Annual of Sofia University "St. Kliment Ohridski"
Faculty of Biology
Book 4 - Scientific Sessions of the Faculty of Biology
2019, volume 104, pp. 288-300
International Scientific Conference "Kliment's Days", Sofia 2018

MORPHOLOGICAL AND HISTOPATHOLOGICAL CHANGES IN THE TESTIS OF HAMSTERS WITH EXPERIMENTALLY-INDUCED MYELOID TUMOR OF *GRAFFI*. HYPOTHESES FOR METASTASES IN THE TESTIS.

ILIANA ILIEVA*, RENETA TOSHKOVA, ISKRA SAINOVA, ANI GEORGIEVA, IVELIN VLADOV

Institute of Experimental Morphology, Pathology and Anthropology with Museum (IEMPAM) - Bulgarian Academy of Sciences (BAS), Sofia, Bulgaria
**Corresponding author: iilieva@abv.bg*

Keywords: myeloid *Graffi* tumor, myeloid leukemia, testicular metastases, spermatogenesis, peritubular capillaries and intratubular blood vessels

Abstract: Metastatic malignant tumors in the testis are rare phenomenon, probably due to the protection offered by the blood–testis barrier by metastatic developing tumor mass. In this aspect, the current investigation was the first morphological study on testes, using experimental model of *Graffi* myeloid tumor (GMT) in hamsters. The purpose of the present investigation was to examine the early and late histopathological changes and *in vivo*-effects of the transplantable GMT on the testicular morphology and spermatogenesis in tumor-bearing hamsters (TBHs). Significant pathological changes in the TBHs from days 10 and 15 post transplantation (p. t.) were not established in comparison to the control group. The results indicated significant changes in the lumen diameter of capillaries in the testes of TBHs from day 25 and day 30 of the experiment, compared with the controls. In parallel with these data, significant extension of the interstitial space between the seminiferous tubules, as well as the presence of many atypical myeloid cells in the lumen of the whole blood vessels' length in the gonads of the TBHs, were established. Destructive changes in germinal epithelium organization were found in the TBHs from days 25th to 30th p. t. Strong injury and/or suppression of the spermatogenesis were observed in most of the tubules. In the cases of day 30 p. t., proliferation of atypical cells, as well as their infiltration in both tubule lumen and testicular interstitial spaces, near to small blood vessels (neo-angiogenesis), were assessed. The dissemination of these cells additionally injured the seminiferous tubules and probably formed metastases. According to these results, the established changes in the testicular tissue in the blood vessels (in particular capillaries) and in the spermatogenesis process were probably caused by the developing GMT in the organism of the TBHs.

INTRODUCTION

Metastases in the testes can most often derive from hematological malignancies as leukemia and lymphoma, but also from primary tumors with non-hematological origin (Shaffer *et al.,* 1992; Lu *et al.,* 2000). Microscopic evidence of disseminated neoplastic diseases, involving infiltration of the testis by myeloid leukemia cells has been detected in 64% of patients with acute myeloid leukemia (AML) and in 22% of cases with chronic myeloid leukemia (CML) (Giver, 1969). Higher frequency of leukemic cell infiltration in the testis has been observed in patients with acute monocytic (monoblastic-, myelomonocytic) leukemia (AMoL), followed by patients with AML (Kamiyama and Funata, 1976; Peterson *et al.,* 1981). Other myeloproliferative and/or myelodysplastic disorders as CML, myeloid sarcoma (MS) and myelodysplastic syndrome (MDS), involving the testis, have also less commonly been reported (Valbuena *et al.,* 2005; Yuceturk *et al.,* 2014).

Murine retroviruses provoke a large variety of hematopoietic tumors through complex mechanisms involving insertional activation of cellular proto-oncogenes in the tumor cell (Van Lohuizen and Berns, 1990; Kung *et al.,* 1991). The *Graffi* murine leukemia virus (MuLV) has been isolated in 1954 by Arnold Graffi, who has characterized it as a myeloid leukemia-inducing retrovirus (Voisin *et al.,* 2006). The tumor cells show a very low-differentiated blast cell morphology, which suggests that they are probably immature precursors (Van Lohuizen and Berns, 1990; Kung *et al.,* 1991). In 1957, Graffi has reported about induced "chloroleukemias" with a high frequency in various inbred and outbred mouse strains in cell-free supernatants of Ehrlich sarcoma and derivative tumors (Graffi, 1957). He and his team have also observed a large spectrum of leukemic cell types after repeated passages of the putative viral agent (phenomenon has been described as "hematological diversification") (Graffi *et al.,* 1966). *Graffi* virus has been characterized as multipotent and it has been found to induce both lymphoid and nonlymphoid, including megakaryocytic leukemias, which are quite rare in MuLV-induced pathologies (Voisin *et al.,* 2006). Taking in consideration these findings, the morphological and histochemical analysis of the leukemic cell phenotype have suggested a probable mixed nature of viral components of this virus (Ru *et al.,* 1993).

