular blood vessels were observed, compared to healthy animals, suggesting inhibition of the spread of the tumor process in the testicular tissue during EPC3 and DOX treatment.

Histological light microscopic examinations of testicular tissue from Gr-2 and Gr-3 show normal structure and function of the seminiferous tubules (or spermatogenesis) similar to the control group of healthy animals (Fig. 2, A–C). In contrast, untreated GMT-bearing hamsters (Gr-1)

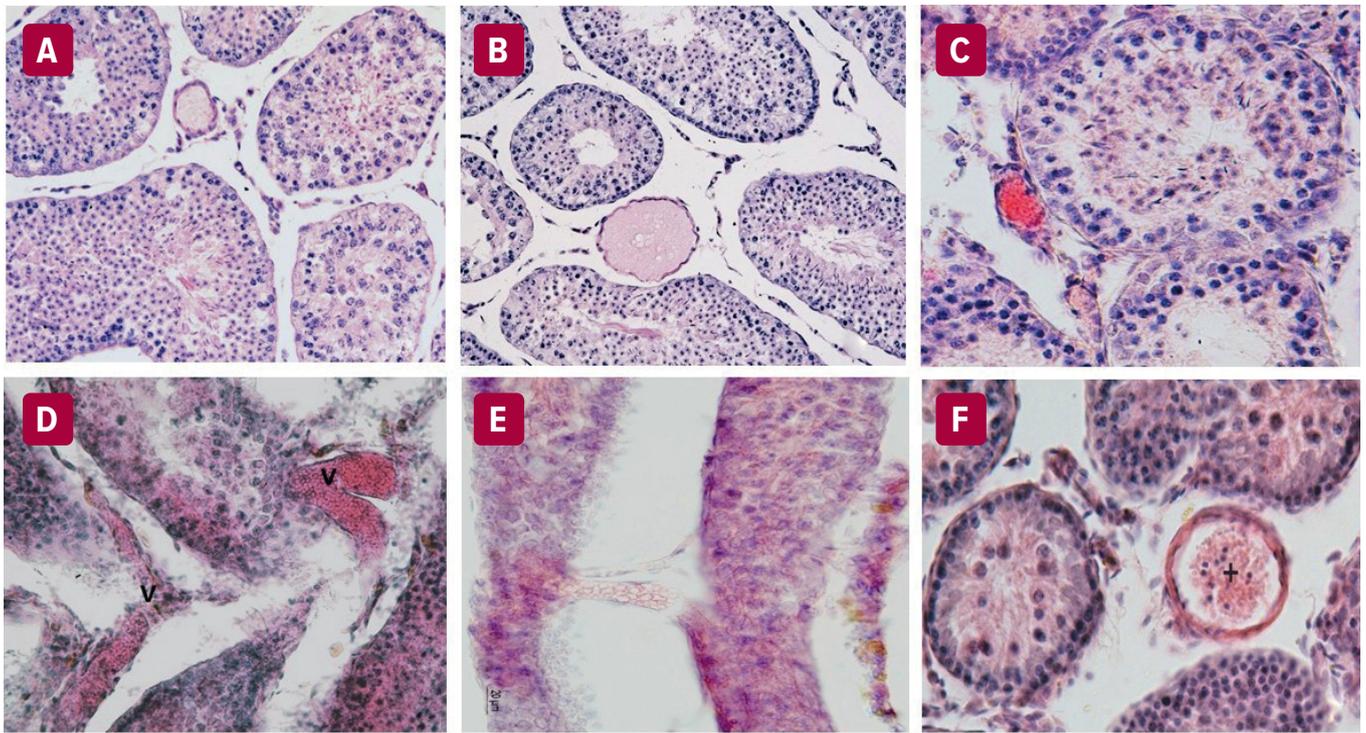


Fig. 3. Testicular cross-sections: (A) TBHs treated with EPC3; (B) EPC3 + DOX treated TBHs; (C) control animals; (D–F) sections of untreated TBHs. (A–C) Region from the testicular tissue, slight enlargement of the interstitial space (in the normal values), as well as normal micro-vasculature with different size of vessel diameter (capillaries and venules); (D, F) blood vessels with many erythroid and atypical myeloid cells in the lumen, and (D) two sinusoidally extended and branched capillaries between seminiferous tubules; (E) thin capillary “bridges” located between two peritubular capillaries were observed. H&E, $\times 200$, $\times 400$
v — microvessels; + — atypical tumor/GMT cells.

showed profound destruction in the seminiferous tubules and surrounding interstitial tissue (Fig. 2, D–F).

