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Original Article

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The Influence of Platelet-Derived Growth Factors on the Proliferation of Germinal Epithelium After Local Irradiation with Electrons

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Abstract

Background: At present, the damage of male reproductive function caused by electron irradiation, as well as the development of methods for its correction are the relevant topics for further research. In fact, the effect of leukocyte-poor platelet-rich plasma (LP-PRP) growth factors are poorly investigated on different aspects of male fertility.

Methods: In this study, Wistar rats were divided into four groups; I) control which were injected with saline; II and III) groups (n=65) whose testes were locally irradiated with electrons to a dose of 2 Gy (linear accelerator "NOVAC-11"); III) the group (n=30) which received LP-PRP for 11 weeks after irradiation; and IV) animals (n=30) which received only LP-PRP (conditional control). The testes were studied by histological, immunohistochemical (IHC), western blotting, and TUNEL methods using Ki-67, Bcl-2, and p53. Comparison between groups was performed and $p<0.01$ was set as the level of significance.

Results: The results showed a decrease in the expression levels of Ki-67 and Bcl-2 besides an increase in p53-positive cells by the end of the experiment ($p<0.01$). After injection of LP-PRP, a gradual restoration of the proliferative activity of gametes was noted, which was confirmed by an increase in the proportion of Ki-67- and Bcl-2-positive germ cells (46.4±2.3, $p<0.01$ and 23.5±1.1, respectively, $p<0.01$).

Conclusion: Ki-67 expression and TUNEL analysis in the testes revealed a modulation of the proliferative-apoptotic balance towards apoptosis of germ cells after 2 Gy local electron irradiation. A tendency to restore the proliferative-apoptotic balance was noted after LP-PRP injections as indicated by increase in Ki-67-positive germ cells.

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Introduction

Currently, the number of cases of male infertility is steadily increasing. Difficulties in the diagnosis, treatment, and prevention of pathological forms of spermatogenesis are associated with its polyetiology and complexity of its mechanism (1).

In modern radiobiology, new diagnostic and therapeutic methods are emerging, the safety of which remains unproven for the human reproductive system, and their effects are poorly understood. In this regard, the selection of optimal radiation doses and the search for methods to reduce

their toxicity remain relevant topics for research. This will optimize the diagnosis and treatment in oncology. Moreover, the data derived through investigation of the scientific literature, which is devoted to the study of irradiation effects on the testicle, are ambiguous. Some authors believe that radiotherapy inevitably entails the risk of infertility (2). On the other hand, researchers argue that spermatogonia A remain in the seminiferous tubules after irradiation and in some cases, these cells are able to restore spermatogenesis (3).

Various models of laboratory animals are used for studying the pathologic mechanisms of male infertility, followed by extrapolation of the results to humans. For this purpose, chemotherapy drugs from the group of cytostatics or ionizing radiation (β-, γ-, X-rays, *etc*.) are often used. They lead to disruption of DNA replication and RNA transcription, suppressing the cell cycle of male germ cells (4). In our study, electron irradiation, as a new and poorly studied type of irradiation, was applied.

Recently, preclinical studies of various drugs and their analogues have been widely performed, the function of which is restoring male reproduction (2, 4, 5). One of the promising directions can be the use of leukocyte-poor platelet-rich plasma (LP-PRP) containing many growth factors (PDGF, TGF-β, VEGF, EGF, *etc*). It is involved in the regulation of various phases of the cell cycle of male germ cells (6, 7). Some authors have induced azoospermia by cytostatic agents. After PRP administration, they noted an increase in the number of spermatogonial stem cells, sperm motility, and testosterone levels (8). Data on the study of the effect of LP-PRP on spermatogenesis after irradiation are nearly absent.

Reproduction and death of male germ cells are regulated by Ki-67 proteins and caspases, which result in proliferation and apoptosis, respectively (9, 10). The testis is highly sensitive to radiation as manifested by the decrease in mitotic division and the death of germ cells, especially spermatogonia (11). Apoptosis is activated via extrinsic and intrinsic pathways, and caspase-3 is responsible for the terminal phase. Other cell cycle regulators are pro-apoptotic (p53) and anti-apoptotic (Bcl-2) proteins (9).