The experimental model of transplantable *Graffi* myeloid tumor (GMT) in hamsters is very similar in nature and presents basic features of the acute monocytic (monoblastic-, myelomonocytic) leukemias in humans (Zvetkova and Toshkova, 2006). According to many literature data, this tumor has been found to arise in many extra-medullar regions of the skin, brain, lymph nodes, etc. (Jakimov et al., 1979; Toshkova *et al.,* 2010; Ormandzhieva and Toshkova, 2015). These features make this model very appropriate for the aim of the presented study.

GMT cells have been spread by hematogenous (arterial–retrograde venous) and retrograde lymphatic mechanisms. The functions of the blood-testis barrier (BTB), especially of testicular microcirculation (microvasculature) and Sertoli cells, are of

great importance for the protection of the testicular interstitial tissue and developing male germ cells in the process of spermatogenesis against tumor/leukemia cell invasion (dissemination) (Bart *et al.,* 2002; Holstein *et al.,* 2003; Dave *et al,* 2007; Yuceturk *et al.,* 2014).

In this aspect, further studies on the histopathological changes in the testicular microcirculation (micro-vessel dilatation and neo-angiogenesis) in the course of neoplastic diseases could characterize the first steps of malignant cell metastasis through the BTB and elucidate, at cellular and molecular levels, the etiology and pathogenesis of neoplastic blast cell dissemination. Moreover, the used experimental model gives a possibility for investigation on the influence of myeloid malignant diseases on the morphology of seminiferous tubules and spermatogenic process.

The purpose of the present study was to examine the early and late histopathological changes and *in vivo*-effects of the transplantable GMT on the testicular morphology and spermatogenesis in tumor-bearing hamsters (TBHs). The used transplantable myeloid tumor originated as a *Graffi* murine leukemia virus-induced tumor in newborn hamsters, adapted and maintained to mature Golden Syrian hamsters (Jakimov *et al.,* 1979; Toshkova, 1995; Toshkova *et al.,* 2010).

MATERIALS AND METHODS

Experimental hamster model of GMT: Golden Syrian hamsters, 2 months old, were used in the experiments. The experimental *GMT* was primary created by the *Graffi* virus in new-born hamsters, and maintained monthly *in vivo* by subcutaneous transplantation of live tumor cells (2×10^6/ml PBS) in the inter-scapular area of hamsters, to maintain the tumor (Jakimov *et al.,* 1979, Toshkova, 1995; Toshkova *et al.,* 2010). Between days 10 and 15 post transplantation (p. t.) of the tumor cells, appearance of solid subcutaneous tumor formation in the spinal region of the hamsters was observed. This tumor formation progressively increased and caused death of the experimental rodents around the day 30 p. t. The tumor is 100% carcinogenic, with 100% mortality (lethality) for the experimental animals, and lack of spontaneous regression in them has also been assessed. The animals were kept under standard conditions with free access to food and water. Samples of testes were stained by hematoxylin-eosin (H&E) and examined under light microscope *Leica DM5000B.* The morphometric data were obtained by using eyepiece micrometer at 400 x magnification. The luminal diameter was measured as perpendicular distance across the maximum chord axis of each vessel in the control (n = 6) and TBHs (n = 4 at the 10^{th}, n = 2 at the 15^{th}, n = 6 at the 25th and n = 3 at the 30^{th} day p. t.) groups.

Statistical analysis: The results are reported as mean values ± SEM and statistically analyzed by Student's t-test. All studies were performed in accordance to 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.

RESULTS

In the current study, morphological/morphometrical examinations were performed on the testes from the control group (untreated) hamsters and from the group of GMT-bearing hamsters (at 10th, 15th, 25th and 30th days p. t.). These findings are shown in Table 1, Figures 1, 2 and 3. The results reveal normal testicular structure and function (spermatogenesis) in healthy animals (controls) (**Table 1, Fig. 1 and Fig. 2A**).