In general, the pathological morphological changes occurring in the seminiferous tubules in untreated TBHs are characterized by: suppressed/incomplete spermatogenesis, disorganization of the seminiferous epithelium with “detachment” of the germ cells from the basal membrane of the tubules (related probably to their degeneration) formation of multinucleated (giant) cells and clusters of degenerative (apoptotic) spermatogenic cells in the lumen of the seminiferous tubules; depletion of differentiated (spermatocytes and spermatids), but an increase in the number of undifferentiated (blast-like) germ cells located near the basal membranes or tubule lumen. As a result of altered spermatogenesis, elongated spermatids, and spermatozoa are not visualized (Fig. 2, D–F and Fig. 3, A–C). At this stage of GMT development in hamsters, no morphological changes in the Leydig cells were established. In the testes, sections containing blood vessels (mainly capillaries located in the peritubular and intratubular space) with sinusoidal expansions of the lumen with branches arising from them (thin growths) directed to the main blood vessel are found (Fig. 3, D). Short and thin capillary “bridges” (probably anastomoses) between peritubular and/or peritubular and intratubular blood vessels (capillaries, arterioles, and venules) are also observed (Fig. 3, D, E). In the untreated TBHs, in the lumen of blood vessels, in addition to erythrocytes, numerous nuclei of atypical myeloid cells are visualized, and in cases with impaired integrity of the vascular wall, cell infiltrates are observed in the surrounding tissue (Fig. 2, D and Fig. 3, D, F).

Changes in the peritubular blood vessels are combined with enlarged interstitial spaces, as well as with a damaged structure of the nearby seminiferous tubules. Moreover, it is observed that the malignant myeloid cells invade the tubules, disrupting the basal membrane and thus affecting spermatogenesis, probably with the participation of the tumoricidal toxic factors that alter the nuclear chromatin of the spermatogenic cells, turning them into “atypical” degenerative cells (Fig. 2, D–F and Fig. 4, A, B). It is assumed that precisely neoangiogenesis in neoplastic processes helps to carry out the exchange of various metabolic substances, including pathological cell factors (by near inter-cellular contacts), inducing initial tumorigenesis (in this case — early development of metastases in the testis). According to some authors, the injured spermatogenesis and the subsequent disintegration of germ cells in tumor-bearing rats can be induced under the influence of body fluids containing damaging humoral cell-tissue factors/substances (produced and secreted by tumor cells) to whose pathological influence the germ cells in the testes are most sensitive and susceptible [30].

Human melanoma cells are known to produce various angiogenic factors which promote tumor angiogenesis through in-growth in the tumor vasculature from pre-existing blood vessels [3, 31]. Similarly to acute leukemic diseases in humans, the primary GMT is just as malignant and leads to early death in the animals before the development of late tumors (metastatic) foci in the testes. The cells of these organs are also rich in DNA, RNA (respectively DNA, RNA proteins), as well as monoblastic and/or myelomonocytic cells (depending on the type of AML), which probably accounts for their stronger resistance