In some studies, radiation-induced proliferative-apoptotic imbalance of germ cells was accompanied by a decrease in the cell count due to the modulation of GSK3-, ERK-, and Ras/Raf/MEK-1 signaling pathways. In turn, this led to Bcl-2 deactivation and p53 induction (12, 13).

At present, the post-radiation imbalance between proliferation and apoptosis of germ cells is caused by damage to cell cycle at various stages and remains an appropriate topic for research. However, the main purpose of the current research was to study the effect of platelet-derived growth factors on the restoration of the morphological characteristics of the germinal epithelium, and the method and dose of irradiation served only as a model for the experiment. Therefore, an attempt was made to evaluate the proliferation and apoptosis of germ cells against injection of LP-PRP after electron irradiation and to assess the expression levels of the universal proliferation factor (Ki-67), the apoptosis termination factor (caspase-3), the key factors of anti-apoptosis (Bcl-2) and pro-apoptosis (p53), as well as DNA fragmentation (TUNEL).

Methods

An experimental morphological study was carried out at A. Tsyb Medical Radiological Research Centre and Sechenov University in Russia. As the first step, Wistar rats (n=105, 9–10 weeks) were divided into four groups: I) control (n=10) group which were injected with saline; II and III) groups (n=65) whose testes were locally irradiated with electrons to a dose of 2 Gy (2IR; dose rate: 1 *Gy/min*, energy: 10 MeV, frequency: 9 *Hz*, field size: Ø 100 *mm*, linear accelerator: NOVAC-11 (Sordina IORT Technologies, Italy), exposure time: 2 *min*); III) group (n=30) which received LP-PRP for 11 weeks after irradiation; and IV) animals (n=30) which received only LP-PRP (conditional control). Animals were gradually removed from the experiment by injecting high doses of anesthetic (ketamine, 50 *mg/kg*, intramuscularly, and xylazine, 5 *mg/kg*, intraperitoneally) over 11 weeks; in total, 5 animals from group II were removed one week after irradiation, and then 5 animals of all experimental groups were removed once every 2 weeks.

All interventions were carried out in accordance with the International Guiding Principles for Biomedical Research Involving Animals (1985), the European Convention for the Protection of Vertebrate Animals used for Experimental or other Scientific Purposes (1986), Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Research, US), and Declaration of Helsinki (1964). The study was approved by the

local ethics committee of Sechenov University (protocol No. 043, dated August 11, 2020).

Testes were prepared according to the standard protocol after fixation in Bouin's solution for immunohistochemical studies. Antigens were unmasked in citrate buffer with pH=6.0. Monoclonal antibodies, Ki-67 (Clone MM1; 1:200), Bcl-2 (Clone bcl-2/100/D5; 1:50), and p53 (Clone DO-7; 1:200), were applied as primary, using HiDef Detection™ HRP Polymer System (Cell Marque, USA), and mouse/rabbit anti-IgG, horseradish peroxidase (HRP), and DAB substrate were applied as secondary antibodies. Cell nuclei were counterstained with Mayer's hematoxylin. The number of immunopositive cells was counted in 10 randomly selected fields of view at ×400 magnification (in percentage). Microscopic analysis was performed using a video microscopy system (DM2000 microscope, ICC50 camera, Leica Biosystems, Germany).

Western blotting: The protein concentration in the homogenized samples was determined by the Bradford method. Then, 25 *μg* of the protein was separated on 12% SDS-PAGE and transferred to a nitrocellulose membrane (clone GTX110543, 1:600; GeneTex, US). The membrane was blocked with 5% nonfat dry skimmed milk (70166; Sigma-Aldrich, US) in TBS. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-tubulin were used as western blotting standards. The unit of measurement was nmol pNA/mg protein C. Visualization was performed using Novex™ ECL Chemiluminescent Substrate Reagent Kit (Invitrogen, US).

The TUNEL assay was used to study the DNA fragmentation of germ cells, which were stained with a TDT solution (Thermo Fisher Scientific, US) and incubated for 60 *min*. The nuclei were counterstained with DAPI (blue spectrum) (Thermo Fisher Scientific, US) and the luminous intensity was controlled in a fluorescence microscope with a set of FITC filters (green spectrum).

Statistical analysis: The obtained data were processed using the SPSS *vs.* 12 (IBM, USA). Arithmetic mean values with limiting deviations and standard error were calculated. Comparison between groups was performed using ANOVA, Post Hoc with post hoc test and $p<0.01$ was set as the level of significance.