Morphometric examinations of the testicular tissue in GMT-bearing hamsters

The results from the performed study indicate statistically significant changes in the mean luminal diameter, with dilatation of capillaries, but also of the larger blood vessels (arterioles and venules), in tumor progression process in the testes of TBHs from day 25th (mean luminal diameter of capillaries/vessels < 15.0 μm and vessels of 15.5-30.0 μm in diameter: 11.80 ± 1.89 μm; 24.82 ± 3.32 μm; and large vessels of 30.5-50.0 μm and >50.0 μm: 39.30 ± 6.06 μm; 76.40 ± 9.10 μm in diameter, respectively) and day 30th of the experiment (mean luminal diameter of capillaries/vessels < 15.0 μm and vessels of 15.5-30.0 μm in diameter: 12.93 ± 1.66 μm; 25.72 ± 3.26 μm; and large vessels of 30.5-50.0 μm and >50.0 μm: 43.33 ± 4.95 μm; 82.65 ± 7.77 μm in diameter, respectively), in comparison with the controls (Table 1).

Table 1. Morphometric data of blood vessels in the testes of tumor-bearing and healthy (control) hamsters (diameter of blood vessels lumen in μm)

Blood vessels (Luminal diameter)	6.0 – 15.0 μm	15.5 – 30.0 μm	30.5 – 50.0 μm	> 50 μm
Control	***9.19 ± 2.05***	***23.25 ± 2.36***	***34.62 ± 1.22***	***60.95 ± 6.11***
10 day	9.13 ± 3.38	23.97 ± 3.21	35.44 ± 2.41	62.36 ± 5.42
15 day	9.34 ± 3.45	24.11 ± 3.34	35.50 ± 2.89	63.38 ± 7.37
25 day	11.80 ± 1.89	24.82 ± 3.32	39.30 ± 6.06*	76.40 ± 9.10**
30 day	12.93 ± 1.66*	25.72 ± 3.26*	43.33 ± 4.95**	82.65 ± 7.77**

*p< 0.01, **p< 0.001

Significant changes in the blood vessels diameter in the testes of hamsters from 10th and 15th days p. t. were not established in comparison to the control group (**Table 1, Fig.1 and Fig. 2 B, C**).

The percentage distribution of blood vessels with different size indicates an increase in the number of the large blood vessels – venules, arterioles or/and dilated capillaries (vessels of 30.5 - 50.0 μm and > 50.0 μm in diameter) in the testes of TBHs at day 25 p. t. (8.09 % and 2.86%, respectively), and at day 30th p. t. (9.35 % and 5.14 %), in comparison with these from days 10/15 p. t. (3.88 %/4.22 % and 1.46 %/1.10 %) and the controls (3.43 % and 1%). In the same

cases, the number of blood vessels with lumen rate 15.5 - 30.0 μm in diameter (at day 25 – 40.48 %, and at day 30 – 41.77 %) was significantly increased, while the percent of these with the smallest size - decreased (at day 25 - 48.57 %, and at day 30– 43.74 %), in comparison with the hamsters from the control group (69.11 % and 26.46 %, respectively) (**Fig. 1**).

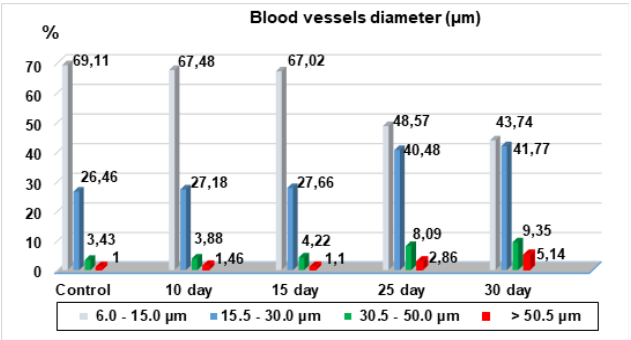

Fig. 1. Comparison of morphometric data of testis blood vessels in tumor-bearing and untreated (control) hamsters (relative part in %)

Regions from the testicular tissue with blood vessels (mainly capillaries), located in the peritubular and intratubular space, respectively, with sinusoidally extensions of the lumen and branched, or thin excrescences derived from them, which are parallel directed to the main blood vessel could be noted (**Fig. 2D-I**). Short and thin capillary "bridges" (probably anastomoses) between nearly-located peritubular capillaries, as well as between peritubular and intratubular blood vessels (capillaries, arterioles and venules) could also be observed (**Fig. 2D**).

In the blood vessel lumen, including in the whole vessel in some cases, many nuclei of atypical myeloid cells are often visualized (**Fig. 2G-I**). The capillaries are filled mainly with erythroid cells. In the cases from day 30 p. t., blood vessels with injured integrity of their walls and with cell infiltrates in the surrounding area could also be established in the testis.

The changes in the blood vessels are combined with extended interstitial spaces (especially in the peritubular tissue), as well as with injured structure of the most seminiferous tubules. The results from the morphometric measures of the peritubular tissue thickness in the TBHs at day 25 p. t. (60.30 ± 26.78 μm) and at day 30 p. t., respectively (60.70 ± 28.79 μm) show considerable increase in comparison with the animals, treated at days 10 (16.39 ± 4.73 μm) and 15 (16.65 ± 3.31μm) p. t. and in the controls (14.16 ± 4.34 μm) ($p < 0.05$).