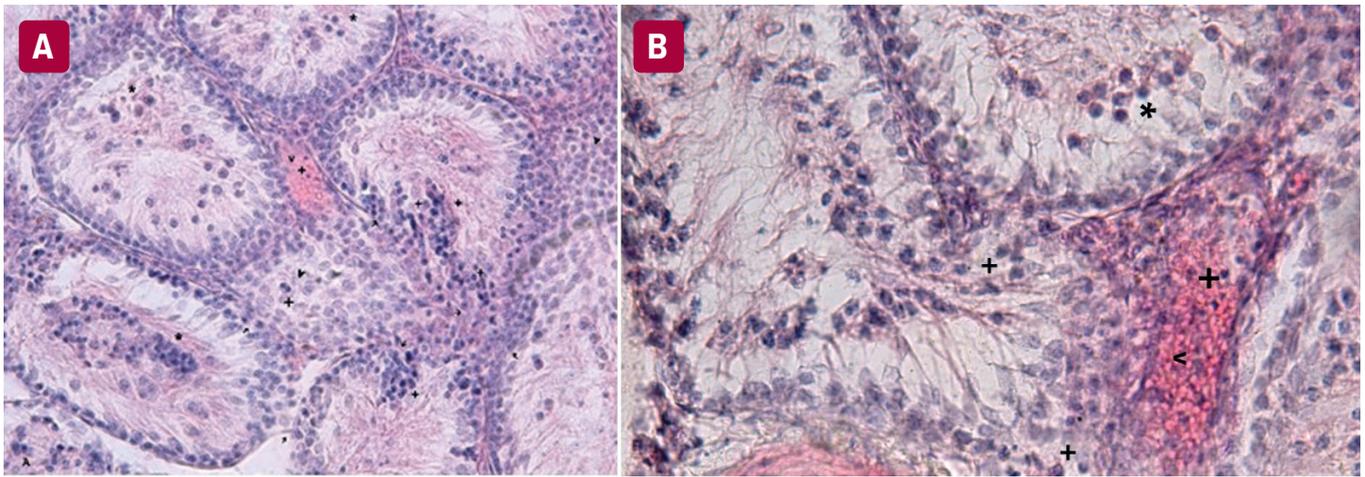


Fig. 4. (A, B) Transverse section of testes of untreated TBHs (terminal day). Along with the damaged tubular basal membranes, clusters of tumor cells/metastases were observed spreading from the microvessels into the testicular interstitium and infiltrating the walls and lumen of the adjacent seminiferous tubules. Highly invasive GMT cells form tubular and interstitial, micrometastases are formed. The seminiferous tubule is observed with “detachment” of abnormal germ cells and atypical cells from the basement membrane into the lumen of the tubule. H&E, $\times 200$, $\times 400$

arrowhead — interstitium; small arrows — destroyed basal membranes; v — microvessels; + — tumor cells; * — atypical germ cells.

against the invasion of tumor cells, unlike other tissues and organs [26]. According to many literature data, BTB is very important in the spreading of neoplastic diseases, related to the male reproductive tract [32–34].

The mechanism of blood flow microcirculation in the testes is by the active vasomotor function of the terminal afferent and efferent blood vessels (arterioles and venules), as well as anastomoses between small vessels of both types. Thus, through adjacent peritubular and intertubular capillaries, blood reaches the seminiferous tubules and Leydig cells. The exchange of substrate/metabolite substances between the bloodstream and the testicular cells takes place through the walls of these blood vessels, including through the BTB [34]. This is the main reason our studies are directed to the changes in the diameter of the blood vessels lumen (especially of the capillaries), as morphological proof about the early sign of tumor-induced neovascularization, while pathological changes in testicular tissue are associated with invasion and development of testicular metastases in untreated TBHs. The presented data were in confirmation of literature findings, according to which the spreading of malignant myelogenous disease could influence male fertility by influencing the spermatogenesis process [35, 36]. Cases of relapse after systemic chemotherapy in patients with malignant diseases, especially cases of AML are often discussed in the scientific literature. According to one of the widely discussed hypotheses, this poor prognosis is associated with the ability of cancer cells to “hide/harbor” in places with a microenvironment that protects from chemotherapy, where they can remain quiescent or exert their resistance to cytotoxic agents. Metastases and/or relapses can occur in extramedullary sites such as the brain and testes, which are designated as “sanctuary sites” for cancer cells because of the relative protection they seem to offer and where chemotherapeutic drugs could reach, but in more low doses (concentrations) compared with their plasma levels. This is probably due to the protective function of the tissue-blood barriers in these organs, which hinder the process of drug supply [3, 33, 37].