Results

Histological examination: In histological examination, spermatogenesis was observed in the control group. In the testes of groups II and III, a morphological image of azoospermia, a violation of histoarchitecture, and signs of hypospermatogenesis were observed a week after irradiation, which persisted in the group of irradiated animals without LP-PRP correction until the end of the experiment (Figure 1). By injection of LP-PRP, a gradual increase in the number of germ cells was observed, starting from the 14th day of the experiment: number of spermatogonia increased by 7.3 times (15.3±0.6 cells per field of view, $p<0.01$); spermatocytes , by 4.5 times (69.2±3.2 cells per field of view, $p<0.01$); and spermatids , by 4.1 times (94.5±4.1 cells per field of view, $p<0.01$) in comparison with group II (2.1±0.1 cells per visual field, $p<0.01$; 15.3±0.6 cells per visual field, $p<0.01$; 28.2±1.2 cells per visual field, $p<0.01$, respectively). By the end of the study in group III, the number of germ cells increased to values close to those in the control group (spermatogonia: 24.3±1.0 cells per field of view, $p<0.01$; spermatocytes: 103.1±4.8 cells per field of view, $p<0.01$; spermatids: 129.1±5.4 cells per field of view, $p<0.01$), and spermatozoa were present in the lumen of the seminiferous tubules (at week 12) (Figure 1). Animals in group IV did not show any significant differences in all studied parameters after the injections of LP-PRP compared with the control group (Figure 1).

Immunohistochemic al (IHC) study: When analyzing the distribution of Ki-67 after irradiation (group II), a decrease in the studied marker by 4.6

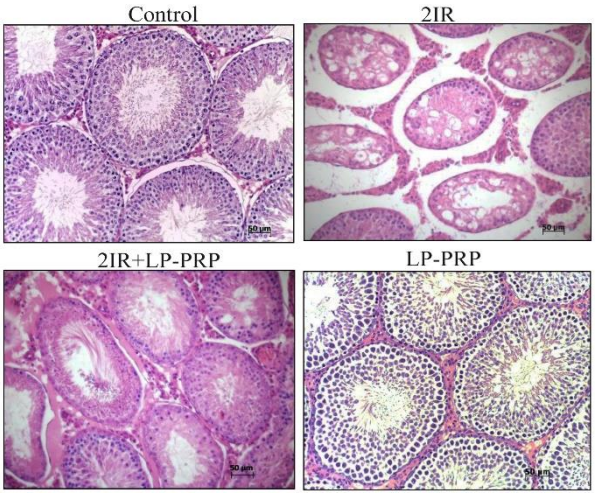

Figure 1. Morphological study of the testes at the 12th week of the experiment in the groups of control, irradiation (2IR), irradiation+LP-PRP (2IR+LP-PRP), and LP-PRP. Hematoxylin and eosin stain, magnification ×200

Table 1. The proportion of IHC-positive germ cells in the seminiferous tubules of the control and experimental groups (percentages)

Groups	Ki-67 M±STD	Bcl-2 M±STD	p53 M±STD
Control	76.0±3.7	53.4±2.6	54.3±2.7
2IR 7 day	53.7±2.6 *	29.3±1.3 *	57.1±2.8 *
2IR 14 day	34.2±1.6 *	15.2±0.7 *	65.4±3.1 *
2IR 56 day	21.4±1.0 *	12.5±0.6 *	69.9±3.4 *
2IR 84 day	16.3±0.6 *	9.1±0.4 *	74.8±3.6 *
2IR+LP-PRP 14 day	39.4±1.8 **	17.8±0.8 **	62.9±3.0 **
2IR+LP-PRP 56 day	43.2±2.1 **	20.3±1.0 **	57.6±2.7 **
2IR+LP-PRP 84 day	46.4±2.3 **	23.5±1.1 **	44.1±2.1**
LP-PRP	73.6±3.6	49.8±2.4	51.3±2.6

* Control and 2IR, ** 2IR, and 2IR+LP-PRP; p<0.01

times (16.3±0.6%, p<0.01) was noted by the end of the experiment compared with control values (76.0±3.7%, p<0.01). After LP-PRP injections, animals of group III showed a gradual increase in Ki-67-positive germ cells compared to group II which was 2.8 times higher by the end of the experiment (46.4±2.3%, p<0.01). In group IV, no significant differences were found compared to control values (Table 1, figure 2).