Morphological/histopathological examinations of the testicular tissue in GMT-bearing hamsters

The results from the light microscopy observation of testes from GMT-bearing hamsters at days 25 and 30 p. t. are shown in Figs. 2 and 3. The seminiferous tubules and the interstitial tissue in the testes of TBHs at days 10 and 15 p. t. were not affected by myeloid tumor cell growth. At this early stage of GMT-proliferation and probable micro-dissemination in the experimental animals, no destructive changes were observed in the tissue of the testes in the tumor-bearing animals (**Fig. 2B,C**)

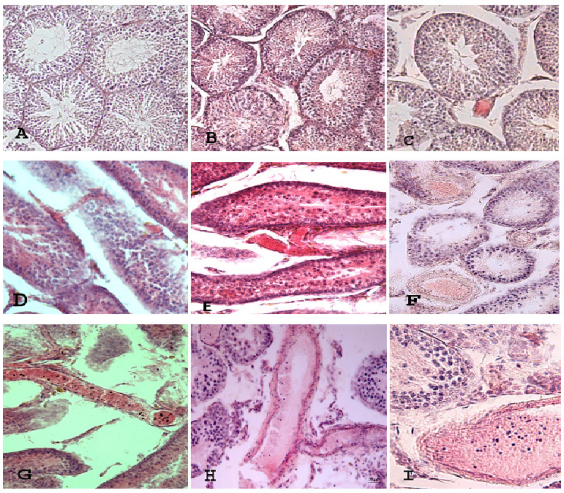

Fig. 2. Testicular cross-sections of: **A)** control and **B, C)** sections of TBHs from days 10 and 15. Normal micro-vasculature and testicular tissue with preserved spermatogenesis. **D-F)** Day 25th: **D)** Thin capillary "bridges" between two nearly-located peritubular blood vessels (capillaries); **E)** Sinusoidally-extended capillary, with two branches, deriving from it and located between the seminiferous tubules; **F)** Enlarged interstitial space with three blood vessels (arterioles). **G-I)** Day 30: In all three slides, a lot of erythroid cells, as well as nuclei of atypical myeloid cells in the lumen of the whole blood vessel were observed; **G)** Two branched blood vessels (sinusoidally extended capillaries), tightly put close to each other, in the upper part of the micrograph; H) Two blood vessels (venules); **I)** One blood vessel (venula). H&E, 200 x,400 x

The seminiferous tubules and the interstitial tissue in the testes of TBHs at the 25th day p. t. showed severe changes in testicular morphology and progression of spermatogenesis (**Fig. 3A**). Intact spermatogonia were seen on the basal membranes of the seminiferous tubules together with some degenerating cells, probably primary spermatocytes. Single germ cells, differentiated as spermatocytes and round spermatids, can also be found, in contrast to the controls. At this stage of

GMT development in hamsters no morphological changes in Sertoli and Leidig testicular cells were established. In the testes of the experimental animals at 30th day p. t., profound destruction in the seminiferous tubules and the surrounding interstitial tissue was observed (**Fig. 3B-D**). In the most of the tubules, strongly suppressed spermatogenesis was found together with many degenerative germ cells. The seminiferous epithelium is disorganized, showing their depletion. As a result of injured spermatogenesis elongated spermatids and spermatozoa were not visible (**Fig. 3B-C**).