The compared results of the performed morphological investigations in groups of TBHs treated with EPC3 and EPC3 + DOX showed for the most part normal structure of the seminiferous epithelium and spermatogenesis in the tubules, i.e. they are not affected by the growth and proliferation of myeloid tumor cells. In the light microscopic observations in the lumen of the blood vessels (capillaries, arterioles, and venules) of the testes, besides erythrocytes were not noted nuclei of atypical myeloid cells (Fig. 2, A–C). This is probably due to the positive effect of antitumor agents that inhibit secondary invasion, as well as suppression of proliferation and micro-spread of tumor cells in the male gonads in these experimental groups of animals. However, in one of the cases from Gr-3, a higher percentage of seminiferous tubules with impaired spermatogenesis (69.3 %) was assessed compared to Gr-2 (> 75 %) and control healthy animals (> 80 %). The probable reasons, in this case, may be different, including a more pronounced sensitivity to one of the therapeutic agents (e.g., DOX), having a toxic effect on the development of germ cells. The results of the present study complement and confirm the previously published data proving the significant antitumor effect of EPC3 and EPC3 + DOX on Graffi tumor by inhibiting tumor growth (significantly reducing the size of the primary tumor), reducing the transplantability of tumor cells, inducing apoptotic changes (apoptosis) in them, suppress metastatic activity (by blocking cell migration) and prolong the average survival time of experimental animals [38]. According to many literature findings, the positive therapeutic effect of APC derivatives (including erufosine) is explained by their ability to influence lipid metabolism, membrane permeability (fluidity, impaired elasticity of the cell membrane), modulation of intracellular signaling pathways transduction and subsequent induction of apoptosis in cancer cells [39–41]. Data have been presented showing that caspase 3 activity is doubled under the action of erufosine, which is probably the main apoptosis pathway for cancer cells [5].

In relation to therapeutic practices, new data are being accumulated related to the helpful effects that in-

frared thermal fields and electromagnetic fields can have on malignant diseases. Their biological impact on water, saline, and blood serum was investigated by analyzing the parameters of electromagnetic hydrogen bonds [42]. During the study of physiological saline observed a 5-times increase of hydrogen ions (H^+) and a change in membrane conductivity, which is evidence of restoring the ion balance. The increase in the number of H^+ ions reveals a process of restoring the potential of the cancer cell to a healthy state. The processes of improvement of ion exchange and electrical potential in the tumor cells and the synthesis of basic proteins, support the body protection and the immune status, and they hold up the tumor process. Results show the positive effect of near-infrared radiation on the structure and function of the erythrocyte membrane in normal and pathological conditions. Positive results related to DNA synthesis have also been reported [42]. In addition, studies have been conducted related to the influence of electrolyzed alkaline water (catholyte) on the development of tumors, including in developing experimental GMT in hamsters [43]. Analysis shows significant differences in the values of some hematometric indices and morphological changes in some of the blood cells. Hamsters treated with catholyte were also found to develop tumors with some delay, have slow tumor growth, and increased survival rates compared to untreated (drinking tap water). Catholyte water (investigated *in vivo* — in an experimental model of hamsters bearing GMT) can improve cellular immunity through an immunomodulating and/or immunostimulating influence. The results suggest that in this experimental model the treatment of tumor-bearing animals with catholyte, such as drinking water, improved the hematometric indices to the normal values and give a reason for the possible use of catholyte to support the non-invasive therapy of cancer diseases [43].

CONCLUSION

The presented results show the protective anti-malignant effect of erufosine drug, which is expressed as suppressed metastatic activity in the testes of hamsters with Graffi myeloid tumor as an appropriate experimental *in vivo* model. The morphological assessment of the degree of injury in the spermatogenesis, as well as of the cytological changes in the germ cells in the seminiferous tubules in pathological and/or experimental (as GMT model) conditions, could be helpful for the development of new methods and directions for therapy and prevention of the male fertility in malignancy development and neoplastic metastases in the testes.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

FINANCIAL SUPPORT

This work research was supported by the Bulgarian Science Fund, grant No. BO2/5-2014.

AUTHOR CONTRIBUTIONS

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ACKNOWLEDGMENT

The authors would like to thank Prof. Detelina Kulchitskaya (National Medical Research Center for Rehabilitation and Balneology of the Ministry of Health of Russia, Moscow, Russian Federation) for her helpful partnership, advice, recommendations, and consultation on the present research publication.

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