The anti-apoptotic Bcl-2 protein showed a similar immunohistochemical image a week after irradiation and a decrease in positively stained germ cells by 1.8 times (29.3±1.3%, p<0.01) was noted compared with the control group (53.4±2.6%, p< 0.01). After the LP-PRP injections, a gradual increase in the number of Bcl-2-positive germ cells starting from the 2nd week after irradiation was found, which by the 12th week was 2.6 times more (23.5±1.1%, p<0.01) in comparison to group II (9.1±0.4%, p<0.01). The Bcl-2 values in the testes of group IV were close to control values (Table 1, figure 2).

The number of p53-stained germ cells varied at different stages of the experiment (Table 1). Therefore, a week after electron irradiation at a dose of 2 Gy, a slight increase in the number of p53-positive germ cells (57.1±2.8%, p<0.01), mainly spermatogonia, was revealed compared with the control group (54.3±2.7%, p<0.01), where p53-stained spermatids and spermatozoa predominated. After the LP-PRP injections, the animals of group III showed a gradual decrease in the proportion of positive germ cells by 1.7 times (44.1± 2.1%, p<0.01) by the 12th week compared with group II. In group IV, throughout the experiment, there were no statistically significant differences

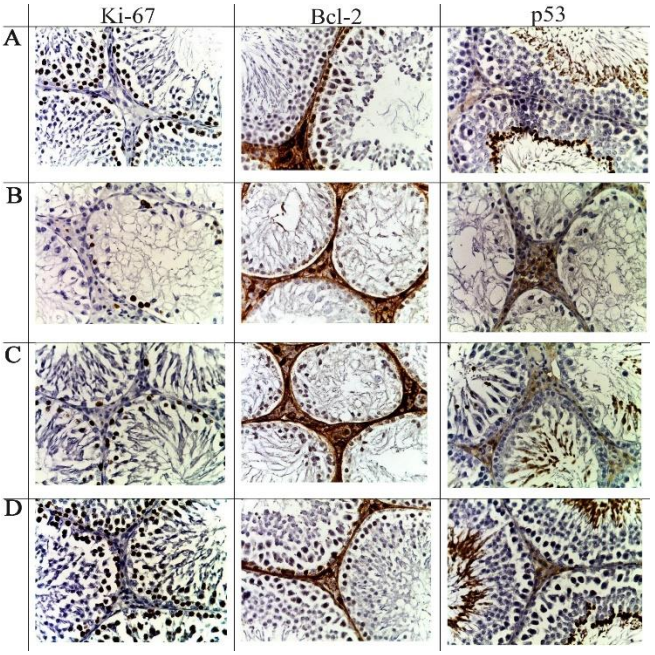

Figure 2. Seminiferous tubules at week 12 of the experiment. Immunohistochemical reactions with antibodies of Ki-67, Bcl-2, p53; core staining with hematoxylin, magnification ×400. A–control; B–after electron irradiation at a dose of 2 Gy (2IR); C–after electron irradiation at a dose of 2 Gy and administration of platelet-rich plasma (2IR+LP-PRP); and D–after administration of platelet-rich plasma (LP-PRP)

in the number of p53-positive germ cells compared with the control group (Table 1, figure 2).

Thus, based on the results of immunohistochemical studies, an increase in the proliferative activity of germ cells, especially spermatogonia, after the injection of LP-PRP was clearly observed.

Western blotting: When analyzing the content of Ki-67 (315 *kDa*) and caspase-3 (19 *kDa*) proteins by western blotting, statistically significant differ-

ences were found between the control and group II by the end of the experiment. At the same time, there were no differences in the concentration of these proteins between the control and groups III and IV.

The amount of Ki-67 protein after irradiation in group II was higher (more intense luminescence; value -1.6 c.u.) than in the control group. On the contrary, during electrophoresis of the testis homogenate of group II in a polyacrylamide gel, a decrease in the content of caspase-3 protein ("dull" reaction, value -0.20 c.u.) was found compared to the animals of the control group (Figure 3).