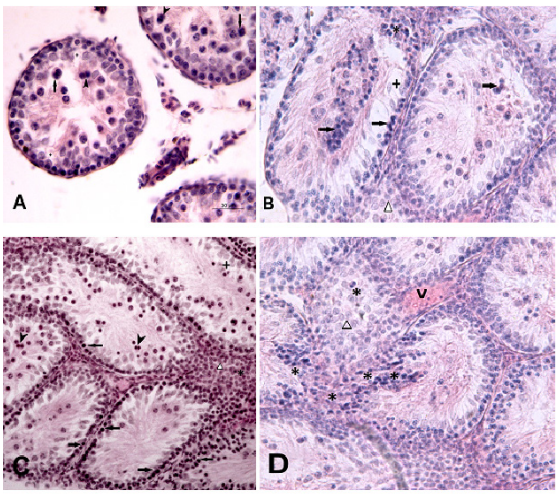

Fig. 3. Testicular cross-section of TBHs from day 25 **(A)** and day 30 p. t. **(B-D)**. The germinal epithelium is disorganized, with many cavities and showing depletion of germ cells, degenerating primary spermatocytes (St) with abnormal chromatin condensation/ fragmentation, immature spermatids, atypical GMT-blast cells and giant multinuclear cells. Seminiferous tubules with damaged basal membranes, as well as "detachment" and amassment of abnormal germ cells and atypical cells from the basal membrane in the lumen of tubule were observed (*slide B - in the left*). Clusters of tumor cells/metastases (*slide D*) were seen spreading from the micro-vessels to the testicular interstitium, and infiltrating the wall and lumen of the adjacent seminiferous tubules. The highly invasive GMT-cells form the tubular and interstitial regions, micro-methastases, were formed. *(arrows) – abnormal germ cells/St, (arrowheads) – giant multinuclear cells, (*) - atypical tumor/GMT cells, (+) –cavities, (Δ) – interstitium, (v) - microvessels. H*&E, 200 x, 400x

From the morphological point of view, interesting findings are the cases of "detachment" of germ cells from the basal membrane of the tubules, which probably leads to degeneration of the germ cells and formation of clusters of degenerative spermatogenic cells in the lumen of seminiferous tubules (**Fig. 3B**). Seminiferous tubules with injured structure and integrity of the basal membranes were visualized very nearly or in direct contact to the new vessels

(neo-vascularization), leading to the penetration of atypical GMT cells into the testicular interstitium and formation of metastases (**Fig. 3D**).

In summary, in both groups of experimental hamsters - examined at days 25 and 30 p. t., the morphological changes, occurring in the seminiferous tubules with suppressed spermatogenesis are expressed with three main features: depletion of differentiated spermatogenic cells (spermatocytes and spermatids) and formation of multinuclear (giant) cells in the tubular lumen; increased number of degenerative germ cells mainly in the tubular regions localized near to the basal membranes; and increased number of undifferentiated germ (blast-like) cells in the lumen of the seminiferous tubules.

DISCUSSION

Microscopically, the testicular metastases are disseminated as nets of tumor cells in the intersticium (interstitial tumor growth). In other cases, a simultaneous invasion of metastatic carcinoma in the interstitium and/or seminiferous tubules (intratubular tumor growth) of the testis have been observed (Nistal *et al.,* 1989; Lu *et al.,* 2000). In all cases of testicular relapse in AML (mainly at monoblastic – and/or myelo-monocytic-cell differentiation, according to the sub-type of AML), leukemic infiltrates have been located in the interstitial spaces reaching the testis through the enlarged, permeable and injured capillaries (as a result of neoplastic process and neo-angiogenesis related) (Shaffer *et al.,* 1992; Zvetkova and Toshkova, 2006).

In our study, the blood-testes barrier (BTB) is accepted as a main factor about the secondary invasion and dissemination of the tumor cells in the male gonads. This suggestion is in support of some literature data (Kusumakumari et al., 1994; Bart *et al.,* 2002; Dave *et al.,* 2007; Yuceturk *et al.,* 2014) for the importance of this barrier about the early dissemination of neoplastic disease in the male reproductive system. The blood stream regulation in the microcirculation of the testes takes place by active vasomotor function of the terminal afferent and efferent vessels – arterioles, venules, as well as anastomoses between small vessels from both types. The blood reaches to the seminiferous tubules by adjacent peritubular and intertubular capillaries. The substance metabolism between the blood, Leidigh cells and seminiferous tubules occurs namely through the walls of these vessels (Kawashima *et al.,* 1988; Yuceturk *et al.,* 2014). This is the main reason for the investigation on changes in the blood vessels (especially in the capillaries), taking in consideration its role as early sign for invasion and development of metastases in the testes.

In this aspect, the data from the morphometric studies of the blood vessel lumen, as well as the microscopic observations, revealing the presence of casual short, "blind" capillary excrescences and sinusoidal extensions, could be accepted to be in result of neo-vasculature (neo-capillaries and enlarged small blood vessels,

including venules). Moreover, the establishment of small "bridges" (anastomoses) between the capillaries, the presence of blood vessels with injured integrity of their wall, as well as the extended interstitial space between the seminiferous tubules, wholly change the normal look of the testicular tissue, and could be supplied as signs of initial tumor-induced neoangiogenesis. The establishment of many atypical myeloid cells in the blood vessels' lumen suggests the initiation of tumor cell invasion in the testes in TBHs at days 25 and 30 p. t.

Analogical processes of blast cells interstitial infiltration have been described by Yuceturk *et al.* (2014) in cases of CML. Similar morphological destruction changes were also observed in the blood vessels from the choroid plexus of the brain (Ormandzhieva and Toshkova, 2015), as well as presence of atypical myeloid cells with multiple mitochondria inside the cytoplasm and nucleus of periphery blood cells (Zvetkova & Toshkova, 2006) in the same experimental model of Graffi TBHs. Other authors, who have used similar experimental models, have explained the observed by them tumor blood vessel-like and capillary-like structures formation in vivo (with the presence of erythrocytes in their lumens) by expression of several tumor vasculogenic- and angiogenic genes, as well as of genes for cell growth factors (including VE-cadherin, FGFR1, VEGFA, etc.), from the neoplastic cells, e. g. in cases of human melanoma and ovarian (Hey 1B) cancer (Maniotis *et al.,* 1999; Cao *et al.,* 2013).

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 (Maniotis *et al.,* 1999). To verify and confirm whether blood vessels in the course of malignant cell dissemination are a part of the prime organ vasculature or they grow as tumor cell "vasculogenic mimicry channels" (Liu *et al.,* 2012), probably as in our experimental model, further studies and observations on VE-cadherin expression in GMT cells, accompanied by co-expression of tumor cell marker proteins should be performed. The existence of new tools, suppressing tumor neo-angiogenesis and VE-cadherin expression in melanoma- and other malignant cells (Liu *et al.,* 2012; Nair *et al.,* 2016) should be also discussed by us in the course of our further experimental studies on GMT-models.

In the presented research, we suggested a possible disintegration of the BTB (disrupted by neoplastic process) in the cases of detachment (ablation) of the germinal cells from the basal membrane of seminiferous tubules. Our results demonstrated the appearance of leukemic infiltrates of monoblastoid cells in the interstitial space through the disruption of blood vessel walls and injuries in the BTB (Ilieva *et al.,* 2017). These data were in agreement with findings of other authors (Shaffer, 1992), who have also identified leukemic infiltrates, located in the testicular interstitial tissue, with secondary atrophy of the seminiferous tubules. We also observed light-microscopically the secondarily involved seminiferous tubules, due to the local spread of monocytoid-like blasts (blasts

of monocytoid appearance - see Shaffer,1992), simultaneously with disruption of the BTB. On the other hand, our histological studies showed a disorganized seminiferous epithelium together with many degenerative germ cells and strongly suppressed spermatogenesis with depletion of differentiated spermatogenic cells (spermatocytes and spermatids) in the testes of TBHs at days 25 and 30 p. t. These results were in support of literature data, according which the dissemination of malignant myelogenous disease could affect the man's fertility by influencing spermatogenesis (Hallak *et al.,* 2000; Agarwal & Allamaneni, 2005).

Pioneer microscopically investigations on the elevated testes in tumor-bearing rats (concerning Yoshida – and-MTK-sarcomas), undertaken by K. Kano in early 1952, have also shown similar results (Kano, 1952). The histological studies on the seminiferous tubules showed attenuated – suppressed- or absent spermatogenic activity probably due to germinal cell degeneration. Cytological features of the nuclear chromatin and chromosomal abnormalities in all phases of the cell cycle have also been described. The general consideration of K. Kano (1952) has been that abnormal spermatogenesis and the subsequent germ cell disintegration in the tumor-bearing rats might be induced on the influence of body fluids, containing produced and secreted by tumor cells injurious substances (humoral cell and tissue factors), to which pathological effects the testicular germinal cells are particularly sensitive and susceptible. Similar changes in the spermatogenesis have been observed by other authors only in cases of primary testicular germ cell tumors (TGCTs) and in some sarcomas types (Kano, 1952; Gainetdinov *et al.,* 2016). The authors suggested that spermatogenesis could be retained (suppressed) in the pre-malignant testis tissues adjacent to the more aggressive nonseminomas, sarcomas, etc., but not those adjacent to less invasive seminomas. Moreover, DNA-methylation level is higher in the preneoplastic testis tissue adjacent to the nonseminomas. An interesting fact is that essentially all TGCTs arrise due to failure of normal spermatogenesis undergoing.

However, because the causes of poor semen quality in cancer- and leukemia patients are not yet well understood, our contributions in this field tended to be concerned about cell- and tissue (humoral) factors involved in impaired spermatogenesis in the testes of leukemia and cancer patients. These data could be discussed in different aspects and in relationship to the premalignant/malignant testicular tissue adjacent to the seminiferous tubules and influenced (suppressed) intratubular spermatogenesis in the conditions of the testicular tubular involvement by differently aggressive and invasive myeloid malignancies.