TUNEL assay: In control group, TUNEL-positive cells accounted for 16.0±0.8% of the cells ($p< 0.01$) throughout the experiment. In groups II and III, their number (mainly spermatogonia) increased 1.3 times (21±1.0%, $p<0.01$) a week after electron irradiation compared with the control group. In the testes of group II, the number of TUNEL-positive germ cells increased 4.6 times by the end of the experiment (73.0±3.5%, $p<0.01$) compared with control group. A gradual increase in the number of TUNEL-positive Leydig cells was also observed. In group III, a decrease in the proportion of TUNEL-positive cells by 2.9 times in the spermatogenic epithelium (25.0±1.2%, $p< 0.01$) was observed 12 weeks after irradiation compared with group II. Group IV showed no significant differences compared to the control (Figure 4).

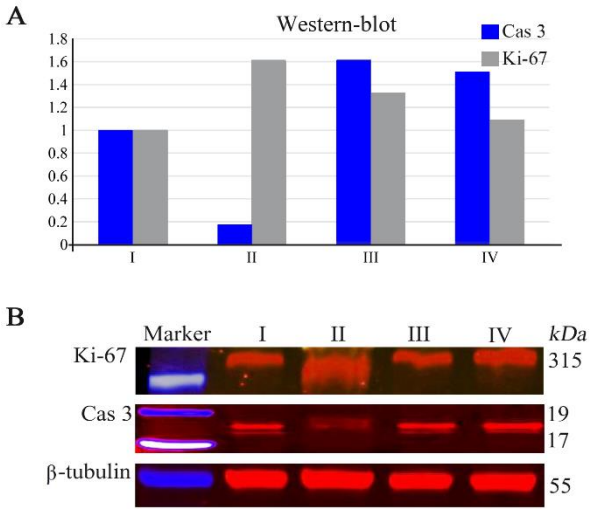

Figure 3. Western blotting of Ki-67 and caspase-3 (Cas 3) proteins in the testes of the control and experimental groups. Three testicular tissue samples from each group were pooled and subjected to western blotting. Ki-67 and caspase-3 expression in testicular tissues was measured as nmol pNA/mg protein C: control (I); 2IR (II); 2IR+LP-PRP (III); and LP-PRP (IV). β-tubulin immunostaining was used as a standard. A–densitometric analysis of western blotting, B–visualization (immunochemiluminescence)

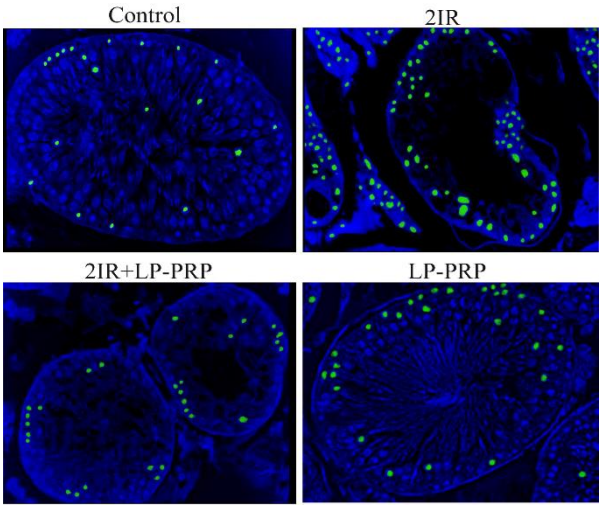

Figure 4. TUNEL and confocal microscopy of the testes on the 84th day. The luminescence was observed mainly in the basal compartment of the tubules and in the Leydig cells. Magnification ×400

Discussion

The purpose of this research was to study the cell cycle of germ cells after electron irradiation, which was modulated by growth factors of platelet-rich plasma and the second objective was to evaluate such effect on spermatogenesis.

After local electron irradiation with a dose of 2 Gy in groups II and III, a pronounced decrease in the number of Ki-67-positive germ cells was observed. It is associated with a direct toxic effect of radiation on actively proliferating spermatogenic cells, primarily spermatogonia. According to studies of the molecular mechanisms of exposure to radiation, it leads to damage to cell membranes and structure of macromolecules (DNA, RNA, proteins, lipids, *etc.*), modulation of MAPK-, PI3K-, and NFκB-signaling pathways and proteins of the ErbB family (14). Disruption of cellular respiration at the level of electron transport chain in mitochondria leads to an imbalance of the antioxidant and prooxidant systems. In addition, the observed inhibition of germ cell differentiation is associated with damage to such signaling pathways such as PI3-kinases/Akt and Ras/Raf/MEK-1, as well as glycogen synthase kinase-3 (GSK-3) and extracellular signal-regulated kinase (ERK) (12).