CONCLUSION

The current (pilot) study showed for the first time the histopathological changes in the testes in experimental model of induced transplantable myeloid *Graffi* tumor in hamsters. Also, this investigation clearly indicates a presence of destructive pathological changes in the testicular tissue and blood vessels (in

particular capillaries), including formation of metastases, as well as, a possibility about significant suppression and/or injuries in the spermatogenesis process in result of the noted morphological changes. In this way, the hypothesis for incipient metastatic process in testes of TBHs could be confirmed. The simultaneous expression of several cancer/testis antigens (CTAs) in the course of both processes – spermatogenesis and carcinogenesis (Cheng *et al.,* 2011), should be evaluated in future investigations on Graffi myeloid tumor model. This experimental model, as other similar models, could be helpful for the development of new methods and directions about therapy and prevention of the male fertility in malignancy development and neoplastic metastases in the testes.

Acknowledgements: This research was financially supported by the Bulgarian National Science Fund Grant BO2/5-2014). The authors wish to thank M. Pavlova, medical laboratory assistant, for the excellent technical assistance of the histological preparation of tissue.

DECLARATION OF INTEREST:
The authors declare no existing conflict of interest.

AUTHOR CONTRIBUTION STATEMENT:
I I performed investigation on the morphological changes in the testicular histology and in the male germ cells in different phases
R T provided the experimental model of *Graffi* tumor in hamsters, inoculated with tumor cells, derived by infection with *Graffi* virus
I S performed investigation on the morphological changes in other cellular types (blood cells, epithelial cells, etc.) in male germ tract
A G performed *in vitro*-incubation of the malignant cells, used for inoculation of hamsters
I V provided a possibility about light-microscopy observation of histological preparations

REFERENCES

1. Agarwal, A., Allamaneni, Sh. S. R. 2005. Disruption of Spermatogenesis by the cancer disease process. *J. Natl. Cancer Inst. Monogr.,* 34: 9-12.
2. Bart, J., Groen, H. J., van der Graaf, W. T., Hollema, H., Hendrikse, N. H., Vaalburg, W., et al. 2002. An oncological view on the blood-testis barrier.*Lancet Oncol.*, 36): 357-363.
3. Cao, Z., Yu, D., Fu, S., Zhang, G., Pan, Y., Bao, M., Tu, J., Shang, B., Guo, P., Yang, P., Zhou, Q. 2013. Lycorine hydrochloride selectively inhibits human ovarian cancer cell proliferation and tumor neovascularization with very low toxicity. *Toxicology Letters,* 218: 174-185.
4. Cheng, Y. H., Wong, E., Cheng, P. 2011. Cancer/testis (CT) antigens, carcinogenesis and spermatogenesis.*Spermatogenesis*, 1(3): 209-220.
5. Dave, D. S., Leppert, J. T., Rajfer, J. 2007. Is the testis a chemo-privileged site? Is there a blood-testis barrier? *Rev. Urol.,* 9: 28-32.