It is known that the action of ionizing radiation leads to a decrease in the expression of key growth factors that have a direct positive effect on the proliferation and differentiation of germ cells. In this regard, it was advisable to use platelet-rich plasma in which α-granules contain a high concentration of growth factors that ultimately led to an increase in the regenerative activity of germ cells. The biologically active substances included in LP-PRP, primarily transforming growth factor-β, epidermal growth factor, and insulin-like growth factor-1, induce the mitotic activity of intact spermatogonial stem cells and, thereby, restore the proliferative-apoptotic balance (8, 15-17).

In our study, LP-PRP injections resulted in a significant increase in proliferating germ cells as measured by an IHC study with antibodies of Ki-67. This is likely due to the modulation of MAPK-, PI3K-, and NFκB-signaling pathways, restoration of antioxidant protection and the electron transport chain, which was similar to the results of molecular biology studies (8, 16). Also, exogenous growth factors increased the synthetic activity of germ cells and the restoration of the pool of macromolecules and cell membranes. This is probably due to the specific action of growth factors described by many researchers (16, 17).

The increase in the number of Bcl-2-positive cells detected after LP-PRP introduction is associated with the induction of mRNA translation of the Mcl-1 protein and its rapid restoration as a result of proteasomal degradation slowdown mediated by growth factors based on the decrease in the activity of phosphodegron and GSK-3 signaling pathway. This indicates an increase in the activity of the antiapoptosis system in the testes of animals after exposure to growth factors (13).

Modulation of the activity of the above cascades also leads to inhibition of apoptosis, which was confirmed by a decrease in the proportion of p53-positive germ cells in treated animals. It should be noted that after irradiation, a decrease in the number of mitotically dividing cells was observed, while during normal spermatogenesis, the pool of meiotically dividing gametes mainly decreased due to their active selection during crossing over (18).

Thus, the shift of the proliferative-apoptotic balance towards proliferation of germ cells was confirmed by the results of immunohistochemical studies; there was a decrease in the proportion of positive germ cells for p53 against an increase in the number of Ki-67- and Bcl-2-immunostained cells in the testes of animals of the III group.

At the same time, based on the results of additional western blotting, an increase in the expression of Ki-67 and a decrease in the expression of caspase-3 in 2 Gy irradiated testicular tissue were found. However, the results were contradictory. On the contrary, a decrease in Ki-67 expression and an increase in caspase-3 expression were noted after LP-PRP injections. Western blotting results of Ki-67 and caspase-3 proteins were inconsistent with IHC results due to the use of tissue homogenate, which contains not only germ cells, Sertoli and Leydig cells, but also somatic cells (endotheliocytes, cells of connective tissue, *etc*.).

Taking into account the high levels of DNA fragmentation in the germ cells according to the TUNEL results and the histological image of hypospermatogenesis after electron irradiation with a dose of 2 Gy, it is highly probable to assume the activation of caspase cascade. At the same time, given the state of the somatic and endocrine testicular compartments, other types of non-apoptotic cell death cannot be ruled out, such as mitochondrial permeability transition (MPT)-driven necrosis or paraptosis (19); yet, all these arguments would not be confirmed unless further research be conducted in the future.

Thus, the use of LP-PRP growth factors after electron irradiation increases the proliferation and antiapoptosis of germ cells (Ki-67 and Bcl-2), and decreases apoptosis of germ cells (p53) and DNA fragmentation. The limitations of this study were the small number of experimental animals and the lack of a molecular genetic study to uncover the molecular mechanisms of restoration of spermatogenesis by growth factors. This may serve as a basis for further research in regenerative medicine.

Conclusion

Immunohistochemical study of Ki-67 expression and TUNEL analysis in the testes revealed a modulation of the proliferative-apoptotic balance towards apoptosis of germ cells after 2 Gy local electron irradiation. A tendency to restore the proliferative-apoptotic balance was noted after LP-PRP injections which was confirmed by the increase in Ki-67-positive germ cells.

Conflict of Interest

The authors declare no conflict of interest.

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