6. Gainetdinov, I. V., Kondratieva, S. A., Skvortsova, Y. V., Zinovyeva, M. V., Stukacheva, E. A., Klimov, A., Tryakin, A. A., Azhikina, T. L. 2016. Distinguishing epigenetic features of preneoplastic testis tissues adjacent to seminomas and nonseminomas. – *Oncotarget*, 7(16): 22439-47.
7. Giver, R. L. 1969.Testicular involvement in leukemia and lymphoma.*Cancer*, 23: 1290-1295.
8. Graffi, A. 1957. Chloroleukemia in mice. *Ann NY Acad Sci,* 68:540–558.
9. Graffi, A., Fey, F., Schramm, T. 1966. Experiments on the hematologic diversification of viral mouse leukemias. *Natl Cancer Inst Monogr,* 22:21–31.
10. Hallak, J., Mahran, A., Chae, J., Agarwal, A. 2000. Poor semen quality from patients with malignancies does not rule out sperm banking.*Urol. Res.,* 28: 281-284.
11. Holstein, A.F., Schulze, W., Davidoff, M. 2003. Understanding spermatogenesis is a prerequisite for treatment.*Reproductive Biology Endocrinology*, 1(107):1-16.
12. Ilieva I., Toshkova R., Zvetkova E., Sainova I., Vladov I. 2017. Morphological studies on the spermatogenesis and Graffi myeloid tumor cell dissemination (metastasis) in the testes of tumor-bearing hamsters. *Acta morphologica et anthropologica,* 24(1-2): 33-40.
13. Jakimov, M., Mladenov, Z., Konstantinov, A., Yanchev, I. 1979. Transplantable myeloid tumor in hamsters (MTH) induced by Graffi virus. *Gen. Compar. Pathol.,* 6: 24-35.
14. Kamiyama, R., Funata, N. 1976. A study of leukemic cell infiltration in the testis and ovary. *Bull. Tokyo Med. Dent. Univ.,* 23: 203-210.
15. Kano, K. 1952. Degeneration of Germ Cells in the Testes of Tumor-bearing Rats (Studies on Abnormal Nuclear Divisions. *Cytologia*, 16: 341-346.
16. 16. Kawashima, H., Sakamoto, W., Nishijima, T., Hanada, M., Mori, K., Maekawa, M. 1988. Granulocytic sarcoma of the testis preceding acute myeloid leukemia. *Urol. Int.,* 43: 310-312.
17. Kung, H.J., Boerkoel, C., Carter, T.H. 1991. Retroviral mutagenesis of cellular oncogenes: a review with insights into mechanisms of insertional activation. *Curr Top Microbiol Immunol,* 171:1–25.
18. Kusumakumari, P., Kumar, S. R., Pillai, G. R. 1994. Unusual course of chronic myeloid leukemia.*A report. Am. J. Clin. Oncol.,* 17: 19-21.
19. Liu, R., Cao, Z., Tu, J., Pan, Y., Shang, B., Zhang, G., Bao, M., Zhang, S., Yang, P., Zhou, Q. 2012. Lycorine hydrochloride inhibits metastatic melanoma cell-dominant vasculogenic mimicry. *Pigment Cell & Melanoma Research,* 25: 630-638.
20. Lu, L. Yi., Kuo, J. Y., Lin, T. L. A., Chang, Y. H., Chen, K. K., Pan, C. C., Chang, L. S. 2000. Metastatic Tumors Involving the Testes. *J. Urol. Roc.,* 11(1): 12-16.
21. Maniotis, A. J., Folberg, R., Hess, A., Seftor, E. A., Gardner, L.M., Pe'er, J., Trent, J. M., Meltzer, P. S., Hendrix, M. J. 1999. Vascular channel formation by human melanoma cells in vivo and in vitro:vasculogenic mimicry. A*merican Journal of Pathology,* 155: 739-752.
22. Nair, J. J., Bastidab, J., van Stadena, J. 2016. In vivo effects of Amaryllidaceae alkaloids. *Natural Product Communications,* 11(1): 121-132.
23. Nistal, M., Gonzalez-Peramato, P., Paniagua, R. 1989. Secondary testicular tumors. *Eur. Urol.,* 16: 185-188.

24. Ormandzhieva, V., Toshkova, R. 2015. Morphometrical study of the choroid plexus blood vessels in experimental hamster graffi tumor model. *Acta morphologica et anthropologica,* 22: 31-36.
25. Peterson, L., Dehner, L. P., Brunning, R. D. 1981.Extramedullary masses as presenting features of acutemonoblastic leukemia.Am. J. Clin. Pathol., 75: 140-148.
26. Ru, M., Shustik, C., Rassart, E. 1993. Graffi murine leukemia virus: molecular cloning and characterization of the myeloid leukemia-inducing agent. *J. Virol.,* 67:4722–4731.
27. Shaffer, D. W., Burris, H. A., O'Rourke, T. J. 1992. Testicular relapse in adult acute myelogenous leukemia. *Cancer*, 70: 1541-1544.
28. Toshkova, R. 1995. Attempts for immunomodulation in hamsters with transplanted myeloid tumor, previously induced by Graffi virus. PhD Thesis, , Sofia, Bulgaria, p. 168.
29. Toshkova, R., Manolova, N., Gardeva, E., Ignatova, M., Yossifova, L., Rashkov, I., Alexandrov, M. 2010.Antitumor activity of quaternized chitosan-based electrospun implants against Graffi myeloid tumor. *J. Intern. Pharm.,* 400(1-2): 221-233.
30. Valbuena, J. R., Admirant, J. H., Lin, P., Medeiros, L. J. 2005. Myeloid sarcoma involving the testis. *Am. J. Clin. Pathol.,* 124: 445-452.
31. Van Lohuizen, M., Berns, A. 1990. Tumorigenesis by slow-transforming retroviruses - an update. *Biochim Biophys Acta,* 1032: 213–235.
32. Voisin, V., Barat, C., Hoang, T., Rassart, E. 2006. Novel Insights into the Pathogenesis of the Graffi Murine Leukemia Retrovirus. *Journal of virology,* 80(8): 4026–4037.
33. Yuceturk, C. N., B. C. Ozgur, H. Sarici, P. Borcek, O. Telli. 2014. Testicular Involvement of Chronic Myeloid Leukemia 10 Years after the Complete Response. *J. Clin. Diagn. Res.,* 8(4): ND03-ND04.
34. Zvetkova, E., Toshkova, R. 2006. Photos of monoblast from the peripheral blood of Graffi myeloid tumor-bearing hamster. In: BloodMed – Slide Athlas, Oxford – UK.
35. (http://www.bloodmed.com/home/slide.asp?id=741)